

**Investigating the molecular mechanism
whereby auxin modulates *Arabidopsis thaliana*
growth under salinity stress conditions**

by

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PLAGIARISM DECLARATION

I, Paul Thomas Ferrandi, hereby declare that this thesis, submitted in fulfilment of the requirements for the degree of Doctor of Philosophy, is my own unaided work, both in concept and in execution. No assistance was received in the writing hereof, other than the appropriate guidance from my supervisor(s).

All contributions to this thesis from the work of others has been correctly cited and referenced.

No part of this thesis has previously been submitted for a degree at the University of Cape Town, or any other University.

Signature:

Date:

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ABSTRACT

Soil salinization is detrimental to plant growth and yield, and an increasing global problem in irrigated agricultural land. Salinity imposes both an osmotic and ionic stress on plants, the latter of which is unique to salt stress. Previous work has indicated that the plant growth hormone auxin could play an important role in ionic stress tolerance: transcriptome profiling revealed that the genes differentially expressed in response to ionic stress were enriched with the gene ontology term ‘Response to auxin’. Additionally, expression of the auxin biosynthesis gene, *Nitrilase 2* (*NIT2*), was also specifically up-regulated in response to ionic stress. A *NIT2* overexpressing line (35S::*NIT2*) has been shown to display improved germination and growth, as well as increased auxin levels compared to wild-type plants under saline conditions. The hypothesis thus is that salt stress induces *NIT2* expression, thereby increasing auxin biosynthesis, which results in the activation of the plasma membrane (PM) H⁺-ATPase pump and subsequent activation of expansion growth mediated by expansins. Additionally, the pump activation could enhance the proton motive force for ion transport across the PM, thereby affecting ion homeostasis during salt stress.

The aim of this study was to investigate this proposed molecular mechanism whereby auxin modulates plant growth in response to salt stress. Measurement of Na⁺ and K⁺ content revealed that 35S::*NIT2* plants had improved root and shoot Na⁺/K⁺ ratios compared with wild-type (No-0 ecotype) plants, under saline conditions. This altered ion homeostasis might be a consequence of differences in the expression of several Na⁺ and K⁺ transporter/channel genes between the two lines. Of particular interest, under saline conditions, the 35S::*NIT2* line had increased root *HKT1;1*, *AKT1*, and *SKOR* expression, as well as decreased *GLR2.3* expression; and increased shoot *NHX3*, *SOS1*, and *KUP1* expression. Together these results provide evidence that improved Na⁺/K⁺ homeostasis underlies the increased salinity tolerance of the 35S::*NIT2* line. Proteomic analysis of enriched PM protein fractions isolated from the shoots of salt-stressed and untreated 35S::*NIT2* and No-0 plants revealed genotype-specific differences in salt-responsive PM protein abundance, as well as differences between the two lines under both conditions. Gene ontology analysis revealed that several of the identified salt-responsive proteins were assigned with GO terms related to auxin and the ‘response to salt stress’. This included *NIT2*, which was up-regulated in response to salt in both 35S::*NIT2* and No-0 plants, supporting the hypothesis that auxin biosynthesis is increased via *NIT2* to modulate growth in saline conditions. Notably, far fewer salt-responsive proteins were identified in the 35S::*NIT2* line than in No-0. A subset of the salt-responsive proteins identified

in No-0 displayed higher constitutive expression in the 35S::*NIT2* line under control conditions, and were not differentially expressed in response to salt (ERD7, PATL1, ABCC4, DRP2B). I propose that the processes that these proteins regulate, or are involved in, could be constitutively active in the 35S::*NIT2* line, and that they could contribute to the increased salt-tolerance of the 35S::*NIT2* line. This is the first report of salt-responsive changes in the PM proteome of Arabidopsis shoots, as well as the differences in the PM proteome of the 35S::*NIT2* line that could contribute towards its improved salinity tolerance.

Finally, Expansin 11 (EXPA11) was identified as a candidate growth effector acting downstream of auxin-induced PM H⁺-ATPase activation, as its expression was shown to be significantly higher in the 35S::*NIT2* line than in No-0, and was further up-regulated in response to salt stress. Attempts to knock-down *EXPA11* expression in the 35S::*NIT2* line proved unsuccessful, with the transgenic 35S::*EXPA11*amiRNA lines not displaying any reduction in *EXPA11* expression, or an altered phenotype. Transgenic lines overexpressing *EXPA11* (35S::*EXPA11*) displayed increased hypocotyl elongation, improved seedling growth in untreated, salt, and osmotic stress conditions, and increased shoot fresh weight in older plants under saline conditions. Furthermore, the 35S::*EXPA11* plants displayed a greater leaf surface area, with increased epidermal and palisade mesophyll cell areas. Collectively, these results indicate that EXPA11 plays a role in promoting growth in multiple tissues across different developmental stages, both in control conditions and in response to salt stress, and that overexpression of *EXPA11* improves salinity tolerance by increasing shoot growth under saline conditions without causing a growth penalty under control conditions. Overall, this study presents evidence that NIT2 likely affects both ion homeostasis and plant growth through the auxin-induced activation of the PM H⁺-ATPase, and downstream growth modulating activity of EXPA11, during salt stress.

LIST OF ABBREVIATIONS

ABA	abscisic acid
ABC	ammonium bicarbonate
ABC(X)	ATP-binding cassette superfamily type (X)
ACN	acetonitrile
AHA	Autoinhibited H ⁺ -ATPase
amiRNA	artificial microRNA
ANOVA	analysis of variance
Asp	asparagine
At	<i>Arabidopsis thaliana</i>
ATP	adenosine 5-triphosphate
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CaMV	<i>Cauliflower mosaic virus</i>
CBL	calcineurin B-like protein
cDNA	complementary DNA
CDS	coding DNA sequence
CHX	cation/H ⁺ exchanger
CIPK	CBL-interacting protein kinase
Col-0	<i>Arabidopsis</i> ecotype Columbia
CNGC	cyclic nucleotide-gated channel
CPK	Calcium-dependent protein kinase
CWI	cell wall integrity
DEG	differentially expressed gene
dH ₂ O	deionized water
DNA	deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dsRNA	double-stranded RNA
DW	dry weight
EDTA	Ethylenediaminetetraacetic acid

ePM	enriched plasma membrane
EtBr	Ethidium bromide
EV	empty vector
EXL	expansin-like
EXPA	α -expansin
EXPB	β -expansin
F	forward primer
FA	formic acid
FC	fold-change
FDR	false discovery rate
FV	fast-vacuolar channel
gDNA	genomic DNA
GB	glucobrassicin
GWAS	genome-wide association studies
GFP	green fluorescent protein
GFSA	glufosinate ammonium
GLR	glutamate-like receptor
GO	gene ontology
GUS	β -glucuronidase
HKT	High affinity K ⁺ transporter
IAA	indole-3-acetic acid
IAM	indole-3-acetamide
IAN	indole-3-acetonitrile
IAOx	indole-3-acetaldoxime
IBA	indole-3-butyric acid
IPyA	indole-3-pyruvic acid
ICP-OES	inductively coupled plasma-optical emission spectroscopy
KOR	outward-rectifying K ⁺ channel
LB	Luria-Bertani
LC-MS/MS	liquid chromatography tandem mass spectrometry

LR	lateral root
LRR RLK	leucine-rich repeat receptor-like kinase
LSD	least significant difference
LUC	luciferase
mRNA	messenger RNA
miRNA	microRNA
MOPS	3-(N-morpholino)propanesulfonic acid
MS	mass spectrometry
NCX	Na ⁺ /Ca ⁺ exchanger
ND	no detection
NHX	Na ⁺ /H ⁺ exchanger
NIT2	<i>Nitrilase2</i>
No-0	Arabidopsis ecotype Nossen
NSCC	non-selective cation channel
Os	<i>Oryza sativa</i>
PAGE	polyacrylamide gel electrophoresis
PAT	polar auxin transport
PCR	polymerase chain reaction
PIP	plasma membrane intrinsic protein
PM	plasma membrane
PN	Plant Nutrient
PR	primary root
PTM	post-translational modification
R	reverse primer
RLK	receptor-like kinase
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT-qPCR	quantitative reverse transcriptase PCR
SAUR	Small Auxin Up-RNA

SCB	sodium cacodylate buffer
SDS	sodium dodecyl sulphate
Ser	serine
Sorb	sorbitol
SOS	Salt-Overly-Sensitive
SV	slow-vacuolar channel
sRNAs	small RNAs
siRNAs	small interfering RNAs
T1/2/3	First, second, and third transgenic generation
TAE	Tris-Acetate-EDTA
T _A	annealing temperature
TAIR	The Arabidopsis Information Resource
TD	tryptophan-dependent
T-DNA	transfer DNA
TE	Tris-EDTA
TF	transcription factor
TFBS	transcription factor binding site
Th	<i>Thellungiella halophila</i>
Thr	threonine
TI	tryptophan-independent
TIP	tonoplast intrinsic protein
Trp	tryptophan
UTR	untranslated region
UV	Ultraviolet
WMD3	Web MicroRNA Designer 3 tool
WT	wild-type
XPC	xylem parenchyma cell
XyG	xyloglucan
Zm	<i>Zea mays</i>

UNITS AND SYMBOLS

°C	degrees Celsius
bp	base pair
g	gravity constant (9.81 m.s^{-1})
kb	kilobase
kDa	kilodalton
MW	molecular weight
g	gram
µg	microgram
ng	nanogram
L	liter
mL	milliliter
µL	microliter
mm	millimeter
µm	micrometer
M	molar
mM	millimolar
µM	micromolar
p	probability
ppm	parts per million
h	hour
min	minute
s	second
v/v	volume/volume
w/v	weight/volume
V	volts

CHAPTER 1: INTRODUCTION

1.1. The global impact of soil salinization

Soil salinization is one of the most detrimental abiotic factors limiting global crop yields. Saline soils, which account for more than one billion hectares of land worldwide, are characterised as having high concentrations of soluble salts, the most common of which is NaCl (> 40 mM NaCl is considered saline, and in well drained soils this is increased to 80 mM NaCl) (Munns, 2005; Munns & Tester, 2008; Ivushkin et al., 2019; Munns et al., 2020). Natural salinity arises due to the release of soluble salts from the gradual weathering of parental rock in arid areas, or the deposition of oceanic salt by wind and rain in coastal regions (Rengasamy, 2002; Munns & Tester, 2008). However, the salinization of cultivated agricultural land can largely be attributed to secondary salinization. This arises due to irrigation with poor quality water, as well as the clearing of natural vegetation, both of which raise the water table and cause the subsequent concentration of salts in the root zone (Rengasamy, 2010, Munns and Tester, 2008). In arid areas or in times of drought, this effect is worsened, with the increased reliance on irrigation, increased evaporation, and lack of rainfall to leach salts from the soil, all of which greatly increase soil salinization (Kotze & Rose, 2015). Currently, between 20-50% of irrigated agricultural land, responsible for one third of global food production is classified as saline (Qadir et al., 2014; Al-shareef & Tester, 2019), and it has been estimated that this could increase by as much as 10% annually, due chiefly to worsened drought, and poor farming and irrigation practices (Shrivastava & Kumar, 2015). Clearly, soil salinization is one of the main threats to global food security, and the need for the development of salt-tolerant crops is crucial. This requires a greater understanding of the molecular basis of the plant salinity stress response.

1.2. The classical two-phase growth response of plants to salinity stress

The classical view in plant salt stress literature is that salinity elicits both an osmotic and ionic stress on plants, and it has been well established that plant growth inhibition by salt occurs in two distinct temporal phases, corresponding with these two stresses (Munns & Tester, 2008; Tilbrook & Roy, 2014; Al-shareef & Tester, 2019). Subsequently, the nomenclature has been changed from ‘osmotic’ to ‘ion independent’ stress (Hairmansis et al., 2014; Negrão, Schmöckel & Tester, 2017; Morton et al., 2019), but for the purposes of this thesis, these terms will be used interchangeably. The first growth response phase is termed the ion independent phase, and is characterised by a rapid, and large reduction in shoot growth, upon perception of high salt concentrations (> 40 mM NaCl) in the soil solution surrounding the roots (Munns & Tester, 2008). Interestingly, unlike the shoots, the roots do not display a large growth reduction;

however, the reason for this is still unclear. The reduction in shoot growth is largely attributed to the osmotic effect of the salt, and it is thought that the reduction in leaf area decreases water use, thus limiting water uptake by the roots and preventing a further concentration of salt in the soil. Importantly, the osmotic stress is independent of Na^+ ions entering the transpiration stream (Tester & Davenport, 2003). The plant implements several responses in order to restore osmotic homeostasis, after which growth is resumed at a lower rate.

The second growth response phase is the ionic phase, which is characterised by a further, but less severe reduction in shoot growth due to the accumulation of Na^+ ions to toxic levels within the older, transpiring leaves (Munns, 2002). Depending on the rate of Na^+ accumulation, and the degree of tolerance (both of which vary between species), this ionic phase can occur days or even weeks after the initial exposure to salt stress (Munns & Tester, 2008). The further reduction of growth occurs when the rate of leaves dying due to toxic Na^+ accumulation exceeds the rate of new leaf growth, and the plant no longer has the photosynthetic capacity to supply the required carbohydrates for growth (Munns & Tester, 2008). Recently, the idea that the osmotic and ionic stresses imposed by salinity are spatially and temporally separated has been challenged by the findings that rapid salt-specific signal transduction events, as well as rapid Na^+ -induced growth responses, occurred in *Arabidopsis* roots after a period of time too short for Na^+ to accumulate to toxic levels within the shoots (Galvan-Ampudia et al., 2013; Choi et al., 2014; van Zelm, Zhang & Testerink, 2020). The effect of the two stresses, as well as the responses implemented by the plant will be discussed hereafter. In short, the mechanisms of salinity tolerance utilised by plants can be split into three categories: osmotic stress tolerance, Na^+ exclusion, and tissue Na^+ tolerance, with the latter two being a response to the ionic stress imposed by salinity (Munns and Tester, 2008, Tilbrook and Roy, 2014). However, given the recent evidence for rapid sodium-specific responses in the root, the osmotic/‘ion independent’ and ionic stresses are not as spatially or temporally separate as had been previously thought, and there is significant overlap between the early and downstream responses to these two stresses (van Zelm, Zhang & Testerink, 2020).

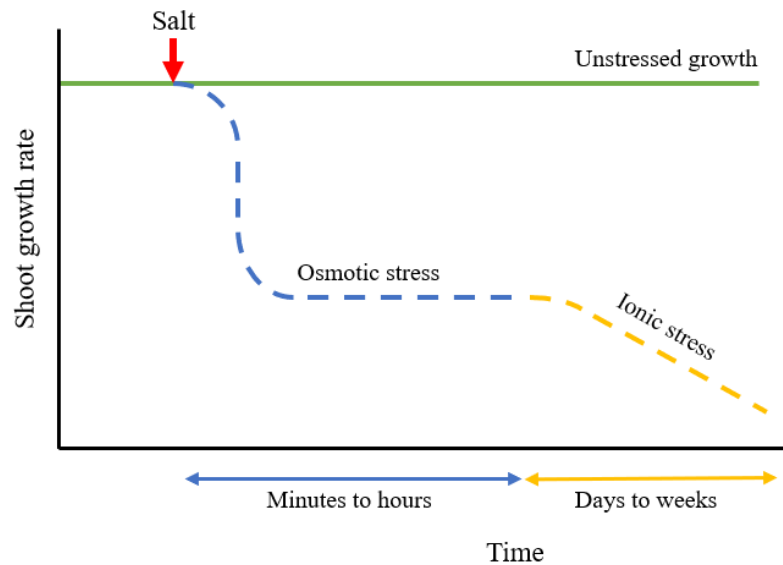


Figure 1.1: The two-phase shoot growth response to the osmotic and ionic stresses imposed by salinity

Exposure to salinity induces a rapid, large reduction in shoot growth due to the osmotic stress imposed by Na^+ ions in the soil surrounding the roots. Osmotolerance mechanisms are employed to restore osmotic homeostasis, and growth is resumed at a lower rate. After prolonged exposure to salinity, the ionic stress caused by the accumulation of Na^+ to toxic levels within the leaves causes a further, gradual reduction in shoot growth as the rate of leaves dying exceeds the rate of new leaf growth. Image adapted from Munns & Tester (2008).

1.3. Ion independent effects of salt stress and the plants response

1.3.1. Ion independent effects of salt stress on plant growth and physiology

Exposure to high levels of salinity causes an immediate reduction in shoot growth, leading to delays in flowering time as well as lower crop yield (Tilbrook & Roy, 2014). In the case of monocot cereal crops, this is observed as a reduction in the number of tillers, and in dicotyledonous plants, as a reduction in the number branches and the size of individual leaves (Munns & Tester, 2008). On a cellular level, the osmotic gradient generated by a high concentration of Na^+ in the soil solution drives water out of the root cells into the external environment (Shabala & Cuin, 2007). In order to maintain metabolic activity, the cytosolic water that is lost is replaced by water from the vacuole, resulting in an immediate loss of turgor. This loss of water availability within the cells causes the initial rapid reduction in leaf and root elongation that is observed within minutes of salt stress perception (Yeo et al., 1991). Following the initial growth reduction, the plant seeks to restore osmotic homeostasis, and the growth rate increases steadily until a new, lower growth rate is observed. The osmotic gradient generated by the high salt levels, not only reduces water uptake, but also the subsequent uptake of key growth-limiting nutrients, further compounding the growth reduction (Silberbush & Ben-Asher, 2001). In addition to the rapid reduction in growth, an immediate closure of the

stomata occurs in order to further limit water loss (James et al., 2008). Stomatal closure has two negative impacts on growth. Firstly, it restricts CO₂ uptake, which results in a reduction of carbon fixation and subsequently photosynthetic carbohydrate production, which directly impacts plant growth (Tilbrook & Roy, 2014). Secondly, the reduction in photosynthesis causes an over-reduction of the electron transport chain, resulting in a build-up of reactive oxygen species (ROS), which become deleterious to the plant at high concentrations. It must be noted that the ion independent/osmotic effects of rapid growth reduction and stomatal closure are not unique to salinity stress, but also occur in response to drought, and other osmotic stresses, indicating that this is a result of decreased cellular water levels, and not Na⁺ (Munns, 2002, Huang et al., 2012). Recent evidence has also emerged that the salt-induced reduction in photosynthesis is not solely due to the reduction in available CO₂ following stomatal closure, but also that excessive accumulation of Na⁺ within the chloroplast has a negative effect on chloroplast function (van Zelm, Zhang & Testerink, 2020). Excessive Na⁺ has an inhibitory effect on the activity of CO₂-fixing enzymes, such as fructose-1,6-bisphosphatase, as well as disturbing the balance between pH and electro-potential changes across the thylakoid membranes thus disrupting the proton-motive force required for photosynthetic processes (Bose et al., 2017). Aside from the effects of reduced photosynthesis, the reduction in growth during the osmotic phase is also caused by a decrease in cell cycle activity, cell expansion, and cell wall rigidity, although the genetic basis for these remains largely unknown (Awlia et al., 2021).

1.3.2. The balance between osmotolerance and tissue tolerance mechanisms

Osmotolerance, or the ability to tolerate osmotic stress, is one of the three salinity tolerance mechanisms mentioned previously. Osmotolerant plants are capable of maintaining shoot and root growth, as well as stomatal conductance when exposed to salt stress (Tilbrook & Roy, 2014). As such, plants implement several osmotolerance mechanisms, with varying degrees of success between species. In order to balance the osmotic potential generated by salt stress outside of the cell, plants accumulate compatible organic solutes, known as osmolytes, within the cytosol of the cell (Park, Kim and Yun, 2016, Munns and Tester, 2008). These osmolytes include, but are not limited to, simple sugars (sucrose and fructose), sugar alcohols (glycerol and sorbitol), complex sugars (trehalose and raffinose), and other charged metabolites (proline, glycine, betaine) (Cheong & Yun, 2007). While different plant species accumulate different osmolytes, they are all capable of high levels of accumulation without inhibition of the metabolic activities of the cell (Park, Kim & Yun, 2016). Additionally, during salt stress,

osmolytes function as osmoprotectants of proteins, signalling molecules, and as antioxidants to redress redox balance (Cheong & Yun, 2007). While osmolyte accumulation is an effective mechanism against the osmotic stress during both salinity and drought, the synthesis of these organic solutes is energetically expensive and redirects energy sources that are already lower on account of the reduction in photosynthesis, thus the enhanced survival comes at a further growth penalty (Munns & Tester, 2008; van Zelm, Zhang & Testerink, 2020). The energetic cost of osmotic adjustment has been the focus of recent studies, and it is thought that these energetically expensive organic solutes are only used for balancing osmotic pressure in the cytoplasm, and the majority of the osmotic adjustment of the cell is achieved by the compartmentation of Na^+ (and Cl^- ions) as osmotica in the vacuole (Munns, Passioura, et al., 2020). Vacuolar compartmentation of Na^+ is a key component of the ‘tissue tolerance’ salinity tolerance mechanism which will be discussed in depth later on. While Na^+ can be used as ‘cheaper’ osmotica in the root, there is also a fine balance between taking up enough Na^+ for osmotic adjustment, and excluding Na^+ from the root (the third aforementioned salinity tolerance mechanism) in order to prevent toxic accumulation thereof in the shoot tissues (Munns, Day, et al., 2020).

Another direct way to address the osmotic imbalances generated in the cell is by controlling water transport via aquaporins. Aquaporins are integral membrane proteins present in the plasma and tonoplast membranes that are known to play a role in water, solute and gas transport, and have been widely implicated in the response to salinity as well as other abiotic stresses (Bhardwaj et al., 2013). A notable example is the finding that salt stress causes the down-regulation of expression of 20 plasma membrane intrinsic protein (PIP) and tonoplast membrane intrinsic protein (TIP) aquaporin transcripts in *Arabidopsis* roots, as well as causing a change in the sub-cellular localisation of both PIPs and TIPs, which presumably aids in cellular water retention by limiting avenues whereby water can leave the cell (Boursiac et al., 2005). In response to the water loss and subsequent decrease in turgor pressure that occurs following exposure to salinity, it is crucial for the cell to implement membrane rearrangement processes to maintain membrane integrity and combat cellular leakage (Park, Kim & Yun, 2016). An example of this was the finding that overexpression of the maize phosphatidylinositol synthase gene (*ZmPIS*), which encodes a key enzyme in the biosynthesis of the structural membrane protein phosphatidylinositol (PtdIns), caused an alteration in membrane lipid composition that conferred improved drought/osmotic stress tolerance in maize and tobacco (Liu et al., 2013, Zhai et al., 2012). Another key osmotolerance mechanism,

that has a direct effect on maintaining membrane integrity, is the regulation of the ROS that accumulate following stomatal closure. Increased ROS production in response to salinity is known to enhance lipid peroxidation as well as cause oxidative damage to proteins, both of which negatively affect the permeability of the plasma membrane and functioning of associated proteins, thereby causing tissue damage and ultimately cell death (Møller, Jensen & Hansson, 2007; Mansour, 2013). The plant implements a number of antioxidant mechanisms to counteract ROS, including enzymatic ROS-scavengers such as superoxide dismutase, catalase and various peroxidases, as well as the production of non-enzymatic ROS-scavenging compounds such as glutathione, ascorbic acid and flavonoids (Park, Kim & Yun, 2016). As low levels of ROS act as signalling molecules in the regulation of many biological processes, maintaining the balance between necessary ROS production and excessive ROS accumulation is key to the plant's salt stress response.

1.4. Ionic effects of salt stress and the plants response

1.4.1. Ionic effects of salt stress on plant growth and physiology

After osmotic homeostasis has been restored and growth resumes, the plant starts to take up water, and along with it, Na^+ ions. The toxicity of Na^+ is chiefly due to its physiochemical similarity to K^+ , allowing for its uptake into cells via K^+ transport channels, and competition for binding sites on various enzymes that require K^+ as a co-factor, thereby interfering with many cytosolic metabolic processes, protein synthesis and ribosomal functioning (Shabala and Cuin, 2007, Tilbrook and Roy, 2014). While the majority of plants exclude between 94-98% of Na^+ from the surrounding soil solution, the Na^+ that is taken up by the roots enters the transpiration stream of the plant, and is transported to transpiring leaves where it accumulates (Munns, 2005). This accumulation occurs as there is very little Na^+ re-distribution via the phloem (Tester & Davenport, 2003). Older leaves that are no longer expanding, lack the ability to dilute the Na^+ they are receiving like younger expanding leaves do, and thus die once toxic concentrations are reached (Munns & Tester, 2008). Cell death arises due to an inability to further compartmentalize Na^+ in the vacuole, which results in an inhibition of metabolic enzymes due to excessive cytosolic Na^+ , or cell dehydration as a result of salt-deposition in the cell wall (Munns, 2005). If the rate of salt-induced leaf death exceeds the rate of new leaf production, the photosynthetic capabilities of the plant will be insufficient to provide the carbohydrates required by the younger leaves, which leads to premature leaf senescence, and the further reduction in plant productivity/growth characterised by the ionic phase of salinity stress (Munns & Tester, 2008; Awlia et al., 2021).

1.4.2. K^+ maintenance and the importance of K^+/Na^+ ratios

One of the major constraints on plant growth during salinity is K^+ deficiency. Negative ionic interactions caused by high concentrations of Na^+ in the soil reduces the availability of K^+ (and other essential nutrients) for uptake by the roots (Fageria, Gheyi & Moreira, 2011). Na^+ also competes with K^+ for uptake across the plasma membrane via K^+ channels and transporters (Shabala & Cuin, 2007). Furthermore, when Na^+ is taken up it causes significant depolarisation of the plasma membrane (Shabala et al., 2005), which prevents the passive uptake of K^+ via inward-rectifying K^+ channels while concomitantly increasing K^+ leak via outward-rectifying K^+ channels (KOR) (Shabala & Cuin, 2007). As mentioned previously, Na^+ toxicity is largely due to its physiochemical similarity with K^+ and the resulting inhibitory effect it has on enzyme activation and other metabolic processes in the cytoplasm. The ability of a plant to maintain cytosolic K^+ levels while limiting the uptake of Na^+ , thereby maintaining a high cytosolic K^+/Na^+ ratio, is thus a key determinant of its salinity tolerance (Maathuis & Amtmann, 1999; Isayenkov & Maathuis, 2019). For example, the salt-tolerant relative of Arabidopsis, *Thellungiella halophila*, has been shown to increase leaf mesophyll K^+ content in response to salinity stress, whereas a decrease was observed in Arabidopsis (Volkov et al., 2003). Below I will focus on the transporters and channels that contribute towards K^+/Na^+ homeostasis during the ionic response to salt stress.

1.4.3. Na^+ transport

Given the toxic effect of Na^+ in the aerial tissues of plants, the control of Na^+ transport within the transpiration stream as well as the uptake of Na^+ by cells is a key determinant of salinity tolerance. There are several avenues, at the plasma membrane, by which Na^+ uptake into cells is thought to occur. At the root-soil boundary, Na^+ uptake is largely thought to occur via non-selective cation channels (NSCCs) such as cyclic-nucleotide gated channels (CNGCs) and glutamate-like receptors (GLRs), and there is also evidence that it could occur via aquaporins (Hanin et al., 2016; Assaha et al., 2017; Byrt et al., 2017). Additionally, non-specific Na^+ uptake is also thought to be mediated by K^+ transporters, such as the high-affinity K^+ uptake transporter (HAK5) and the Arabidopsis K^+ transporter (AKT1) (Mian, Senadheera & Maathuis, 2011; Wang, Guan, et al., 2015). The plant also has several Na^+ transporters that allow it to control Na^+ transport, and thus are crucial contributors to salt-tolerance, including High Affinity K^+ Transporters (HKTs), and Na^+/H^+ antiporters such as intracellular Na^+/H^+ exchangers (NHXs) and the plasma membrane located Salt Overly Sensitive 1 (SOS1) (Assaha et al., 2017; Zhao et al., 2020).

1.4.3.1. Non-selective cation channels (NSCCs)

As NSCCs are thought to be the main port for the uptake of Na^+ at the root-soil boundary, their role in salinity tolerance has been widely reviewed (Demidchik & Maathuis, 2007; Kronzucker & Britto, 2011). One of the main groups of NSCCs implicated in Na^+ uptake at the plasma membrane are the CNGCs, which are permeable to Na^+ , K^+ and Ca^{2+} . While the main physiological role of CNGCs in the root is the nutritional uptake of Ca^{2+} (and to a lesser extent K^+ and Na^+) (Demidchik & Maathuis, 2007), their permeability to Na^+ has implicated them in the Na^+ influx that occurs in the roots during salt stress. There are several studies showing a down-regulation of CNGCs to provide improved salinity tolerance: down-regulation of *OsCNGC1* in the roots contributed to the superior salinity tolerance of multiple rice cultivars (Senadheera et al., 2009; Mekawy et al., 2015). Furthermore, Arabidopsis *atcngc10* null mutants, and *atcngc3* T-DNA insertion mutants were found to display enhanced growth and increased resistance to toxic levels of NaCl respectively, under salt stress when compared to the wild-type (Jin et al., 2015; Gobert et al., 2006). At the tonoplast membrane, two NSCCs have been implicated in the transport of Na^+ between the cytosol and the vacuole; the slow vacuolar (SV) and fast vacuolar (FV) channels, both of which display K^+/Na^+ selectivity (Demidchik & Maathuis, 2007). While the primary roles of the SV and FV channels are in vacuolar Ca^{2+} release and K^+ homeostasis respectively (Demidchik & Maathuis, 2007), it is thought that they also mediate the leakage of vacuolar Na^+ , thereby affecting cytosolic Na^+ levels (Hanin et al., 2016; Assaha et al., 2017).

1.4.3.2. Salt Overly Sensitive 1 (SOS1)

The Salt Overly Sensitive 1 (SOS1) locus was identified in a genetic screen for salt-sensitive Arabidopsis mutants, where *sos1* mutants were found to have a growth reduction 20 times more severe than the wild-type under salt stress (Wu, Ding & Zhu, 1996). AtSOS1 was found to encode a putative plasma membrane Na^+/H^+ antiporter, homologous to those in bacteria and fungi, and expression thereof was shown to be activated in response to salt stress (Shi et al., 2000). Subsequently, a GFP-SOS1 fusion protein was shown via confocal imaging to localise to the plasma membrane in transgenic Arabidopsis, and *SOS1* promoter-GUS analyses showed that *AtSOS1* was preferentially expressed in the parenchyma cells at the xylem/symplast boundary in roots, stems, and leaves, as well as in the epidermal cells at the root tip (Shi et al., 2002). Importantly, *sos1* mutants were found to accumulate higher levels of Na^+ as well as greater xylem sap Na^+ contents than the wild-type under severe salt stress conditions (100 mM NaCl), while under mild salt stress (25 mM NaCl), they accumulated less (Shi et al., 2002).

These findings led to the model that SOS1 functions, depending on the level of salt stress, in either the retrieval or loading of Na^+ into the xylem, thereby controlling the xylem sap Na^+ concentration and thus playing a crucial role in the control of long-distance Na^+ transport from the roots to the shoots (Shi et al., 2002). Additionally, AtSOS1 has been suggested to function in the direct extrusion of Na^+ into the external environment from the mature epidermal zones of the root, thus is key to counter-acting Na^+ uptake during salt stress (Shabala et al., 2005). SOS1 is the final component of the widely studied SOS pathway, which is considered to be one of the most crucial salt tolerance pathways in plants. In brief, the AtSOS1 antiporter is regulated by other SOS family proteins that were identified in the initial genetic screen, including AtSOS2, which is a protein kinase belonging to the Calcineurin B-like interacting protein kinase (CIPK) family, and AtSOS3, which is a Ca^{2+} binding protein belonging to the Calcineurin B-like proteins (CBL), as well as additional SOS proteins, and the calcium binding protein SCaBP8, all of which function together to activate AtSOS1 in response to increased cytosolic Ca^{2+} levels that occur during salt stress (Liu & Zhu, 1998; Halfter, Ishitani & Zhu, 2000; Liu, 2000; Shi et al., 2000; Lin et al., 2009; Luan, 2009). The increased cytosolic Ca^{2+} is sensed and bound by SOS3 and SCaBP8, whereafter SOS3 recruits SOS2 to the SOS1 Na^+/H^+ antiporter at the plasma membrane, which is then phosphorylated by SOS2, activating the extrusion of Na^+ out of the cell. Importantly, these SOS family proteins have been identified in many plants including wheat (Mullan, Colmer & Francki, 2007; Xu et al., 2008), barley (Rivandi et al., 2011), rice (Martínez-Atienza et al., 2007), and tomato (Olías et al., 2009), indicating that the transport of Na^+ via SOS1 is a conserved mechanism.

1.4.3.3. High Affinity K^+ Transporters (HKTs)

The HKTs have also been demonstrated to play a key role in the transport of Na^+ and/or K^+ into plant cells. HKTs can be divided into two subfamilies, Class I and II, based on amino acid sequence (Platten et al., 2006). In general, Class I HKTs are Na^+ specific uniporters, and Class II are Na^+/K^+ symporters (Munns and Tester, 2008; Tilbrook and Roy, 2014). The ion selectivity of the two classes has been linked to the identity of a specific residue at the filter position in the first pore-forming region of the protein, with the serine residue present in Class I HKTs being replaced with a glycine residue in Class II, resulting in the increased K^+ permeability (Hauser & Horie, 2010). Here I will focus on Class I HKTs. The transport of Na^+ by Class I HKTs is widely accepted as a conserved salt tolerance mechanism for sodium exclusion from the shoot during salinity stress (Hauser & Horie, 2010). The Arabidopsis AtHKT1;1 is by far the most studied Class I subfamily member. AtHKT1;1, which is the only

HKT transporter found in *Arabidopsis*, has been shown to display a strong preference for Na^+ selective transport in heterologous *S. cerevisiae* and *Xenopus laevis* oocyte systems (Uozumi et al., 2000). Furthermore, *athkt1;1* mutants are hyper-sensitive to salinity stress, displaying severe leaf chlorosis as well as extreme shoot Na^+ accumulation, but reduced root Na^+ accumulation under saline conditions (Mäser et al., 2002; Berthomieu et al., 2003; Sunarpi et al., 2005).

While AtHKT1;1 is clearly crucial for the regulation of Na^+ homeostasis under salinity stress conditions, there was initially some contention as to the precise manner in which this occurs. The early ‘re-circulation’ model proposed that AtHKT1;1 functions in the outward loading of Na^+ from companion cells into the phloem, allowing for the elimination of Na^+ accumulated in the shoots via the subsequent long-distance transport of Na^+ from the shoots to the roots within the phloem (Berthomieu et al., 2003). However, the now widely accepted ‘exclusion’ model states that AtHKT1;1 rather functions in the retrieval of Na^+ from the xylem into the XPC in the roots, thus reducing the amount of Na^+ in the transpiration stream of the plant, and reducing Na^+ transport to the aerial tissues, thereby protecting the leaves from salinity stress (Sunarpi et al., 2005; Davenport et al., 2007; Hauser and Horie, 2010). This is based on a large body of evidence: Immunoelectron microscopy studies found AtHKT1;1 to be targeted to the plasma membrane in xylem parenchyma cells (XPC) in leaves, and promoter-GUS reporter analyses confirmed *AtHKT1;1* expression in XPC in *Arabidopsis* roots and leaves (Sunarpi et al., 2005). Additionally, *athkt1;1* null mutants displayed large increases in xylem sap and leaf Na^+ content compared with the wild-type (Sunarpi et al., 2005). This finding was supported by $^{22}\text{Na}^+$ flux measurements in the *athkt1;1* mutant, which also found no evidence to support the Na^+ re-circulation model involving the phloem (Davenport et al., 2007). Importantly, AtHKT1;1 orthologues have been identified in rice (OsHKT1;5) and wheat (TmHKT1;5-A) where they were found to perform the same function (Ren et al., 2005; Byrt et al., 2007), indicating that xylem Na^+ exclusion in the roots is a widely used salt tolerance mechanism in monocot plants. Additionally, TmHKT1;4-A has been shown to play an exclusive role in preventing Na^+ accumulation in the leaf blade of wheat by unloading Na^+ from the xylem into the leaf sheath as it enters the shoot, thereby retaining the Na^+ in the base of the leaf (Huang et al., 2006; James, Davenport & Munns, 2006). This suggests that the xylem unloading activity of HKT1s is not limited to the roots.

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

While SOS1 and HKT function in the transport of Na^+ across the plasma membrane, another mechanism for plant cells to deal with the high cytosolic Na^+ levels that arise during salt stress is the sequestration thereof into the vacuole, which is achieved by intracellular Na^+/H^+ exchangers (NHXs). *AtNHX1* was identified by sequence homology to the *Saccharomyces cerevisiae Nhx1*, and could rescue *nhx1* mutant yeast (Gaxiola et al., 1999). Subsequently, overexpression of *AtNHX1* was shown to improve Arabidopsis growth under salt stress (200 mM NaCl) conditions, and this improved salinity tolerance was correlated with increased *AtNHX1* transcript and protein expression in wild-type Arabidopsis (Apse et al., 1999). The Arabidopsis genome encodes eight NHX isoforms, which are split into three classes: AtNHX1 to 4 are vacuolar, AtNHX5, 6 are vesicular, and AtNHX7 (SOS1) and AtNHX8 are a divergent plasma membrane class (Bassil & Blumwald, 2014). Vacuolar NHXs function by transport of Na^+ (or K^+) across the tonoplast membrane into the vacuole in exchange for the efflux of H^+ into the cytosol, thereby affecting intracellular K^+/Na^+ and pH homeostasis (Jiang et al., 2010). Importantly, it has been suggested that vacuolar NHXs only transport Na^+ under salinity stress conditions, whereas under normal conditions they function primarily as K^+/H^+ exchangers (Jiang et al., 2010). Orthologues of all three of the Arabidopsis NHX classes have been identified in a wide range of plant species including maize, rice, sorghum, soybean, and tomato (Bassil & Blumwald, 2014). Additionally, overexpression of NHXs has been shown to lead to improved salinity tolerance in tomato (Zhang & Blumwald, 2001; Gálvez et al., 2012), sugar beet (Liu et al., 2008), rice (Ohta et al., 2002), and wheat (Brini et al., 2006).

1.4.4. K^+ transport during salinity stress

In order to maintain optimal K^+/Na^+ ratios during salinity stress, uptake of K^+ by the root, loading of K^+ into the xylem for transport to the shoot, as well as K^+ influx into the leaf must be increased, while cytosolic K^+ efflux must be restricted (Assaha et al., 2017). This regulation of K^+ transport involves many proteins, which can be divided into two main groups: potassium permeable channels (consisting of Shaker-type potassium channels, ‘two-pore’ potassium channels, and NSCCs), and potassium transporters (consisting of KUP/HAK/KT transporters, HKTs, and K^+/H^+ antiporters) (Shabala & Cuin, 2007). I will focus on K^+ transport during salt stress specifically.

1.4.4.1. Shaker-type potassium channels

Shaker-type potassium channels are plasma membrane located channels involved in the low affinity uptake of K^+ . They are divided into three functional subfamilies: hyper-polarisation-

activated inward-rectifying channels (AKT1, KAT1/2), weakly inward-rectifying channels (AKT2/3), and depolarisation-activated outward-rectifying channels (SKOR, GORK) (Shabala & Cuin, 2007). In *Arabidopsis*, AKT1 is one of the main channels for low-affinity K^+ uptake by the roots (Hirsch et al., 1998), however during salinity stress conditions when K^+ availability in the soil is effectively reduced it has been suggested that the majority of root K^+ uptake is achieved instead by high-affinity transporters like HAK5 (Nieves-Cordones et al., 2010; Wu et al., 2018). While it has been suggested that AKT1 could allow for non-specific uptake of Na^+ (Mian et al., 2011), it is unclear what the extent of this might be given that low affinity K^+ uptake is down-regulated under saline conditions. AKT2/3, which is proposed to function in the loading and unloading of K^+ to the phloem in shoots (Marten et al., 1999; Lacombe et al., 2000), has been shown to be up-regulated during salinity (Maathuis, 2006). Additionally, SKOR, which functions in the outward loading of K^+ into the xylem in stelar root cells (Gaymard et al., 1998), has also been shown to be up-regulated during salinity (Maathuis, 2006). Together, the up-regulation of AKT2/3 in the shoots, and SKOR in the roots are thought to contribute towards the long-distance root to shoot redistribution of K^+ within the vascular tissues of the plant under salinity stress, making these channels important for improved shoot K^+ retention (Maathuis, 2006; Shabala & Cuin, 2007). The cytosolic efflux of K^+ that occurs due to the plasma membrane depolarisation following increased Na^+ uptake into cells is facilitated largely by KORs, such as GORK. *Gork1* *Arabidopsis* mutants were found to display significantly reduced K^+ efflux under saline conditions compared with the wild-type, strongly implicating a negative role for GORK in plant salt tolerance (Shabala & Cuin, 2007).

1.4.4.2. KUP/HAK/KT transporters

The KUP/HAK/KT family of K^+ transporters are plasma membrane transporters involved in high affinity K^+ uptake. AtHAK5 has been shown to be crucial for the uptake of K^+ in the roots under conditions of low K^+ availability, both in the absence and presence of salinity (Nieves-Cordones et al., 2010). While HAKs are generally high affinity K^+/H^+ symporters (Assaha et al., 2017), there is some evidence to suggest that under very mild salt stress conditions (25 mM NaCl) AtHAK5 can also mediate low-affinity Na^+ uptake in *Arabidopsis* roots (Wang, Guan, et al., 2015). Interestingly, the expression of AtHAK5 was found to be down-regulated in *Arabidopsis*, whereas the expression of the orthologue ThHAK5 in the salt-tolerant *T. halophila*, was maintained under salinity stress (Alemán et al., 2009). ThHAK5 has a higher affinity for K^+ than AtHAK5, so it was thought that the downregulation of AtHAK5 in *Arabidopsis* was to limit the uptake of toxic Na^+ , whereas the high levels of expression of

ThHAK5 maintained a higher level of K^+ uptake that contributed towards the increased salinity tolerance of *T. halophila* (Alemán et al., 2009). The importance of HAKs for high affinity K^+ transport under saline conditions has also been demonstrated in rice, where both *OsHAK1* and 5 have been linked with xylem K^+ loading and increased salt-tolerance (Nieves-Cordones et al., 2016). *OsHAK1* expression was found to be up-regulated in the root and shoot apical meristem, and epidermis and stele of the root in response to salt stress, with *oshak1* mutants displaying increased sensitivity to salt stress, and *OsHAK1* overexpressing plants displaying increased K^+/Na^+ ratios (Chen, Hu, Luo, et al., 2015). Likewise, *OsHAK5* overexpression increased the shoot K^+/Na^+ ratio and improved growth of rice under salt stress, with the knockout having the opposite effect (Yang et al., 2014). There is also evidence for the role of KUP transporters in the regulation of K^+ during salt stress. Expression of *KUP2* has been found to decrease in Arabidopsis roots under salinity stress, whereas *KUP6* and *KUP11* have both been found to increase, along with the *KUP3*-like homologue in maize roots (Maathuis, 2006). Additionally, *KUP7* has been shown to play a role in both K^+ uptake, as well as xylem K^+ loading (plays an equivalent role to *OsHAK1* and 5) in Arabidopsis roots under K^+ -limited conditions (Han et al., 2016). While this has yet to be shown under salinity stress conditions, the K^+ -limited conditions in which it was observed do occur during salt stress.

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

As mentioned previously, it has been suggested that vacuolar NHXs function primarily as K^+/H^+ exchangers under normal conditions (Jiang et al., 2010). There is also some evidence for their role in cytosolic K^+ homeostasis during salt stress. Transgenic tomato plants overexpressing *AtNHX1* were found to display enhanced salinity tolerance, with increased levels of vacuole-sequestered K^+ (but no difference in Na^+) compared with the wild-type (Leidi et al., 2010). Additionally, overexpression of *TaNHX2* in alfalfa resulted in increased intracellular K^+ retention, and salinity tolerance (Zhang et al., 2014).

1.4.5. Ionic stress tolerance mechanisms

The plant implements a number of mechanisms to deal with the ionic stress imposed by the accumulation of Na^+ . It is widely accepted that these mechanisms fall under two main strategies: the exclusion of Na^+ from the plant, and the tolerance of Na^+ within the plant tissues (Munns & Tester, 2008; Tilbrook & Roy, 2014). Additionally, the importance of maintaining cytosolic K^+ has been shown to be another key ionic stress tolerance mechanism (Wu et al., 2018). These ionic stress tolerance mechanisms, which involve the action of many of the Na^+

and K⁺ transporter and channels discussed above, will be discussed in depth in chapter 2, section 2.1.1.

1.5. Rapid root Na⁺ sensing and signalling

Salinity is known to induce increases in cytosolic Ca²⁺ ([Ca²⁺]_{cyt}), ROS, and 3',5'-cyclic guanosine monophosphate (cGMP) levels, all of which have a broad effect on downstream signalling processes (Kiegle et al., 2000; Donaldson et al., 2004; Miller et al., 2010), as well as the regulation of NSCCs by which Na⁺ enters the root. Calcium signalling in response to sodium is thought to occur via different calcium waves/fluxes over time, and the maintenance of these calcium signatures involves positive feedback with ROS signalling (refer to van Zelm et al. (2020) for a detailed review). In short, perception of salt stress results in an immediate influx of Ca²⁺, which is strengthened by the further release of Ca²⁺ from the vacuole by the TWO-PORE CHANNEL1 (TPC1) (Choi et al., 2014). Additionally, salt stress causes elevated levels of apoplastic ROS, which interact with polyamines to increase [Ca²⁺]_{cyt}, resulting in the activation of RESPIRATORY BURST OXIDASE HOMOLOGs (RBOHs), which are plasma membrane-located NADPH oxidases that produce sustained ROS waves (Miller et al., 2010; Xie et al., 2011). The importance of ROS in the maintenance of Ca²⁺ signalling during salt stress is highlighted by the findings that *RBOHD* and *RBOHF* expression was up-regulated in Arabidopsis in response to salt treatment, and that *rboh* double mutants displayed a reduction in [Ca²⁺]_{cyt} and plasma membrane Ca²⁺ influx, concomitant with increased sensitivity to salt stress compared with the wild-type (Ma et al., 2012). ROS-activated plasma membrane Ca²⁺ influx in response to high extracellular Na⁺ in Arabidopsis roots is also mediated by Annexin1 (ANN1), with both adaptive signalling and salt-responsive growth adaptation found to be impaired in *ann1* mutants (Laohavisit et al., 2013). Ultimately, the salt-induced Ca²⁺ signal results in the activation of many signalling pathways, and is decoded by a wide range of Ca²⁺-dependent proteins, including calcium dependent protein kinases (CPKs), CBLs, and CIPKs (for a detailed review of this refer to Manishankar et al. (2018)). The best characterised of all of these is the salt overly sensitive pathway that was discussed earlier. The SOS pathway not only regulates Na⁺ homeostasis in response to salt-induced Ca²⁺ signalling, but also affects K⁺ transport, with SCA8 being found to negatively modulate the activity of AKT1 following phosphorylation by SOS2 (Ren et al., 2013).

While salinity is known to induce rapid calcium and ROS signals, how the plant actually senses Na⁺ prior to these signals is one of the least understood aspects of the salt stress response, with it being suggested that multiple Na⁺ sensors exist to respond to different aspects of salt stress

(van Zelm, Zhang & Testerink, 2020). Very recently, it has been shown that glycosyl inositol phosphorylceramide (GIPC) sphingoloids, which are lipids present on the outer PM bilayer, are a likely candidate for extracellular salt sensing (Jiang et al., 2019). GIPCs were shown to directly bind to Na^+ (along with other monovalent cations) and regulate the entry of Ca^{2+} into the cytosol. GIPCs were shown to be synthesised by the protein glucuronosyltransferase Monocation-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increases 1 (MOCA1), and *moca1* mutants lacked salt-induced cytoplasmic Ca^{2+} signalling waves, but displayed normal Ca^{2+} signalling in response to other stresses. These findings led the authors to suggest that GIPCs are salt-specific sodium sensors that bind Na^+ and open/activate Ca^{2+} channels to induce downstream signalling responses, although the method of Ca^{2+} channel activation remains unknown. Apart from this recent identification of GIPC, little progress has been made in the identification of other salt-stress sensors. This paucity has been attributed to the complete knockout of candidates resulting in either a lethal phenotype or no phenotype due to functional redundancy, as well as difficulties in studying the root, which as the first organ to encounter salt stress, is thought to be the primary location of such Na^+ sensors (Wang et al., 2022). That being said, while not a direct sensor of Na^+ ions, the receptor-like kinase, FERONIA (FER) can detect salt-induced changes in the integrity of the cell wall (Feng, Kita, et al., 2018). FER was shown to play a crucial role in the recovery of Arabidopsis root growth following exposure to salinity, where it was found to directly bind to pectin and detect salt-induced softening of the cell wall. Furthermore, *fer* mutants displayed increased sensitivity to high salinity, and a reduction in salt-induced Ca^{2+} signalling. Along with FER, a number of other putative receptor-like kinase salt-stress sensors have been suggested to play a role in mediating Na^+ signalling responses at the cell surface (both in the cell wall and plasma membrane), as well as at organelle membranes, with these candidates thought to detect changes in ion concentrations, osmotic potential, ROS levels, and the mechanical tension of the cell surface that occur during salt stress (for a detailed review, refer to (Wang et al., 2022)). While the understanding of calcium signalling in the context of rapid responses to salinity continues to grow, clearly much more work is required to identify proteins involved in Na^+ sensing in the different cellular contexts, as well as to elucidate the complex network of salt stress signalling pathways that act downstream.

1.6. The effect of salinity on root growth and root system architecture

As the organ that is in first, and direct, contact with the salt present in the soil, the root needs to adapt in order to maintain growth and the uptake of water and other nutrients required for the survival of the plant (van Zelm, Zhang & Testerink, 2020). An obvious example of this is

the recently described halotropic root response, which refers to the ability of roots to bend in order to circumvent or avoid high concentrations of salt within the surrounding growth medium, thereby allowing the plant root to grow away from the salt (Galvan-Ampudia et al., 2013). The directionality of bending is established by the active re-distribution of auxin in the root tip, which will be discussed in depth later on. Additionally, the development of automated phenotyping platforms has allowed for the detailed analysis of changes in root system architecture (RSA) in the model plant, *Arabidopsis*, in response to salt stress, where it has been established that salt stress reduces overall root mass, as well as causes a modification of RSA by differentially inhibiting the growth rate of the primary root (PR, which anchors the plant and explores the soil for favourable conditions), and lateral roots (LR, which function largely in nutrient acquisition), as well as causing the inhibition of lateral root formation (Julkowska et al., 2014; Julkowska & Testerink, 2015). Furthermore, the phenotyping of 31 *Arabidopsis* accessions revealed that there was a fair degree of natural variation in these salt-responsive changes in RSA, indicating the existence of different strategies to adapt root growth in response to salinity (Julkowska et al., 2014). Subsequently, genome-wide association studies (GWAS) looking at the natural variation in salt-responsive RSA changes, as well as halotropic responses, among *Arabidopsis* accessions have been used to identify several genetic components of salt-induced root growth modulation: salt-responsive changes in the expression of *CYP79B2* and *CYP79B3* (which function in auxin biosynthesis) were found to be positively correlated with LR development, whereas high *HKT1* expression was found to repress LR development (Julkowska et al., 2017). Similarly, GWAS looking at the natural variation of PR halotropism among *Arabidopsis* accessions identified several genes that contribute to halotropic growth responses (Deolu-Ajayi et al., 2019). However, much more work is required to complete our understanding of the genetic basis underlying salt-responsive changes in root growth, particularly since very few studies to date have focused on crop plants, as opposed to *Arabidopsis* (van Zelm, Zhang & Testerink, 2020).

1.7. Limitations in the development of salt tolerant crops and future perspectives

Given how much is known about how plants respond to salinity stress, it is concerning that to date such limited progress has been made in the development of salt-tolerant crops. The paucity in salt tolerance genes or alleles that have been identified and taken forward in real world applications can largely be explained by the enormous genetic complexity of salt tolerance agronomically important traits (Morton et al., 2019). Recent reviews have highlighted a number of gaps in our knowledge, including how salt is perceived by the plant (both externally at the

plasma membrane, and also by the organelles within the cell), the scant understanding of Na^+ and Cl^- uptake mechanisms, the need for cell and tissue level mapping of ion toxicity, which signalling pathways affect the various salt stress responses, how salt stress induces changes in the cell wall and the effect thereof, and the need to understand the expression of the various genes involved in salt-tolerance in a tissue/cell-specific context throughout the temporal stages following salt exposure (Isayenkov & Maathuis, 2019; van Zelm, Zhang & Testerink, 2020; Zhao et al., 2020). Recent advances in high-throughput phenotyping capabilities has allowed for the detailed genetic mapping of early salt stress responses in 191 different *Arabidopsis* accessions from 1 h to 7 days after treatment (Awlia et al., 2021). Here, GWAS identified 770 loci specific to salt stress. The use of such high-throughput techniques will likely be key to uncovering the genetic basis of temporal salt stress responses in a more expedient manner.

Recently, the importance of understanding the energy costs associated with the many salt-tolerance strategies employed by plants, at both the cellular and whole-plant level, has been highlighted (Munns, Day, et al., 2020). In this review, a detailed comparison between the current knowledge of energy supply (by mitochondrial respiration, photosynthesis, and the action of vacuolar pyrophosphatases), and the energy costs associated with the regulation of water and ion uptake and transport throughout the plant during salt stress, was performed. While the transport processes that require the most energy (along with the associated spatial and temporal information) have yet to be identified, it was suggested that combining physiological studies and computational/biophysical modelling will allow for the identification of the most cost-efficient salt tolerance strategies, and this will inform which genetic components to target in the development of salt-tolerant crops (Munns, Day, et al., 2020).

Another issue that has likely limited salt-tolerant crop development is that many previous transcriptomics studies have failed to adequately separate the osmotic/ion independent and ionic stress response transcriptomes in order to identify true salt-specific response genes. As the osmotic response is activated following challenge by many other biotic and abiotic stresses, transgenic manipulation of osmotic response genes to develop salt tolerant plants will not target salt stress responses specifically and may have other deleterious off target effects. Given that the ionic phase of salinity stress is unique to Na^+ accumulation, ionic response genes perhaps represent the best candidates for transgenic manipulation. Many of these previous transcriptomics studies have treated plants with high concentrations of NaCl (150 mM or higher) that exceed those seen in a natural environment, and for too short a time period for Na^+ accumulation to have occurred (Abogadallah, 2010, Li et al., 2010, Tang et al., 2011, Goyal et

al., 2016, Li et al., 2019). Such treatments do not mimic natural salinity stress, but rather elicit an osmotic shock referred to as ‘salt shock’, which can cause plasmolysis and the subsequent activation of genes involved in the osmotic response and plasma membrane repair (Shavrukov, 2013). As salt shock rarely occurs naturally, or as a result of agricultural irrigation, genes identified in these studies are not particularly relevant for further targeting or application in an agronomic context (Shavrukov, 2013). In contrast, salt stress is induced by exposure to low concentrations of NaCl for longer periods of time, or exposure to a gradual increase in NaCl, as would be the case in saline field environments (Shavrukov, 2013). In the broadest sense, the concentration of NaCl required to elicit the different responses can be delineated as follows: 25-50 mM represents mild salt stress, 80-100 mM represents intermediate salt stress, and 150-300 mM represents salt shock (Shavrukov, 2013). However, recently, there have been some transcriptomics studies performed in which plants were exposed to physiologically relevant NaCl concentrations for an extended period of time (Wang et al., 2018, Luo et al., 2019), indicating that there is perhaps progress towards the identification of true salt stress response genes.

1.8. The link between auxin and the salt stress response

In order to delineate the osmotic/ion independent and ionic stress responses and identify true salt-specific response genes, our research group conducted a microarray experiment on *Arabidopsis* seedlings that were germinated and grown for two weeks on concentrations of NaCl ranging from 50-125 mM – this represented a NaCl concentration low enough, and a period of time long enough to induce a physiologically relevant salt-stress response (Shavrukov, 2013). In addition, seedlings were grown on the iso-osmolar concentrations of the sugar-alcohol, sorbitol. NaCl induces both an osmotic and ionic stress, whereas sorbitol only induces an osmotic stress equivalent to that of NaCl. Genes that were differentially expressed in the NaCl and sorbitol treatments were designated as osmotic stress response genes, whereas those that were differentially expressed in the NaCl treatment only were designated as salt-specific, ionic stress response genes. This transcriptomics study allowed for the identification of genes involved in the salt stress response during early development (germination and seedling development) (Cackett et al., 2022). Gene Ontology (GO) analysis found that the salt-specific gene list was enriched for the GO term ‘response to auxin stimulus’ (Cackett et al., 2022). Auxin is the primary plant growth hormone, which plays a regulatory role in cell division and expansion, and thus is involved in almost all aspects of plant growth and development (Du, Spalding & Gray, 2020). The majority of the ‘response to auxin stimulus’

genes were *SMALL AUXIN UP-RNAs (SAURs)*, including the *SAUR19-24* sub-family genes. Additionally, the auxin biosynthesis gene *Nitrilase 2*, as well as the cell wall modifying *Expansin 11* were also identified as being upregulated specifically in response to salt. These genes and their role in auxin-induced expansion growth, as well as in the salt stress response, will be discussed in depth later. Importantly, in this same study, the levels of the predominant form of auxin, indole-3-acetic acid (IAA), as well as IAA biosynthetic intermediates were found to be elevated in a salt-specific manner (Cackett et al., 2022). Collectively, these results strongly suggest that auxin is being produced to modulate growth in response to salt stress.

1.9. The role of auxin during plant growth

1.9.1 Auxin is the major growth hormone in plants

Auxin was the first major plant growth hormone discovered, and the field of auxin biology is one of the oldest in experimental plant research, dating as far back as tropic shoot elongation experiments performed by Charles Darwin in the 1880s (Woodward & Bartel, 2005). IAA, the predominant form of auxin, plays a role in the regulation of cell division and expansion, and thus is involved in almost all aspects of plant growth and development, including stem elongation, root and shoot lateral branching, embryogenesis, vascular tissue differentiation, the regulation of tropisms (modulation of growth in response to the environment), as well as playing a role in stress responses (Woodward and Bartel, 2005, Di et al., 2016, Spartz et al., 2012, Du, Spalding and Gray, 2020). The regulation of growth by auxin involves the accumulation of auxin at specific sites, known as auxin maxima (Ryu & Cho, 2015), the subsequent initiation of auxin signal transduction pathways, and ultimately a change in the activity of growth related proteins (Majda & Robert, 2018). Auxin has also been shown to have both a growth promoting and inhibitory effect (Collett, Harberd & Leyser, 2000), and given its important role in plant growth and development, the regulation of auxin production, distribution and signalling needs to be precisely controlled. Here, I will discuss aspects of auxin biosynthesis, with a particular focus on the role of *Nitrilase 1* family genes, as well as how auxin biosynthesis, transport, and signalling is affected during salt stress.

1.9.2. Auxin is central to the acid growth theory

Auxin's ability to promote growth by the activation of cell expansion has long been explained by the acid growth theory (Rayle & Cleland, 1970). The basic premise is that auxin causes the extrusion of protons from plant cells into the surrounding apoplast, leading to the acidification thereof (Rayle & Cleland, 1992). The decrease in apoplastic pH activates proteins that 'loosen' the cell wall via modulation of intercellular load-bearing bonds (Rayle & Cleland, 1992). These

cell wall loosening proteins have subsequently been identified as expansins (McQueen-Mason and Cosgrove, 1994, Cosgrove 2016). The proton extrusion is achieved by the plasma membrane (PM) H⁺-ATPase pump, which couples ATP hydrolysis and proton pumping to generate an electrochemical gradient across the plasma membrane (Takahashi, Hayashi & Kinoshita, 2012). This gradient drives the uptake of solutes, such as K⁺ ions, and subsequently water into the cell, increasing the turgidity thereof, which when combined with the cell wall loosening, drives expansion growth. The role of the PM H⁺-ATPase pump and expansins in driving auxin-mediated expansion growth will be explored in depth in section 1.9 and 1.10, respectively.

1.9.3. Auxin biosynthesis

1.9.3.1. The tryptophan-dependent and -independent pathways

Currently, the mechanism of *de novo* auxin biosynthesis in plants is not well understood, however it is clear that it is extremely complex, and involves multiple pathways (Zhao, 2010). In addition to this, while the core mechanisms for auxin biosynthesis seem to be evolutionarily conserved in plants, it is likely that different species adopt unique strategies to optimise auxin production (Zhao, 2010). As it stands, there are two main strategies that have been proposed for auxin biosynthesis in plants – tryptophan-dependent (TD) and tryptophan-independent (TI) biosynthesis (Di et al., 2015). The TD biosynthetic pathway has been extensively studied, and many of the genes involved, as well as the intermediate metabolites have been identified; however, this is not the case for the TI pathway, of which little is known (Di et al., 2015). Both biosynthetic pathways begin with indole-3-glycerol phosphate (IGP), which is produced from chorismate via a multi-step process in the chloroplast (Radwanski & Last, 1995). This IGP serves as a branch point (Ouyang, Shao & Li, 2000): firstly, it can be converted into tryptophan (Trp), via an indole intermediate, by the Trp synthase complex (TS-complex) and subsequently used in the TD pathway (Radwanski & Last, 1995). Alternatively, it can be used in the TI pathway, however this is poorly understood (Wang, Chu, et al., 2015), and will not be discussed further. The TD pathway has been further refined into four separate branches, all of which result in the conversion of Trp to IAA by a series of intermediates (refer to Figure 1.2). The names of these pathways are derived from the chief intermediate produced in each: the indole-3-acetamide (IAM) pathway, the indole-3-pyruvic acid (IPyA) pathway, the tryptamine (TAM) pathway, and the indole-3-acetaldoxime (IAOx) pathway (Woodward and Bartel, 2005, Zhao, 2010, Di et al., 2015). The IPyA pathway, involving the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) enzymes and YUC-family of flavin

monooxygenase-like enzymes (YUCCA), is the only fully elucidated pathway, and is thought to be the primary auxin biosynthesis pathway as it is required for all major developmental processes, and is highly conserved in plants (Won et al., 2011, Zhao, 2018). However, of particular interest to this study is the IAOx pathway, which involves the Nitrilase1 family (consisting of NIT1, 2 and 3) of enzymes that have only been identified in *Brassicaceae* (Lehmann et al., 2017). It has thus been suggested that the IAOx pathway could be *Brassicaceae*-specific (Lehmann et al., 2017), however, a nitrilase capable of converting indole-3-acetonitrile (IAN) to IAA has also been identified in maize (Park et al., 2003), casting some doubt on this suggestion.

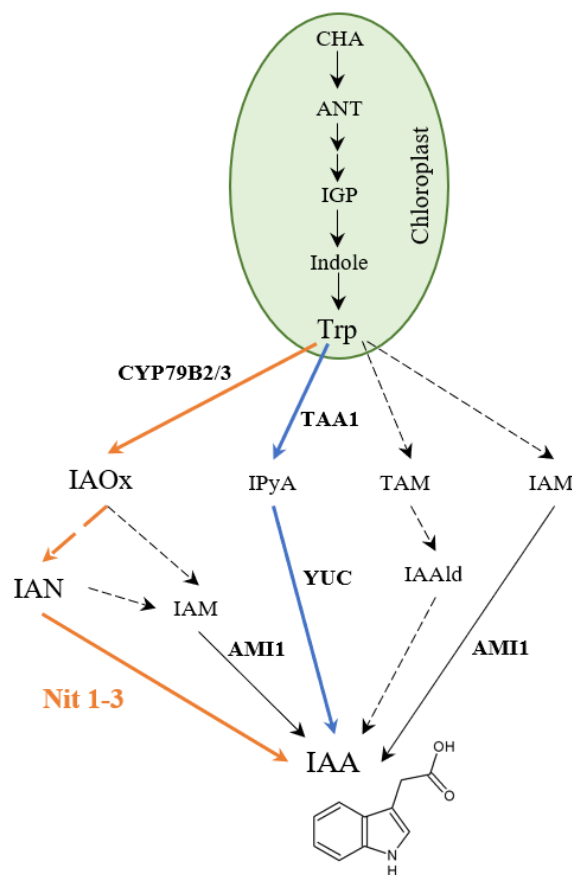


Figure 1.2: The Tryptophan-dependent IAA biosynthesis pathway

The tryptophan-dependent biosynthesis pathway comprises four different pathways by which Trp is converted to IAA via various intermediates. Solid arrows indicate reactions with identified enzymes, and the dashed arrows indicate unconfirmed reactions. Confirmed enzymes are written next to their respective reaction in bold. The orange arrows represent the IAOx pathway, in which the Nitrilase 1 family genes are involved. The blue arrows indicate the IPyA pathway. CHA: chorismite, ANT: anthranilate, IGP: indole-3-glycerol phosphate, Trp: tryptophan, CYP79B2.3: Cytochrome P450 family 79B2/3, IAOx: indole-3-acetaldoxime, IAN: indole-3-acetonitrile, IAM: indole-3-acetamide, Nit 1-3: Nitrilase 1/2/3, AMI1: Amidase 1, IAA: indole-3-acetic acid, TAA1: Tryptophan aminotransferase of Arabidopsis 1, IPyA: indole-3-pyruvic acid, YUC: Flavin monooxygenase-like enzyme, TAM: tryptamine, IAAld: indole-3-acetaldehyde. Image adapted from Wang et al. (2015).

1.9.3.2. *The role of the Nitrilase1 family in auxin biosynthesis*

Plant nitrilases were identified as enzymes that can hydrolyse nitriles to their corresponding carboxylic acid, an example of this being the hydrolysis of IAN to IAA (Thimann & Mahadevan, 1964). Subsequently, four nitrilase genes (NIT1-4) have been identified in *Arabidopsis* (Bartling et al., 1992; Bartel & Fink, 1994). These nitrilases have been shown to convert IAN to IAA in heterologous *E. coli* (Vorwerk et al., 2001). The compound IAN, along with IAM, has been shown to be an intermediate in the conversion of IAOx to IAA in *Arabidopsis* (Sugawara et al., 2009), which suggests a role for NIT1 family genes in catalysing this key step in the IAOx pathway of TD auxin biosynthesis. It must be noted, however, that the role of these plant nitrilases is not limited to auxin biosynthesis, as they have also been shown to play a role in glucosinolate catabolism and cyanide detoxification (Piotrowski, 2008). Of the four Nitrilase genes identified in *Arabidopsis*, AtNIT1-3, which are arranged in tandem on chromosome three, share 84-90% amino acid similarity, whereas AtNIT4, which is located on chromosome five, only has 66-68% similarity (Janowitz, Trompetter & Piotrowski, 2009). Thus AtNIT1-3 have been grouped into the NIT1 family, whereas AtNIT4 is its own family. Importantly, most other plants have been found to have *Nitrilase* genes that are homologous to AtNIT4, whereas the NIT1 family genes are found only in *Brassicaceae*, leading to the hypothesis that the NIT1 family genes arose from an early gene duplication of NIT4 during evolution in *Brassicaceae* (Piotrowski, 2008). Additionally, the typical plant NIT4 homologue is known to be involved in β -cyanoalanine conversion during cyanide detoxification, whereas the NIT1 family nitrilases cannot use β -cyanoalanine, but rather hydrolyse nitriles that result from the decomposition of glucosinolates, the typical secondary metabolites of *Brassicaceae* (Janowitz, Trompetter & Piotrowski, 2009).

There is a sizeable body of evidence for the role of NIT1 family genes in the conversion of IAN to IAA: transgenic *Arabidopsis* plants overexpressing *NIT2* have been shown to display an increased turnover of exogenously applied IAN, as well as a sensitivity to exogenous IAN application, as seen in the alteration in root elongation, an auxin-related growth phenotype (Normanly et al., 1997). Transgenic tobacco plants overexpressing *AtNIT2* were found to be able to convert IAN to IAA, whereas untransformed plants could not (Schmidt et al., 1996). Additionally, upon exogenous application of IAN, these *AtNIT2* overexpressing tobacco plants displayed the auxin-overproduction phenotype of increased lateral root formation. The *NIT2* overexpressing *Arabidopsis* plants have also been shown to display increased levels of free IAA, whereas anti-sense *NIT2* and *NIT1* lines both displayed a 75% decrease in free IAA

compared with the wild-type (Grsic et al., 1998). In the latter study, the *NIT2* overexpressing plants also displayed a large increase (22-fold) in IAA content upon exogenous IAN application, with a much lower increase in IAA content observed in the wild-type and anti-sense lines. Like the *NIT2*-overexpressor, a *NIT1* overexpressing Arabidopsis line has also been found to display an increase in free IAA and IAN levels, as well as increased sensitivity to exogenous IAA and IAN application, in response to which it displayed the similar auxin-overproduction phenotypes of an increase in the number of LRs, as well as number of root hairs (Lehmann et al., 2017). In contrast, a *nit2* RNAi line, capable of suppressing all *NIT1* family genes, was found to be insensitive to exogenous IAA or IAN application, as well as displaying no change in free IAA or IAN levels (Lehmann et al., 2017). Furthermore, the conversion of IAN to IAA by nitrilases is not limited to Arabidopsis. The maize (*Zea mays*) nitrilase, ZmNIT2, has been shown to convert IAN to IAA with an efficiency seven times greater than the AtNIT1 family (Park et al., 2003). Interestingly, ZmNIT2 shares the highest homology with AtNIT4, but displays the AtNIT1 family-associated specificity to IAN as opposed to any activity towards β -cyanoalanine (Park et al., 2003). ZmNIT2 has subsequently been renamed ZmNIT4A and has been shown to be highly homologous to SbNIT4A from *Sorghum bicolor* (Piotrowski, 2008). In line with this, maize and sorghum are the only plants other than Arabidopsis that have been found to have nitrilases capable of converting IAN to IAA (Piotrowski, 2008).

It has been proposed that the NIT1 family and the IAOx pathway does not play a role in general auxin biosynthesis, but rather functions in a more specific manner in the roots, during the early stages of seedling development (Lehmann et al., 2017). Additionally, there is evidence that could suggest that the IAOx pathway is utilised to produce IAA during stress responses, as *NIT1* family gene expression has been shown to be induced in Arabidopsis in response to both biotic and abiotic stresses; *NIT1* and 2 were induced in roots during *Plasmodiophora brassicae*-associated Clubroot disease development, with *nit1-3* mutants found to have reduced disease symptoms, and lower IAA contents (Grsic-Rausch et al., 2000). Sulphate-starvation has been found to induce *NIT3* expression as well as result in auxin-associated alterations in root morphology, such as an increase in root length, and the number of LRs, strongly suggesting that increased IAA via *NIT3* modulates root system architecture in search of sulphur in the surrounding soil (Kutz et al., 2002). Furthermore, *NIT2* expression has been shown via transcriptomics experiments to be up-regulated in response to both drought, and very recently by our research group, salt stress (Rasheed et al., 2016, Cackett et al., 2022), both of which

result in large-scale changes in auxin-induced root and shoot growth. Importantly, the salt-induced expression of *NIT2*, was concomitant with increased IAA levels, providing strong evidence that *NIT2* is responsible for IAA biosynthesis during salt stress (Cackett et al., 2022).

1.9.4. The role of auxin in the plant response to salt stress

1.9.4.1. Salt-induced changes in auxin levels and biosynthesis

Modulation of auxin levels and the resulting effect on plant growth under salt stress has been assessed in several ways. Firstly, salt stress has been shown to alter endogenous IAA levels. IAA levels have been shown via LC-MS/MS analysis to be increased in whole two-week-old *Arabidopsis* seedlings in response to salt stress (Cackett et al., 2022). Additionally, increased IAA levels have been reported in the leaves of salt-stressed maize (*Zea mays*) (Veselov et al., 2008). Furthermore, IAA has been shown to accumulate in the root tip of transgenic *DR5::GUS* *Arabidopsis* seedlings in response to salt stress (Wang, Li & Li, 2009). The DR5 promoter is a highly active synthetic auxin response element, which is routinely used to drive the expression of *GUS* to allow for an indirect assay of where IAA is present in the plant (Ulmasov et al., 1997). In contrast, GC-MS analysis revealed decreased IAA levels in the roots of seven-day-old *Arabidopsis* seedlings following exposure to salt stress (Liu et al., 2015). IAA levels have also been shown to decrease by up to 75% in the roots of tomato (*Lycopersicon esculentum*) plants (Dunlap & Binzel, 1996), and a marked reduction in IAA has been observed in the leaves of rice (*Oryza sativa*) plants (Prakash & Prathapasanen, 1990) in response to salt stress. It is thus clear that salt stress has a complex effect on endogenous auxin levels, in both the root and the shoot.

One likely way in which salt stress alters endogenous auxin levels, is by affecting the expression of IAA biosynthesis genes, and thus IAA production. There is evidence that salt stress alters the expression of genes in both the IAOx and IPyA TD-dependent IAA biosynthesis pathways. For the IAOx pathway, the expression of both *NIT1* and *NIT2* has been found to be increased in the roots of three-week-old, *Arabidopsis* plants following exposure to salt stress for 48 hours (Jiang & Deyholos, 2006a). Additionally, as mentioned earlier, *NIT2* has been found to be up-regulated in response to salt stress in two-week-old *Arabidopsis* seedlings (Cackett et al., 2022). *Cytochrome P450 family 79B2 and 3* (*CYP79B2* and *CYP79B3*) have been shown to have high expression in the differentiation zone of LR, and changes in the expression of *CYP79B2* during salt stress has been positively correlated with LR development in a detailed study comparing natural variation in root system architecture between *Arabidopsis* accessions (Julkowska et al., 2017; Korver, Koevoets & Testerink, 2018).

Furthermore, *cyp79b2 cyp79b3* double mutants developed fewer and shorter LR_s when grown under salt stress than in control conditions (Julkowska et al., 2017). Collectively, this suggests a strong role for CYP79B2/3 and the IAOx pathway in the regulation of LR development during salt stress. In terms of the IPyA pathway, there is evidence that *YUCCA* gene expression is affected by salt stress. In cucumber (*Cucumis sativus* L.) seedlings, exposure to 100 mM NaCl resulted in the up-regulation of *CsYUC10b*, and the down-regulation of *CsYUC10a* and *CsYUC11*, suggesting an antagonistic regulatory mechanism to maintain IAA levels during salt stress (Yan et al., 2016). Interestingly, overexpression of *CsYUC11* enhanced the salinity tolerance of transgenic *Arabidopsis* plants (Yan et al., 2016). However, the contribution of YUCCAs during salt stress in *Arabidopsis* remains unknown (Korver, Koevoets & Testerink, 2018). Collectively, the studies discussed above provide evidence that auxin biosynthesis, by both the IAOx and IPyA TD dependent pathways, is likely altered in response to salt stress.

1.9.4.2. Effect of salt on auxin transport and distribution

The ability of auxin to modulate tissue specific growth via the creation of local auxin maxima is reliant on its transport and redistribution throughout the plant. Unsurprisingly, there is a growing body of evidence that auxin transport is altered during salt stress, with most of these studies focusing on auxin transport in the root. Changes in polar auxin transport (PAT), the main mechanism of auxin flow within the root, have been found to occur in response to salt stress, with the PIN-FORMED (PIN) family of auxin efflux carriers being largely implicated in these changes (Korver, Koevoets & Testerink, 2018). *PIN1*, *PIN3* and *PIN7* have been shown to be down-regulated, thereby reducing auxin levels and causing a reduction in root meristem growth in *Arabidopsis* in response to salt stress (Liu et al., 2015). This forms part of a mechanism of salt-mediated inhibition of root growth whereby the root meristem size is reduced by nitric oxide (NO)-mediated modulation of auxin levels, involving the reduction in *PIN* expression, as well as the stabilisation of auxin-resistant 3 (AXR3)/indole-3-acetic acid 17 (IAA17), which reduces auxin signalling (Liu et al., 2015). Another example of PIN-mediated changes in auxin levels, resulting in altered root growth in response to salt stress, is the aforementioned, halotropism. This, recently described, tropism is the ability of roots to bend in order to circumvent or avoid high concentrations of salt within the surrounding growth medium, thereby allowing the plant root to grow away from the salt (Galvan-Ampudia et al., 2013). The directionality of bending is established by the active re-distribution of auxin in the root tip by *PIN2*. It has been shown that increased phospholipase D activity, and subsequently increased clathrin-mediated endocytosis of *PIN2* on the side of the root tip facing higher salt

concentrations, resulted in the redistribution and accumulation of auxin on the opposite side of the root (Galvan-Ampudia et al., 2013). Additionally, computational modelling of auxin distribution within the root tip, combined with detailed fluorescent microscopy experiments has revealed the expression of *AUX1*, the major auxin importer involved in tropisms, to be decreased on the salt-exposed side, and increased on the opposite side of the root, suggesting that auxin uptake is increased by the cells on the non-exposed side (van den Berg et al., 2016). Furthermore, a transient increase in *PIN1* expression in the root tip in response to salt has been suggested to enhance the auxin asymmetry that arises during the early stages of the halotropic response (van den Berg et al., 2016). In general, PIN-mediated auxin transport activities are clearly required for the remodelling of root system architecture in response to salt stress.

Aside from the PIN family of auxin efflux carriers, the other large family involved with auxin transport to the root, namely the ATP-binding cassette superfamily type B (ABCB) (Geisler et al., 2017), has also been shown to play a role in the response to salt stress. Transcriptomics experiments performed to assess detailed, tissue-specific changes in *OsABCB* expression in rice plants (*Oryza sativum*) revealed that 20 of the 22 *OsABCB* genes were salt-responsive (Chai & Subudhi, 2016). In the root, *OsABCB1* and *19* were found to be up-regulated, whereas *OsABCB4* was found to be downregulated upon exposure to salt stress (Chai & Subudhi, 2016). These results suggest a large potential role for ABCB-mediated transport in altering auxin levels to allow for growth modulation in response to salt stress. The studies discussed here are by no means a complete picture of auxin transport under salt stress – for a detailed review, refer to Han et al. (2017).

1.9.4.3. Effect of salt on auxin signalling

At the sites of auxin maxima, auxin signalling is controlled by SCF^{TIR1/AFB}-Aux/IAA (SKP-Cullin-F box, TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALLING F-BOX, AUXIN/INDOLE ACETIC ACID) receptor complexes leading to the transcriptional activation of auxin-responsive genes. This signalling process has been reviewed in detail (Salehin, Bagchi & Estelle, 2015; Strader & Zhao, 2016). In short, auxin is perceived by the TIR1/AFB receptors (Dharmasiri, Dharmasiri & Estelle, 2005; Parry et al., 2009; Calderon-Villalobos et al., 2010), and this interaction is stabilised by the binding of Aux/IAA repressors, forming a transient co-receptor complex. This binding recruits the Aux/IAA repressors to the SCF^{TIR1/AFB} ubiquitin ligase complex, leading to their subsequent degradation (Gray et al., 1999, 2001; dos Santos Maraschin, Memelink & Offringa, 2009). This Aux/IAA degradation results in the de-repression of Auxin Response Factors (ARFs), leading to the transcription of auxin-responsive

genes (Salehin, Bagchi & Estelle, 2015). Unsurprisingly, there is evidence that signalling cross-talk occurs between the complex SCF^{TIR1/AFB}-Aux/IAA auxin-signalling cascade and salt stress responses. In particular, *tir1 afb2* Arabidopsis double mutants displayed increased root elongation, chlorophyll content, and germination rate compared with the wild-type when grown under saline conditions, suggesting that suppression of auxin signalling may enhance salinity tolerance (Iglesias et al., 2010). Subsequently, it has been shown that salt stress increases the expression of *miRNA393*, which causes a reduction in *TIR1/AFB2* transcript abundance via RNA silencing (Iglesias et al., 2014). Furthermore, *mir393* mutants displayed reduced inhibition of LR initiation, emergence and elongation compared with the wild-type in response to salinity (Iglesias et al., 2014). Together, these results suggest that *miRNA393*-mediated reduction in *TIR1/AFB2* receptor levels, and the subsequent down-regulation of auxin-signalling, is a key mechanism that allows the plant to modulate root system architecture in response to salt stress. In addition, transgenic Arabidopsis over-expressing a *miRNA393*-resistant *TIR1* gene (*mTIR1*) had increased germination, delayed senescence, lower water loss, and reduced inhibition of root elongation compared with the wild-type under saline conditions (Chen et al., 2015).

There is also some evidence that salt stress effects auxin signalling through the modulation of Aux/IAA repressor levels. In germinating Arabidopsis seeds, salinity was found to induce the binding of the NAC transcription factor, NTM2, to the promoter of the Aux/IAA co-receptor family gene, *IAA30*, resulting in the transcriptional activation thereof (Park et al., 2011). Increased expression of *IAA30* is proposed to alter downstream auxin transcriptional responses that ultimately lead to negative regulation of seedling germination under high salinity. Additionally, Arabidopsis *ntm2-1* mutants were found to display enhanced germination compared with the wild-type under salt stress conditions, but this increased resistance was abolished in *ntm2-1* mutants in which *IAA30* was overexpressed (Park et al., 2011). The authors proposed that NTM2, and its modulation of *IAA30* expression, serves as a molecular link integrating salt stress and auxin signalling during seedling germination.

These studies provide some evidence that auxin signalling cascades are affected during salt stress; however, much more work is required to understand the extent of cross talk between auxin signalling and the salt stress response. A question remains whether the activation of auxin-responsive processes is only to modulate growth in response to salt, or whether there could be some other advantage in terms of salinity tolerance. One such auxin-responsive process that could have dual effects in terms of salt-tolerance is the activation of the plasma membrane H⁺-ATPase.

1.10. The plasma membrane H⁺-ATPase and its role in auxin-mediated expansion growth

The plant plasma membrane (PM) H⁺-ATPase utilises the energy released from the hydrolysis of ATP to pump protons across the plasma membrane from the cytoplasm into the apoplast (Palmgren et al., 1991). This generates an electrochemical gradient across the plasma membrane, and, due to the fact that almost all other PM transporters function using the proton motive force created by such electrochemical gradients, it is widely accepted that the PM H⁺-ATPase is the power-house for plant growth (Sondergaard, Schulz & Palmgren, 2004).

1.10.1. Structure of the PM H⁺-ATPase

Plant PM H⁺-ATPases are members of a larger family of membrane proteins known as P-type ATPases (Palmgren, 2001), which are named as such as they all exploit a phosphorylated reaction cycle intermediate of ATP hydrolysis (Pedersen & Carafoli, 1987). These P-type ATPases generate plasma membrane potential and energise secondary transport systems in all eukaryotes (Pederson et al., 2007). The plant PM H⁺-ATPase is further classified as a member of the type III P-type ATPase subfamily (Axelsen & Palmgren, 1998), whereas other P-type ATPases such as the Na⁺ and K⁺-ATPases found in animals (Skou & Esmann, 1992) are classified as type II. It must be noted here that referring to the plant PM H⁺-ATPase in the singular is somewhat of a misnomer, as there are multiple PM H⁺-ATPase genes present in plants, and different cell types can express different isoforms (Falhof et al., 2016). However, while *Arabidopsis* has 11 PM H⁺-ATPase genes, there are two predominant isoforms that are very well characterised, namely Autoinhibited H⁺-ATPase isoform 1 and 2 (AHA1/AHA2) (Harper, Surowy & Sussman, 1989; Harper et al., 1990).

The crystal structure of an active form of AHA2, lacking the C-terminal autoinhibitory domain, has been determined in complex with the non-hydrolysable ATP analogue, adenosine 5'-(β,γ-methylene)-triphosphate (AMPPCP) (Pederson et al., 2007). The AHA2 protein is 948 residues long (Falhof et al., 2016). It has a transmembrane domain consisting of 10 hydrophobic α-helices, three cytosolic domains designated N (nucleotide binding - residues 338-448), P (phosphorylation – residues 308-337 and 489-625), and A (actuator – residues 12-57 and 129-233) (Pederson et al., 2007), as well as a C-terminal domain consisting of approximately 100 amino acids referred to as the R domain (Palmgren et al., 1991). Refer to Figure 1.3 for a two-dimensional representation of the structure of AHA2. The catalytic activity of the protein is attributed to the three cytosolic domains, where the AMPPCP (and presumably ATP) substrate is bound with the adenosine portion at the N domain, and the triphosphate group oriented towards the P domain, thereby assembling the catalytic site at which the Asp-329 residue within

the P domain is phosphorylated, and subsequently dephosphorylated by the A domain, once per pumping cycle (Pederson et al., 2007). The C-terminal R domain, also known as the auto-inhibitory domain, is thought to inhibit PM H⁺-ATPase function, as removal thereof results in the activation of H⁺ pumping (Palmgren et al., 1991). The auto-inhibitory domain is thought to work in concert with a short hydrophilic N-terminal domain to interfere with the catalytic domains and prevent pump activity (Ekberg et al., 2010). Finally, the transmembrane domain forms a cavity that is lined by conserved hydrophilic residues, and is thought to be large enough to accommodate 12 water molecules, as well as containing a conserved Asp-684 residue that is thought to be the likely candidate for proton binding – this structural arrangement allows for proton transport against a high membrane potential (Pederson et al., 2007).

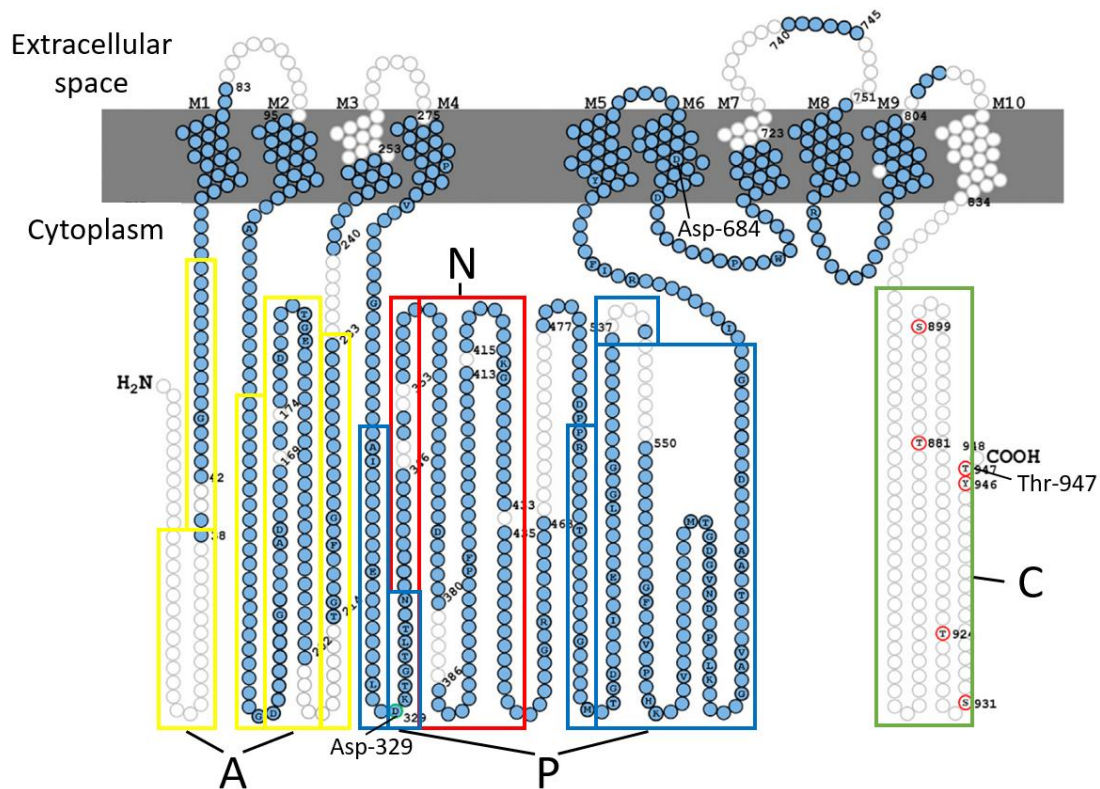


Figure 1.3: Overall structure of the plasma membrane H⁺-ATPase isoform AHA2

A 2D model of Autoinhibited PM H⁺-ATPase 2 (AHA2) adapted from Falhof et al. (2016). The protein consists of ten transmembrane domains (M1-10), the actuator domain (A), phosphorylation domain (P), nucleotide binding domain (N), and C-terminal autoinhibitory domain (C). The Asp-329 residue present in the P domain which is phosphorylated within the catalytic site is shown, as well as the Asp-684 residue that is thought to be crucial for proton binding during transmembrane proton transport, and the Thr-947 residue in the C-terminal autoinhibitory domain, whose phosphorylation allows for the binding of 14-3-3 proteins which stabilise the PM H⁺-ATPase in an active conformation.

1.10.2. Regulation of PM H⁺-ATPase activity occurs at the post-translational level

The PM H⁺-ATPase exists in either an active state, where there is tight coupling between ATP hydrolysis and proton pumping, or an autoinhibited state where there is only loose coupling. Importantly, the alternation between these two states is determined by the post-translational modification (PTM) status of the cytosolic C-terminal auto-inhibitory domain as well as the short N-terminal tail (Falhof et al., 2016). Phosphorylation is the PTM that has been found to play the largest role in the regulation of the PM H⁺-ATPase, with several phosphorylation sites being characterised *in planta* (Rudashevskaya et al., 2012). The most studied site is the penultimate threonine residue (Thr-947 in AHA2 and Thr-948 in AHA1) in the C-terminal auto-inhibitory domain. Phosphorylation of this Thr-947 residue allows for the direct binding of 14-3-3 proteins to the three terminal residues of the C-terminal domain of AHA2 (Tyr-946, Thr-947, and Val-948) (Fuglsang et al., 1999). The binding of 14-3-3 proteins stabilises the active, upregulated state of the PM H⁺-ATPase by displacing the C-terminal R domain, thus preventing its auto-inhibitory effect on the three catalytic domains described previously (Jahn et al., 1997). The 14-3-3 proteins, are abundant cytoplasmic proteins that are known to play a role in signal transduction in eukaryotic cells by tightly binding to other proteins, thus stabilising them in either active or inactive conformations (Fertl, 2004). Very recently, the transmembrane kinase (TMK) has been identified as the protein kinase responsible for the phosphorylation of the penultimate threonine residue during auxin-induced H⁺-ATPase activation (Lin et al., 2021). TMK1 was found to directly interact with the PM H⁺-ATPase, and this interaction was rapidly induced by auxin. Once interacting, TMK1 was shown to directly phosphorylate the penultimate Thr-948 residue of AHA1, resulting in the rapid activation of the PM H⁺-ATPase. Dephosphorylation of this penultimate residue, and thereby inhibition of PM H⁺-ATPase activity, is achieved by PP2C.D family protein phosphatases, which have been shown to directly interact with and dephosphorylate the Thr-947 residue of AHA2 (Spartz et al., 2014). Specifically, the negative regulation of PM H⁺-ATPase activity in this manner during growth regulation has been attributed to the three plasma membrane-localised PP2C.D family members, PP2C.D2, PP2C.D5, and PP2C.D6, with the remaining six family members functioning in other subcellular locations (Ren et al., 2018). The larger family of type 2C protein phosphatases, of which PP2C.D is a sub-family, has also previously been implicated in the regulation of PM H⁺-ATPase activity via the de-phosphorylation of the penultimate threonine residue during stomatal opening in response to blue light (Hayashi et al., 2010). Aside from regulation at the penultimate threonine residue, phosphorylation of the Thr-881 residue in the C-terminal autoinhibitory domain of AHA2 of Arabidopsis (Niitylä et al., 2007; Fuglsang

et al., 2014), as well as phosphorylation of the corresponding Thr-889 residue in the plasma membrane H⁺-ATPase isoform 2 (PMA2) of Tobacco (*Nicotiana tabacum*) (Piette et al., 2011), has been shown to activate the PM H⁺-ATPase in a 14-3-3 protein-independent manner. In Arabidopsis, the Thr-881 residue is phosphorylated by the intracellular domain of the receptor kinase, plant peptide containing sulphated tyrosine receptor (PSY1R), following binding of the growth hormone, plant peptide containing sulphated tyrosine (PSY1) (Fuglsang et al., 2014). In contrast, the PM H⁺-ATPase has also been found to be inactivated by the phosphorylation of certain residues in the C-terminal auto-inhibitory domain, such as Ser-899 (Haruta et al., 2014). This inhibition occurs as a result of the binding of the peptide hormone, rapid alkalization factor (RALF), to the receptor-like kinase, FERONIA, which activates a signalling cascade that ultimately results in the phosphorylation of Ser-899 by an unidentified kinase. This FERONIA-induced inhibition of the PM H⁺-ATPase plays a role in the suppression of cell elongation in the primary root. While it is clear that phosphorylation plays a crucial role in the regulation of the PM H⁺-ATPase, more work needs to be done to elucidate all of the kinases involved, as well as their respective signalling pathways. Furthermore, it is not clear how the different phosphorylation sites interact, and under what conditions this could occur. It must also be noted that the studies of PM H⁺-ATPase PTM have centred around the identification of phosphorylation sites, but it is very likely that other PTMs may also play a role in its regulation.

1.10.3. Auxin-induced activation of the PM H⁺-ATPase

1.10.3.1. Evidence for PM H⁺-ATPase regulation by IAA

It was widely accepted that the activation of the PM H⁺-ATPase pump by the classical growth hormone, auxin (IAA), underpinned the acid growth theory (Rayle & Cleland, 1992), however, the specific molecular mechanism whereby IAA activates the PM H⁺-ATPase has only recently been revealed. Multiple studies have shown that the exogenous application of IAA affects the phosphorylation status, and thereby activity of the PM H⁺-ATPase. Phosphoproteomic studies, involving time course experiments in which IAA was applied to Arabidopsis cell cultures, revealed that the presence of IAA resulted in an increased phosphorylation of the penultimate Thr-948 and Thr-947 residue in AHA1 and 2, respectively (Chen, Hoehenwarter & Weckwerth, 2010). In addition to this, exogenous application of IAA to auxin-depleted Arabidopsis hypocotyls (endogenous auxin induces early-phase hypocotyl elongation) induced phosphorylation of the penultimate threonine residue of the PM H⁺-ATPase, increased H⁺-ATPase activity, and stimulated hypocotyl elongation (Takahashi, Hayashi & Kinoshita, 2012).

To deplete endogenous auxin, sections were excised from etiolated hypocotyls, and grown for several hours in complete darkness, during which time all hypocotyl elongation ceased, and the PM H⁺-ATPase was completely dephosphorylated. With the IAA application, the changes in Thr-947 phosphorylation as well as the IAA-induced elongation were found to be concentration dependant. Furthermore, IAA application did not result in a change in *AHA1* and *AHA2* transcription, meaning that the hypocotyl elongation induced was not due to increased levels of the PM H⁺-ATPase (Takahashi, Hayashi & Kinoshita, 2012). Furthermore, the IAA-induced PM H⁺-ATPase phosphorylation was observed in a *tir1-1 afb2-3* double mutant, which is severely defective in auxin-mediated transcriptional regulation, as well as in the presence of α-(phenylethyl-2-one)-IAA, the auxin antagonist specific for the nuclear auxin receptor TIR1/AFBs (Takahashi, Hayashi & Kinoshita, 2012). Collectively, this evidence strongly suggests that auxin's ability to activate the PM H⁺-ATPase occurs at the post-translational, rather than the transcriptional level.

1.10.3.2. The role of SAUR 19-24 sub-family genes

Central to the molecular mechanism whereby auxin mediates changes in PM H⁺-ATPase activity is the *SMALL AUXIN UP-RNA (SAUR)* 19-24 sub-family of genes. The Arabidopsis genome contains 79 *SAUR* genes, all of which lack introns, and have auxin response elements in their promoters (Ren & Gray, 2015). In Arabidopsis, SAUR19 has been shown to promote cell expansion, and over-expression thereof results in a number of auxin-related phenotypes, including increased leaf size, increased vegetative biomass, hypocotyl elongation, and altered tropic responses (Spartz et al., 2012). Furthermore, amiRNA knockdown of *SAUR19* resulted in plants with reduced leaf size and hypocotyl length, although it must be noted that the amiRNA construct targeted several *SAUR* genes along with *SAUR19* (Spartz et al., 2012). *SAUR19* plays a crucial role in increasing the activity of the PM H⁺-ATPase, as it has been shown to bind to, and inhibit the protein phosphatases, PP2C.D2, PP2C.D5, and PP2C.D6, which in turn are known to inhibit PM H⁺-ATPase activity by the specific de-phosphorylation of the Thr-947 residue in its C-terminal domain (Spartz et al., 2014; Ren et al., 2018). Furthermore, *SAUR19*-overexpressing plants have been shown to display both an increase in *AHA2* Thr-947 phosphorylation, and increased levels of PM H⁺-ATPase activity compared with wild-type controls. Importantly, *SAUR19*'s role in H⁺-ATPase activation is not limited to Arabidopsis, but has also been demonstrated in tomatoes, indicating that this system is likely a universal plant mechanism (Spartz et al., 2017). Here, transgenic tomatoes overexpressing *AtSAUR19* displayed the same phenotypes observed in Arabidopsis, and *SAUR* proteins were

found to also inhibit tomato PP2C.D family phosphatases in *in vitro* phosphatase assays using yeast-expressed AHA2 that was phosphorylated on Thr-947 (Spartz et al., 2017). Further evidence for the role of SAURs in auxin-mediated cell expansion are the findings that constitutive GFP-AtSAUR19 expression increased the mechanical extensibility of hypocotyls thereby bypassing the normal requirement for auxin to stimulate elongation growth, and that auxin-induced *SAUR* expression was closely correlated with auxin-dependent elongation growth (Spartz et al., 2017). Collectively, there is strong evidence for a molecular mechanism, whereby auxin up-regulates *SAUR19* expression, thereby inhibiting PP2C.D protein phosphatases, resulting in an increase in PM H^+ -ATPase activity.

1.10.3.3. Downstream effect of auxin-induced PM H^+ -ATPase activation

The auxin-induced activation of the PM H^+ -ATPase causes the pumping of protons from the cytoplasm across the PM and into the apoplast, causing the acidification thereof. This has two main effects. Firstly, by a still unknown mechanism, it causes the activation of α -expansins, which act non-enzymatically to loosen cell walls (Cosgrove, 2016) (expansins will be discussed in greater depth later on in section 1.10). Secondly, the electrochemical gradient generated across the plasma membrane provides the proton motive force to drive the transport of solutes by many PM transport proteins (Sondergaard, Schulz & Palmgren, 2004). An example of this is the uptake of K^+ ions, which has been shown to be absolutely necessary to drive auxin-induced coleoptile elongation in maize (Claussen et al., 1997). This transport is mediated by the inwardly rectifying K^+ channel protein, *Zea mays* K^+ channel 1 (ZMK1), expression of which has also been shown to be induced by auxin (Philippar et al., 1999). Subsequently, the inwardly rectifying K^+ channel protein, K^+ transporter of *Arabidopsis thaliana* (KAT1), was identified as playing a similar role in the regulation of auxin-induced elongation growth in *Arabidopsis* (Philippar et al., 2004). Both ZMK1 and KAT1 facilitate the inward transport of K^+ across the plasma membrane from the extracellular space into the cytosol. The increased K^+ uptake facilitated by these channels increases the osmotic potential of the cell, which in turn stimulates the uptake of water, and a subsequent increase in turgor pressure (Philippar et al., 2004). This increase in turgor pressure, combined with the cell wall loosening activity of expansins is what drives cell expansion and ultimately leads to auxin-mediated expansion growth (Majda & Robert, 2018).

1.10.4. Role of the PM H^+ -ATPase in regulation of PM ion transport during salt stress

The ability of the PM H^+ -ATPase to energise the plasma membrane, due to the electrochemical gradient it generates via proton extrusion, places it in a central role in many plant physiological

processes (Falhof et al., 2016). Aside from powering the uptake of K^+ by inwardly rectifying channels during auxin-induced elongation growth discussed above, the electrochemical gradient generated by the PM H^+ -ATPase essentially provides the proton motive force for all transport across the plasma membrane. Included, is the powering of K^+ and Na^+ transport via H^+ antiporters (SOS1, NHXs, CHXs) and H^+ symporters (HAKs). As plants lack a primary active transport system to export Na^+ , they are fully reliant on the PM H^+ -ATPase-energised SOS1 Na^+/H^+ -antiporter to extrude Na^+ during salt stress (Falhof et al., 2016). The maintenance of plasma membrane potential is also crucial for controlling K^+ homeostasis in the root during salinity stress, as the influx of Na^+ that occurs depolarises the plasma membrane past the equilibrium potential for K^+ (E_K), resulting in K^+ efflux via KORs (Pottosin & Dobrovinskaya, 2014). Increased activity of the PM H^+ -ATPase is required to restore membrane polarity to prevent this depolarisation-induced K^+ leakage (Falhof et al., 2016; Zhao et al., 2020). Additionally, increased PM H^+ -ATPase activity is crucial in providing the proton motive force to power root K^+ uptake via HAK K^+/H^+ symporters during salinity stress (Assaha et al., 2017).

Unsurprisingly then, the PM H^+ -ATPase, and its regulation at both the transcriptional and post-translational level, has been shown to play a role in the response of plants to salt stress. It has been found that salt stress can cause increased expression of the PM H^+ -ATPase; increased transcript accumulation has been observed in the roots and leaves of the salt-tolerant saltbush, *Atriplex nummularia*, as well as in tobacco (*Nicotiana tabacum*) (Morsomme & Boutry, 2000), and increased *CsAHA10* expression has been observed in cucumber (*Cucumis sativus*) seedlings in response to salt stress (Janicka-Russak et al., 2013). Furthermore, the salt-tolerant poplar variety, *Populus euphratica*, has been found to display increased PM H^+ -ATPase expression in response to salt stress compared with the salt-sensitive, *Populus popularis* (Ding et al., 2010). A tobacco (*Nicotiana tabacum*) mutant with a constitutively active PM H^+ -ATPase had increased salinity tolerance compared with the wild-type (Gévaudant et al., 2007). This Δ PMA4 mutant, which lacks the auto-inhibitory domain of the PM H^+ -ATPase4, displayed improved germination, increased biomass yield, and improved root growth compared with the wild-type under salt stress conditions. Likewise, transgenic Arabidopsis overexpressing *PeHA1*, from the salt-tolerant *Populus euphratica*, have been found to display improved germination, root length, and greater biomass yield compared with the wild-type under salt stress conditions (Wang, Wang, et al., 2013). Additionally, ectopic expression of *PeHA1* resulted in an increased capacity to control ion homeostasis via enhanced hydrogen peroxide signalling in Arabidopsis under salt stress (Wang, Wang, et al., 2013).

There is also evidence that regulation of the PM H⁺-ATPase occurs at the post-translational level in response to salt stress. The enhanced salt tolerance displayed by two halophyte species, *Atriplex lentiformis* and *Chenopodium quinoa*, compared with *Arabidopsis*, was found not to be linked with a change in expression of PM H⁺-ATPase genes but rather was speculated to be due to the ability to rapidly up-regulate PM H⁺-ATPase activity through PTM in response to salt stress (Bose et al., 2014). The halophyte species maintained greater membrane potential, while displaying much lower *AHA* expression than *Arabidopsis*, under both control and salt-stress. This suggests that the higher PM H⁺-ATPase activity in the halophytes under salt stress is not linked to changes in transcription, but rather must occur at the post-translational level. However, this study did not identify the precise PTM involved, or the effector protein responsible. In cucumber (*Cucumis sativus*) roots, salt stress has been shown to induce the phosphorylation of the PM H⁺-ATPase, as well as cause increased expression of 14-3-3 proteins, and increased binding of 14-3-3 proteins to the PM H⁺-ATPase (Janicka-Russak et al., 2013). Again, this study did not identify the specific residues that were phosphorylated, but rather a general increase in the phosphorylation of the threonine residues of the PM H⁺-ATPase was detected using anti-phosphothreonine antibodies. With there being evidence for an increase in transcription, activity, and regulation of PTM, it is clear that the PM H⁺-ATPase plays a key role in plant salt tolerance.

The PM H⁺-ATPase thus plays a central role in energising ion transport to affect Na⁺ and K⁺ homeostasis, as well as in the activation of expansin proteins during auxin-induced expansion growth. What is still unclear, however, is how altered ion homeostasis and expansin-mediated expansion growth contribute towards altered growth in response to salt stress, and to what extent.

1.11. The role of expansins during plant growth

1.11.1. Expansins are central to auxin-mediated expansion growth

The activation of cell wall loosening proteins, due to apoplast acidification following auxin-induced activation of the plasma membrane H⁺-ATPase, is a key facet of the acid growth theory. These proteins have been identified as α -expansins, which mediate the acid-induced extension of cell walls without causing any mechanical weakening thereof (Cosgrove, 2016). Expansins were first discovered as proteins that promoted the acid-induced extension of cucumber cell walls (*Cucumis sativus*) (McQueen-Mason & Cosgrove, 1994). Although our understanding of expansins has since improved, the precise mechanism by which apoplast acidification activates α -expansins remains unknown.

1.11.2. Expansin classification

Plant expansins are classified into two major families by sequence-based phylogeny: the aforementioned α -expansins (EXPA), which mediate acid-induced cell wall loosening, and β -expansins (EXPB) (Cosgrove, 2015). While the activity of EXPB proteins has mainly been characterised for a single clade that is highly expressed in grass pollens (known as pollen β -expansins, or ‘group I grass pollen allergens’) which are known to facilitate pollen tube invasion of the stigma by dissolving the middle lamella, there are several EXPBs that also mediate cell wall-loosening (Cosgrove, 2015, 2016). Furthermore, many EXPBs remain to be studied. Plant expansins also include two minor families, namely expansin-like A and expansin-like B (EXLA and EXLB), however the function of these proteins is largely unknown (Cosgrove, 2015). In addition to plant expansins, several plant pathogenic bacteria have been found to encode bacterial expansins, the most prominent of which, is the *Bacillus subtilis* protein BsEXLX1, which was found to be homologous in structure to pollen β -expansins, and was found to be able to promote plant cell wall extension, which is suggested to enable *B. subtilis* to colonise maize roots (Kerff et al., 2008).

1.11.3. Expansin structure and mechanism of action

The early dogma for expansin activity, was that in the growing cell wall, expansins act non-enzymatically to disrupt the hydrogen bonds between cellulose microfibrils and the polysaccharide matrix, causing stress relaxation (cell wall ‘loosening’), which is followed by wall surface expansion and the subsequent enlargement of the cell (McQueen-Mason & Cosgrove, 1994). This was supported by the findings that expansins did not hydrolyse any hemicelluloses or pectins in the cucumber (*Cucumis sativis*) cell wall, or cause any weakening thereof associated with hydrolase activity. Furthermore, expansins were found to bind crystalline cellulose at sites consistent with their proposed functioning (McQueen-Mason & Cosgrove, 1995). These early studies involved α -expansins, but it has subsequently been found that all plant and bacterial expansins examined to date lack detectable lytic activity (Cosgrove, 2015). Canonical plant expansins consist of two domains: An N-terminal six-stranded double-psi beta-barrel (DPBB) domain, and a C-terminal β -sandwich containing domain (classified as a family-63 carbohydrate binding molecule (CBM63)), which are spanned by an open surface for polysaccharide binding (Georgelis, Yennawar and Cosgrove, 2012). Studies to determine the cell wall binding capabilities of expansins in complex with various cell wall polysaccharide components have largely utilised BsEXLX1, as it has proven easier to purify at concentrations high enough to perform the requisite crystallography and nuclear magnetic resonance (NMR)

analyses (Georgelis et al., 2011; Georgelis, Yennawar & Cosgrove, 2012; Wang, Bum, et al., 2013; Cosgrove, 2015). The cell wall loosening activity of BsEXLX1 has been shown to coincide with selective binding to specific, uncommon, regions of cellulose, that display slightly altered conformation and are enriched with xyloglucans (XyG), as well as having a lower pectin content (Wang et al., 2013). Additionally, the extensibility of the cell wall is proposed to be controlled at specific, limited sites of direct cellulose microfibril contact that are rich in XyG, that are referred to as ‘mechanical hotspots’ (Park & Cosgrove, 2015). The dogma on the mechanism of action of expansins has thus subsequently been refined: In the current cell wall model, α -expansins are thought to bind to the specific XyG ‘mechanical hotspots’ where the cellulose microfibrils are in direct contact (Park & Cosgrove, 2015), where they act non-enzymatically to initiate cell wall loosening. On the other hand, β -expansins are thought to employ other unique mechanisms of action (Tabuchi, Li & Cosgrove, 2011), although there is a larger degree of uncertainty as to how the majority of them act (Cosgrove, 2016), so they will not be discussed further. It must be noted that the precise mechanism whereby expansins loosen the cell wall remains unresolved, and the current understanding of the XyG-rich ‘mechanical hotspots’ between cellulose microfibrils, and how expansins might interact with them, is extremely limited (Cosgrove, 2022).

1.11.4. Role of expansins during plant development

Due to the ubiquitous nature of expansin genes present in plant genomes, it stands to reason that different expansin genes are expressed throughout the varying stages of morphological development, as well as in a cell/tissue-specific manner, and that they modulate plant growth in varying ways.

1.11.4.1. Role in leaf development

Many expansins have been shown to play a role in leaf development. A study assessing the expression pattern of α -expansin genes in *Arabidopsis thaliana* (AtEXPA) throughout leaf development, found that 12 of the 26 annotated AtEXPA genes were expressed at some stage of development. In the earliest stage of development studied (day 12-14), seven AtEXPA genes were expressed. Expression of these genes was maintained with further AtEXPA genes being expressed later on. Notably, the period when the greatest number of ten AtEXPA genes was expressed (days 20-28), correlated with the period of maximum leaf growth rate (Goh et al., 2012). As expected, manipulation of endogenous expansin expression influences leaf growth and development. In *Arabidopsis*, anti-sense RNA knockdown of AtEXPA10, which is maximally expressed at the base of the pedicel in growing leaves, resulted in plants with smaller

rosettes due to shortening of the petiole, as well as a curling of the leaf blade (Cho & Cosgrove, 2000). The petiole cells of these *atexpa10* antisense plants were smaller than controls, and the cell walls were less extensible *in vitro*. In contrast, plants in which *AtEXPA10* was overexpressed displayed larger leaf blades, slightly longer petioles, and larger petiole cell size than the control (Cho & Cosgrove, 2000). Together these findings suggest a role for *AtEXPA10* in control of both leaf/organ size and morphology. Similarly, the simultaneous knockdown of *AtEXPA1*, 5, 3, and 10 in Arabidopsis (all of which are expressed during the early to mid-stage of leaf development) via an inducible artificial microRNA (amiRNA) system, resulted in greatly reduced plant growth, as well as a decrease in the number of vegetative leaves compared with the control (Goh et al., 2012). While the overall rate of rosette expansion, as well as the overall rate of leaf expansion was lower in these *amiREXPA10,1,5,3* knockdown lines, different regions of the leaf displayed varying degrees of growth repression; for instance, the restriction of petiole extension was more pronounced than the reduction in lamina expansion, suggesting that these two tissue types display a different sensitivity to manipulation of the same expansin gene expression – it must, however, be noted that without actual measurements of expansin activity in the different leaf regions, it is perhaps unclear whether the changes in growth repression can be attributed to varied sensitivities to the expression of the different expansins, or rather to differences in the localisation of their activity. Nonetheless, when induction of amiRNA knockdown was performed at a later stage of plant development, in 12-day old plants, only leaves in a particular stage of development displayed growth repression when measured eight days later. At 12 days, leaves 6 and 7 have just initiated, and it was observed that only these leaves displayed a decreased size, whereas the developmentally older (1-5) and younger (8) leaves did not. Taken together, these findings suggest that the growth response to alterations in expansin expression is also dependent on the developmental stage and the specific tissue of the plant (Sloan et al., 2009).

1.11.4.2. Role in root development

Both α - and β -expansins are known to play a role in root development. Expression of *AtEXPA7* and *AtEXPA18* have been shown, via promoter reporter analyses, to be specific to root hair cells in Arabidopsis, and to be tightly linked to the initiation of root hairs, as well as their subsequent elongation (Cho, 2002). Specifically, RNAi-silencing of *AtEXPA7* resulted in the shortening of root hairs compared with controls (Lin, Choi & Cho, 2011). Interestingly, the reduction in root hair length in the various RNAi transformed lines (25-48%) was seemingly correlated with the reduction in *AtEXPA7* transcript levels observed in these lines (25-48%).

Expansins have also been shown to be required for root hair elongation in rice (*Oryza sativa* L.) (Zhiming et al., 2011). A short root hair mutant rice was found to have a point mutation in a gene encoding OsEXPA17, and subsequent promoter::reporter analyses showed that expression of OsEXPA17 was exclusive to root hair cells. OsEXPA17 RNAi transformed lines displayed a very similar reduction in root hair length (3-4 mm) to the mutant, confirming its role in root hair development. Importantly, root hair elongation could be restored in the OsEXPA17 mutant by complementation with the Arabidopsis AtEXPA7, as well as an additional rice expansin, OsEXPA30 (Zhiming et al., 2011). OsEXPA17, OsEXPA30, AtEXPA7, as well as AtEXPA18, all belong to the EXPA Clade-X (Sampedro et al., 2005). This, together with the fact that they can functionally complement one another, indicates that there is functional conservation of these root hair EXPAs in both monocots and dicots (Zhiming et al., 2011).

Other than the root hair-specific expansins, there is a group of α -expansins that has been shown to play a role in the emergence of LR primordia in Arabidopsis, namely AtEXPA4, 14, and 17. Promoter reporter analyses using *GUS* fused to the AtEXPA14 promoter revealed *GUS* expression in the LR primordium and overlying endodermal and cortical tissues upon induction (Lee et al., 2012). Furthermore, overexpression of AtEXPA14 stimulated LR emergence, whereas AtEXPA14 loss-of-function plants displayed reduced LR formation. Likewise, AtEXPA17 expression is detectable in the LR primordium, whereas AtEXPA4 expression is detectable in the cortical cell layers overlying the primordium (Lee & Kim, 2013). AtEXPA17 RNAi transformed lines displayed a significant decrease in LR emergence when compared with the wild-type, whereas overexpression of AtEXPA17 resulted in the opposite effect, with increased LR density observed. Additionally, the AtEXPA17 RNAi plants displayed a delay in LR emergence compared with the wild-type during gravitropic induction experiments. Together, these results suggest a key role for AtEXPA4, 14, and 17 in the enhancement of cell separation or expansion to promote LR emergence (Lee & Kim, 2013). Recently, AtEXPA1 has been shown to be crucial for controlling the asymmetric division of pericycle cells that is required for marking where auxin-driven LR initiation will occur (Ramakrishna et al., 2019). LR initiation requires co-ordination of asymmetric cell division in pericycle cells, involving the auxin-induced asymmetric swelling of these cells. However, in *expa1* mutants, the whole length of the pericycle cells was swollen, resulting in defects in the first asymmetric pericycle cell divisions, thus affecting the onset of LR formation (Ramakrishna et al., 2019).

There is also evidence that AtEXPA4 is involved in primary root (PR) elongation in Arabidopsis. Promoter reporter analyses using *GUS* fused to the AtEXPA4 promoter revealed

expression in the root tip of three-day-old seedlings which, by seven days had spread throughout the root (Liu et al., 2021). Furthermore, *atexpa4* knockout mutant seedlings, and *EXPA4*-overexpressing seedlings displayed reduced and increased PR length compared with the WT, respectively. AtEXPA1, 10, 14, and 15 have also been shown to contribute to the control of root cell differentiation, by driving the elongation of meristematic cells in the transition zone of the root (Pacifici et al., 2018). The contribution of AtEXPA1 is likely greater, as *expa1* mutants display larger meristems, and a longer primary root than the wild-type, whereas these phenotypes were not observed in single mutants of the other three expansins, but only in double mutants (*expa10expa14*, *expa10expa15*, *expa14expa15*), suggesting that they act redundantly. From the studies mentioned above, it is clear that individual expansins have both overlapping, and specific roles in root development.

1.11.5. Role of expansins in the plant response to salt stress

Several studies have shown that transgenic manipulation of *EXPA* expression has an effect on salinity tolerance in Arabidopsis; overexpression of *RhEXPA4*, a rose α -expansin gene, resulted in increased germination rate, greater LR development, as well as higher chlorophyll a content, when compared with the wild-type under salinity stress conditions (Lü et al., 2013). Additionally, overexpression of *AtEXPA2* has also been shown to improve germination when compared with the wild-type under salinity stress conditions (Yan et al., 2014). *AtEXPA2* was shown, via RT-qPCR analyses, to be mainly expressed in the seed, and subsequent *GUS* assays performed on seeds following water imbibition displayed promoter activity throughout the seed and in the emerging radicle, suggesting a role in germination, and possibly in root function. Furthermore, *atexpa2* mutants displayed decreased germination compared with the wild-type under salt stress (Yan et al., 2014). Collectively, this suggests that *AtEXPA2* plays a key role in controlling germination under salt stress conditions. Conversely, expansin overexpression has also been shown to be detrimental to plant salt tolerance. Transgenic Arabidopsis lines overexpressing *AtEXPA3* and *AtEXPB1* have both been found to display a lethal phenotype in response to salt stress, despite growing larger than the wild-type under control conditions (Kwon et al., 2008). This suggests that the increased cell wall remodelling action by different expansins has a varying effect on the salt tolerance of the plant.

Expansins have also been found to play a role in the response of agriculturally relevant plants such as maize and rice to salinity stress. A comparison between the salt-sensitive maize (*Zea mays*) cultivar, Lector, and the salt-resistant cultivar, SR03, under salt stress, showed that the salt-sensitive cultivar displayed a 50% reduction in β -expansin protein expression which was

found to be positively correlated with a 65% reduction in shoot growth, whereas the salt-resistant cultivar maintained shoot growth, and maintained β -expansin protein levels when compared with the control (Geilfus, Zörb & Mühling, 2010). A study of the growth-related changes in the epidermal cells of the leaves of the two cultivars under salt stress revealed that the salt-resistant SR03 cultivar had increased epidermal cell wall extensibility, with a concomitant reduction in epidermal apoplastic pH into the range associated with acid growth, whereas the sensitive Lector cultivar displayed stiffer epidermal cell walls, with no change in apoplastic pH (Zörb et al., 2015). Ultimately, *ZmEXPB6* was identified as the specific β -expansin responsible for the difference in salt-tolerance of the Lector and SR03 maize cultivars, as its expression was completely lacking in growth inhibited salt-stressed leaves of the sensitive Lector cultivar, and exogenous application of purified *ZmEXPB6* protein to these leaves that lack native *ZmEXPB6* expression under salt stress, was found to partially restore leaf growth (Geilfus et al., 2015).

In rice (*Oryza sativa* L.), the root-specific α -expansin *OsEXPA3* has been found to be highly induced in response to salt stress (Qiu et al., 2014). RNAi silencing of *OsEXPA3* resulted in a severe impairment in growth of the primary root, as well as a reduction in LR density, plant height, and leaf number. While these effects were not tested under salt stress conditions, the fact that *OsEXPA3* expression is greatly up-regulated in response to salt stress would suggest that it potentially plays a key role in modulating rice plant growth in response to salt stress. Additionally, overexpression of *OsEXPA7* has also been shown to greatly enhance the salinity tolerance of transgenic rice (Jadamba et al., 2020). Following salt-treatment, *OsEXPA7* overexpressing plants displayed significantly increased root and shoot length, increased tiller number, improved chlorophyll content, and decreased leaf damage compared with the wild-type. Additionally, the *OsEXPA7* overexpressing plants were found to have improved ion homeostasis compared to the wild-type under saline conditions, with decreased Na^+ accumulation, significantly improved K^+ retention, and as a result, significantly lower Na^+/K^+ ratios in both the root and the shoot. Overexpression of *OsEXPA7* also resulted in leaf and root morphological changes under control conditions, with increased leaf cell size, number, and overall biomass, as well as elongated root metaxylem cells compared with the wild-type. RT-qPCR analysis, as well as *OsEXPA7::GUS* promoter reporter assays revealed that *OsEXPA7* was highly expressed in the shoot apical meristem as well as in the vascular tissues of both the root and the shoot. Clearly then, overexpression of this particular expansin had a largescale effect on cell wall-loosening, in both root and shoot vascular tissues, which conferred not only improved growth, but also improved ion homeostasis under saline conditions.

It is important to note, however, that most of the evidence to date implicating expansins in the plant response to salt stress is based off observational studies correlating expansin gene and protein expression with improved salt stress phenotypes, as opposed to measuring actual changes in expansin activity and the accompanying modifications of cell wall architecture in response to salt stress.

1.12. Conclusion and aims of this study

Our research group has shown that the salt-specific up-regulation of the auxin biosynthesis gene, *NIT2*, as well as a large number of genes assigned with the GO term ‘response to auxin stimulus’, occurred in salt-stressed *Arabidopsis* seedlings, concomitant with elevated IAA, and IAA biosynthetic intermediates (Cackett et al., 2022). Additionally, we have shown that a *NIT2*-overexpressing line has improved salt-tolerance compared with the wild-type. Included in the salt-specific up-regulated genes were the SAUR19 sub-family, which are known to play a role in the auxin-induced activation of the PM H⁺-ATPase. Additionally, this list included two EXPA genes (*EXPA5* and *EXPA11*), which play a role in mediating cell wall loosening to allow for expansion growth in response to apoplast acidification that occurs following auxin-induced PM H⁺-ATPase activation. Expression of *EXPA11* was also found to be massively increased in the *NIT2*-overexpressing line compared with the wild-type, with a further up-regulation in response to salt (Cackett, 2019). Based on these data, we propose a model whereby normal auxin-mediated acid growth modulation is co-opted in response to salinity (see Figure 1.4 below): Salt stress induces the expression of *NIT2*, leading to an increase in IAA biosynthesis. The elevated IAA levels result in the activation of canonical SCF^{TIR1/AFB}-mediated auxin signalling, which promotes *SAUR* gene expression. Consequently, the plasma membrane-localised PP2C.D members are inhibited, allowing for the PM H⁺-ATPase to be phosphorylated at Thr-947 and stabilised in an active conformation. The resulting increase in proton pumping has two physiological effects: firstly, it hyperpolarises the plasma membrane (which can attenuate salinity-induced depolarisation), providing the proton motive force to power ion transport via H⁺-coupled anion/cation symporters and antiporters, and thereby having an effect on K⁺/Na⁺ homeostasis as well affecting solute uptake to allow for sustained cellular water levels (or increased water uptake) and turgor pressure. Secondly, it acidifies the apoplast, resulting in the activation of cell wall loosening EXPA proteins which, in conjunction with increased turgor pressure, drive expansion growth. Together, an improvement in K⁺/Na⁺ homeostasis, and altered growth, constitute a mechanism for improved plant salt-tolerance.

Furthermore, in the *NIT2*-overexpressing line, we hypothesise that increased IAA levels may result in an increased activation of this system.

From the review of the literature seen above (see section 1.9.4.), as well as the previous findings by our research group, it is clear that there is a link between auxin and salt. Furthermore, it is well established that the PM H⁺-ATPase plays a central role in the regulation of ion transport during salt stress (see section 1.10.4), and there is a growing body of evidence implicating expansin proteins in growth modulation in response to salinity (see section 1.11.5). I thus believe that there is good evidence to support this proposed model. The overall aim of this thesis was to test various aspects of this model and thus further our understanding of the molecular mechanism whereby auxin modulates *Arabidopsis* growth in response to salt stress. A particular emphasis was placed on further characterising the increased salinity tolerance of the *NIT2*-overexpressing line. Aims to achieve this objective included: 1) To determine if the *NIT2*-overexpressing line displays beneficial alterations in Na⁺/K⁺ homeostasis during salt stress (chapter 2). 2) To determine if salt-responsive changes in the shoot PM proteome occur, and if there are genotype-specific differences in the shoot PM proteomes of the *NIT2*-overexpressing line and the wild-type that contribute towards its improved salt-tolerance (chapter 3). 3) To assess the contribution of *EXPA11* to the growth and overall salt-tolerance of the *NIT2*-overexpressing line by knocking down its expression, thereby delineating the contributions of ion homeostasis and expansion growth (chapter 4). A final aim was to generate and characterise an *EXPA11*-overexpressing line under saline conditions, in order to determine if improved plant salt-tolerance can be achieved by altering expansion growth only, without affecting ion homeostasis (chapter 5).

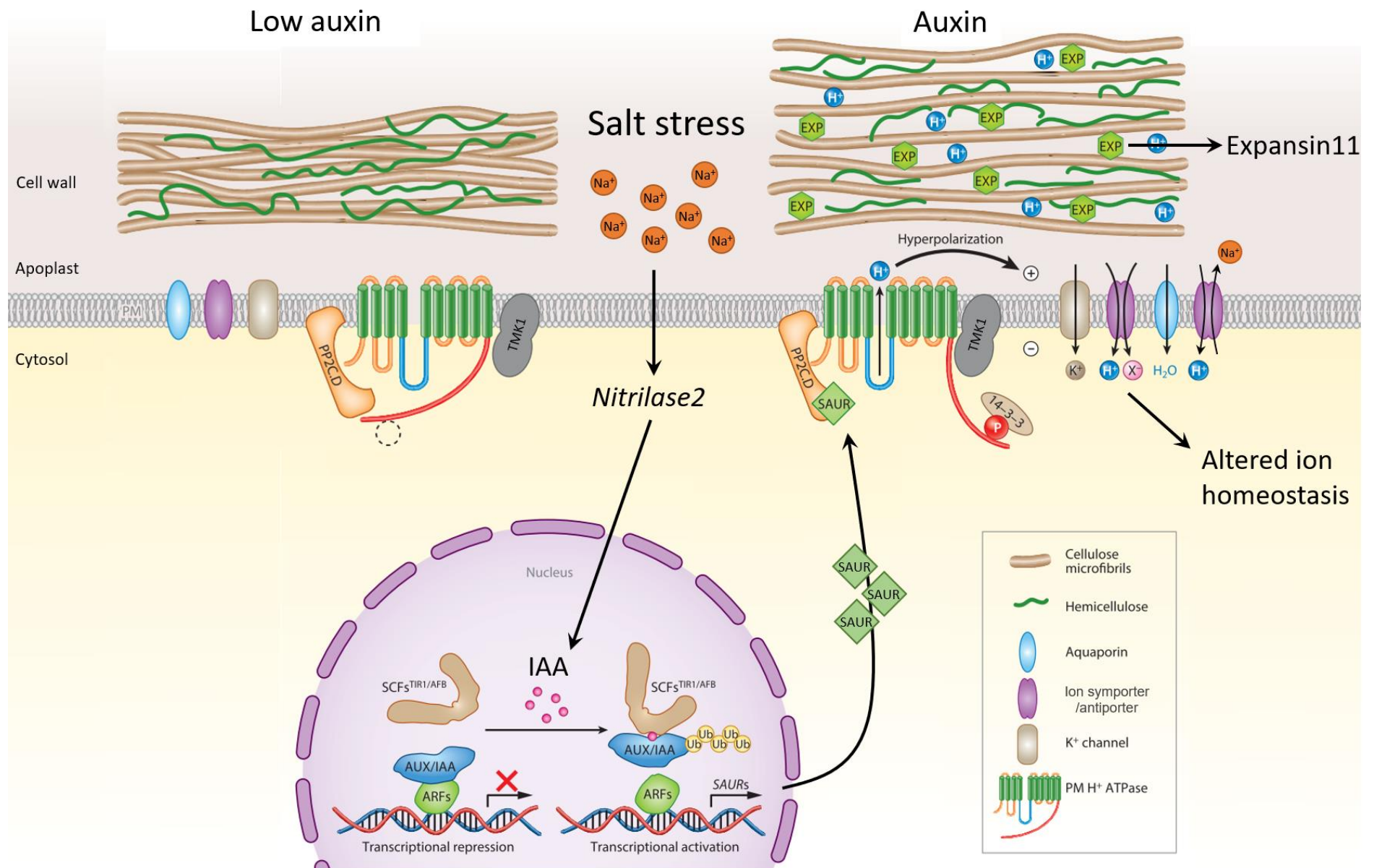


Figure 1.4: Hypothesised model for auxin-mediated acid growth modulation during salinity stress

Salt stress induces the expression of *Nitrilase2*, resulting in increased IAA biosynthesis. Subsequently, canonical SCF^{TIR1/AFB}-mediated signalling promotes *SAUR* gene expression. SAUR proteins interact with and inhibit PP2C.D2, D5, and D6, which prevents the dephosphorylation of Thr-947 of the PM H⁺-ATPase. The PM H⁺-ATPase is phosphorylated by TMK1, allowing for the interaction of 14-3-3 proteins that stabilise the pump in an active state. Increased proton pumping by the active PM H⁺-ATPase acidifies the apoplast, which activates cell wall loosening expansin proteins. Additionally, the proton pumping hyperpolarises the PM, thereby activating inward-rectifying K⁺ channels, as well as providing the proton motive force to power ion transport via other H⁺-coupled anion/cation symporters and antiporters. The resulting uptake of solutes allows for sustained water uptake, resulting in an increase in turgor pressure of the cell, which combined with the cell wall loosening actions of expansins drives expansion growth. We hypothesise that this process is co-opted during salinity stress: *Nitrilase2* expression is increased and the subsequent increase in IAA activates the PM H⁺-ATPase. The resulting increase in proton pumping could have two effects on growth: 1) The activation of expansins (in particular, EXPA11). 2) The energising of various PM Na⁺ and K⁺ symporters and antiporters, thereby affecting ion homeostasis. Image adapted from Du, Spalding and Gray, (2020).

CHAPTER 2: ALTERATIONS IN ION PARTITIONING BY *NITRILASE2* OVEREXPRESSING PLANTS UNDER SALINITY STRESS CONDITIONS

Note: The ion content data presented in Figure 2.1 has been published:

Cackett, L., Cannistraci, C. V., Meier, S., **Ferrandi, P.**, Pěňčík, A., Gehring, C., Novák, O., Ingle, R. A., & Donaldson, L. (2022). Salt-Specific Gene Expression Reveals Elevated Auxin Levels in *Arabidopsis thaliana* Plants Grown Under Saline Conditions. *Frontiers in Plant Science*, 13(February). <https://doi.org/10.3389/fpls.2022.804716>

Note: The ICP-OES analysis herein was conducted by the analytics laboratory at the Chemical Engineering Department at the University of Cape Town. I provided them with air-dried plant samples.

2.1. Introduction:

2.1.1. Ion homeostasis under salinity stress

As previously discussed in chapter 1, the negative effect of salinity on plant growth and physiology can be partly attributed to the ionic effects of Na^+ (and Cl^-) ions accumulating within the metabolically active tissues of the plant. The toxicity of Na^+ ions is attributed to its physiochemical similarity to K^+ , allowing for its uptake into cells via K^+ transport channels, and competition for binding sites on various enzymes that require K^+ as a co-factor, thereby interfering with many cytoplasmic metabolic processes (Shabala and Cuin, 2007, Tilbrook and Roy, 2014). Furthermore, negative ionic interactions caused by high concentrations of Na^+ in the soil reduces the availability of K^+ (and other essential nutrients) for uptake by the roots (Fageria, Gheyi & Moreira, 2011). Na^+ also competes with K^+ for uptake across the plasma membrane via K^+ channels and transporters (Shabala & Cuin, 2007). When Na^+ is taken up, it causes significant depolarisation of the plasma membrane (Shabala et al., 2005), which prevents the passive uptake of K^+ via inward-rectifying K^+ channels, while concomitantly increasing K^+ leak via outward-rectifying K^+ channels (KOR) (Shabala & Cuin, 2007). It is clear that the ability of a plant to maintain cytosolic K^+ levels while limiting the uptake of Na^+ , thereby maintaining a high cytosolic K^+/Na^+ (or low Na^+/K^+) ratio, is a key determinant of its salinity tolerance (Maathuis & Amtmann, 1999). The homeostasis of Na^+ and K^+ is thus ultimately what underlies the ability of a plant to tolerate salt stress. This in turn is achieved by the action of the large number of Na^+ and K^+ transporters and channels present in both the plasma and tonoplast membranes, as well as the P-type and V-type H^+ -ATPases that energize

transport across these respective membranes. The control of ion homeostasis via these proteins is incredibly complex, with many of them transporting both Na^+ and K^+ interchangeably under different cytoplasmic and extra-cellular conditions, as well as in the different tissues of the plant. Herein I will discuss the role of these channels in the context of Na^+ and K^+ homeostasis during salinity stress.

2.1.1.1. Na^+ homeostasis

The understanding of how plants control Na^+ homeostasis in order to limit Na^+ accumulation under salt stress has evolved in recent times, with there being evidence for five main strategies, two of which occur in the roots, and three in the photosynthetically active shoot tissues (Zhao et al., 2020). Together these strategies form the basis of Na^+ exclusion and tissue Na^+ tolerance (Munns & Tester, 2008). The first strategy is the exclusion of Na^+ at the root-soil interface. A number of plasma membrane ion transporters have been implicated in symplastic root Na^+ uptake; the majority of Na^+ uptake is thought to occur via non-selective cation channels, including glutamate receptor-like (GLR) channels and cyclic nucleotide-gated channels (CNGCs), but uptake is also thought to occur via various low- and high-affinity K^+ transporters, including ARABIDOPSIS K^+ TRANSPORTER 1 (AKT1), HIGH AFFINITY K^+ TRANSPORTER 5 (HAK5), and HIGH AFFINITY K^+ TRANSPORTER 2 (HKT2) (although Arabidopsis does not have this gene) (Mian, Senadheera & Maathuis, 2011; Wang, Guan, et al., 2015; Assaha et al., 2017; Zhao et al., 2020). In order to counter-act this Na^+ uptake, the SALT OVERLY SENSITIVE 1 (SOS1) Na^+/H^+ antiporter actively extrudes Na^+ from the roots back into the surrounding soil solution (Shi et al., 2002; Shabala et al., 2005). The second strategy to limit Na^+ accumulation in the cytosol is the sequestration of Na^+ in the vacuole, which occurs in both root cortex cells, and shoot mesophyll cells (Mian, Senadheera & Maathuis, 2011; Zhao et al., 2020). Vacuolar Na^+ sequestration is achieved by intracellular Na^+/H^+ exchangers (NHXs) which transport Na^+ across the tonoplast membrane in exchange for the efflux of H^+ into the cytosol (Bassil & Blumwald, 2014). It has also been suggested that vacuolar NHXs only transport Na^+ under salinity stress conditions, whereas under normal conditions they function primarily as K^+/H^+ exchangers, thereby affecting both intracellular Na^+/K^+ and pH homeostasis (Jiang et al., 2010). Recently, the importance of vacuolar Na^+ retention as part of the Na^+ sequestration process during salt stress has been highlighted (Shabala et al., 2019; Zhao et al., 2020). This is thought to be achieved through the efficient control of Na^+ -permeable slow- (SV) and fast-vacuolar (FV) channels, which mediate the leakage of Na^+ back into the cytosol. The third way in which plants control Na^+ homeostasis is

through the regulation of xylem Na^+ loading. Active loading of Na^+ into the xylem from root xylem parenchyma cells (XPC) is largely attributed to the SOS1 Na^+/H^+ antiporter (Shi et al., 2002), but it is also thought to be mediated by Class II HKT Na^+/K^+ symporters (Jabnoune et al., 2009), as well as cation-chloride co-transporters (CCCs), which symport Na^+ along with K^+ and Cl^- (Colmenero-Flores et al., 2007). By controlling how much Na^+ is loaded into the xylem from the roots, the plant can limit the amount of Na^+ that can be transported to the aerial, photosynthetically active tissues. The two remaining Na^+ homeostasis strategies which seek to reduce the amount of Na^+ present in the shoot, namely xylem Na^+ retrieval and phloem Na^+ re-circulation, are both mediated by Class I HKTs, with the Arabidopsis HIGH AFFINITY K^+ TRANSPORTER 1 (AtHKT1;1) being by far the most extensively studied (Mäser et al., 2002; Berthomieu et al., 2003; Sunarpi et al., 2005). It must be noted that the role of AtHKT1;1 in phloem Na^+ re-circulation is contended, with significantly more evidence pointing to its primary role being the unloading or extrusion of Na^+ from the xylem into the XPC in the roots (Sunarpi et al., 2005; Davenport et al., 2007), thus reducing the amount of Na^+ in the transpiration stream of the plant, thereby reducing Na^+ transport to the aerial tissues, and protecting the leaves from salinity stress (Hauser & Horie, 2010). Clearly, the control of Na^+ homeostasis under salinity stress involves a complex network of different channels and transporters, whose expression and function are tissue-dependent.

2.1.1.2. K^+ homeostasis

Along with the limitation of Na^+ accumulation, the cytosolic retention of K^+ is critically important to plant salt tolerance. Cytosolic K^+ retention in both the root and the shoot/leaf tissues has been shown to be an important salt-tolerance trait in Arabidopsis, as well as in many agriculturally important crop species (Wu et al., 2018). As mentioned previously, salinity causes significant depolarisation of the plasma membrane (Shabala et al., 2005), which triggers K^+ efflux via outward-rectifying K^+ channels (KOR) (Shabala & Cuin, 2007). The most notable examples of this K^+ efflux being that which occurs from root epidermal cells into the surrounding soil solution via GATED OUTWARDLY RECTIFYING K^+ CHANNEL (GORK), and the efflux from root XPCs into the xylem via STELAR K^+ OUTWARD RECTIFIER (SKOR) (Demidchik, 2014). In order to maintain cytosolic K^+ during salinity stress, this efflux needs to be balanced by K^+ uptake into the cell, which is achieved by several high- and low-affinity K^+ transporters. Under saline conditions, K^+ availability in the soil is effectively reduced, thus the majority of cellular K^+ uptake in the roots is mediated by the high-affinity KT/KUP/HAK-family K^+ transporters, HAK5, and POTASSIUM UPTAKE

PERMEASE 7 (KUP7) (Nieves-Cordones et al., 2010; Han et al., 2016; Wu et al., 2018). Root K^+ uptake is also mediated by the low-affinity Shaker-type inward rectifier K^+ channel, AKT1. However, as AKT1 mediates passive K^+ uptake, the plasma membrane depolarisation induced by salinity makes K^+ transport via AKT1 thermodynamically unfavourable, and thus its contribution to K^+ uptake in the roots is likely less significant than HAK5 (Wu et al., 2018). Assuming that K^+ uptake under saline conditions is largely mediated by HAK/KUP transport, the rate of K^+ uptake via these transporters is likely insufficient to compensate for the GORK-mediated K^+ efflux that occurs following membrane depolarisation, as the transport capacity of ion transporters is far lower than that of ion channels, such as GORK (K^+ efflux) or AKT1 (K^+ influx) (Gadsby, 2009). Thus, aside from the uptake of K^+ , replenishment from the vacuolar K^+ pool is crucial for the maintenance of cytosolic K^+ . This vacuolar K^+ release can be mediated by three different types of tonoplast channels: K^+ -selective tonoplast two-pore potassium (TPK) channels, and slow- (SV) and fast- (FV) activating non-selective cation channels (Pottosin & Dobrovinskaya, 2014). In Arabidopsis, vacuolar K^+ release has been attributed to TPK1, which has been shown to be activated in response to salt stress (Latz et al., 2013). While rapid vacuolar K^+ release can occur via SV and FV channels, their non-selective nature means that they are also permeable to Na^+ , and thus, as discussed previously, effective control of these channels is required to prevent the leakage of Na^+ that has been sequestered into the vacuole under salt stress conditions (Assaha et al., 2017; Wu et al., 2018). Another crucial component of K^+ homeostasis during salinity stress is the control of xylem K^+ loading and unloading, which allows for the K^+ , absorbed by the roots, to be transported to the shoots within the transpiration stream of the plant (Rubio et al., 2020). In the root, loading of K^+ from the XPCs into the xylem is achieved by the depolarisation-activated outward rectifying SKOR channel, as well as the H^+ -coupled K^+ transporters, KUP7 and NITRATE TRANSPORTER 1.5 (NRT1.5) (Gaymard et al., 1998; Han et al., 2016; Li et al., 2017). Little is known about the mechanisms controlling xylem K^+ unloading in the shoots, but it has been suggested that this process could be controlled by inward-rectifying K^+ channels (AKT/KAT channels), or potentially by low-affinity K^+ -efflux transporters, such as CATION/HYDROGEN EXCHANGER 14 (CHX14) (Zhao et al., 2015; Wu et al., 2018). Once in the leaf, K^+ is loaded into the phloem, where it is either distributed to the surrounding mesophyll tissues, or re-circulated back to the root. The distribution of K^+ to the surrounding leaf mesophyll tissues requires transport from the vascular tissues through the bundle sheath cells, however this process, and the K^+ transporters involved is not well understood (Wigoda, Moshelion & Moran, 2014; Assaha et al., 2017). The re-circulation of K^+ back to the root via the phloem is thought

to act as a feedback control mechanism to control K^+ uptake, as well as K^+ release into the xylem via SKOR in the roots (Wu et al., 2018; Rubio et al., 2020). Phloem K^+ loading for recirculation is controlled by AKT2/3, but under saline conditions where the plasma membrane is depolarised, it is likely that KT/KUP/HAK transporters that can operate in a voltage-independent manner, are also involved in this process (Lacombe et al., 2000; Wu et al., 2018). As with Na^+ , the control of K^+ homeostasis during salinity stress is a complex process, involving several K^+ transporters, many of which have yet to be identified.

2.1.2. Rationale for measuring ion contents in the 35S::*NIT2* line

Previous research from our group has shown that the 35S::*NIT2* line, overexpressing *Nitrilase2*, displays increased survival and improved growth phenotypes compared with its genetic background, No-0, under salt stress (Cackett et al., 2022). We propose that *Nitrilase2* is responsible for IAA biosynthesis in salt stress conditions because *Nitrilase2* expression, as well as the levels of the auxin intermediate, IAN, and bioactive auxin, IAA, increase in response to salt (Cackett et al., 2022). Furthermore, IAA content was significantly increased in the 35S::*NIT2* line compared with No-0 under salt stress conditions. In addition to the improved salt-stress growth phenotypes displayed by the 35S::*NIT2* line, transcriptomics analysis performed to identify differentially expressed genes (DEGs) between 35S::*NIT2* and No-0 two-week-old seedlings, grown under salt stress and control conditions, revealed a number of ion transporter genes that were differentially expressed (Cackett, 2019). This finding suggests that altered ion homeostasis could play an important role in the salt-tolerance of the 35S::*NIT2* line. As such, our current hypothesis for how growth can be modulated under saline conditions is that perception of salt stress induces *Nitrilase2* expression, resulting in the synthesis of IAA, which in turn causes the activation of auxin-mediated acid growth via the activation of the plasma membrane H^+ -ATPase pump (Rayle & Cleland, 1992; Takahashi, Hayashi & Kinoshita, 2012). The activation of the PM H^+ -ATPase causes the acidification of the apoplast, providing the proton motive force to drive the uptake of solutes and subsequently water into the cells, as well as the activation of cell wall-loosening expansin proteins leading to cell expansion (Cosgrove, 2016). Concurrently, the proton motive force generated can be used to power the transport of Na^+ and K^+ ions across the plasma membrane via various transporters and channels. It is important to note that PM H^+ -ATPases, which have been described as the powerhouse for plant growth (Falhof et al., 2016), presumably always maintain a certain level of activity, given their key role in energising plasma membrane transport. I thus hypothesise that a constitutive production of IAA, and hence increased activation of the PM H^+ -ATPase,

and its resulting effects, underlies the increased salt-tolerance of the 35S::*NIT2* line. Part of testing this hypothesis is to determine if there is a difference in Na⁺ and K⁺ ion homeostasis between the two lines, and if so, to measure the expression of Na⁺- and K⁺-transporters that could be responsible for facilitating ion transport that occurs as a result of this hypothesised auxin-induced PM H⁺-ATPase activation.

2.1.3. Chapter aims

The overall aim was to determine if alterations in Na⁺ and K⁺ ion partitioning contribute towards the improved salt-tolerance phenotype displayed by the 35S::*NIT2* line. This was split into two components; 1) To determine if there is a difference in the Na⁺ and K⁺ content in the root and shoot of 35S::*NIT2* and No-0 plants in untreated conditions, and when exposed to salt-stress later on in development, 2) To determine if there are differences in the expression of candidate Na⁺- and K⁺-transporter genes in the root and shoot between 35S::*NIT2* and No-0 plants in the same untreated and salt-stress conditions.

2.2. Materials and methods:

2.2.1. Stock solutions and chemicals used

All plant nutrient (PN) media components were obtained from Sigma-Aldrich (St Louis, USA). The Trizol® Reagent (catalogue no. 15596026) and SuperScript™ III Reverse Transcriptase (catalogue no. 18080-093) were obtained 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). The KAPA SYBR® FAST qPCR Kit (catalogue no. KK4600) was obtained from Sigma-Aldrich (Gauteng, South Africa).

2.2.2. Arabidopsis seed stocks

Seeds for a *Nitrilase2* overexpressing plant line (35S::*NIT2*) and its wild type genetic background No-0 were obtained from Bonnie Bartel (Rice University, Houston, Texas) (Normanly et al., 1997).

2.2.3. Arabidopsis growth conditions

Arabidopsis plant growth was carried out under standard conditions in a plant growth room (100 µM photons.m⁻².s⁻¹, 16-h light/8-h dark, 22°C, 50 – 60% relative humidity).

2.2.4. Seed sterilisation

Seeds were surface-sterilised by shaking in 70% (v/v) ethanol for five minutes on a nutator. The ethanol was aspirated off and replaced with 15% (v/v) bleach, 0.02% (v/v) Triton X-100 solution, followed by ten minutes of shaking. The seeds were washed five times with sterile dH₂O, then resuspended in sterile 0.1% (w/v) phyto agar. Sterilised seeds were stored for at least two days at 4°C, to allow for vernalisation.

2.2.5. Plant Nutrient (PN) media

Plants were grown in PN media that contains 5 mM KNO₃, 2 mM MgSO₄·7H₂O, 2 mM Ca(NO₃)₂·4H₂O, 0.5 mM Fe.Na.EDTA as well as micronutrients (70 µM H₃BO₃, 14 µM MnSO₄·H₂O, 0.5 µM CuSO₄·5H₂O, 1 µM ZnSO₄·6H₂O, 0.2 µM Na₂MoO₄, 10 µM NaCl and 0.01 µM CoCl₂·6H₂O). Following autoclaving, KPO₄ buffer (pH 5.7) was added to a final concentration of 2.5 mM (Haughn & Somerville, 1986).

2.2.6. Arabidopsis growth in hydroponics

Plants were grown hydroponically using the Araponics system (Liège, Belgium). This system comprises of seed holders, seed supports, 1.8 L tanks, and lids. The seed holders were filled with 7% (w/v) bacteriological agar, then placed into the seed holder support. Sterilised seed was sown individually onto the agar. The 1.8 L tank was filled with ¼ strength PN liquid media, and the seed support placed into the tank such that the base of the seed holders was submerged in the liquid media. The lid was placed on the tank for the first seven days to increase humidity for germination. The system was kept under constant growth room conditions, with the media replaced every seven days. The seedlings were grown for three weeks in ¼ strength PN media, and then were either treated with ¼ strength PN media supplemented with 75 mM NaCl for one week, or maintained in ¼ strength PN media (untreated control).

2.2.7. Measurement of ion content in salt-stressed No-0 and 35S::*NIT2* plants via ICP-OES

2.2.7.1. Experimental design

The 35S::*NIT2* and No-0 Arabidopsis plants were grown hydroponically for three weeks in ¼ strength PN media, followed either by treatment with ¼ strength PN media supplemented with 75 mM NaCl for one week, or maintenance in ¼ strength PN media for one week (control). Root and shoot tissue from four plants per treatment or control was harvested and pooled from

both genotypes. The entire experiment was repeated six times to give six biological replicates (i.e., 48 samples in total).

2.2.7.2. Sample preparation

Intact plants were removed from the Araponics system, and the roots were immediately rinsed three times with dH₂O to remove surface NaCl, then blotted dry. Root and shoot tissue from untreated (control) and 75 mM NaCl treated plants was harvested separately. Tissue from four plants was pooled to constitute one sample. All samples were collected into envelopes and placed in an oven at 60°C for five days until completely dried. The samples were then submitted to the Analytical Laboratory, Department of Chemical Engineering (University of Cape Town, South Africa). Here, the samples were ashed, weighed into Teflon containers, and acid digested as follows: 4 mL of 55% H₂O HNO₃ was added to the samples which were then boiled to almost dryness using a hot plate. This process was repeated until the samples were completely digested. The digested samples were diluted to 100 mL using dH₂O. A Mars 5 Microwave digester (CEM Corporation, Charlotte, NC, USA) was used to heat the samples under pressure at 180°C for 30 minutes. Following digestion, samples were diluted accordingly in dH₂O to account for the initial mass of sample that was digested. All samples were then filtered using a 0.2 µm filter.

2.2.7.3. ICP-OES analysis

Sodium, Potassium, Magnesium, and Calcium ion content was measured in the root and shoot tissue of the untreated and 75 mM NaCl treated plants using a Varian ES 730 Inductive Coupled Plasma – Optical Emission (ICP-OES) Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The ICP-OES spectrophotometer was calibrated using five multi-element standards containing the ions of interest, at concentrations of 0.2; 0.5; 1; 2 and 5 ppm.

2.2.7.4. Data processing and statistical analysis

Average ion contents in µg.g⁻¹ were determined from the six replicates for each treatment for both genotypes, as well as the standard error surrounding these averages. Graphs comparing the ion contents between the two genotypes in the root and shoot tissue were generated in Excel for both untreated and 75 mM NaCl treated plants. Graphs of the potassium ion/sodium ion content were also generated. All statistical analyses were performed using Statistica Software version 13.5 (Statsoft, Tulsa, USA). Two-sample t-tests were conducted to determine

statistically significant differences between sample groups, Bonferroni correction for multiple testing was performed. For all analyses performed, p -values $\leq 0,05$ were considered significant.

2.2.8. RNA extraction, quantification, and cDNA synthesis

2.2.8.1. RNA extraction

Total RNA was extracted using 1 mL of Trizol® Reagent (Invitrogen, Paisley, UK) per ± 100 mg plant tissue (fresh weight) according to the manufacturer's guidelines. For large scale tissue homogenisation of hydroponically grown plants, homogenisation was performed using a pestle and mortar, and liquid nitrogen. Following this, ± 100 mg of homogenised tissue was added into a 1.5 mL microcentrifuge tube along with 1 mL of Trizol reagent. RNA extraction was then performed according to the manufacturer's protocol (Invitrogen, Paisley, UK). The final RNA pellet was resuspended in 88.5 μ L of nuclease free H₂O and incubated at 60°C for 10 min to allow for proper resuspension.

2.2.8.2. dNase treatment and RNA clean up

All RNA samples were dNase-treated using the rNase-free dNase Set (Qiagen, Germany), followed by a column clean-up using the rNeasy® Mini kit (Qiagen, Germany). Both were performed according to the manufacturer's protocol. In order to ensure complete elution from the column, the purified RNA was eluted twice in 30 μ L of nuclease-free water (total 60 μ L), which was collected in a single microcentrifuge tube.

2.2.8.3. RNA quality and integrity assessment

The RNA concentration and quality were assessed using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). A 260:280 nm absorbance ratio of > 1.8 was considered sufficient quality to use for downstream applications. The RNA integrity was assessed by electrophoresis on formaldehyde-agarose denaturing gel containing 1 x MOPS (0.4 M 3-(*N*-morpholino)propanesulfonic acid, 0.1 M sodium acetate (NaOAc), 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7), 1.2% (w/v) agarose, and 6% (v/v) formaldehyde). Samples were heat denatured at 65 °C for five minutes in RNA loading dye (4 x MOPS, 2.7% (v/v) formaldehyde, 30.8% (v/v) formamide, 0.01 mg/mL ethidium bromide and bromophenol blue) then cooled on ice for one minute prior to loading. Electrophoresis was performed in 1 x MOPS running buffer. Alternatively, RNA was assessed by electrophoresis on a 1 x Tris-acetate-EDTA (TAE) 1% (w/v) agarose gel. The RNA bands were visualised using a Gel Doc™ XR UV Transilluminator (Bio Rad Laboratories, UK).

2.2.8.4. cDNA synthesis

The cDNA was synthesised from 1 µg of RNA using Oligo(dT) primers and Superscript® III Reverse Transcriptase (Invitrogen, Paisely, UK), following the manufacturers protocol. In RNA samples with an insufficient concentration, 500 ng of RNA was used in cDNA synthesis (this was later accounted for by halving the dilution of these samples prior to RT-qPCR). 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).

2.2.9. Gene expression analysis via RT-qPCR

All aspects of the gene expression analysis via RT-qPCR, as well as the prior RNA extraction, quantification, quality assessment, and cDNA synthesis, were performed in compliance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).

2.2.9.1. RT-qPCR primer design

Most of the primers were obtained from the literature (see Table 2.1 for references); however, primers were designed for *KAT1*. The genomic and cDNA sequences of *KAT1* were obtained from The Arabidopsis Information Resource, TAIR (<https://www.arabidopsis.org/>). Primers were designed using NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), and checked for self-complementarity and two-primer complementarity using DNAMAN v 4.13 (Lynnon Biosoft, San Ramon, CA, USA). The primers were checked for specificity to the target gene using TAIR BLAST (<https://www.arabidopsis.org/Blast/>). Where possible, RT-qPCR primers were designed to span exons in order to avoid detection of any contaminating gDNA.

2.2.9.2. RT-qPCR experiments

All RT-qPCR was performed on a Corbett Rotor-Gene 6000 HRM Real Time PCR machine (Qiagen, Netherlands) using the KAPA SYBR® FAST Universal qPCR Kit (without 50X ROX High/Low) (Sigma-Aldrich, South Africa). Standard cycling parameters for KAPA SYBR® FAST were utilised: 95 °C for 3 min; 40 cycles of 95 °C for 3 s, 60 °C for 20 s, 72 °C for 1 s; 72 °C for 5 min. For all primer pairs, an annealing temperature (T_A) of 60 °C was used. Three biological repeats were used per sample, each with three technical repeats.

2.2.9.3. RT-qPCR data analysis

Quality control of RT-qPCR results was performed using the Rotor-Gene 6000™ Series Software 1.7. Only RT-qPCR experiments yielding melt curves with a single peak, and standard curves with an R^2 -value > 0.97 and an efficiency value between 0.9 and 1.1 were considered accurate and taken forward for further analysis. Technical replicates were discarded where the quantification cycle/threshold cycle (C_q/C_T) values were different from the median by 0.5 or more. The data was exported into Microsoft Excel (2017), where the calculated concentration/expression-value of each of the candidate genes was extrapolated from the RT-qPCR standard curve, and then normalised to that of the reference gene, *MONENSIN SENSITIVITY1 (MON1)* (Hong et al., 2010) using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). Our group has utilised *MON1* as a reference gene as it has consistently and reliably been shown not to be salt-responsive (Cackett, 2019; Cackett et al., 2022), and thus is suitable for this study. The normalised data was averaged and plotted with error bars representing standard error of the mean. All qPCR primers used can be found in Table 2.1.

2.2.10. Ion channel expression analysis in salt-stressed No-0 and 35S::*NIT2* plants

2.2.10.1. Experimental design

This experiment was set up in a similar manner to the ICP-OES analysis (see section 2.2.7). The 35S::*NIT2* and No-0 Arabidopsis plants were grown hydroponically for 3 weeks on $\frac{1}{4}$ strength PN media, followed either by treatment with $\frac{1}{4}$ strength PN media supplemented with 75 mM NaCl for four days, or maintenance on $\frac{1}{4}$ strength PN media for four days (control). This treatment period was shorter than that used for the ICP-OES analysis (seven days), but was deemed to be sufficient to measure transcriptional responses associated with long-term salt stress. Root and shoot tissue from four plants for each treatment from both genotypes was harvested and pooled. Each treatment had six biological replicates (i.e., 48 samples in total).

2.2.10.2. Statistical analysis of RT-qPCR data

All statistical analyses were performed using Statistica Software version 13.5 (Statsoft, Tulsa, USA). Two-way ANOVAs were conducted to determine the effect of genotype and the effect of salt treatment on gene expression, as well as to assess the interaction between these two effects. Bonferroni post-hoc analysis was performed to correct for multiple testing and assign significant differences. For all analyses performed, p -values ≤ 0.05 were considered significant.

Table 2.1: Primers used for gene expression analysis via RT-qPCR

Note: F: Forward primer; R: Reverse primer

Gene name	TAIR accession	Primer name	Primer sequence (5'→3')	cDNA amplicon size (bp)	Source
<i>MONENSIN SENSITIVITY1 (MON1)</i>	AT2G28390	MON1 F MON1 R	CAGACAAGGCGATGGCGATA GCTTTCTCTCAAGGGTTTCTGGGT	240	(Hong et al., 2010)
<i>HIGH-AFFINITY K⁺ TRANSPORTER 1 (HKT1;1)</i>	AT4G10310	qHKT1;1 F qHKT1;1 R	CAATCACCGAAAGGCAAAAT CGTCCTGCAAACCCATAACT	178	(An et al., 2017)
<i>POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 (KAT1)</i>	AT5G46240	qKAT1 F qKAT1 R	CACAGCACCATTTTCAGCCAC CTCAAGCCTTGCAAATAGCGA	127	Designed
<i>TONOPLAST INTRINSIC PROTEIN 2;2 (TIP2;2)</i>	AT4G17340	TIP2;2 F TIP2;2 R	TAGGAGCTATTGAAGGAGTAG ATGAGTCCAGCAAGTGCACCA	283	(Feng, Xu, et al., 2018)
<i>CATION/H⁺ EXCHANGER 17 (CHX17)</i>	AT4G23700	CHX17 F CHX17 R	ATAGAAGCATCCCGAGGCATA ACGCACTGAGACACGGCTG	225	(Sun et al., 2015)
<i>GLUTAMATE RECEPTOR 2.3 (GLR2.3)</i>	AT2G24710	GLR2.3 F GLR2.3 R	AGCGAAAGACCAGGTTTC TGGTCATGAACCTTGAGACATA	114	(Roy et al., 2008)
<i>HIGH AFFINITY K⁺ TRANSPORTER 5 (HAK5)</i>	AT4G13420	HAK5 F HAK5 R	CATCGCTGTTACCCTTGCCCTTATG TATGGAGCCAAAGACGACCAAGAA	172	(Sun et al., 2015)
<i>POTASSIUM UPTAKE TRANSPORTER 1 (KUP1)</i>	AT2G30070	KUP1 F KUP1 R	GGCGATACCCGAGCCTGTGTT CCGCCAGGCAAAGACACATCA	221	(Sun et al., 2015)
<i>POTASSIUM UPTAKE PERMEASE 7 (KUP7)</i>	AT5G09400	KUP7 F KUP7 R	TGGCGGAGGAAAGCAGTATGG AGTCCAGTACCGTCTGAATCCATCA	175	(Han et al., 2016)
<i>NA⁺/H⁺ EXCHANGER 1 (NHX1)</i>	AT5G27150	NHX1 F NHX1 R	CTACCTATTACCGCACCAGAACG CTCAATGAACGAGTCTTGGTCC	103	(Sun et al., 2015)
<i>NA⁺/H⁺ EXCHANGER 3 (NHX3)</i>	AT5G55470	NHX3 F NHX3 R	CTCAAGATGCAAGTCACAACACC GAAGCAGACTCGTCAAAGGAAAG	106	(Bassil et al., 2019)
<i>SALT OVERLY SENSITIVE 1 (SOS1)</i>	AT2G01980	SOS1 F SOS1 R	ATGATGGGACGGCGATTGT TGCCAAACGCCAGACCAAT	135	(Sun et al., 2015)
<i>ARABIDOPSIS K⁺ TRANSPORTER 1 (AKT1)</i>	AT2G26650	AKT1 F AKT1 R	AAAGGTCTCACTCATCAACAACGA TCGGCAAAAGAGGCAAAATAAG	89	(Wang et al., 2019)
<i>GATED OUTWARDLY-RECTIFYING K⁺ CHANNEL (GORK)</i>	AT5G37500	GORK F GORK R	TGTTCTTCAGTTCTTTGTTGCCTAT ACTTTGCGAACCCGAAATAGC	207	(Sun et al., 2015)
<i>STELAR K⁺ OUTWARD RECTIFIER (SKOR)</i>	AT3G02850	SKOR F SKOR R	ACCGAAACAAACTCGGTAGGAA TTAGCACGGATAGAGACAGGAATG	121	(Wang et al., 2019)
<i>Autoinhibited H⁺-ATPase 2 (AHA2)</i>	AT4G30190	AHA2 F AHA2 R	TGCTCAAAGGACACTTCACG GCCCTTTAGCTTCACGACTG	181	(Pacifici et al., 2018)
<i>Autoinhibited H⁺-ATPase 1 (AHA1)</i>	AT2G18960	AHA1 F AHA 1 R	CACAACCAAGAAAGATTACGG CTTCTCTTGGCTTGCTCTG	159	(Wang, Papanatsiou, et al., 2012)

2.3. Results

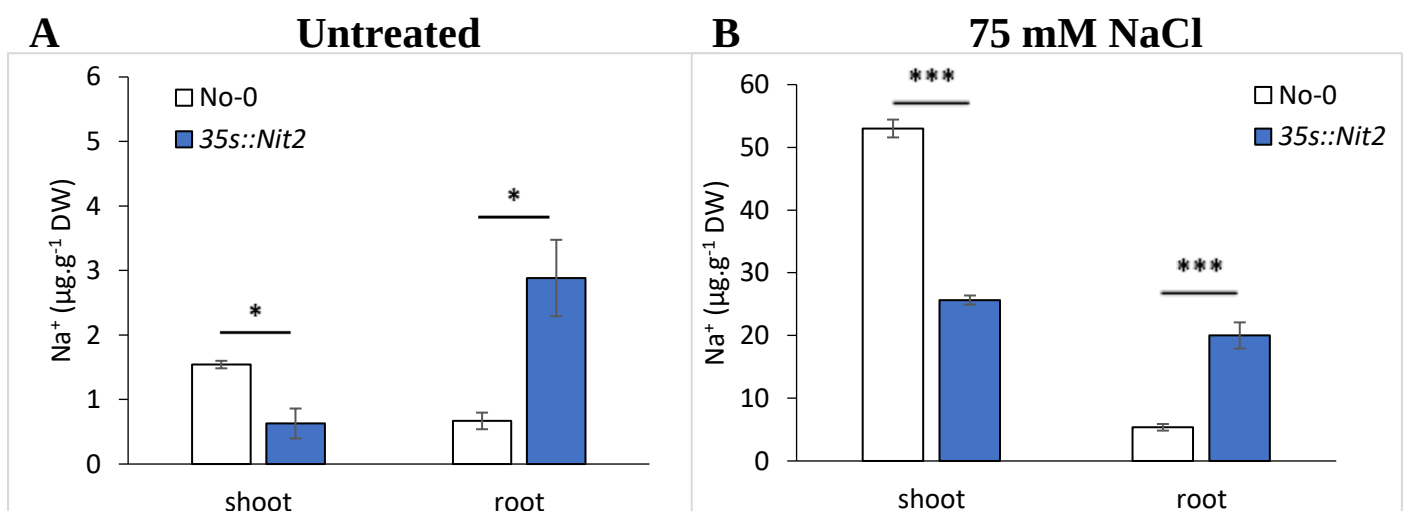
2.3.1 35S::*NIT2* plants have altered Na⁺ and K⁺ ion partitioning in the root and shoot

The homeostasis of Na⁺ and K⁺ is a key component of the plant salt stress response (Chen et al., 2007). As such, the Na⁺ and K⁺ (and Ca⁺) contents in the roots and shoots of 35S::*NIT2* and No-0 plants, following exposure to salt stress for one week, or maintained growth under control conditions, was measured via ICP-OES. This analysis was performed by the analytics laboratory at the UCT Chemical Engineering Department. When looking at the Na⁺ content (Figure 2.1 A and B), a clear difference was observed in partitioning in both the root and shoot between the 35S::*NIT2* and No-0 lines under both control and salt-stress conditions. In the shoot, the 35S::*NIT2* line accumulated significantly less Na⁺ (~2-fold) than No-0 in both control and salt treatments. By contrast, in the root, the 35S::*NIT2* had significantly higher Na⁺ content (~4-fold) than No-0 in both conditions. As expected, the Na⁺ content was greatly increased in all tissues in response to salt treatment. An interesting observation was that while the pattern of Na⁺ accumulation in the root and shoot between the two lines was consistent in both treatments (35S::*NIT2* had less Na⁺ in shoots and more in roots than No-0), the distribution of the Na⁺ between the two tissue types differed; In untreated conditions, the 35S::*NIT2* line had a higher Na⁺ content in the roots than in the shoot, with the opposite observed for No-0. However, under salt-stress conditions, while No-0 accumulated a proportionally much larger amount of Na⁺ in its shoots, the 35S::*NIT2* appeared to partition the Na⁺ evenly between the shoot and root. It is clear though, that under salt-stress conditions, the 35S::*NIT2* line was able to limit Na⁺ accumulation in the shoot, which is a mechanism of improved salt tolerance. Furthermore, the total Na⁺ content in the 35S::*NIT2* plants as a whole ($45.66 \pm 1.71 \mu\text{g.g}^{-1}$ dry weight) was significantly lower ($p = 0.00026$) than that of No-0 plants ($58.37 \pm 1.24 \mu\text{g.g}^{-1}$ dry weight) under salt-stress conditions.

While Na⁺ exclusion is crucial for salinity tolerance, the maintenance of cytosolic K⁺ is very important to maintain cellular function under salt-stress conditions, particularly given that K⁺ uptake is more difficult due to the reduction in plasma membrane potential, and the competitive effect of Na⁺ on K⁺ transport that occurs under saline conditions (Chen et al., 2007). Referring to Figure 2.1 C, no significant difference was observed in K⁺ content in either the root or shoot between the two lines under control conditions (the apparent lower K⁺ content in 35S::*NIT2* roots was not statistically significant). However, following salt-treatment (Figure 2.1 D), the K⁺ content was significantly higher (~6-fold) in the roots of the 35S::*NIT2* line compared with No-0, whereas it was slightly (but significantly) decreased in the shoots. In No-0, salt-treatment

resulted in a significant reduction in root K^+ ($p = 0.0034$), whereas salt-treatment had no effect on root K^+ in the 35S::*NIT2* line. Furthermore, the total K^+ content in the whole 35S::*NIT2* plants ($39.11 \pm 3.92 \mu\text{g.g}^{-1}$ dry weight) was significantly higher ($p = 0.00064$) than that of No-0 plants ($17.88 \pm 0.55 \mu\text{g.g}^{-1}$ dry weight) under saline conditions. Clearly then, the 35S::*NIT2* line is much better able to maintain K^+ in the roots under saline conditions, however it was unexpected that the K^+ content was reduced in the shoots, given the importance of K^+ maintenance in salinity tolerance.

It is, however, important to note that it is not necessarily the absolute K^+ content that is important, but rather the maintenance of an optimal intracellular Na^+/K^+ (or K^+/Na^+) ratio, with this ratio widely regarded as a measure of plant salinity tolerance (Jadamba et al., 2020). In both the root and the shoot, a significantly lower Na^+/K^+ was observed in the 35S::*NIT2* line compared with No-0 in response to salt-treatment, which strongly suggests an improved salinity tolerance (Figure 2.1 F). However, in untreated conditions, no difference in Na^+/K^+ was observed in the shoots between the two lines, and a significantly higher ratio was observed in the roots of the 35S::*NIT2* (Figure 2.1 E). Collectively, these data suggest that the 35S::*NIT2* line has a superior ability to retain Na^+ in the root and prevent its transport to the shoot, and under saline conditions, it has an improved ability to prevent K^+ loss from the root, which altogether translates to improved Na^+/K^+ ratios in both the root and the shoot.



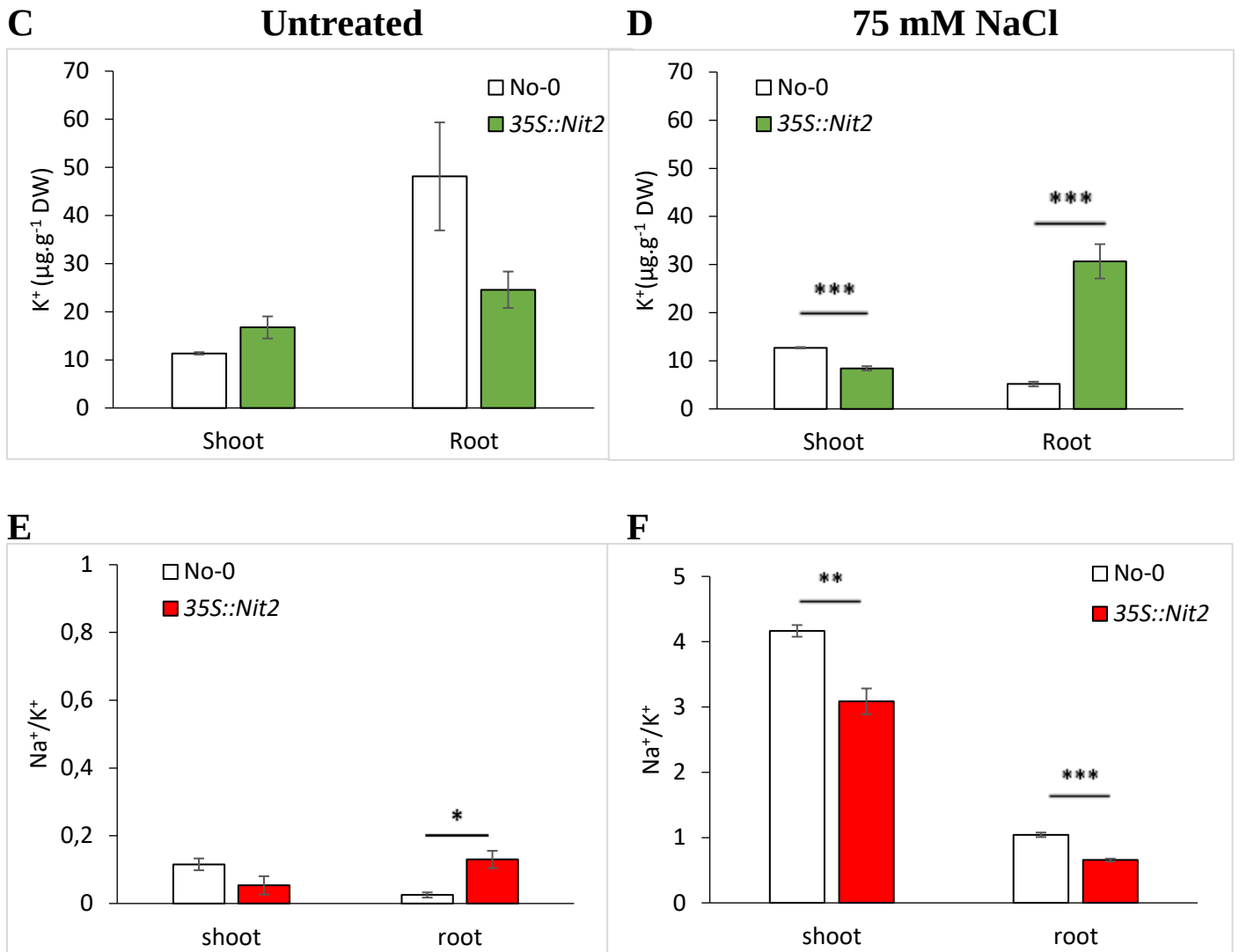


Figure 2.1: Na⁺ and K⁺ content in root and shoot tissue of 35S::NIT2 and No-0 plants exposed to salt stress

The No-0 and 35S::NIT2 plants were grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 75 mM NaCl, or maintained on ¼ strength PN media (untreated control) for one additional week. Root and shoot tissue were harvested, oven dried, and then ashed and acid-digested. The ion content (expressed in μg.g⁻¹ dry weight (DW)) was determined via ICP-OES analysis. Untreated (A, C, E) and 75 mM NaCl treatment (B, D, F). (A and B) Na⁺ content in roots and shoots of No-0 and 35S::NIT2 plants grown in untreated conditions, and following 75 mM NaCl treatment, respectively. (C and D) K⁺ content in roots and shoots of No-0 and 35S::NIT2 plants grown in untreated conditions, and following 75 mM NaCl treatment, respectively. (E and F) Na⁺/K⁺ in roots and shoots of No-0 and 35S::NIT2 plants grown in untreated conditions, and following 75 mM NaCl treatment, respectively. The results shown are an average of six biological repeats (n = 6), each consisting of four pooled plants. Asterisks (*) denote a statistically significant difference between No-0 and the 35S::NIT2, as determined by two-sample t-tests (* $p \leq 0.05$, ** $p < 0.005$, *** $p < 0.0005$). Bonferroni correction for multiple testing was performed. Error bars indicate standard error.

2.3.2. 35S::*NIT2* plants have altered ion channel expression in the root and shoot

In order to understand the altered ion homeostasis observed in both the root and the shoot of the 35S::*NIT2* line under salinity stress conditions, it naturally followed to measure the expression of Na⁺- and K⁺-transporters and channels under the same experimental conditions. To this end, 35S::*NIT2* and No-0 plants were grown hydroponically as described in section 2.3.1. Root and shoot tissue from four plants was pooled to give a single sample, with three biological replicates. RNA was then extracted, cDNA synthesised, and the expression of a number of candidate ion transport channels was measured via RT-qPCR analysis, and normalised to the expression of the housekeeping gene, *MON1* (Hong et al., 2010). A number of the candidate ion transporters whose expression was measured were previously identified as differentially expressed in a transcriptomics experiment comparing 35S::*NIT2* and No-0 seedlings grown under control and 100 mM NaCl conditions (Cackett, 2019). These included HKT1;1 and CHX17, which have both Na⁺ and K⁺ transport activity, KAT1, which has K⁺ transport activity, and GLR2.3, which is thought to play a role in Na⁺ and Ca²⁺ transport. Additionally, the expression of the tonoplast membrane aquaporin, TIP2;2, which was also found to be differentially expressed in this transcriptomics experiment, was measured. The other candidates whose expression was measured were not identified as DEGs in the transcriptomics experiment but were selected as they are classical Na⁺-transporters (NHX1, NHX3, SOS1/NHX7), or K⁺-transporters (HAK5, AKT1, KUP1, KUP7, SKOR, GORK), some of which have a known role in salinity tolerance. For all the genes whose expression was measured, two-way ANOVAs were performed separately on the shoot and root data to assess the effect of both genotype and salt treatment on gene expression, and to assess the interaction between these two effects.

2.3.2.1. Expression of Na⁺ transporters

In both No-0 and the 35S::*NIT2* line, *HKT1;1* was more highly expressed in the roots than the shoots (Figure 2.2 A). There was a significant effect of both genotype ($p < 0.001$) and salt treatment ($p < 0.001$) on root *HKT1;1* expression, as well as a significant interaction between these two effects ($p < 0.001$). *HKT1;1* expression was significantly down-regulated in No-0 roots in response to salt-treatment. Under control conditions, *HKT1;1* expression was significantly lower in the 35S::*NIT2* line than No-0, however, salt-treatment had no effect on *HKT1;1* expression in 35S::*NIT2* roots, such that under saline conditions, *HKT1;1* expression was significantly higher in the roots of the 35S::*NIT2* line than in No-0. In the shoots, *HKT1;1* expression was very low. Furthermore, there was no significant effect of genotype on *HKT1;1*

expression ($p = 0.061$). However, there was a significant effect of salt treatment ($p < 0.001$), with both lines found to have lower expression under saline conditions.

The expression of the plasma membrane located Na^+/H^+ exchanger (*NHX*), *SOS1/NHX7* as well as the tonoplast membrane located, *NHX1* and *NHX3*, was measured. For *SOS1* (Figure 2.2 B), there was a significant effect of both genotype ($p = 0.02$) and salt treatment ($p = 0.007$) on shoot expression, but no significant interaction between these two effects ($p = 0.536$). The expression of *SOS1* was found to decrease significantly in response to salt treatment in the shoots of No-0, however, the *SOS1* expression observed under control conditions in the 35S::*NIT2* line was maintained under saline conditions, and as such, *SOS1* expression was significantly higher in the shoots of the 35S::*NIT2* line compared with No-0 under saline conditions, with no difference observed between the two lines under control conditions. In contrast, in the root, there was no effect of either genotype ($p = 0.703$) or salt treatment ($p = 0.109$) on *SOS1* expression, and no interaction between these two effects ($p = 0.948$).

For the vacuolar Na^+/H^+ antiporter, *NHX3* (Figure 2.2 C), there was a significant effect of both genotype ($p < 0.001$) and salt treatment ($p = 0.005$) on shoot expression, as well as a significant interaction between these two effects ($p < 0.001$). Here, *NHX3* expression was significantly reduced in response to salt treatment in No-0 shoots. By contrast, *NHX3* expression was unaffected in 35S::*NIT2* shoots, and as such, *NHX3* expression was significantly higher in 35S::*NIT2* shoots than in No-0 under saline conditions. Under control conditions, no difference in *NHX3* expression was observed between the two lines in the shoots. In the roots, there was no effect of genotype ($p = 0.07$) or salt treatment ($p = 0.052$) on *NHX3* expression, and there was also no interaction between these two effects ($p = 0.558$). Overall, this expression pattern is highly similar to that observed for *SOS1*.

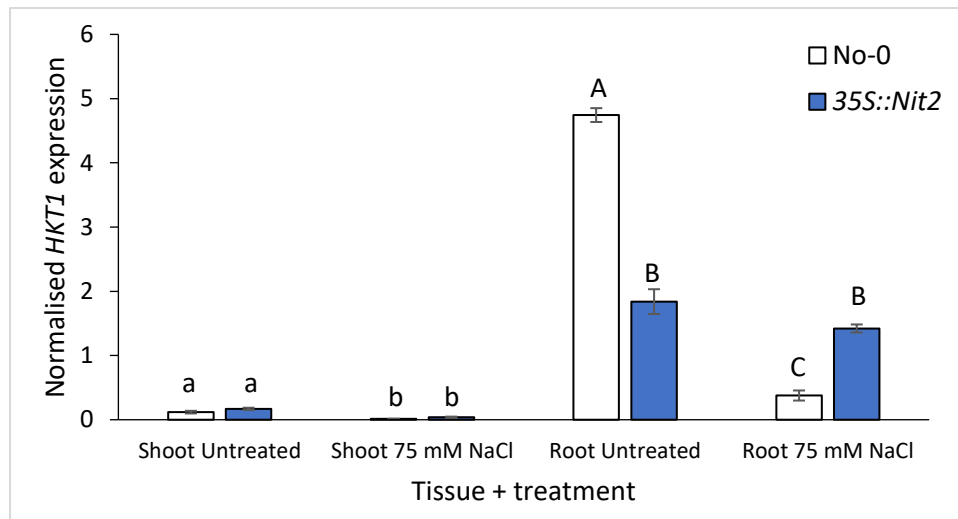
For *NHX1* (Figure 2.2 D), there was a significant effect of both genotype ($p = 0.029$) and salt treatment ($p < 0.001$) on shoot expression; however, in the roots, there was no significant effect of genotype ($p = 0.556$) or salt treatment ($p = 0.061$). For both tissues, there was no significant interaction between genotype and salt treatment ($p = 0.887$ and $p = 0.087$, respectively). As such, while salt-treatment resulted in a significant decrease in the expression of *NHX1*, in the shoots of both No-0 and 35S::*NIT2* plants, it had no effect on expression in the roots of either line. Furthermore, no difference in expression was observed when comparing the two lines in either tissue type for both treatments.

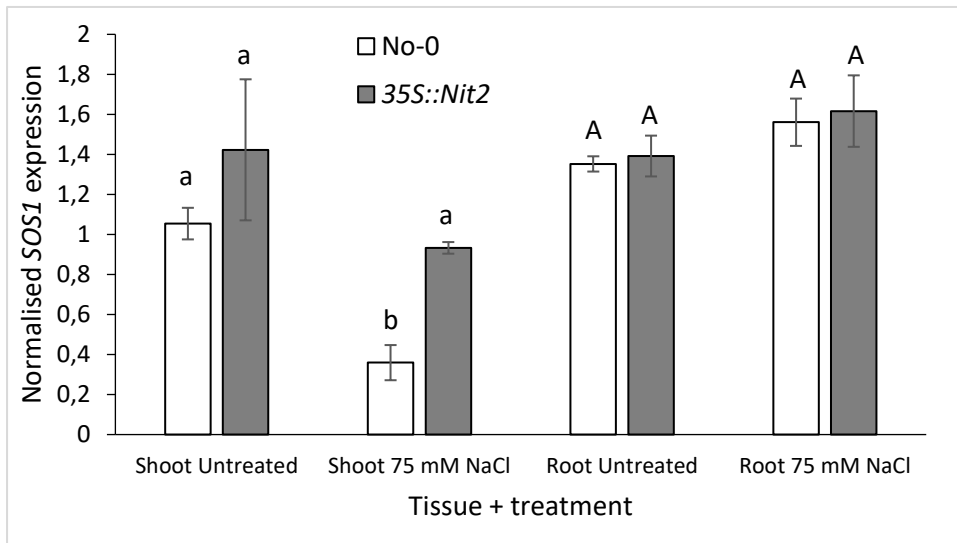
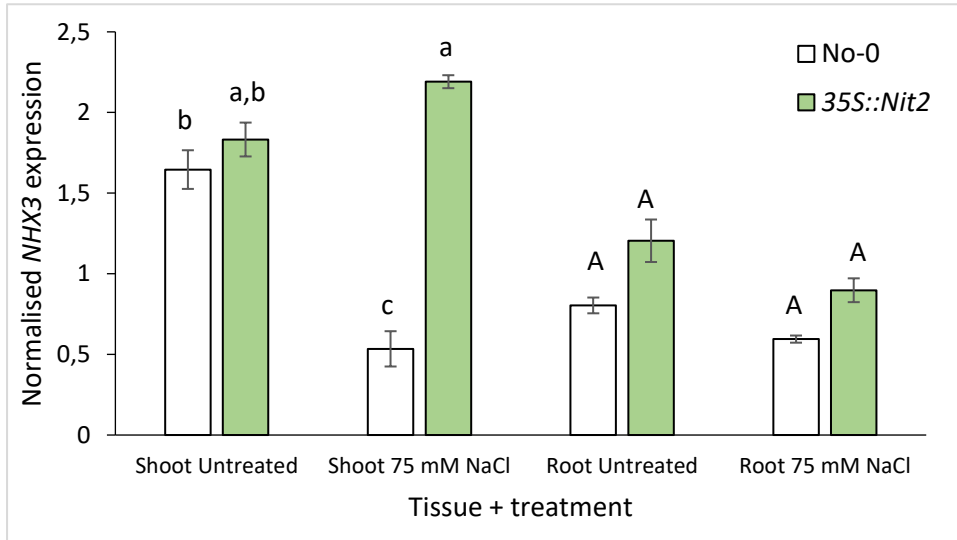
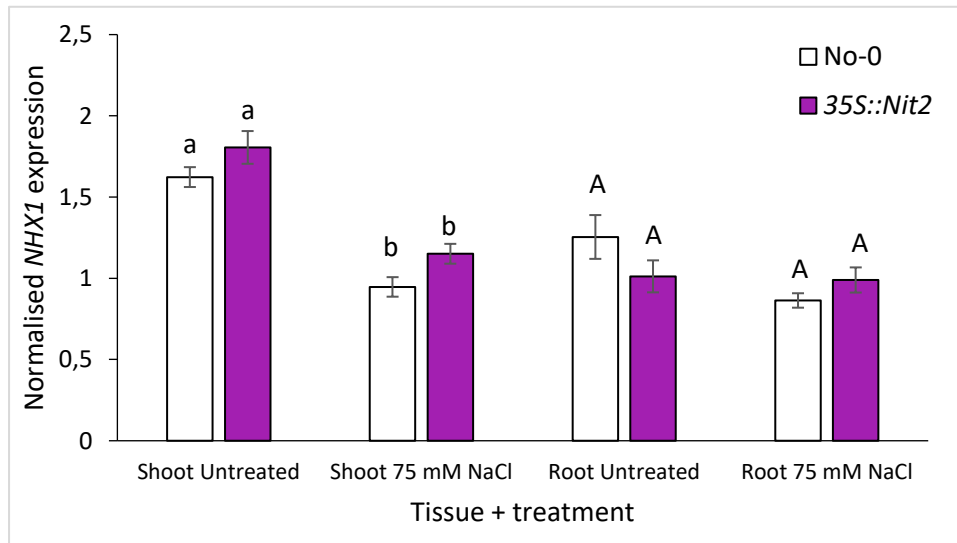
Expression of the cation transporter, *GLR2.3* was detected only in the roots (Figure 2.2 E). Here, both genotype ($p < 0.001$) and salt treatment ($p = 0.001$) was found to have a significant effect on *GLR2.3* expression, and there was a significant interaction between these two effects

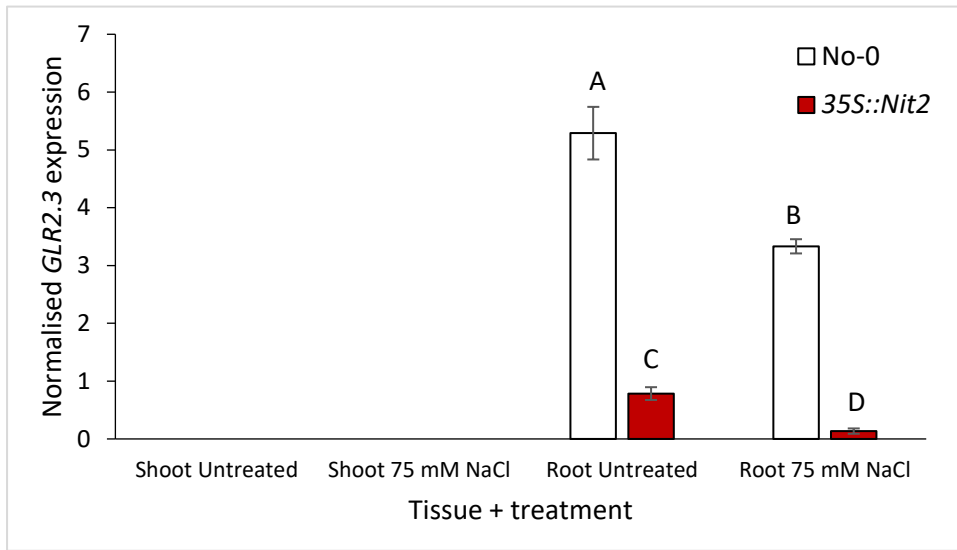
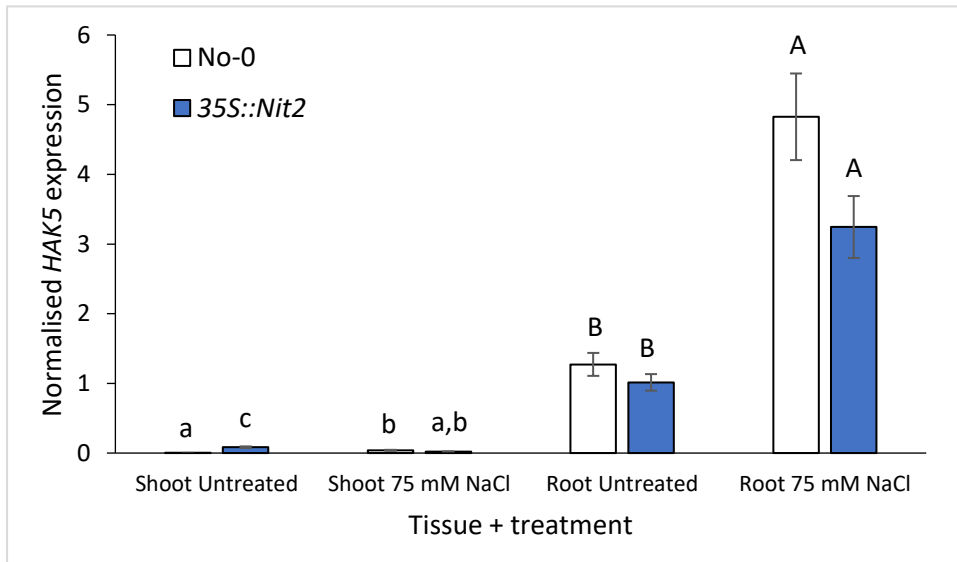
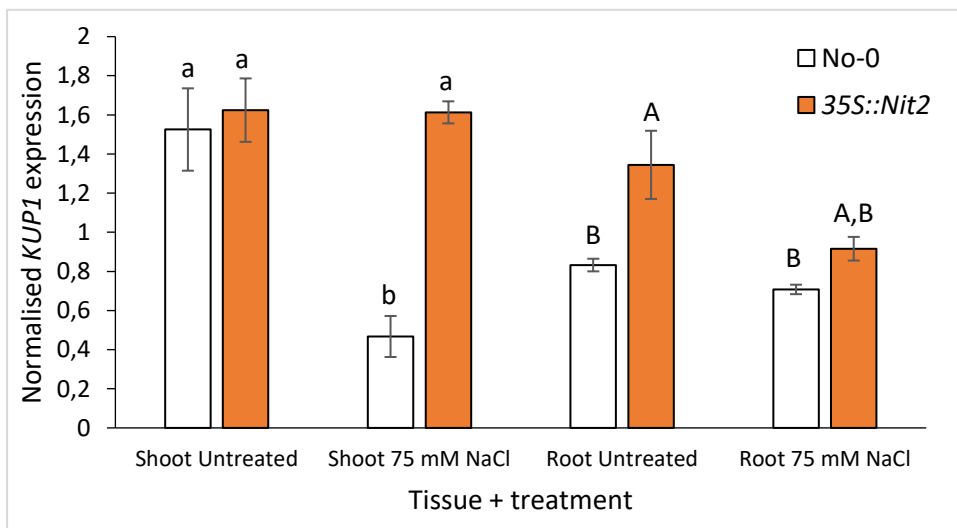
($p = 0.027$). Salt treatment caused a significant decrease in expression in both No-0 and the 35S::*NIT2* line. When comparing the two lines, *GLR2.3* expression was significantly decreased in the 35S::*NIT2* line compared with No-0 under both untreated and salt-stress conditions (~7-fold and ~24-fold, respectively).

In summary, both *HKT1;1* and *GLR2.3* were found to be root specific, and their expression decreased in response to salinity stress. For both genes, expression was significantly higher in No-0 under control conditions, however, *HKT1;1* expression was not regulated in response to NaCl in the 35S::*NIT2* line, and as a result its expression was greater than that of No-0, whereas *GLR2.3* expression was significantly lower in the 35S::*NIT2* line under saline conditions. Both *SOS1* and *NHX3* were down-regulated in response to NaCl in the shoot in No-0, but had maintained expression in the 35S::*NIT2* line, resulting in higher expression in the 35S::*NIT2* line under saline conditions. Collectively, the 35S::*NIT2* line had greater root *HKT1;1* expression, greater shoot *SOS1* and *NHX3* expression, and lower root *GLR2.3* expression than No-0 under saline conditions. Perhaps the most striking observation made was that *HKT1;1*, *SOS1*, and *NHX3* expression appeared to be mis-regulated in the root (*HKT1;1*) or shoot (*SOS1* and *NHX3*) of the 35S::*NIT2* line. All three of these genes were down-regulated in the respective tissues in response to salt in No-0, but were not responsive in the 35S::*NIT2* line.

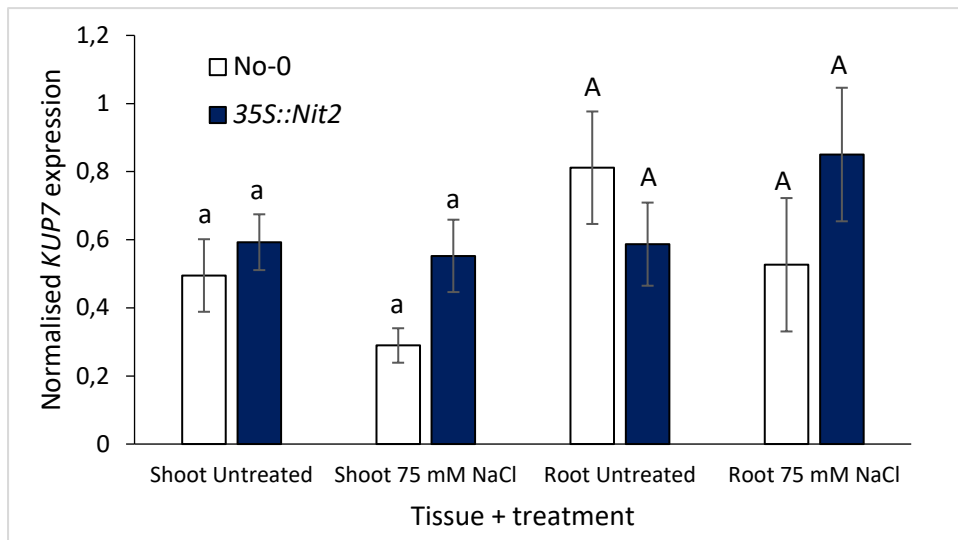
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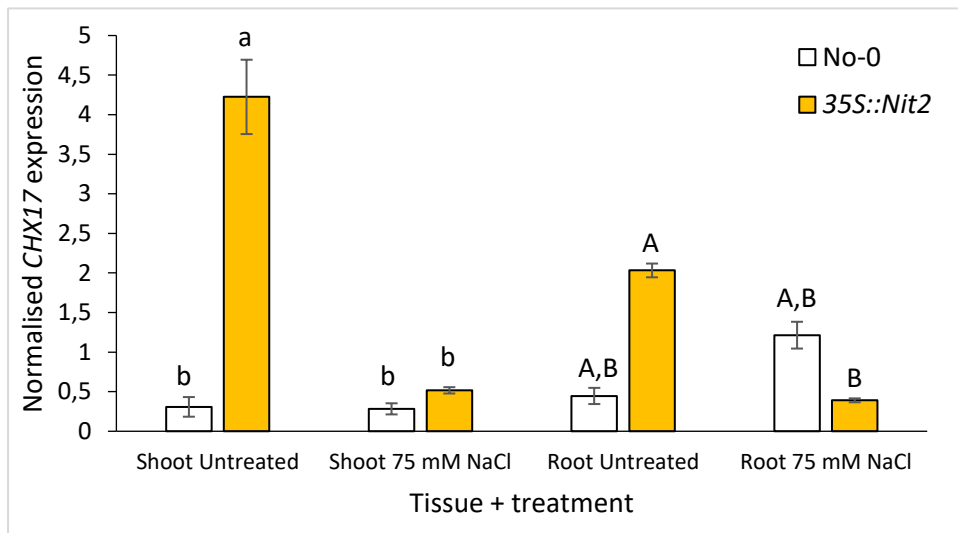
B**C****D**

E**F****G**

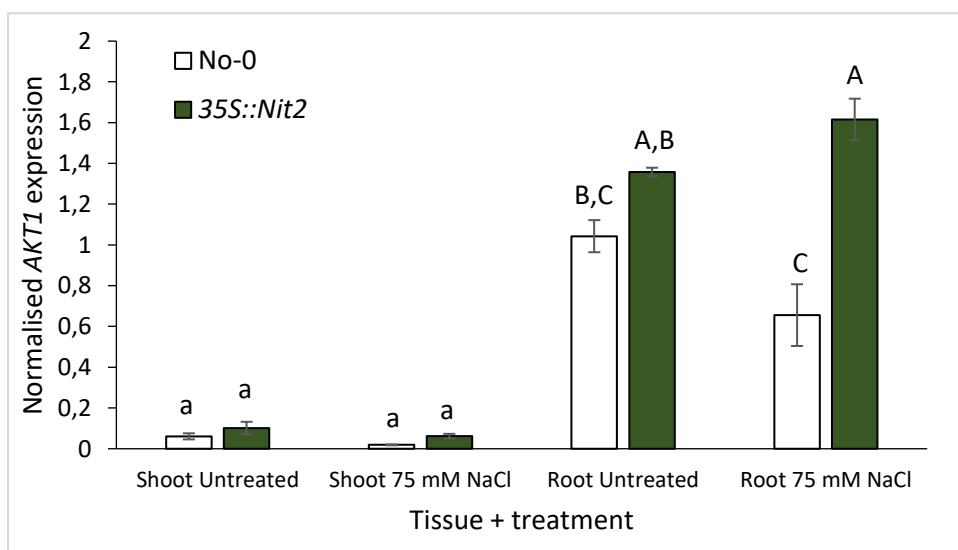
H

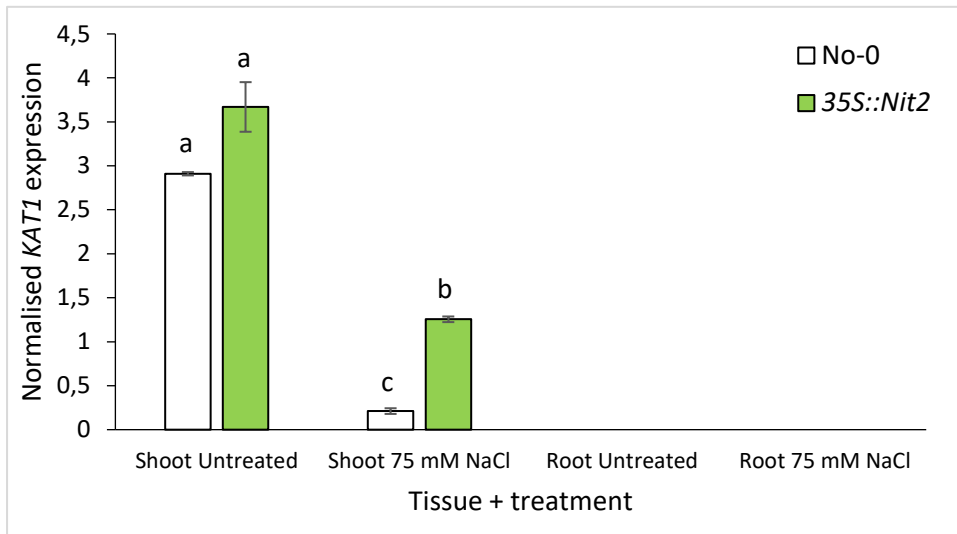
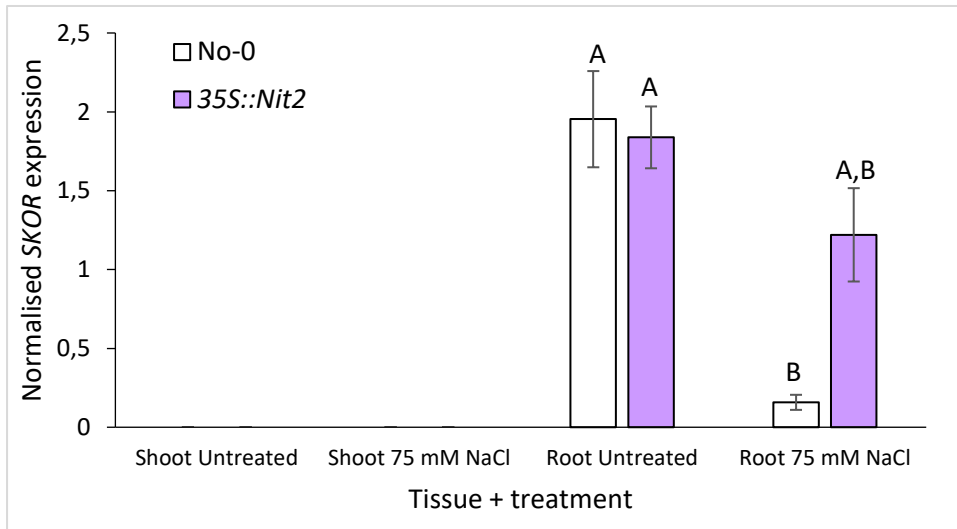
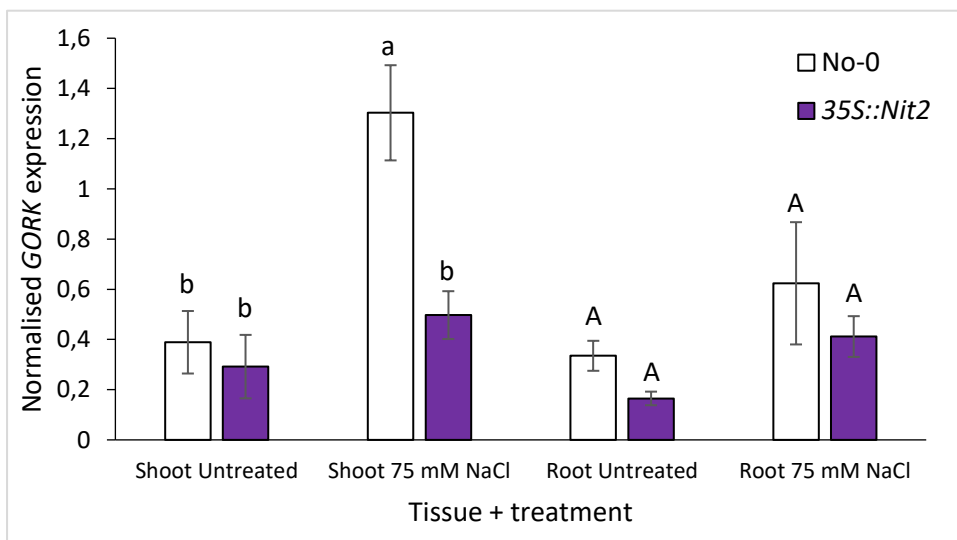


I



J



K**L****M**

N

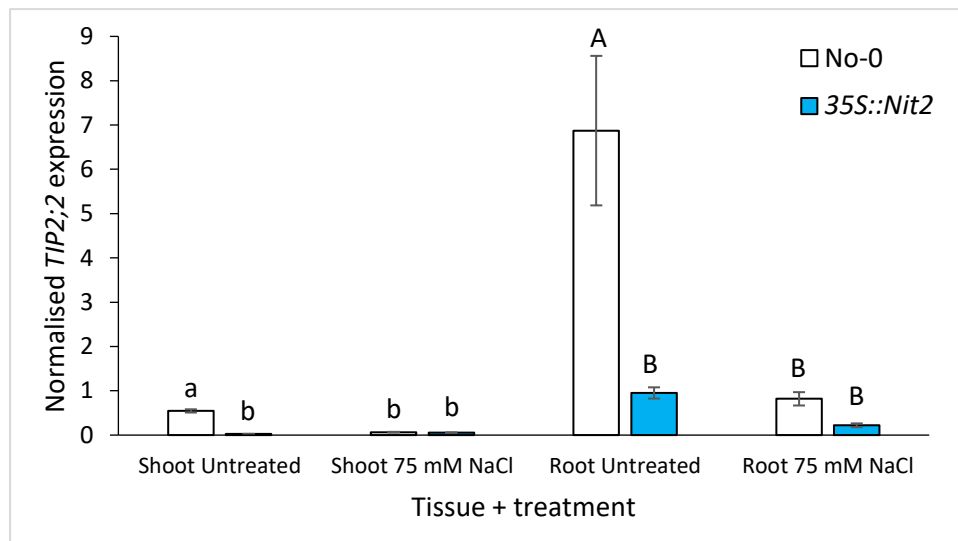


Figure 2.2: Ion channel expression in root and shoot tissue of 35S::NIT2 and No-0 plants exposed to salt stress

Gene expression was measured by RT-qPCR using the qPCR primers in Table 2.1, on cDNA synthesised from RNA extracted from No-0 and 35S::NIT2 plants that were grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 75 mM NaCl, or maintained on ¼ strength PN media (untreated control) for one week. Expression was normalised to the reference gene *MON1*. (A) *HKT1;1*, (B) *SOS1*, (C) *NHX3*, (D) *NHX1*, (E) *GLR2.3*, (F) *HAK5*, (G) *KUP1*, (H) *KUP7*, (I) *CHX17*, (J) *AKT1*, (K) *KAT1*, (L) *SKOR*, (M) *GORK*, (N) *TIP2;2* in roots and shoots of No-0 and 35S::NIT2 plants grown in untreated conditions, or following 75 mM NaCl treatment. Root and shoot tissue from four plants for each treatment from both genotypes was harvested and pooled separately. The experiment was repeated three times to produce three biological replicates (n = 3). The letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Bonferroni post-hoc analysis following a two-way ANOVA. Separate two-way ANOVAs were performed on the shoot and root data with lower case letters (i.e. a) indicating the shoot analysis and upper case letters (i.e. A) indicating the root analysis. Error bars indicate standard error.

2.3.2.2. Expression of K^+ transporters

The expression of both high- and low-affinity plasma membrane K^+ transporters, as well as the cation:proton antiporter, *CHX17*, was measured. The expression of three KUP/HAK/KT family high-affinity transporters, namely *HAK5*, *KUP1*, and *KUP7* was measured. In general, very low levels of *HAK5* expression were detected in the shoot, whereas much higher expression was observed in the root (Figure 2.2 F). While there was a significant effect of genotype ($p = 0.003$) on shoot *HAK5* expression, as well as a significant interaction between genotype and salt treatment ($p < 0.001$), the importance of this finding seems limited given the low expression levels. In the root, where *HAK5* expression was higher, there was a significant effect of salt treatment on expression ($p < 0.001$), although there was no genotype effect ($p = 0.058$), and no

interaction between genotype and salt treatment ($p = 0.132$). As such, salt treatment resulted in significantly increased root *HAK5* expression in both lines. However, no difference in *HAK5* expression was observed between the two lines in either condition.

The expression of *KUP1* in both the shoot and the root (Figure 2.2 G) was significantly affected by genotype ($p = 0.003$ and $p = 0.005$, respectively) and salt treatment ($p = 0.006$ and $p = 0.019$, respectively). However, there was only a significant interaction between the two effects when considering *KUP1* expression in the shoot ($p = 0.007$) and not in the root ($p = 0.146$). Here, the expression of *KUP1* was significantly decreased in response to salt treatment in the shoots in No-0. By contrast, salt-treatment had no effect on shoot *KUP1* expression in the 35S::*NIT2* line, the result of which is that the 35S::*NIT2* line had significantly higher shoot *KUP1* expression than No-0 under saline conditions, with no difference observed between the two lines in the control. In the roots, salt treatment had no effect on *KUP1* expression in either line. No difference in expression was observed between the two lines in saline conditions; however, there was slightly increased expression in the 35S::*NIT2* line under control conditions.

In both the shoot and the root, there was no effect of genotype ($p = 0.078$ and $p = 0.781$, respectively) or salt treatment ($p = 0.206$ and $p = 0.951$, respectively) on *KUP7* expression (Figure 2.2 H), and there was also no interaction between these two effects ($p = 0.382$ and $p = 0.151$, respectively). As such, salt treatment had no effect on *KUP7* expression in the root or shoot of either of the two lines. Furthermore, no difference in *KUP7* expression was observed between the two lines in either the root or shoot under both saline and control conditions.

For the cation:proton antiporter *CHX17* (Figure 2.2 I), there was a significant effect of both genotype ($p < 0.001$) and salt treatment ($p < 0.001$) on expression in the shoot, as well as a significant interaction between the two effects ($p < 0.001$). In control conditions, *CHX17* expression was found to be significantly higher (~13,5-fold) in the shoot of the 35S::*NIT2* line compared with No-0. However, salt-treatment resulted in a significant decrease in *CHX17* expression in the 35S::*NIT2* line, which, combined with the fact that *CHX17* expression was not affected by salt treatment in No-0 shoots, resulted in no difference between the two lines under saline conditions. However, in the root, genotype ($p = 0.495$) and salt treatment ($p = 0.37$) had no effect on *CHX17* expression.

The expression of four shaker-type potassium channels involved in low-affinity transport was also measured: two hyper-polarisation-activated inward-rectifying channels, *AKT1* and *KAT1*, as well as the depolarisation-activated outward-rectifying channels, *SKOR* and *GORK*. In general, very low levels of *AKT1* expression were observed in the shoots, whereas in the roots, expression was much higher (Figure 2.2 J). In the shoots, there was no effect of genotype ($p =$

0.059) or salt treatment ($p = 0.054$) on *AKT1* expression, and no interaction between these two effects ($p = 0.949$). As a result, there was no difference in shoot *AKT1* expression for each line in response to salt, and no expression difference was observed between the two lines in either treatment. In contrast, in the roots where *AKT1* expression was much higher, there was a significant effect of genotype on *AKT1* expression ($p < 0.001$), as well as a significant interaction between genotype and salt treatment ($p = 0.012$), although there was no significant effect of salt treatment ($p = 0.535$). As a result, no difference in expression was observed in the roots between the two lines under control conditions, but in response to salt-treatment, a slight decrease in expression was observed in No-0, and a slight increase was observed in the 35S::*NIT2* line (although neither of these differences were significant), such that *AKT1* expression was significantly higher in the roots of the 35S::*NIT2* line than No-0 under saline conditions.

As expected for a guard cell-specific gene, *KAT1* expression was only detected in the shoots, and was not detected in the roots in either treatment (Figure 2.2 K). Here, there was a significant effect of both genotype ($p = 0.001$) and salt treatment ($p < 0.001$) on *KAT1* expression. There was no significant interaction between the two effects ($p = 0.415$). As such, salt treatment resulted in a significant decrease in *KAT1* expression in the shoots of both No-0 and the 35S::*NIT2* line. However, *KAT1* expression did not decrease as much in the 35S::*NIT2* line, which was found to have significantly higher expression compared with No-0 in saline conditions. With no significant difference observed between the two lines in control conditions, the implication is that the 35S::*NIT2* line is better able to maintain *KAT1* expression under saline conditions.

SKOR expression was only detected in the roots (Figure 2.2 L). The effect of salt treatment on *SKOR* expression was significant ($p = 0.001$), but the effect of genotype was not ($p = 0.079$). Additionally, there was a significant interaction between genotype and salt treatment ($p = 0.037$). Under control conditions, no difference in *SKOR* expression was observed between the two lines. In response to salt treatment, *SKOR* expression in No-0 was found to be significantly decreased, whereas in the 35S::*NIT2* line, *SKOR* expression was maintained, resulting in a significantly higher level of expression when compared with No-0 under salt stress conditions. Referring to the *GORK* expression data (Figure 2.2 M), there was a significant effect of both genotype ($p = 0.011$) and salt treatment ($p = 0.004$) on shoot expression, as well as a significant interaction between the two effects ($p = 0.033$). The expression of *GORK* was significantly increased in response to salt treatment in No-0 shoots. By contrast, *GORK* expression was unaffected in 35S::*NIT2* shoots, and as such, *GORK* expression was significantly higher in the

shoots of No-0 than in the 35S::*NIT2* line under saline conditions. Under control conditions, no difference in *GORK* expression was observed in the shoots between the two lines. Unlike what was observed in the shoots, there was no significance in any of the three analyses performed on the root *GORK* expression data ($p = 0.187$, $p = 0.078$, and $p = 0.877$, respectively). As such, salt treatment had no effect on *GORK* expression in either line, and no difference in expression was observed between the two lines under either control or saline conditions.

In summary, no difference in the expression of the high-affinity K^+ transporters, *HAK5*, *KUP1*, or *KUP7* was observed between the two lines in the roots under either untreated or saline conditions. Of these three, only *KUP1* was found to be differentially expressed between the two lines in the shoots, where the 35S::*NIT2* line was found to maintain a higher level of expression than No-0 under saline conditions. In terms of low-affinity K^+ transporters, a higher level of expression of the inward-rectifying channel, *AKT1*, was observed in the roots of the 35S::*NIT2* line under saline conditions, with no difference observed between the two lines in the control. Salt-treatment resulted in a significant decrease in the expression of the outward-rectifying channel, *SKOR*, in the roots of No-0, but expression in the 35S::*NIT2* line was maintained, resulting in higher expression under saline conditions. In the shoots, salt-treatment caused a significant decrease in the expression of the inward-rectifying channel, *KAT1*, and an increase in the outward-rectifying channel, *GORK*, in No-0, while in the 35S::*NIT2* line, the same level of *GORK* expression observed under control conditions was maintained, and a lesser reduction in *KAT1* expression was observed. This resulted in significantly higher *KAT1* expression, and significantly lower *GORK* expression in the 35S::*NIT2* line under saline conditions. Finally, as seen for several of the Na^+ transporters, the observation was made again that the expression of a number of K^+ transporters/channels was mis-regulated in both the root and the shoot of the 35S::*NIT2* line. *SKOR* and *AKT1* expression in the root, along with *KUP1*, *KAT1*, and *GORK* expression in the shoot, was not salt-responsive in the 35S::*NIT2* line, whereas it was in No-0.

2.3.2.3. Expression of other transporters

The expression of the tonoplast aquaporin, *TIP2;2*, was also measured, as it has previously been linked with a role in salinity tolerance, and was found to be differentially expressed in the transcriptomics experiment comparing No-0 and 35S::*NIT2* seedlings (Boursiac et al., 2005; Cackett, 2019). Referring to Figure 2.2 N, for both the shoot and the root, there was found to be a significant effect of genotype ($p < 0.001$ and $p = 0.005$, respectively) and salt treatment (p

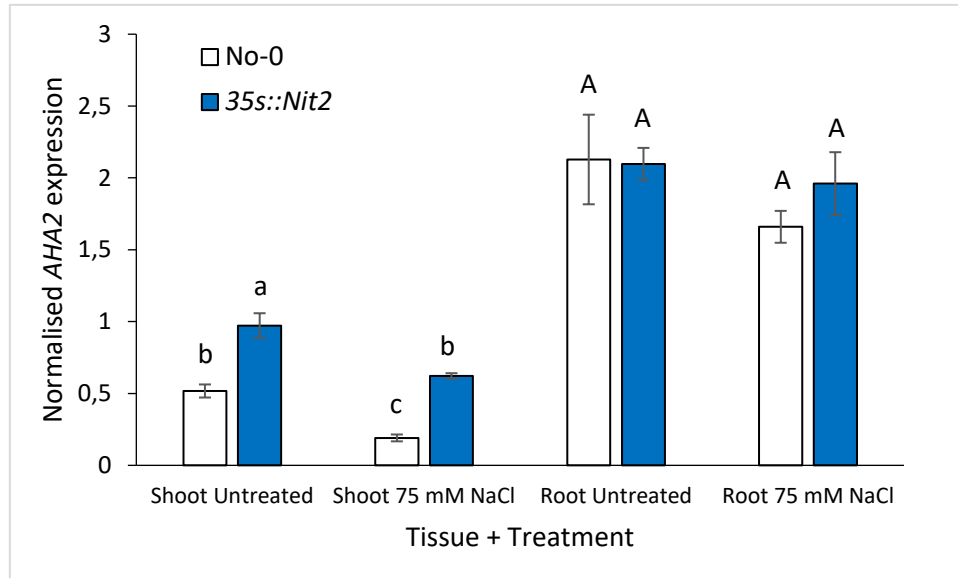
< 0.001 and $p = 0.004$, respectively) on *TIP2;2* expression, as well as a significant interaction between these two effects ($p < 0.001$ and $p = 0.014$, respectively). Here, *TIP2;2* was found to be significantly decreased in response to salt treatment in both the root and shoot of No-0, but not in either tissue in the 35S::*NIT2* line. In control conditions, *TIP2;2* expression was significantly higher in both the shoot and root of No-0 compared with the 35S::*NIT2* line, but as a result of the decrease in expression in response to salt treatment observed for No-0, no difference in expression was observed between the two lines in either tissue under saline conditions.

2.3.3. 35S::*NIT2* plants have increased plasma membrane H^+ -ATPase expression in the shoots under both salt stress and control conditions

Given the importance of the plasma membrane H^+ -ATPase in generating the proton motive force to allow for the transport of Na^+ and K^+ across the plasma membrane via H^+ exchangers, as well as its role in auxin-induced expansion growth, it was logical to assess any differences in gene expression that might exist between the 35S::*NIT2* and No-0 lines (Falhof et al., 2016; Assaha et al., 2017). Expression of the two predominant isoforms, *AHA1* and *AHA2* (*Autoinhibited H^+ -ATPase 1/2*), was measured by RT-qPCR in the same cDNA utilised for the transporter expression analysis (section 2.3.2). As before, two-way ANOVAs were performed separately on the shoot and root data to assess the effect of both genotype and salt treatment on gene expression, and the interaction between these two effects. There was a significant effect of both genotype ($p < 0.001$) and salt treatment ($p < 0.001$) on shoot *AHA2* expression, but no interaction between these two effects ($p = 0.819$). Referring to Figure 2.3 A, *AHA2* expression was found to be significantly increased in the shoots of the 35S::*NIT2* line compared with No-0 under both untreated and salt stress conditions. Additionally, *AHA2* expression was found to be significantly decreased in response to salt treatment in both No-0 and 35S::*NIT2* shoots. However, there was no significant effect of genotype ($p = 0.531$) or salt treatment ($p = 0.18$) on root *AHA2* expression, or a significant interaction between the two ($p = 0.444$), with no differences in expression observed between the two lines under either of the two conditions. It should be noted though that *AHA2* was expressed at a higher level in the roots in both lines when compared with the shoots. For the other predominant isoform, *AHA1*, no difference in expression was observed between the two lines in either the shoot or the root under saline and control conditions (Figure 2.3 B). Furthermore, salt treatment also had no effect on *AHA1* expression in the shoot or root in both lines, with the apparent reduction in expression in No-0 shoots and roots not being found to be statistically significant. Here, for both the shoot and the

root, there was no significant effect of genotype ($p = 0.056$ and $p = 0.337$, respectively) or salt treatment ($p = 0.051$ and $p = 0.129$, respectively) on *AHA1* expression, and no interaction between these two effects ($p = 0.278$ and $p = 0.053$, respectively).

A



B

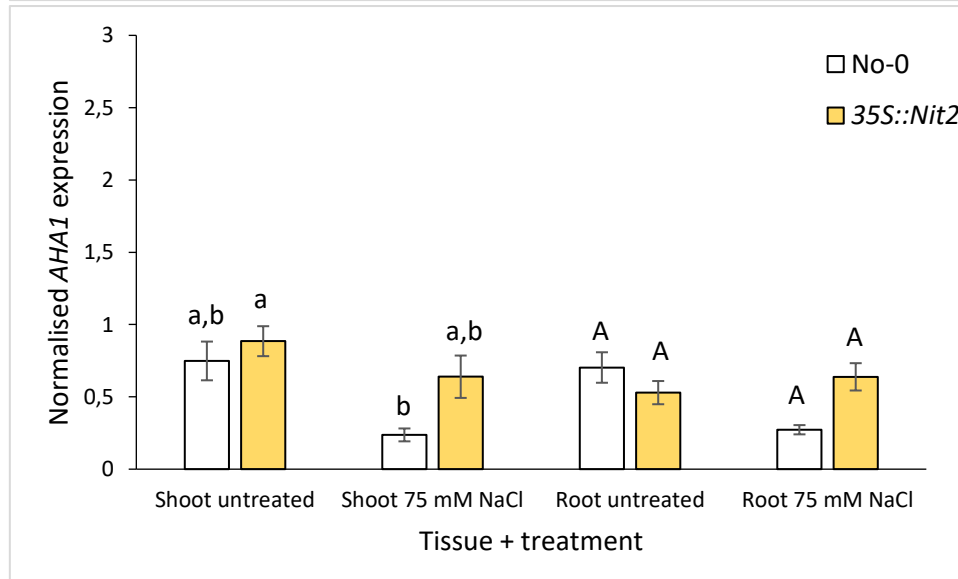


Figure 2.3: *AHA2* and *AHA1* expression in root and shoot tissue of 35S::NIT2 and No-0 plants exposed to salt stress

Gene expression was measured by RT-qPCR using cDNA synthesised from RNA extracted from the roots and shoots of No-0 and 35S::NIT2 plants that were grown hydroponically for three weeks on $\frac{1}{4}$ strength PN media, then, either treated with $\frac{1}{4}$ strength PN media supplemented with 75 mM NaCl, or maintained on $\frac{1}{4}$ strength PN media (untreated control) for one week. Expression was normalised to the reference gene *MON1*. **(A)** *AHA2* expression **(B)** *AHA1* expression in roots and shoots of No-0 and 35S::NIT2 plants grown in untreated conditions, or following 75 mM NaCl treatment. Root and shoot tissue from four plants for each treatment from both genotypes was harvested and pooled separately. The experiment was repeated three times to produce three biological replicates ($n = 3$). The letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values

as determined by Bonferroni post-hoc analysis following a two-way ANOVA. Separate two-way ANOVAs were performed on the shoot and root data with lower case letters (i.e. a) indicating the shoot analysis and upper case letters (i.e. A) indicating the root analysis. Error bars indicate standard error.

2.4. Discussion

2.4.1. The 35S::*NIT2* line displays alterations in ion homeostasis beneficial for salt tolerance

The results of the ICP-OES analysis show that the 35S::*NIT2* line has altered Na⁺ and K⁺ ion homeostasis compared with No-0, under both control and salt-stress conditions, and importantly, these alterations are beneficial for salt tolerance (Figure 2.1). Most notably, under saline conditions, the 35S::*NIT2* line was much better able to limit the accumulation of Na⁺ within the sensitive shoot tissue than No-0, and instead maintained significantly more Na⁺ in the roots. In terms of salinity tolerance, this has two effects; firstly, the toxic effect of Na⁺ accumulation within the photosynthetically active aerial tissues of the plants is reduced (Munns & Tester, 2008). While the mechanistic basis of Na⁺ toxicity and the specific cellular targets affected by Na⁺ remain unknown, the most common explanation is that Na⁺ has an inhibitory effect on enzymes, thereby interfering with primary metabolic processes in the cytosol, such as the Calvin cycle, glycolysis and starch synthesis, as well as potentially affecting the chloroplastic photosynthetic machinery (Munns & Tester, 2008; Zhao et al., 2020). Secondly, maintaining the Na⁺ within the roots allows for its active extrusion back into the surrounding soil solution via the SOS1 Na⁺/H⁺ antiporter (Shi et al., 2000, 2002). However, if the extrusion of Na⁺ was increased in the 35S::*NIT2* line, I would perhaps expect to see a lower root Na⁺ content. Given that this was not observed, a possible explanation is that the excess Na⁺ can be used as vacuolar osmoticum in the expanding cells in the elongation zone of the roots of the 35S::*NIT2* line. Na⁺, along with other inorganic ions, has been shown to play an important role in turgor regulation in osmotically stressed *Arabidopsis* root epidermal cells, and it has been suggested that Na⁺, K⁺, and Cl⁻ are the major contributors to maintenance of cell turgor during osmotic adjustment (Shabala & Lew, 2002; Shabala & Shabala, 2011). Furthermore, the observation that the total Na⁺ content within the whole plant was significantly reduced in the 35S::*NIT2* line compared with No-0, strongly suggests that it has an improved ability to limit Na⁺ uptake via the roots, especially given that the contribution of Na⁺ extrusion from the roots is unclear. In addition to improved Na⁺ homeostasis, the 35S::*NIT2* line also maintained significantly higher K⁺ in the roots, as well as increased whole plant total K⁺ content, compared

with No-0 under saline conditions. This suggests that it is either better able to limit the detrimental loss of K^+ into the surrounding solution via plasma-membrane KOR channels following the membrane depolarisation that occurs due to Na^+ uptake under saline conditions (Shabala et al., 2005; Shabala & Cuin, 2007), or that it is better able to maintain K^+ uptake (or perhaps both of these things). Ultimately, the ion homeostasis is altered in such a way that the 35S::*NIT2* line has improved (lower) Na^+/K^+ ratios in both the root and the shoot under saline conditions when compared with No-0. This finding agrees with the large body of evidence associating reduced Na^+/K^+ ratios with improved salinity tolerance. Another interesting finding is that the pattern of reduced shoot, and increased root Na^+ content in the 35S::*NIT2* line that is observed under saline conditions, also occurred in untreated conditions, suggesting possibly that the 35S::*NIT2* line has constitutively active mechanisms for beneficial Na^+ partitioning that pre-disposes it to tolerate salt stress.

2.4.2. The 35S::*NIT2* line has altered expression of Na^+ and K^+ transporters in the root and shoot

It is necessary to preface the discussion surrounding the effect that differences in expression of the various ion transporters and channels may have on Na^+/K^+ homeostasis, by acknowledging that the abundance of these transporters was only measured at the transcript level. The protein levels or activity of the various transporters was not measured, and it is understood that changes in transcript abundance do not report directly on either of these things. In this instance, transcript levels were used as a proxy to infer effects on ion transport. In some instances, increased transcription is required to make more protein, and presumably alterations in protein synthesis would occur as the levels of active transporter/channel proteins change in response to salt.

2.4.2.1. Na^+ transport

HKT1;1 was the most interesting of the Na^+ transporters that was found to be differentially expressed in the transcriptomics experiment comparing salt-stressed No-0 and 35S::*NIT2* seedlings (Cackett, 2019), due to its widely established role in xylem Na^+ retrieval in root XPCs, and thus importance in regulating root to shoot Na^+ transport and protecting the aerial tissues of the plant from salinity stress (Mäser et al., 2002; Sunarpi et al., 2005). As anticipated, I observed a difference in *HKT1;1* expression in the roots between the 35S::*NIT2* and No-0 lines under both saline and untreated conditions. What was surprising was that under control conditions, No-0 had significantly higher *HKT1;1* expression than the 35S::*NIT2* line, with the

opposite observed in response to salt-treatment. The increased *HKT1;1* expression in the roots of the 35S::*NIT2* line helps to explain the altered Na^+ partitioning observed under saline conditions; increased retrieval of Na^+ from the xylem would increase the amount of Na^+ maintained in the roots, while also reducing the amount of Na^+ transported to the shoots, thus limiting shoot Na^+ accumulation, and aiding in salinity tolerance. The observation of higher levels of *HKT1;1* expression in No-0 roots under untreated conditions remains unexplained. Previous studies have detected *HKT1;1* expression in the shoot, (Mäser et al., 2002; Berthomieu et al., 2003; Sunarpi et al., 2005), however, I measured very low levels of expression in both conditions, with no difference in expression between the two lines in either. The *HKT1;1* promoter-*GUS* expression analyses performed in these previous studies showed *HKT1;1* to be widely expressed throughout the shoot vasculature, so it seems unlikely that the low levels of detection I observed can be attributed to having measured expression in the whole shoot, as opposed to in a more tissue-specific manner – although this possibility cannot be precluded. The low shoot expression I observed could possibly be explained by ecotype-specific differences in *HKT1;1* expression patterns between the wild-type used in this study, No-0, and those used in these previous studies, Col-0 and Ws-0. The expression of *HKT1;1* has been shown to be differentially regulated across four different ecotypes (Col-0, Ws-0, Ler-0 and C24), with the ecotypes with the highest root *HKT1;1* expression levels (Col-0 and Ws-0) also being found to have lower shoot Na^+ accumulation under saline conditions (Jha et al., 2010). That study also reported much lower levels of *HKT1;1* expression in the shoot of the four ecotypes than the root, however, no correlation was observed between the expression of *HKT1;1* in the shoot and shoot Na^+ accumulation, suggesting that it is root *HKT1;1* expression that is more important for determining salinity tolerance. No comparative studies of tissue-specific *HKT1;1* expression have been performed involving No-0, but our findings of lower shoot expression agree with the findings of the study comparing expression in the other ecotypes (Jha et al., 2010).

Perhaps the most discussed Na^+ transporter in the literature is the plasma membrane Na^+/H^+ antiporter, *SOS1*, which helps to limit intracellular Na^+ levels via active extrusion thereof into the apoplastic/extracellular space (Shi et al., 2000). Traditionally, the role of *SOS1* in Na^+ transport has been discussed in the context of the root, as it has been shown to be expressed in the root tip epidermal cells, where it is thought to extrude Na^+ into the surrounding soil solution, as well as being expressed in XPCs, where it is thought to play a role in xylem Na^+ loading (Shi et al., 2002). I did not observe any differences in *SOS1* expression in the roots between

the 35S::*NIT2* line and No-0 in either untreated or saline conditions, so it is unclear whether the differences in the root/shoot Na^+ partitioning observed between the two lines can be attributed to increased Na^+ extrusion or xylem Na^+ loading via SOS1 in the root. However, I measured expression in the whole root, not within the individual tissue types, so it could be possible that *SOS1* was differentially expressed only in the root tips, and that did not reflect when looking at expression levels in the whole root – a more detailed, tissue-specific measurement of all these transporters would be a good avenue for future research. Additionally, the possibility does exist that, while there is no difference in *SOS1* gene expression, there could be a difference in SOS1 protein abundance, or perhaps a difference in the activity of SOS1 as a result of a change in phosphorylation status due to altered expression or inhibition of the other SOS-signalling pathway genes between the two lines; for example, SOS2 (kinase that activates SOS1 via phosphorylation) (Halfter, Ishitani & Zhu, 2000) or SOS3 and SCABP8 (which relay salt-induced Ca^+ signals to SOS2) (Lin et al., 2009). While SOS1 is usually discussed for its role in the root, its Na^+ extrusion activity presumably occurs in all tissues in which it is expressed, including the shoot. Not only did I observe *SOS1* expression levels in the shoot that were similar to those seen in the root, I found that the 35S::*NIT2* line displayed increased *SOS1* expression compared with No-0 under saline conditions. This could suggest an increased Na^+ extrusion via shoot tissues, however this poses the question of where the Na^+ is extruded to? *SOS1* expression has been widely reported in the vasculature of many aerial tissues, and has particularly been detected in XPCs (Shi et al., 2002). This could suggest that Na^+ is extruded back into the xylem from the shoot XPCs. However, a clear role for SOS1 in the shoots has not been fully elucidated, as is the case for its role in Na^+ extrusion from the root tip. It is possible that its function in Na^+ transport in the shoot may vary in different tissues, and at different salt concentrations – a notion that has been discussed previously based off studies of SOS1 in tomato (Olías et al., 2009). In order to further explore this notion, it would be necessary to measure *SOS1* expression in a tissue-specific manner (as alluded to earlier), as opposed to in the entire shoot. Additionally, crossing the 35S::*NIT2* line with a *SOS1* reporter line, such as *SOS1>::GUS* (Shi et al., 2002), would allow for visual detection of *SOS1* expression in the various shoot tissues in the 35S::*NIT2* line. It is also worth noting that the increased shoot *SOS1* expression observed in the 35S::*NIT2* line compared with No-0 under saline conditions, coincides with increased shoot *AHA2* expression. Na^+ extrusion via SOS1 is powered by the proton-motive force generated by plasma membrane H^+ -ATPases, so the observation that both of these genes have increased expression could possibly suggest that increased shoot to root Na^+ transport via the xylem might underlie the decreased shoot Na^+ content observed in the

35S::*NIT2* line under saline conditions. However, the strength of this assertion is somewhat limited given that it is based on the similarity in expression patterns of the two genes.

Aside from extrusion of Na^+ from the cell, a key component of maintaining an optimal intracellular K^+/Na^+ ratio is the control of vacuolar K^+ and Na^+ transport achieved by the NHX-type cation/ H^+ antiporter isoforms 1/2/3/4. It is important to note that the ability of these NHXs to transport both Na^+ and K^+ makes the interpretation of their role in ion homeostasis somewhat challenging. A large body of work comparing the cation exchange affinities and growth phenotypes of all the combinations of *nhx1/2/3/4* single, double, triple, and quadruple knockout plants has revealed that the control of vacuolar K^+ and Na^+ exchange involves complex interactions between these four NHXs (Bassil et al., 2019). Furthermore, it has been suggested that the transport activity of NHX1 (and presumably the other vacuolar NHXs), could shift from favouring K^+/H^+ to Na^+/H^+ as vacuolar pH increases; and as salinity causes vacuolar alkalisation, this could represent a mechanism for Na^+ , rather than K^+ , sequestration under saline conditions (Bassil & Blumwald, 2014). Of the four isoforms, NHX1 is the most widely characterised, with suggested roles in both K^+ and Na^+ exchange under saline conditions (Apse et al., 1999; Apse, Sottosanto & Blumwald, 2003), and in K^+ exchange and vacuolar pH control during normal growth and development (Bassil et al., 2011). Furthermore, *NHX1* has the highest expression, and is expressed across most tissues of the plant (Apse, Sottosanto & Blumwald, 2003; Bassil et al., 2011). As such, it is an important isoform to consider. However, I did not observe any differences in *NHX1* expression between the 35S::*NIT2* line and No-0 under untreated or saline conditions, although both lines displayed reduced expression in the shoots in response to salt treatment. This suggests that vacuolar Na^+ or K^+ exchange via *NHX1* is not an important factor underlying the altered ion homeostasis observed between the two lines. While previous evidence had suggested that NHX3 functions in K^+/H^+ exchange during germination and early seedling development under low K^+ conditions (Liu et al., 2010), the experiments involving the *nhx1/2/3/4* single, double, triple, and quadruple knockout plants mentioned previously, revealed that *NHX3*, unlike *NHX1/2* and 4, does not mediate K^+ transport, but rather is only involved in vacuolar Na^+ transport. I observed no difference in *NHX3* expression in the shoots in untreated conditions, however, following salt treatment, *NHX3* expression was significantly reduced in No-0, whereas it was maintained in the 35S::*NIT2* line, resulting in a more than 4-fold higher expression in the 35S::*NIT2* line compared with No-0. This suggests a potentially increased ability of the 35S::*NIT2* line to sequester Na^+ into the vacuole via *NHX3*, specifically under saline conditions, which would

contribute towards maintenance of improved shoot cytosolic K^+/Na^+ ratios. Furthermore, the 35S::*NIT2* line was shown to have lower shoot Na^+ content than No-0, so an improved vacuolar sequestration ability when there is already less Na^+ present in the cytosol could well help to explain the improved salinity tolerance of the 35S::*NIT2* line.

Along with *HKT1;1*, the other putative plasma membrane Na^+ transporter that was differentially expressed in the 35S::*NIT2* and No-0 seedling microarray was *GLR2.3*. The role of *GLR2.3* (and glutamate receptors as a whole) in cation transport during salinity stress is largely uncharacterised. However, *GLR2.3* expression has been shown to be only detectable in the root, where it was down-regulated in response to salt stress, suggesting a possible function in Na^+ uptake (Maathuis et al., 2003; Maathuis, 2006). In agreement with this previous work, *GLR2.3* expression was only detected in the root of the 35S::*NIT2* line and No-0, and expression was also found to be down-regulated in response to salt stress in both lines. Assuming that *GLR2.3* does function in Na^+ uptake, the decreased *GLR2.3* expression observed in the 35S::*NIT2* line compared with No-0 under both control and saline conditions, suggests that Na^+ uptake in the roots via *GLR2.3* might be reduced, which could contribute to the improved Na^+/K^+ homeostasis displayed by the 35S::*NIT2* line during salt stress. It should be noted that *GLR2.3* expression was also found to be decreased in response to Ca^{2+} deficiency (Maathuis, 2006), and as other GLRs, such as *GLR3.2*, have been shown to play a role in Ca^{2+} homeostasis (Kim et al., 2001), it is possible that *GLR2.3* may transport Ca^{2+} as well as Na^+ . However, no difference in root Ca^{2+} levels was observed between the two lines under either control or saline conditions (see appendix chapter 7, Figure 7.1). It must, however, be noted that Ca^{2+} (and Na^+/K^+) was only measured at the whole root level, and a higher resolution measurement of exactly where the Ca^{2+} is present (cytosolic, vacuolar, or extracellular) is required to make any firm conclusions here.

Clearly, there were several Na^+ transporters whose expression changed in response to salt-treatment in both No-0 and the 35S::*NIT2* line, as well as Na^+ transporters that were salt-responsive in No-0 but not in the 35S::*NIT2* line. For a stylised depiction of the cellular compartments in the root and shoot in which these Na^+ transporters are thought to be expressed, based off the literature, refer to Figure 2.4. This figure serves also to highlight the differences in salt-responsive gene expression between No-0 and the 35S::*NIT2* line.

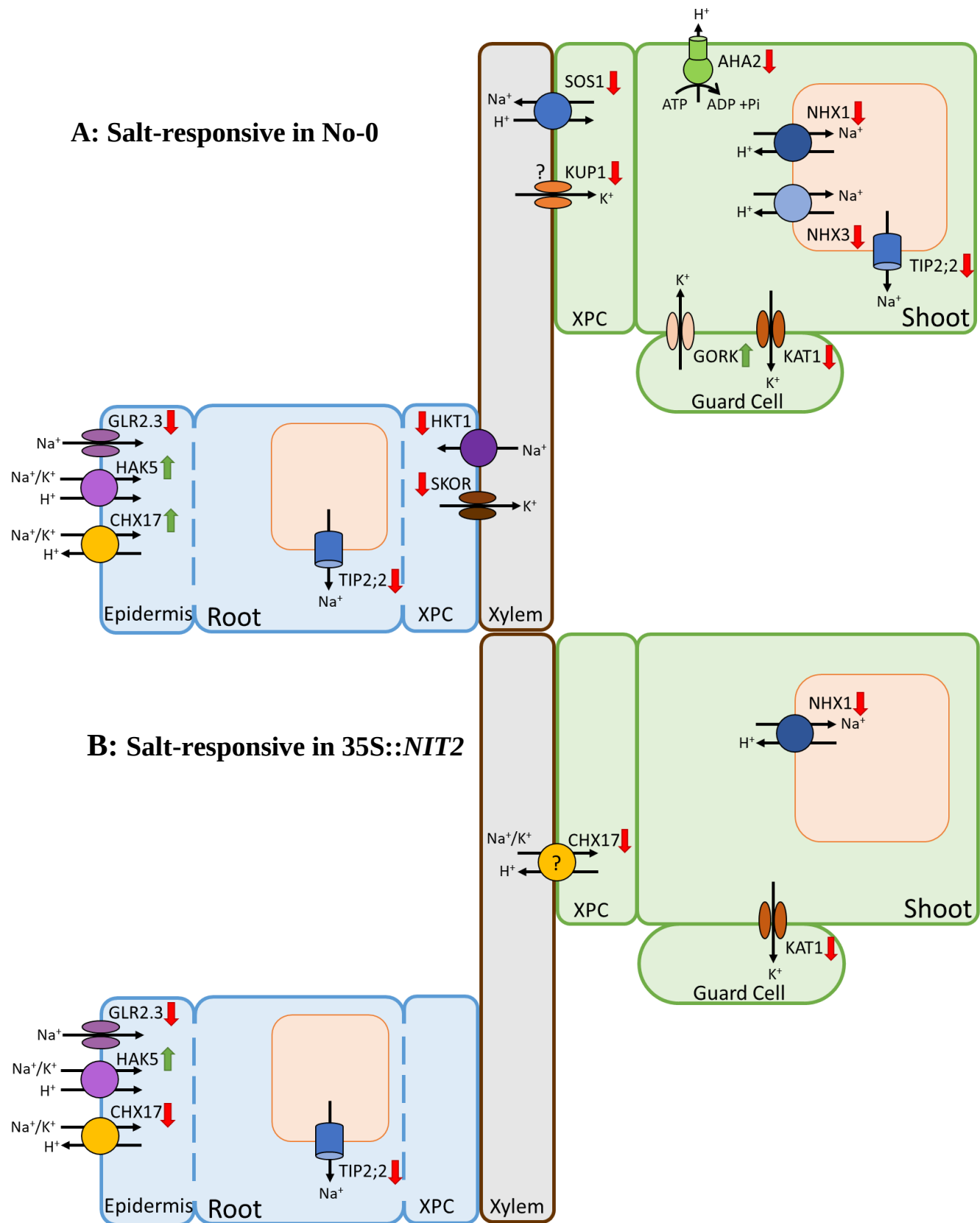


Figure 2.4: Na^+ & K^+ transporters that were differentially expressed in response to salt-treatment in No-0 and 35S::*NIT2* line

The proposed cellular compartments in the root and shoot where the various Na^+ and K^+ transporters and channels are expressed based off the literature. (A) Genes that were differentially expressed in response to salt-treatment in No-0. (B) Genes that were differentially expressed in response to salt-treatment in the 35S::*NIT2* line. The green and red arrows indicate increased or decreased expression in the 75 mM NaCl treatment compared with untreated conditions, respectively. Question marks (?) indicate transporters whose site of expression is unknown. ‘XPC’ stands for xylem parenchyma cells.

2.4.2.2. K^+ transport

The high-affinity K^+/H^+ symporter HAK5, along with AKT1, is the main avenue for K^+ uptake in the roots (Rubio, Santa-María & Rodríguez-Navarro, 2000; Nieves-Cordones et al., 2016), and in particular has been shown to be essential for K^+ uptake under saline conditions (Nieves-Cordones et al., 2010). While an increase in *HAK5* expression was observed in response to salt stress in both lines, there was no difference in expression between the lines in either treatment condition. As such, the increased K^+ observed in salt-stressed 35S::*NIT2* roots cannot be attributed to a change in *HAK5* transcription between the lines; however, I cannot preclude the possibility that alterations in *HAK5* translation, or in the stability of the HAK5 protein, may play a role in affecting K^+ transport, thus contributing to the difference in root K^+ content observed between the two lines. Interestingly, our observation of increased *HAK5* expression in response to salt stress is in contrast to previous findings of reduced expression following salt treatment (Nieves-Cordones et al., 2010), however, it makes sense that the plant would increase the expression of *HAK5* in order to improve high affinity K^+ uptake under the low K^+ availability conditions imposed by NaCl. It must also be noted that, unlike ours, the study by Nieves-Cordones et al. (2010) involved K^+ -starved plants treated with a lower NaCl concentration (30 mM). Aside from HAK5, there are several other KUP/HAK/KT family high-affinity K^+ transporters expressed in Arabidopsis roots that could play a role in K^+ uptake. While KUP7 has not directly been shown to play a role in K^+ transport under saline conditions, it has been shown to function in high-affinity K^+ uptake in the roots during K^+ -limited conditions (Han et al., 2016), and as these conditions arise due to the effect of salinity on soil K^+ availability, it is plausible that KUP7 could be important for K^+ uptake and transport during salt stress. KUP7 has been suggested as a candidate for root K^+ uptake and root xylem K^+ loading, thereby contributing towards root to shoot K^+ translocation (Han et al., 2016). However, our findings do not support this argument, as salt stress did not have any effect on *KUP7* expression in the root or the shoot of either No-0 or the 35S::*NIT2* line. Furthermore, there was no difference in *KUP7* expression between the two lines in either of the tissues under control or saline conditions. Thus the differences in K^+ partitioning between No-0 and the 35S::*NIT2* line cannot be attributed to KUP7. Another KUP family high-affinity transporter that could play a role is KUP1, which has been shown to be expressed in the root tip, root hairs, and leaves (Kim et al., 1998; Ahn, Shin & Schachtman, 2004). Interestingly, along with *HAK5*, elevated levels of *KUP1* expression, as well as improved K^+ retention, has been observed in seedlings of several naturally-occurring salt-tolerant Arabidopsis accessions (Sun et al., 2015). As previously reported, *KUP1* expression was observed in both the root and shoot. The

35S::*NIT2* line maintained a significantly higher level of *KUP1* expression in salt-stressed shoots than No-0, suggesting that K^+ uptake into the shoot tissue via *KUP1* could be maintained under saline conditions. It must, however, be noted that the exact tissue in the shoot in which *KUP1* is expressed is unknown, so precisely how it might contribute to shoot K^+ uptake is unclear. A plausible suggestion could be that it is expressed in the shoot XPC and functions in the retrieval of K^+ from the xylem, however, this would require further investigation.

The plasma membrane cation:proton (Na^+ , K^+/H^+) antiporter *CHX17*, has been suggested to play a role in root K^+ uptake and homeostasis in response to salt stress, as its expression has been shown to be highly up-regulated in the epidermal and cortical tissues of mature roots under saline conditions, and *chx17* mutants were found to accumulate less K^+ in the roots (although they did not display altered growth) (Cellier et al., 2004). *CHX17* was also found to have altered expression in the seedlings of the salt-tolerant *Arabidopsis* accessions discussed previously, as well as in the transcriptomics experiment comparing 35S::*NIT2* and No-0 seedlings under control and 100 mM NaCl conditions (Sun et al., 2015; Cackett, 2019). Contrary to the previous findings, where *CHX17* expression was induced in response to salt stress in the roots only (Cellier et al., 2004), I found *CHX17* expression to be highly expressed in both the root and the shoot in untreated conditions in the 35S::*NIT2* line, and that salt-treatment resulted instead, in a reduction in expression in both tissues. With no change in *CHX17* expression observed in response to salt in either tissue of No-0, it is difficult to surmise what effect this large reduction in expression might have on the K^+ homeostasis of the 35S::*NIT2* line.

The expression of four shaker-type potassium channels involved in low-affinity transport was also measured: two hyper-polarisation-activated inward-rectifying channels, *AKT1* and *KAT1*, as well as the depolarisation-activated outward-rectifying channels, *SKOR* and *GORK*. Along with *HAK5*, *AKT1* is largely responsible for K^+ uptake by the roots, with *HAK5* solely being responsible for high-affinity uptake at external K^+ concentrations lower than 10 μ M, both *HAK5* and *AKT1* being involved between 10 μ M and 200 μ M, and only *AKT1* being responsible for low-affinity uptake at concentrations higher than 500 μ M (Nieves-Cordones et al., 2016; Nieves-cordones et al., 2019). While high concentrations of NaCl does impose low K^+ availability on the roots, I did not exclude K^+ from the hydroponic plant nutrient media in which the plants were grown, so it should be assumed that the K^+ concentration available for uptake by the roots is in the range where low-affinity uptake via *AKT1* could occur. No difference in *AKT1* expression was observed between the two lines in the roots under control

conditions, however, in salt-stressed roots, significantly higher *AKT1* expression was observed in the 35S::*NIT2* line, due to the decreased *AKT1* expression in No-0 and increased expression in the 35S::*NIT2* line (although neither was significant) in response to salt treatment. As no difference in *HAK5* expression was observed between the two lines, these higher levels of *AKT1* expression in the 35S::*NIT2* line following salt treatment, and presumably an increase in K^+ uptake, may well explain the increased K^+ levels observed in the roots of the 35S::*NIT2* line under saline conditions. *KAT1* is an inward-rectifying K^+ channel that is expressed in guard cells, which plays a crucial role in the K^+ uptake that drives stomatal opening (Nakamura et al., 1995). While the expression of *KAT1* was decreased in the shoots of both lines in response to salt stress, the 35S::*NIT2* line maintained a significantly higher level of expression compared with No-0, suggesting that the 35S::*NIT2* line might maintain improved stomatal conductance under saline conditions. Improved stomatal conductance would allow for sustained photosynthesis, which in turn might contribute towards the improved growth observed in the 35S::*NIT2* line under saline conditions.

SKOR is expressed only in the stelar tissues of the root, including the pericycle and XPCs (Gaymard et al., 1998). *SKOR* mediates K^+ efflux from root XPCs into the xylem (Demidchik, 2014), thereby playing a role in root to shoot K^+ transport. This efflux is activated by the depolarisation of the plasma membrane induced by salinity stress, and once plasma membrane potential has been restored via increased plasma membrane H^+ -ATPase activity, this *SKOR*-mediated K^+ efflux into the xylem is suppressed (Wu et al., 2018). It was observed that the 35S::*NIT2* line maintained the same levels of *SKOR* expression in the roots under saline conditions as was observed in untreated conditions, whereas *SKOR* expression was significantly decreased in No-0. This would suggest that increased K^+ efflux into the xylem for root to shoot transport occurs in the 35S::*NIT2* line, however, this does not agree with our findings that the 35S::*NIT2* line maintains higher root K^+ than No-0 under saline conditions.

On the other hand, *GORK* is well known for mediating the depolarisation-activated K^+ efflux from guard cells that results in stomatal closure (Ache et al., 2000). Interestingly *GORK* expression was significantly increased in response to salt stress in No-0 shoots, but not in the 35S::*NIT2* line, suggesting greater stomatal closure in No-0 under salt stress. A further thing to consider is that this increased *GORK* expression in No-0 could be linked with the increased plasma membrane depolarisation that could occur in salt-stressed No-0 shoots as a result of the significantly lower *AHA2* expression than the 35S::*NIT2* line – i.e. in salt-stressed shoots, the 35S::*NIT2* line has higher *AHA2* expression, thus better ability to maintain membrane potential,

and lower *GORK* expression, thus decreased K^+ efflux in the guard cells. This observation of increased *GORK* expression in No-0 shoots, along with the finding that the 35S::*NIT2* line has increased *KAT1* expression compared with No-0 under saline conditions, suggests that the guard cells of the 35S::*NIT2* line have increased K^+ uptake via increased *KAT1* expression, and decreased K^+ efflux due to decreased *GORK* expression, thereby maintaining higher guard cell K^+ and maintaining open stomata even under salt stress. Maintenance of stomatal conductance in the 35S::*NIT2* under salt stress could be advantageous for growth as it allows for continued CO_2 uptake for photosynthesis, however, it comes at the cost of potentially increased water loss. However, none of this aids in the explanation of why No-0 has increased shoot K^+ content compared with the 35S::*NIT2* under saline conditions. This difference could possibly be attributed to a decreased ability of No-0 to recirculate K^+ from the shoot to the root in order to balance Na^+ that is taken up under saline conditions. K^+ recirculation via the phloem has been suggested to be important for the maintenance of root K^+ homeostasis under saline conditions in which root K^+ uptake is reduced (Wu et al., 2018). As mentioned previously, while phloem loading and unloading has been attributed to AKT2 (De Schepper et al., 2013; Sklodowski et al., 2017), under saline conditions where the membrane is depolarised, phloem K^+ loading for recirculation is likely attributed to other KT/KUP/HAK transporters (Wu et al., 2018). While candidates for this process, such as KZM1 (K^+ channel *Zea mays* 1) and VFK1 (*Vicia faba* K^+ channel 1), have been identified in other species (Ache et al., 2001; Philippar et al., 2003), they have yet to be identified in Arabidopsis. It is thus difficult to infer the possible contribution of phloem K^+ recirculation to the difference in shoot K^+ content observed between No-0 and the 35S::*NIT2* line under saline conditions, however, it does not exclude it. Finally, apart from its role in guard cell regulation, *GORK* also mediates the salt-stress induced efflux of K^+ from root epidermal cells (Demidchik, 2014). However, no changes in *GORK* expression were observed in the roots between the two lines in either untreated or saline conditions, so the improved K^+ maintenance observed in the roots of the 35S::*NIT2* line cannot be attributed to altered *GORK* expression.

As discussed previously for the Na^+ transporters, there were several K^+ transporters whose expression changed in response to salt-treatment in both No-0 and the 35S::*NIT2* line, as well as K^+ transporters that were salt-responsive in No-0 but not in the 35S::*NIT2* line. Refer again to Figure 2.4. for a visual depiction.

2.4.2.3. Potential effect of aquaporins

The tonoplast aquaporin, TIP2;2, has been linked with a role in salinity tolerance, with reduced expression observed in Arabidopsis roots in response to salt-treatment (Boursiac et al., 2005). *In silico* analysis of available transcriptomics data has revealed that *TIP2;2* is ubiquitously expressed throughout the root and shoot, and again, that its expression is sensitive to salt stress (Feng, Xu, et al., 2018). Additionally, *tip2;2* mutants were found to display decreased sensitivity to salt-stress, as well as reduced ion leakage, and interestingly, increased expression of SOS-signalling pathway genes (Feng, Xu, et al., 2018). Given its reported ubiquitous expression, it was striking that incredibly low levels of *TIP2;2* expression were detected in the root and shoot of the 35S::*NIT2* line under both control and saline conditions. By contrast, No-0 displayed significantly higher levels of *TIP2;2* expression in both tissues in control conditions, that were then down-regulated in response to salt treatment, as previously reported. It is notable that the *tip2;2* mutant, as well as the 35S::*NIT2* line, which has very low levels of *TIP2;2* expression, display increased salt-tolerance. It is thus plausible that the very low levels of *TIP2;2* expression contributes to its improved growth under saline conditions compared with No-0. To fully understand the potential role of TIP2;2, a proper investigation of its ion and water transport activities is required. Ion transport activity has been reported for a number of other aquaporins (see Tyerman et al., 2021 for a detailed review). In particular, the plasma membrane aquaporin, AtPIP2;1, has been suggested as a candidate NSCC for Na⁺ entry into the roots, where it is highly expressed. AtPIP2;1 has been shown to function in Na⁺ transport as evidenced by its expression in *Saccharomyces cerevisiae* and *Xenopus* oocytes, which resulted in increased cytoplasmic Na⁺ content (Byrt et al., 2017). If TIP2;2 also has Na⁺ transport activity, it is possible that it facilitates Na⁺ leakage from the vacuole into the cytoplasm. If this is the case, the significantly lower *TIP2;2* expression seen in the shoots and roots of the 35S::*NIT2* line in control conditions could suggest an improved ability to prevent vacuolar Na⁺ leakage than No-0, at least in the early stages of salt stress before *TIP2;2* expression is decreased in No-0. This is, however, a highly speculative suggestion.

2.4.2.4. Potential mis-regulation of transporter/channel expression in the 35S::*NIT2* line

As mentioned previously, one of the most striking observations from the ion transporter/channel gene expression analysis was that a number of the Na⁺-/K⁺-transporters that were differentially expressed in response to salt-treatment in No-0, did not respond to salt-treatment in the 35S::*NIT2* line. These included HKT1;1 and SKOR in the roots, as well as SOS1, NHX3, KUP1, and GORK, in the shoots. Given that the 35S::*NIT2* line displays

increased salinity tolerance, this observation raises questions as to whether this mis-regulation of gene expression is the cause, or rather an effect, of the increased salt-tolerance of this line: Does the mis-regulation of transporter expression contribute to the increased salinity tolerance of the 35S::*NIT2* line? Or is it perhaps that the down-regulation of these genes in No-0 reflects that the plants are salt-stressed, and the maintenance of expression of these genes in the 35S::*NIT2* line occurs as the plants are more salt-tolerant as a result of some other contributing factor/s?

Given the function of *NIT2* in auxin biosynthesis, and the very similar expression patterns of the genes of interest, an obvious avenue of further investigation was to try and identify potential binding sites for transcription factors (TFs) that were shared across the promoters of these genes, as this could suggest that they are co-regulated by common TFs. Of particular interest would be the identification of binding sites of TFs involved in auxin/other hormone-related processes, as well as TFs involved in the salinity stress response. Following this, any salt-responsive changes in the expression of the corresponding TFs could be assessed in the two lines. It must be noted that the transcriptional control of stress-responsive genes involved in ion homeostasis in plants is co-ordinated by a vast, complex set of TFs (and their corresponding binding motifs) with varying spatio-temporal expression, including MYBs, MYCs, WRKYs, Dofs, and AREB/ABF/bZIPs (Agarwal, Dave & Agarwal, 2018). In fact, plant transcriptional control as a whole is very complex, with *Arabidopsis* encoding 2296 TFs that are classified into 58 families, according to the Plant Transcription Factor Database v5.0 (Jin et al., 2017). We are also limited in our knowledge of transcription factor binding sites (TFBSs) (only 619 sites have been identified via experimental work in *Arabidopsis* to date (Jin et al., 2017)). Whether these large TF families bind to the same TFBSs, or if there are differences in binding specificities between TFs within each family remains largely undetermined. As such, it must be stressed that this work represented a very preliminary investigation of this topic.

Searching the literature for native TFs that are known to regulate our genes of interest in response to salt stress was an obvious starting point. Unfortunately, there is currently a paucity of studies, such as yeast-one-hybrid screens, performed to identify native *Arabidopsis* TFs that bind to the promoters of our genes of interest, both in general, and in response to salt stress. Therefore, an *in silico* approach was adopted in order to identify potential TFBSs of interest present in the promoters of the transporter/channel genes of interest. Although *AKT1* and *KAT1* were found to be salt-responsive in the 35S::*NIT2* line, unlike the other transporter genes mentioned earlier, they were included in the promoter query set; while their expression did

decrease in response to salt-treatment, *AKT1* and *KAT1* still displayed significantly increased expression in the root and shoot, respectively, of the 35S::*NIT2* line compared with No-0, as was observed for the transporters displaying the mis-regulated expression in the 35S::*NIT2* line. For this *in silico* analysis, the 1000 bp upstream of the transcriptional start site of the genes was considered as most Arabidopsis promoters are assumed to span this region. Motif identification was performed using the Find Individual Motif Occurrences (FIMO) tool of MEME Suite 5.4.1, (Grant, Bailey & Noble, 2011; Bailey et al., 2015), where the promoter sequences were searched using the JASPAR CORE Position Frequency Matrix (PFM) non-redundant plant motif sequence database, which includes 656 motifs. Many MYB, MYC, WRKY, bZIP, BHLH, and NAC (NAM, ATAF1/2, CUC2) domain TF binding sites were identified in the promoters of the genes of interest (refer to appendix chapter 7, Table 7.1). A non-exhaustive search of the data was performed to identify binding sites for TFs with a pre-established link with salt stress, which revealed those for several MYBs (1, 15, 30, 41, 44, 49, 73, 74), WRKYs (11, 17, 25, 33, 46), and NACs (55, 92) in the promoters of one or more of the genes (refer to appendix chapter 7, Table 7.2).

Having identified binding sites for a wide range of TFs in the promoters of the ion transporter genes of interest, including several sites for TFs with a pre-established role in salt stress, it followed to assess whether the corresponding TFs display altered expression in response to salt stress in No-0 and the 35S::*NIT2* line. To do this, the expression of all the corresponding TFs was assessed in the transcriptomics dataset that was used to identify DEGs between 35S::*NIT2* and No-0 two-week-old seedlings, grown under 100 mM NaCl and control conditions (Cackett, 2019). *ANAC054*, which was shown to have binding sites in the promoters of *HKT1;1* and *SKOR*, was found to have significantly increased expression in response to salt-treatment in the 35S::*NIT2* line, but not in No-0. *ANAC054*, also known as CUP SHAPED COTYLEDON 1 (*CUC1*), has been shown to play a key role in regulating embryonic organ phyllotactic patterning, including control of cotyledon separation and shoot apical meristem formation. Furthermore, *CUC1* has been shown to be partially redundant with *CUC2* (*ANAC098*) and *CUC3* (*ANAC031*) (Aida et al., 1997; Vroemen et al., 2003), both of which were found to have binding sites in the promoters of *HKT1;1* and *AKT1*. While *CUC1* expression was increased in the 35S::*NIT2* line in response to salt-treatment in the transcriptomics dataset, as opposed to *HKT1;1*, *SKOR*, and *AKT1*, which displayed maintenance of the expression seen in untreated conditions in the data presented herein, in both scenarios expression of these genes was higher in the 35S::*NIT2* line than No-0 under saline conditions. Thus, it could perhaps be possible that

CUC1 played a role in the regulation of *HKT1;1* and *SKOR* expression, and given the functional redundancy, potentially also *AKT1* via the CUC2/3 binding sites in its promoter. Furthermore, it has very recently been shown that the expression of the auxin biosynthesis genes, *YUCCA1* and *YUCCA4*, during embryonic cotyledon boundary development is dependent on the *CUC* genes (Yamada et al., in press). Given that it plays a role in regulating auxin-responsive processes, it is noteworthy that *CUC1* displayed increased expression in the 35S::*NIT2* line under salt stress conditions, in which it has previously been established to have increased IAA levels (Cackett et al., 2022). Unfortunately, no other TFs corresponding to the sites identified in the FIMO analysis displayed differential expression either in response to salt-treatment in the two lines, or between the two lines under control or 100 mM NaCl conditions.

It must be noted that some obvious caveats prevent a true comparison between this transcriptomics dataset and the ion transporter gene expression dataset. There is a difference in the developmental stage, and salt-treatment of the plants, as well as the tissue in which expression was measured; the ion transporter expression analysis was performed on root and shoot tissue of three-week-old hydroponically-grown plants that were then treated with 75 mM NaCl for one week further, compared to the transcriptomics experiment in which expression was measured in whole two-week-old seedlings germinated and grown on 100 mM NaCl. Given these differences, I cannot preclude the possibility that these TFs, or other MYBs, MYCs, WRKYs, NACs etc. are in fact differentially expressed between the 35S::*NIT2* line and No-0 in hydroponically grown plants in response to 75 mM NaCl treatment, and might potentially play a role in regulating the ion transporter genes of interest, via the many corresponding binding motifs identified in their promoters. The use of a yeast-one-hybrid system to identify TFs capable of binding to the promoters of our Na⁺-/K⁺-transporters of interest is an obvious avenue of future work that would greatly contribute to the current gap in the literature. Additionally, the gene expression of any interacting TFs identified via yeast-one-hybrid screening could then be measured within the ion transporter expression analysis experiment discussed in this chapter. Together, this would hopefully provide insight into the mis-regulated ion transporter gene expression pattern observed within the 35S::*NIT2* line in response to salt stress, as well as provide valuable information as to how the expression of these Na⁺-/K⁺-transporter genes is regulated in response to salt stress, both in No-0 and the 35S::*NIT2* line.

2.4.3. The 35S::*NIT2* line potentially has increased membrane potential in the shoots due to higher *AHA2* expression

The greater *AHA2* expression observed in the roots compared with the shoots of both the 35S::*NIT2* line and No-0 (Figure 2.3) is in agreement with previous reports of significantly higher root *AHA2* expression, that form the basis of *AHA2* being considered the major root PM H⁺-ATPase (Harper et al., 1990; Falhof et al., 2016). Furthermore, the root *AHA1* expression was the same as in the shoot, and was much lower than that of *AHA2* in both lines, which further agrees with this assertion. This comparison of root *AHA1* and *AHA2* expression does, however, assume that the efficiency of the respective qPCR primer sets was the same; a consideration that can prevent the direct comparison of qPCR data between different genes when primer efficiencies differ. Although no difference in expression was observed between the two lines in the roots in either treatment, the 35S::*NIT2* line displayed increased *AHA2* expression compared with No-0 in the shoots in both untreated and saline conditions. While increased *AHA2* gene expression cannot be equated to increased pump activity, and thus improved maintenance of plasma membrane potential under salt stress *per se*, increased PM H⁺-ATPase gene expression has previously been reported in the leaves of the salt-tolerant poplar, *Populus euphratica*, compared with the salt-sensitive, *Populus popularis* (Ding et al., 2010). Perhaps then the increased shoot *AHA2* expression displayed by the 35S::*NIT2* line also contributes towards its improved salinity tolerance compared with No-0. It must also be noted that only the expression of the two major PM H⁺-ATPase isoforms were measured here. Given that *Arabidopsis* encodes eleven isoforms whose expression vary across different cell types (Falhof et al., 2016), it is possible that some of the other isoforms are also differentially expressed between the 35S::*NIT2* line and No-0, thereby potentially affecting membrane potential in a similar way as has been postulated for *AHA2*. However, as they are the major isoforms, only the expression of *AHA1* and *AHA2* was considered here, and the expression of the remaining nine isoforms could be assessed in future work.

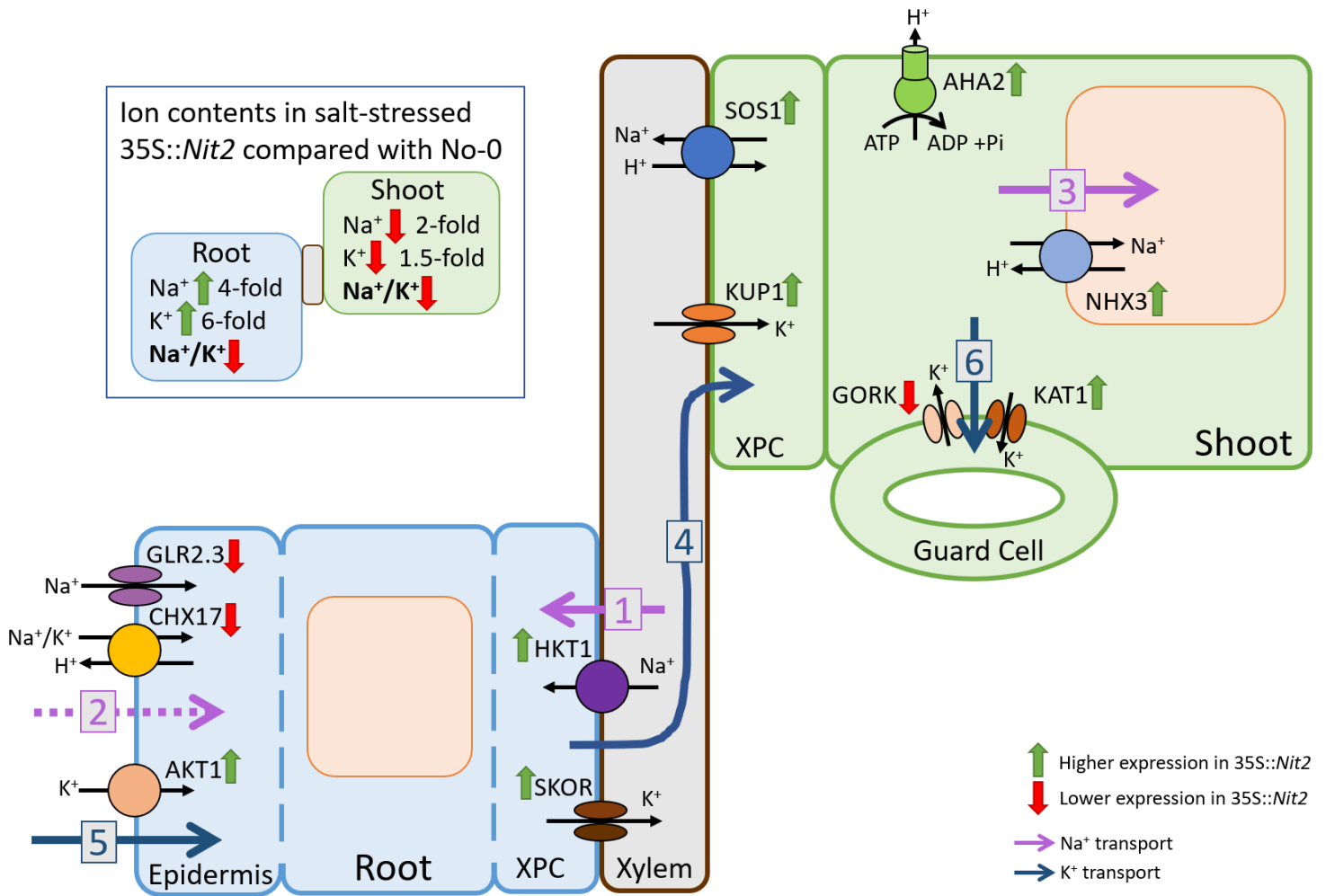


Figure 2.5: Model for mechanisms of improved ion homeostasis in the 35S::NIT2 line under saline conditions

The Na⁺ and K⁺ transporters that were differentially expressed in the 35S::NIT2 line compared with No-0 under saline conditions, and their potential roles in contributing to the improved root and shoot ion partitioning displayed by the 35S::NIT2 line in the presence of salt, are shown. The green and red arrows indicate increased or decreased gene expression, respectively, in the 35S::NIT2 line compared with No-0. The numbered pink and blue arrows indicate the transport of Na⁺ and K⁺, respectively, between cellular compartments, with dotted arrows indicating reduced transport. The inset shows the Na⁺ and K⁺ contents in the root and shoot of the 35S::NIT2 line compared with No-0 under saline conditions.

(1) Increased *HKT1;1* expression in the roots of the 35S::NIT2 line may indicate improved xylem Na⁺ retrieval, and thus an improved ability to limit root to shoot Na⁺ transport. This likely explains the improved ability of this line to limit shoot Na⁺ accumulation, whilst maintaining more Na⁺ within the root. (2) The decreased expression of *GLR2.3*, and *CHX17* in the roots may indicate that the 35S::NIT2 line is able to restrict Na⁺ uptake from the soil via these avenues. (3) Increased *NHX3* expression in the shoot may indicate that the 35S::NIT2 line has an improved ability for vacuolar sequestration of Na⁺ in the shoots, thereby limiting its negative effect on cytosolic metabolic processes. (4) Increased expression of *SKOR* in the root may indicate improved xylem K⁺ loading, and the increased shoot *KUP1* expression improved unloading of K⁺ from the vascular tissues into the shoot tissue.

By the combined action of these transporters it is proposed that the 35S::*NIT2* line has improved root to shoot K^+ transport, which contributes to the reduced Na^+/K^+ ratio observed in its shoot tissue. **(5)** Increased expression of *AKT1* in the root may indicate an improved ability to take up K^+ from the soil in the 35S::*NIT2* line, as suggested by its significantly higher root K^+ levels. **(6)** Finally, the decreased *GORK*, and increased *KAT1* expression suggests the 35S::*NIT2* line has an improved ability to maintain guard cell K^+ levels, thereby increasing stomatal conductance, and allowing for CO_2 uptake for photosynthesis and continued growth under saline conditions.

2.4.4. Summary

The results of this chapter show that the improved salinity-tolerance phenotype displayed by the 35S::*NIT2* line can indeed be explained by alterations in Na^+/K^+ homeostasis in both the root and the shoot under saline conditions compared with No-0. The 35S::*NIT2* line was found to have improved Na^+/K^+ ratios in both the root and the shoot in saline conditions. Furthermore, the altered ion homeostasis can be attributed to changes in the expression of several Na^+ and K^+ membrane transporters and channels. Of the many differences in Na^+ transporter expression, the most interesting were the findings that, under saline conditions, the 35S::*NIT2* line had increased root *HKT1;1* expression, suggesting an improved ability to limit root to shoot Na^+ transport via the xylem, increased shoot *NHX3* expression, suggesting increased vacuolar Na^+ sequestration, and decreased root *GLR2.3* expression, suggesting limited root Na^+ uptake via this channel. Likewise, under saline conditions, the 35S::*NIT2* line had a number of interesting changes in K^+ transporter expression such as increased root *AKT1* expression, suggesting increased root K^+ uptake, as well as increased root *SKOR* expression, suggesting an increased loading of K^+ into the xylem for root to shoot transport. Additionally, the 35S::*NIT2* line displayed alterations in shoot *KAT1* and *GORK* expression, which could suggest improved stomatal conductance compared with No-0 under saline conditions. A proposed model for the mechanism of improved ion homeostasis in the 35S::*NIT2* line under saline conditions is presented in Figure 2.5. Taken together, the findings presented in this chapter provide the first evidence that improved Na^+/K^+ homeostasis underlies the increased salinity tolerance of the 35S::*NIT2* line.

CHAPTER 3: PROTEOMIC ANALYSIS OF PLASMA MEMBRANE PROTEINS FROM *NITRILASE2* OVEREXPRESSING PLANTS

Note: The proteomics analysis herein was conducted at the proteomics unit of the Central Analytical Facility at the University of Stellenbosch. I provided them with Bis-tris polyacrylamide gel slices containing enriched plasma membrane protein samples, and they performed the in-gel tryptic digest, LC-MS/MS, and subsequent initial data analysis.

3.1. Introduction:

3.1.1. The current status of salt-stress proteomics in Arabidopsis

While there are many detailed studies reporting on the transcriptome of salt-stressed Arabidopsis, the number of studies investigating the proteomic (and post-translational modification) changes that occur in response to salt stress is far smaller. The few current studies (Ndimba et al., 2005; Jiang et al., 2007; Hsu et al., 2009; Pang et al., 2010; Chang et al., 2012; Vera-Estrella, Barkla & Pantoja, 2014; Vialaret et al., 2014; Rodriguez et al., 2021) have varied greatly in experimental design, including the way in which the plants were grown (in soil, seedling suspension culture, or hydroponically), the age of the plants prior to salt treatment (18-day-old to seven-week-old), the concentration of NaCl used (50 to 400 mM NaCl), as well as the length of the salt treatment (45 minutes to five days). Furthermore, some of these studies have considered the total proteome (Jiang et al., 2007; Rodriguez et al., 2021) or microsomal/membrane proteome (Vialaret et al., 2014) of the root, while others have focused on the microsomal/membrane proteome of the whole plant (Hsu et al., 2009; Chang et al., 2012) or the leaves (Pang et al., 2010; Vera-Estrella, Barkla & Pantoja, 2014). Notably, none of these have reported on the plasma membrane proteome specifically. This is likely due to the fact that PM proteins are particularly difficult to isolate, and the hydrophobic nature and likely low abundance of these PM proteins makes comparative proteomic analyses challenging (Zhang & Peck, 2011). Another consideration is that many of these earlier proteomics studies relied upon older quantification techniques, such as two-dimensional differential gel electrophoresis (2D-DIGE), which limited the number of proteins that could be identified in subsequent mass spectrometry (MS) analyses. The more recent use of a label-free quantitative approach for both proteomics and phosphoproteomics has allowed for the identification of large numbers of salt-responsive proteins and phosphorylation events. However, the studies that have adopted this approach have either treated the plants with high NaCl concentrations

that exceed physiologically relevant conditions (Shavrukov, 2013), or for too short a period of time to detect true alterations in salt-responsive protein expression. For example, Rodriguez et al. (2021) used 150 mM NaCl, when *Arabidopsis* is a salt-sensitive species that cannot complete its life cycle when grown under constant exposure to 100 mM NaCl (Sickler et al., 2007; Munns & Tester, 2008), and Vialaret et al. (2014) only treated for two hours, which is likely too short a time for Na⁺ to accumulate, thus salt-specific proteomic changes were not measured. However, given that rapid salt-specific signalling and growth responses have been found to occur within the root it is also possible that the two hour treatment period was sufficient to measure true salt-specific proteomic changes (van Zelm, Zhang & Testerink, 2020). Additionally, both of these studies only analysed the root (phospho)proteome. All the previous studies mentioned here have measured the proteome of the Columbia (Col-0), *Arabidopsis* eco-type (except Ndimba et al. (2005), who used *Landsberg erecta* (Ler-0)). Considering the above, there is clearly a gap in the literature reporting on the plasma membrane proteome of salt stressed *Arabidopsis* plants, as well as that of the Nossen (No-0) ecotype, specifically. Furthermore, there is a need to investigate the root and shoot salt stress plasma membrane proteome in response to physiologically relevant salt concentrations and treatment periods.

3.1.2. The current status of plasma membrane proteomics in *Arabidopsis*

Given that the plasma membrane forms the barrier between the cell and the external environment, it functions in a diverse range of cellular processes, including signalling, ion and metabolite transport, response to biotic and abiotic challenges, and cellular differentiation and proliferation (Marmagne et al., 2004, 2007). Understandably, many proteomics studies have been performed to identify the wide range of integral (embedded in the PM bilayer) and peripheral (associated with inner or outer PM surface) proteins that are involved in these plasma membrane processes. Earlier studies have assessed the PM proteome of *Arabidopsis* suspension cell cultures (Marmagne et al., 2004, 2007), in two-week-old seedlings (Nelson et al., 2006), in the leaves of six-week-old soil-grown plants (Alexandersson et al., 2004; Zarrini et al., 2012), and in the leaves and roots of 8-week-old hydroponically grown plants (Bernfur et al., 2013). In all instances, a two-phase partitioning system was used to purify the plasma membranes, however, the type of subsequent MS analysis differed greatly. As the resolving and quantification power of MS technologies has improved, the number of PM proteins identified has increased (for example Alexandersson et al. (2004) detected 114, and Zarrini et al. (2012) detected 304). Despite this, a distinct PM protein signature, consisting of

transporters, Leucine-rich repeat receptor-like kinases (LRR RLKs) and other RLKs, and cell wall-related proteins has emerged from all these studies. Of pertinence to our study is the detection of transporters/channels, as well as proteins involved in energising membrane transport. The PIP/TIP aquaporins are readily detected in large numbers - both Zarrini et al. (2012), and Bernfur et al. (2013) identified nine of the thirteen PIP isoforms encoded by *Arabidopsis*. The two major isoforms of the PM H⁺-ATPase, AHA1 and AHA2, are consistently detected in all the studies mentioned above, and in several cases up to six of the eleven *Arabidopsis* isoforms were identified. Clearly then, AHA2 can be robustly detected by a wide range of MS approaches. Other commonly detected integral PM transporters include those involved in the transport of organic ions such as phosphates and ammonium, inorganic ions such as potassium and copper, as well as sugars, and oligopeptides. The existence of a PM protein signature indicates that there are certain PM proteins that have consistently high abundance in plants at varying developmental stages when grown without stress.

While, to the best of our knowledge, there are no investigations of the salt-stress plasma membrane proteome in *Arabidopsis*, changes in the PM proteome in response to other stresses have been assessed. In a study of temporal changes in the PM proteome during freezing and the subsequent de-hardening of *Arabidopsis* leaves, Miki et al. (2019), identified 873 proteins that changed in abundance during these cold-acclimation and subsequent de-acclimation processes using a label-free semi-quantitative nano-LC-MS/MS approach. Interestingly, they reported that, of all the proteins that they detected, the proportion of transporter, signal-transduction related, and cell growth/division-related proteins was consistent with the earlier studies that were discussed above. Similarly, in a study assessing the effect of ABA treatment on the freezing tolerance of *Arabidopsis* suspension-cultured cells, 841 PM proteins were found to change abundance in response to cold-acclimation and ABA treatments (Li et al., 2020). Additionally, several studies have been performed to assess changes in the PM proteome in response to pathogen challenge. The PM contains pattern recognition receptors (PRRs), which are crucial for the perception of the microbe-associated molecular pattern molecules (MAMPs) associated with the surface of pathogens, resulting in signal transduction events that lead to the activation of immune defence responses (Baloyi, Dubery & Piater, 2017). The PM proteome of *Arabidopsis* leaves has been shown to be responsive to exposure to lipopolysaccharides from *E. coli*, the Gram-negative bacteria, *Xanthomonas campestris*, as well as when treated with ergosterol, the major sterol component of fungal membranes (Baloyi, Dubery & Piater, 2017; Khoza, Dubery & Piater, 2019; Hussan, Dubery & Piater, 2020).

Clearly, large-scale changes in the leaf PM proteome do occur in response to both biotic and abiotic stress, and these changes are readily detectable.

3.1.3. Chapter aims

The overall aim was to assess changes in the PM proteome that occur in response to salt-stress in the shoots of the 35S::*NIT2* line and No-0, thereby filling the current gap in the literature in both salt-stress, and PM proteomics, as well as identifying differences in the PM proteome between the two lines that could help explain the improved salinity tolerance of the 35S::*NIT2* line. This was split into three components; 1) To isolate enriched PM protein fractions from the shoots of 35S::*NIT2* and No-0 plants in control and salt stress conditions, 2) To identify salt-responsive changes in the PM proteomes of both lines, 3) To determine if there were any PM proteins that were differentially expressed between the two lines in both control and salt stress conditions.

3.2. Materials and Methods:

3.2.1. Arabidopsis growth in hydroponics

No-0 and 35S::*NIT2* seeds were sterilised as described in chapter 2, section 2.2.4. The plants were grown hydroponically on 1/4 strength PN media (see chapter 2, section 2.2.5.) as described in chapter 2 section 2.2.6. The plants were grown for four weeks in 1/4 strength PN media, and then were either treated with 1/4 strength PN media supplemented with 75 mM NaCl for 3 days, or maintained on 1/4 strength PN media (untreated control). Standard growth room conditions were used (see chapter 2, section 2.2.3.).

3.2.2. Plasma membrane protein preparation via simplified enrichment

The simplified enrichment protocol described in Collins et al. (2017) was utilised to extract enriched plasma membrane proteins from the rosette tissue of untreated and salt-stressed (75 mM NaCl) No-0 and 35S::*NIT2* plants. The experiment included three biological replicates. Each set of four samples that formed one replicate were processed together. All homogenisation steps were conducted using buffers and equipment pre-cooled to 4 °C, or kept on ice. For each of the samples, 600-700 mg of liquid N₂-snap frozen tissue was homogenised in 3 mL of ice-cold homogenisation buffer (250 mM sucrose, 50 mM HEPES-KOH (pH 7.5), 5% (v/v) glycerol, 50 mM sodium pyrophosphate decahydrate, 1 mM sodium molybdate

dihydrate, 25 mM sodium fluoride, 10 mM Na.EDTA (pH 7.5), 0.5% (w/v) polyvinylpyrrolidone (PVP-10) with the following inhibitors; 3 mM DTT, 1 mM phenylmethanesulfonylfluoride fluoride (PMSF), 10 μ M leupeptin, 10 nM calyculin A (PHZ1044, Thermo Fisher Scientific, USA), and cOmplete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail ((Roche, USA) according to the manufacturer's instruction) using a pestle and mortar. The homogenate was filtered through Miracloth (Merck, Germany) into microcentrifuge tubes, then centrifuged at 8000 x *g* for 10 min at 4°C using a Heraeus Biofuge Pico benchtop microcentrifuge (Kendro Laboratory Products, Germany) to remove cell debris. The supernatant was transferred into 3.5 mL open-top thickwall polycarbonate tubes (#349622, Beckman Coulter, USA), then centrifuged at 100 000 x *g* for 30 min at 4°C to collect the microsomal protein fraction. All high-speed centrifugation was performed using a Beckman Optima™ *TL-100 ultracentrifuge and TLA-100.3 fixed-angle rotor* (Beckman Coulter, USA). An aliquot of the supernatant containing all the soluble and cytosolic proteins was retained for down-stream analysis. The microsomal pellet was resuspended in 100 μ L of Buffer H using a rounded pestle. The concentration of the microsomal fractions was determined via Bradford assay, then they were diluted with Buffer H supplemented with 0.02% Brij-58 (P5884, Sigma-Aldrich)(1 x Brij-58) to a final volume of 2 μ L per μ g of protein. The microsomal fractions diluted with 1 x Brij-58 were incubated for 30 min on ice, then centrifuged at 100 000 x *g* for 30 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 1 x Brij-58 to the same final volume calculated previously, incubated on ice for 30 min, then centrifuged again at 100 000 x *g* for 30 min at 4 °C. The supernatant was discarded, then to remove the Brij-58, the pellet was resuspended in 1.75 mL of Buffer H and centrifuged a final time at 100 000 x *g* for 30 min at 4 °C, yielding the enriched plasma membrane protein fraction. The enriched plasma membrane protein was resuspended in 100 μ L of Buffer H and the final protein concentration of each sample was determined.

3.2.3. Protein quantification

The protein concentrations of the microsomal and enriched plasma membrane fractions were determined via Bradford assay (Bradford, 1976) using the Pierce™ Coomassie protein assay reagent according to the manufacturer's instruction (23200, Thermo Fisher Scientific, Waltham, MA, USA). A bovine serum albumin standard ranging from 0.05 – 0.5 mg.mL⁻¹ was used. Absorbances were measured at 595 nm using a Multiskan EX plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

3.2.4. PAGE analysis

The integrity of the protein samples was assessed via separation with bis-Tris PAGE, which eliminates hydrophobicity-related issues observed when separating plasma membrane protein fractions via SDS PAGE. The samples were heat-denatured at 65°C for 7 min in sample application buffer (0.1 M Tris-HCl pH 6.8, 35% (v/v) glycerol, 4% (v/v) sodium dodecyl sulfate (SDS), bromophenol blue, 1% (v/v) β -mercaptoethanol). For all samples, 15 μ g of protein was used for each of the fractions. Following heat-denaturation, the samples were briefly centrifuged at 10 000 $\times g$, then loaded into 10% (v/v) bis-Tris-polyacrylamide gels (10% (v/v) 37.5:1 acrylamide/Bis solution (Promega, WI, USA), 357.5 mM bis-Tris pH 6.8, 0.1% (w/v) ammonium persulfate, 0.00027% (v/v) TEMED). The samples were separated by electrophoresis at 125 V for 60 min in MOPS-SDS buffer (50 mM Tris, 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 1 mM Na.EDTA pH 8, 0.1% (w/v) SDS, 1 mM sodium bisulfite). The PageRuler™ Prestained Protein Ladder, 10-180 kDa (Thermo Fisher Scientific Inc., MA, USA; 26616) was used. Following electrophoresis, residual SDS was removed by rinsing in dH₂O three times for 10 min. Following this, the gel was stained with filter-sterilised Coomassie solution (0.3% (w/v) Coomassie R-250, 10% (v/v) glacial acetic acid, 45% (v/v) methanol). The staining solution was discarded, and the gels were rinsed with dH₂O twice for 5 min. To remove non-specific/excess staining, the gel was de-stained overnight in de-stain solution (10% (v/v) glacial acetic acid, 40% (v/v) methanol), then rinsed in dH₂O three times for 5 min. The gels were imaged using the white light filter of the Molecular Imager® Gel Doc™ XR System (Bio-Rad, CA, USA).

3.2.5. Protein identification and quantification by label-free mass spectrometry (MS)

The in-gel tryptic digests and subsequent liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis of all the samples was performed at the Mass Spectrometry unit of the Central Analytical Facility (CAF) at the University of Stellenbosch, Tygerberg.

3.2.5.1. Sample preparation

The enriched plasma membrane protein fractions from the untreated and salt-treated No-0 and 35S::NIT2 plants were separated via bis-Tris PAGE, then excised prior to proteomic analysis. 25 μ g of each of the protein samples were prepared and loaded into 10% (v/v) bis-Tris-polyacrylamide gels as outlined in section 3.2.6. Empty lanes were left between samples in the gels to prevent cross-contamination, and the bis-Tris PAGE solutions were filter-sterilised using a 0.2 μ M filter to prevent keratin contamination. The samples were separated via electrophoresis for ~30 min at 125 V. Coomassie staining was performed as outlined in section

3.2.6. Following this, the gels were rehydrated with HPLC-grade H₂O. The protein bands were excised from the gel using a sterile scalpel blade, cut into 1 mm x 1 mm cubes, and placed into microcentrifuge tubes where they were submerged in HPLC-grade H₂O.

3.2.5.2. In-gel tryptic digest

All reagents used were analytical grade or equivalent. The gel pieces were destained with 100 mM ammonium bicarbonate (ABC), 50% (v/v) acetonitrile (ACN) (Sigma-Aldrich) until clear. The gel pieces were then dehydrated and further desiccated under vacuum using a centrifugal evaporator (Speed Vac, Jouan, France) for 20 min at room temperature. Sample reduction was performed with 2 mM Tris (2-carboxyethyl) phosphine (TCEP) (Fluka; Honeywell Research Chemicals) in 25 mM ABC for 15 min at room temperature with agitation. Excess TCEP was removed, and the gel pieces were dehydrated again. Cysteine residues were thiomethylated with 20 mM S-Methyl methanethiosulfonate (Sigma-Aldrich) in 25 mM ABC for 30 min at room temperature. Following thiomethylation, the gel pieces were dehydrated and washed with 25 mM ABC, followed by another dehydration step. Proteins were digested by rehydrating the gel pieces in Pierce™ trypsin protease solution (20 ng.μL⁻¹) (Thermo Scientific) and incubation at 37°C overnight. Peptides were extracted from the gel pieces twice with 150 μL water. The samples were dried down and re-suspended in 30 μL 2% (v/v) ACN:water; 0.1% (v/v) formic acid (FA). Residual digest reagents were removed using an in-house manufactured C₁₈ stage tip (Empore Octadecyl C₁₈ extraction discs; Supelco™ Analytical). The samples were loaded onto the stage tip after activating the C₁₈ membrane with 30 μL methanol (Sigma-Aldrich) and equilibration with 30 μL 2% (v/v) ACN:water; 0.05% (v/v) trifluoroacetic acid (TFA). The bound sample was washed with 30 μL 2% (v/v) ACN:water; 0.1% (v/v) FA before elution with 30 μL 50 % (v/v) ACN:water; 0.1% (v/v) FA. The eluate was evaporated to dryness. The dried peptides were dissolved in 2% (v/v) ACN:water; 0.1% (v/v) FA for LC-MS/MS analysis.

3.2.5.3. Liquid chromatography

Liquid chromatography was performed using a Thermo Scientific Dionex UltiMate™ 3000 RSLCnano (Thermo Scientific, Waltham, MA, USA) equipped with a 5 mm x 300 μm C₁₈ trap column (Thermo Scientific) and a Charged Surface Hybrid (CSH) 25 cm x 75 μm 1.7 μm particle size C₁₈ analytical column (Waters, Munich, Germany). The solvent system employed was as follows; loading 2% (v/v) ACN:water; 0.1% (v/v) FA; Solvent A: water; 0.1% (v/v) FA; Solvent B: 100% ACN:water. The samples were loaded onto the trap column using loading solvent at a flow rate of 2 μL.min⁻¹ from a temperature controlled autosampler set at 7 °C. Loading was performed for 5 min before the sample was eluted onto the analytical column.

Flow rate was set to 300 nL.min⁻¹ and the gradient was generated as follows: 2.0-30% B over 65 minutes and 30-45% B from 65-80 minutes. Chromatography was performed at 45°C and the outflow was delivered to the mass spectrometer through a stainless-steel nano-bore emitter.

3.2.5.4. Mass spectrometry

Mass spectrometry was performed using a Thermo Scientific Orbitrap™ Fusion™ mass spectrometer equipped with a Nanospray Flex™ ionization source (Thermo Scientific, Waltham, MA, USA). Data was collected in positive ion mode with a capillary temperature of 280°C, and electrospray voltage set to 1.8 kV. Spectra were internally calibrated using polysiloxane ions at $m/z = 445.12003$ and 371.10024 . The MS1 scans were performed using the orbitrap detector set at 60 000 resolution over the scan range 375-1500 Da, with the automatic gain control target set to standard. Data was acquired in profile mode. The MS2 acquisitions were performed using monoisotopic precursor selection for ions with charges +2 - +7 with error tolerance set to +/- 10 ppm. Precursor ions were excluded from fragmentation once for a period of 60 s. Precursor ions were selected for fragmentation in higher energy C trap dissociation (HCD) mode using the quadrupole mass analyser with HCD energy set to 30%. Fragment ions were detected in the orbitrap mass analyser set to 15 000 resolution. The AGC target was set to standard and the maximum injection time to auto. The data was acquired in centroid mode.

3.2.5.5. Data analysis

The raw MS/MS files generated by the mass spectrometer were imported into the Proteome Discoverer software (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.1.14) and processed using both SEQUEST and MS Amanda algorithms. Database interrogation was performed against a concatenated database created using the Uniprot Arabidopsis FASTA database (<http://www.uniprot.org>) concatenated with the Contaminant Repository for Affinity Purification (cRAP) contaminant database (Mellacheruvu et al., 2013). The database search parameters were as follows: Semi-tryptic cleavage with 2 missed cleavages was allowed for; precursor mass tolerance was set to 10 ppm and fragment mass tolerance set to 0.02 Da; deamidation (NQ), oxidation (M), and acetylation of protein N-terminal was allowed as dynamic modifications and thiomethyl of C as static modification. Peptide validation was performed using the Target-Decoy peptide-spectrum mass (PSM) validator node. The results files were imported into Scaffold 5 (version Scaffold_5.2.1, Proteome Software Inc., Portland, OR) and identified peptides validated with X!Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)) and the Peptide Prophet (Keller et al., 2002) and Protein Prophet

(Nesvizhskii et al., 2003) algorithms included in Scaffold. Peptide identifications were accepted if they could be established at greater than 84.0% probability to achieve a false discovery rate (FDR) less than 0.5%. Protein identifications were accepted if they could be established at greater than 95.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Only proteins that were identified, according to these criteria, in at least two of the three biological replicates for any sample group were considered for further analysis. Gene Ontology (GO) annotations were assigned using the Uniprot Arabidopsis GOA database version 187 (<https://ftp.ebi.ac.uk/pub/databases/GO/goa/ARABIDOPSIS/>) (Dimmer et al., 2012). Quantitation was performed in Scaffold. For the quantitative comparisons between sample groups, the normalised total spectra number for each identified protein was used, as recommended by Scaffold. The normalised total spectra number of the three biological replicates was compared between sample groups, and a Fisher's Exact Test was employed to identify differentially expressed proteins with $p < 0.05$ considered significant. Benjamini-Hochberg correction for multiple testing was utilised (Benjamini & Hochberg, 1995). Differences in protein abundance between sample groups were reported as Log₂-transformed fold-change values (Log₂FC).

3.3. Results:

3.3.1. Isolation of enriched plasma membrane protein fractions from shoots of untreated and salt-stressed 35S::*NIT2* and No-0 plants

In order to assess the effect of salinity on the shoot plasma membrane proteome of the 35S::*NIT2* line and No-0, enriched plasma membrane (ePM) protein fractions were isolated from the rosette tissue of four-week-old 35S::*NIT2* and No-0 plants that were exposed to salt stress for three days, or maintained under control conditions. Following the initial protein extraction, the microsomal proteins were first separated from the soluble cytosolic proteins, then plasma membrane proteins were enriched in the microsomal fractions via treatment with Brij-58, and a series of high-speed centrifugations (Collins et al., 2017). Referring to Table 3.1, ePM proteins were successfully isolated for all of the untreated and salt-stressed 35S::*NIT2* and No-0 samples. The ePM protein yields obtained for each sample were fairly similar across the three extractions (each set of four samples per biological replicate were processed separately), indicating that the protocol employed was followed with a good level of consistency. As expected, a reduction in protein yield was observed from the microsomal fraction to the final

ePM fraction for each sample, suggesting that treatment with Brij-58 successfully eliminated a large amount of contaminating cytosolic/organellar proteins from the PM vesicles (Collins et al., 2017). It must be noted that for the majority of the samples the ePM yield was between ~40-60% of the microsomal protein present prior to the Brij-58 wash steps, however for the ‘No-0 75 mM NaCl 1’ and ‘35S::*NIT2* untreated 1’ samples, this yield was noticeably higher at 76.69% and 84.89%, respectively. While this could possibly be attributed to a smaller reduction in contaminating cytosolic/organellar proteins during the Brij-58 wash steps for these two samples, it should also be noted that their respective microsomal yields were lower than the other two replicates for each of their respective treatments. It is thus also possible that the initial microsomal protein fraction contained a higher proportion of PM proteins, such that less contaminating cytosolic/organellar proteins were present, and subsequently removed during the Brij-58 washes. Ultimately, the yield of ePM proteins obtained was similar across all the samples.

Table 3.1: Microsomal and enriched plasma membrane protein yields

Sample	Replicate	Microsomal yield (µg)	ePM yield (µg)	ePM yield (%)
No-0 untreated	1	187.8	81.3	43.29
	2	150.7	81.3	53.95
	3	168.7	94.5	56.02
No-0 75 mM NaCl	1	103.4	79.3	76.69
	2	194.4	113.8	58.54
	3	203.3	84.3	41.47
35S:: <i>NIT2</i> untreated	1	176.1	149.5	84.89
	2	233.2	104.4	44.77
	3	227.5	114	50.11
35S:: <i>NIT2</i> 75 mM NaCl	1	313.6	187.8	59.89
	2	249.5	118.4	47.45
	3	320.1	168.8	52.73

3.3.2. Assessment of 35S::*NIT2* and No-0 ePM protein sample integrity

In order to determine if the ePM samples were suitable for further proteomic analysis, their integrity was assessed by PAGE separation. Referring to Figure 3.1, distinct protein bands were observed in all the samples. The No-0 untreated replicate 1 was the only sample that appeared to be slightly different, with fewer obvious protein bands present. Importantly, distinct high molecular weight proteins bands could be observed, suggesting that the ePM samples likely contained larger PM proteins of interest to this study, including the 104 kDa PM H⁺-ATPase.

Based on the presence of distinct protein bands, it was concluded that the ePM samples were not degraded, and thus were suitable for use in downstream proteomic analysis. It had initially been planned to conduct immunoblot experiments to probe the soluble, microsomal and ePM proteins fractions with antibodies against PM (AHA) and other non-PM proteins, however due to unforeseen issues that were out of my control, there was insufficient protein remaining to conduct these supplementary experiments in conjunction with the main proteomics analysis.

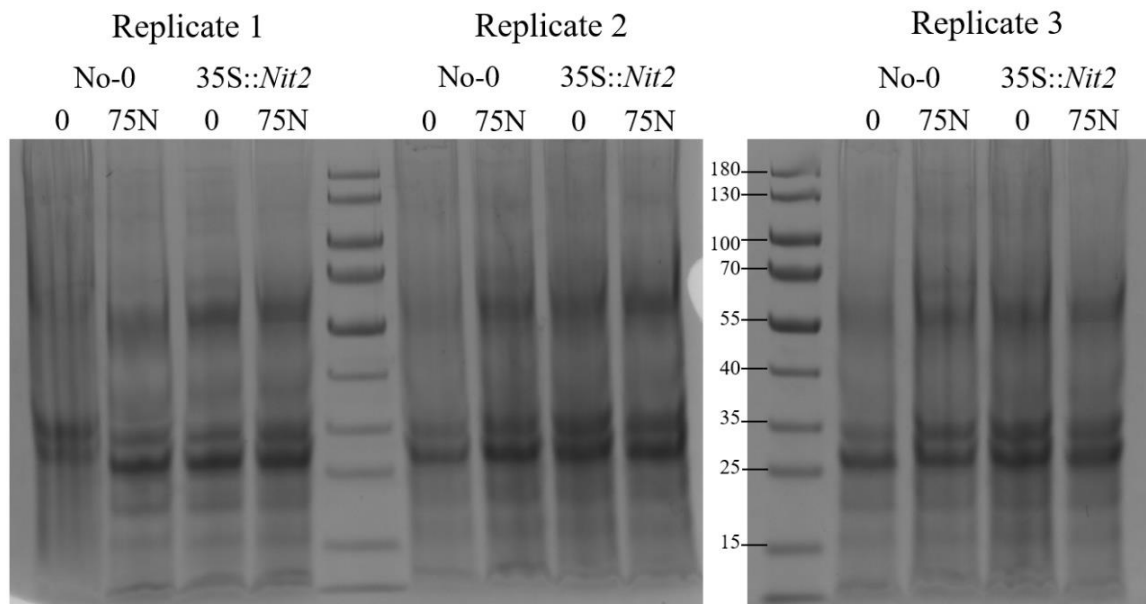


Figure 3.1: Bis-Tris PAGE of enriched plasma membrane proteins from untreated and salt-stressed 35S::*NIT2* and No-0 shoots

Enriched plasma membrane (ePM) proteins extracted from the shoots of untreated and 75 mM NaCl-treated No-0 and 35S::*NIT2* plants via a simplified enrichment protocol. The plants were grown hydroponically for 4 weeks on ¼ strength PN media, then either treated with ¼ strength PN media supplemented with 75 mM NaCl (75N), or maintained on ¼ strength PN media (0) for 3 days further. Three biological replicates were used. Here, 15 µg of the ePM protein samples were separated via 10% bis-Tris PAGE, and visualisation of protein bands was performed by Coomassie stain. The PageRuler™ Prestained Protein Ladder, 10-180 kDa (Thermo Fisher Scientific Inc., 26616) was used.

3.3.3. Protein identification in the 35S::*NIT2* and No-0 ePM samples by LC-MS/MS

The ePM samples were analysed by LC-MS/MS, following an in gel tryptic digest, both of which were conducted at the proteomics unit of the Central Analytical Facility at the University of Stellenbosch. A protein identification was accepted if it contained at least 2 identified peptides, and could be established at greater than 95.0% probability to achieve an FDR less than 1.0%. Referring to Table 3.2, it can be seen that over 2000 proteins were identified in total

in each of the four sample groups, with the lowest number identified in No-0 untreated (2149) and the highest in the 35S::*NIT2* untreated (2625). Gene Ontology terms were assigned to the identified proteins from the Uniprot Arabidopsis GO analysis database, which allowed for the classification of their cellular location, and molecular function. Over 50% of all the proteins identified in each sample group were membrane proteins (See Table 3.2). In turn just under 50% of the membrane proteins identified were assigned as PM proteins, and approximately 40% were identified as organelle membrane proteins. Thus, although the total number of proteins identified differed between the four sample groups, the proportion of PM proteins identified was very consistent.

Table 3.2: Number of proteins identified in the 35S::*NIT2* and No-0 ePM sample groups

Sample	Total	Membrane	Plasma membrane	Organelle membrane
No-0 untreated	2149	1115	553 (49.59%)	437 (39.19%)
No-0 75 mM NaCl	2437	1277	635 (49.73%)	524 (41.03%)
35S:: <i>NIT2</i> untreated	2625	1517	737 (48.58%)	597 (39.35%)
35S:: <i>NIT2</i> 75 mM NaCl	2294	1401	689 (49.18%)	559 (39.90%)

Note: The % represents the proportion of identified membrane proteins assigned as plasma or organelle membrane

For subsequent analyses, only proteins that were identified in at least two of the three biological replicates for any sample group were considered further, reducing the total number of PM proteins identified across the four sample groups to 288. These PM proteins were classified according to the following functional categories based off evidence contained in the Uniprot and TAIR databases: Transporters, Receptor kinases, Other signalling-related proteins, Membrane trafficking and structure, Cell-wall related proteins, and Other proteins. For a complete list of the proteins refer to appendix chapter 7, Table 7.3. In total, 105 transporters were identified, of which 94 were present in more than one sample group. Eight of the eleven PM H⁺-ATPase isoforms were identified, with AHA1, 2, 3, 7, 9, and 11 detected in all four of the sample groups. All thirteen of the PIP isoforms were identified except for PIP2-4. Multiple Calcium transporting ATPases (ACAs), as well as fourteen members of the ATP-binding cassette (ABC) transporter super-family were detected. Of particular interest, several auxin transporters, or transporters involved in auxin-related processes were identified, including Auxin transporter protein 1 (AUX1), Auxin-transporter-like protein 1 (LAX1), PIN3, PIN4, and PIN7, Phospholipid-transporting ATPases 3, 9, 10, and 11 (ALA3, 9, 10, 11), and several

ABC family transporters (ABCC3, 4, 5, 8, 14, ABCB1, 4, 6, 19, 20, 21, and ABCG11, 12, 22, 32, 36). Additionally, the sodium/calcium exchanger, NCL, was identified, along with transporters known to play a role in the salt stress response, such as Sugar transporter ERD6, Sugar transport protein 13, Monosaccharide-sensing proteins 1 and 2, and ABCG11 and 12. The remaining transporters identified have a diverse range of roles, including the transport of organic and inorganic ions, sugars, oligopeptides, amino acids, and other compounds.

Many PM proteins involved in cellular signalling were identified, including 62 receptor kinases. Additionally, 54 proteins involved in other signalling-related processes were detected, including a number of calcium-dependent protein kinases, as well as other kinases acting downstream in the defence response, and in brassinosteroid signalling. Several proteins implicated in auxin signalling were identified: Transmembrane Kinase 1, 3, and 4 (TMK1, 3, 4), Auxin-induced in root cultures protein 12 (AIR12), Calcium-dependent protein kinase 29 (CPK29), and Ras-related protein RABA1a. Likewise, multiple proteins involved in signalling during the salt stress response were detected: MALE DISCOVER1 (MDIS1)-interacting receptor like kinase 2, Serine/threonine-protein kinase BSK5, Calcium-dependent protein kinases 3 and 32, Phosphoinositide phospholipase C 1, Plasma membrane-associated cation-binding protein 1, Remorin 1.4, and the Ras-related proteins RABA1b and RABG3e.

Additionally, 27 PM proteins involved in membrane trafficking and structure were detected. Identified proteins functioning in PM vesicle trafficking included five out of the six clathrin proteins that together form the subunits of vesicular clathrin coats, four syntaxin proteins that function in SNARE-mediated membrane fusion, Patellin-1 and -2, and five Dynamin proteins. Furthermore, the Fasciclin-like arabinogalactan proteins 1, 2, 7, 8, 9, and 13 (FLA1, 2, 7, 8, 9, and 13), which are thought to contribute to PM structure in varied ways, were detected.

A total of 17 PM proteins with cell wall-related function were identified. The majority of these function in cell wall biogenesis, including six cellulose synthase proteins, and six callose synthase proteins. Of particular interest, Cellulose synthase 1 and Endoglucanase 25 have both been implicated in the modification of the cell wall during the hyperosmotic salinity response (Kang et al., 2008).

Finally, most of the remaining 23 PM proteins identified function in the defence response to various biotic and abiotic stresses. Of these, Annexin D1 and Annexin D4 (ANN1 and ANN4), as well as Early-Responsive to Dehydration 7 (ERD7) were of interest as they have all been implicated in the plant salt stress response, with the two Annexins acting in calcium-dependent

signalling during salt and osmotic stress responses (Huh et al., 2010; Laohavisit et al., 2013; Ma et al., 2019), and ERD7 likely playing a role in cell membrane lipid composition remodelling (Barajas-Lopez et al., 2021). Based off the above, it is clear that a broad range of PM proteins were successfully identified by LC-MS/MS analysis across the four ePM protein sample groups, with the vast majority of the identified proteins detected in two or more of the sample groups.

3.3.4. Identification of salt-responsive PM proteins in the 35S::*NIT2* line and No-0

In order to assess the effect of salt stress on the PM proteome of both the 35S::*NIT2* line and No-0, a quantitative comparison of the PM proteins identified in the untreated and 75 mM NaCl samples of the respective genotypes was performed. The normalised total spectra number of the three biological replicates was compared between the two treatments, and a Fisher's Exact Test, was employed to identify proteins that were differentially expressed. Benjamini-Hochberg correction for multiple testing was employed (Benjamini & Hochberg, 1995).

3.3.4.1 Salt-responsive PM proteins in No-0

A list of the salt-responsive PM proteins in No-0, ranked according to Log₂FC values, can be found in Table 3.3. Here, six proteins were found be up-regulated in response to salt stress, and a further two proteins were only detected in the salt-stressed samples. As expected, the up-regulated list included three proteins that have been shown to function in the salt stress response, namely ERD7 (which displayed the highest degree of up-regulation), sodium/calcium exchanger NCL, and Beta-D-glucopyranosyl abscisate beta-glucosidase (BGLU18). Additionally, the IAA biosynthesis protein Nitrilase 2, as well as the Calcium-dependent protein kinase 29 (CPK29), which has been implicated in auxin-related signalling processes, had increased abundance in response to salt (*NIT2* was only detected in the salt-stressed samples).

A total of nine proteins were significantly down-regulated in response to salt stress, and an additional four proteins were found to only be detected in the untreated samples. Two of the most down-regulated proteins, Pyridoxal 5'-phosphate synthase subunit PDX1.3 and ANN1 have been shown to function in the antioxidant response. More specifically, PDX1.3 is involved in Vitamin B6 biosynthesis, and ANN1 has peroxidase activity (Gorecka et al., 2005; Titiz et al., 2006). Additionally, Dynamin-2A, Dynamin-2B (DRP2A and DRP2B), and Patellin-1 (PATL1), all of which play a role in membrane trafficking, were down-regulated. The other notable down-regulated protein was the Nitrate transporter 1.11. Of the proteins only

detected in control conditions, the Probable pectin methylesterase CGR3, and the Cellulose-synthase-like protein G3 (CSLG3), play a role in cell wall modification/biosynthesis.

3.3.4.2. Salt-responsive PM proteins in the 35S::NIT2 line

A list of the salt-responsive PM proteins in the 35S::NIT2 line, ranked according to Log₂FC values, can be found in Table 3.4. Notably, far fewer salt-responsive proteins were identified in the 35S::NIT2 line than No-0. Only four proteins were found to be differentially regulated, with two proteins up-regulated in response to salt stress. The uncharacterized GPI-anchored protein, AT5G19240, was the most up-regulated, followed by Nitrilase 2. The observation of increased NIT2 abundance was interesting as it suggests that salt stress induced a further increase in expression in the 35S::NIT2 line where NIT2 is already highly expressed. Only two proteins were identified as down-regulated, both of which were only detected in untreated conditions. These were the ABCC3 transporter, and Callose synthase 3.

Table 3.3: Plasma membrane proteins with altered abundance in response to salt in No-0

Protein	Accession number*	Molecular Function**	Log ₂ FC	Average total spectra (quantitative value)		Average total unique peptides	
				Untreated	Salt	Untreated	Salt
Up-regulated							
Protein EARLY-RESPONSIVE TO DEHYDRATION 7	O48832	Cell membrane lipid composition remodelling, response to water deprivation, response to salt stress	2.86	1.33	9.67	1.33	7
Calcium-dependent protein kinase 29	Q8RWL2	Calmodulin-dependent protein kinase activity, modulates PIN-FORMED polarity	2.66	1.67	6.33	1.67	5
Mechanosensitive ion channel protein 6	Q9SYM1	Monoatomic anion transport, response to water deprivation	2.12	1	4.33	1	4
ABC transporter C family member 4	Q7DM58	Stomatal aperture regulation, folic acid transmembrane transport, response to water deprivation	0.76	12	20.33	11.33	17.33
Sodium/calcium exchanger NCL	Q8L636	Calcium:sodium antiporter activity, cellular response to salt stress	0.70	10.67	17.33	5.67	6
Beta-D-glucopyranosyl abscisate beta-glucosidase (BGLU18)	Q9SE50	Absciscic acid metabolic process, response to salt stress	0.29	47.33	57	25.67	30.33
Nitrilase 2	P32962	Indole-3-acetic acid (IAA) biosynthesis	ND in unt.	0	2	0	1.33
G-type lectin S-receptor-like serine/threonine-protein kinase AT2G19130	O64477	Regulation of defense response, response to water deprivation	ND in unt.	0	4.67	0	3.67
Down-regulated							
Pyridoxal 5'-phosphate synthase subunit PDX1.3	Q8L940	Vitamin B6 biosynthetic process, response to oxidative stress, response to salt stress	-3.32	8.67	1.33	6	1
Protein NRT1/ PTR FAMILY 1.2 (NPF1.2) / Nitrate transporter 1.11	Q9M817	Nitrate transmembrane transport	-2.72	11	1.67	7.67	1.67
Annexin D1 (ANN1)	Q9SYT0	Peroxidase activity, response to salt stress	-2.52	7.67	1.33	5.67	1
Patellin-1 (PATL1)	Q56WK6	Membrane-trafficking, negatively regulates plant salt tolerance	-1.63	34	18	23.33	13.33
Tubulin alpha-4 chain	Q0WV25	Structural constituent of cytoskeleton	-1.36	39.67	11	20	5.33
Dynamin-2B (DRP2B)	Q9LQ55	Clathrin binding, clathrin-dependent endocytosis	-1.30	28.67	11.67	23	10.33
Tubulin beta-9 chain	P29517	Structural constituent of cytoskeleton	-1.22	54.33	21.67	34.33	18

Dynamin-2A (DRP2A)	Q9SE83	Microtubule binding, trafficking from the trans-Golgi network	-1.17	24	10.67	20.33	9.33
14-3-3-like protein GF14 omega	Q01525	Brassinosteroid mediated signaling pathway	-0.76	30	17.67	25.67	14.67
Sulfate transporter 4.1	Q9FY46	Sulfate transmembrane transporter activity	ND in salt	4.67	0	4	0
Serine/threonine-protein phosphatase 2A (PP2AA1)	Q38845	Auxin polar transport, abscisic acid-activated signaling pathway	ND in salt	5	0	4.33	0
Cellulose synthase-like protein G3 (CSLG3)	Q0WVN5	Cellulose synthase activity, primary cell wall biogenesis	ND in salt	3.67	0	3.33	0
Probable pectin methylesterase (CGR3)	Q0WPN7	Pectin biosynthesis	ND in salt	3.67	0	3.67	0

*Uniprot accession number; ** Information on molecular function obtained from TAIR (<https://www.arabidopsis.org/>); Benjamini-Hochberg adjusted p -value = 0.01088

Table 3.4: Plasma membrane proteins with altered abundance in response to salt in 35S::*NIT2*

Protein	Accession number*	Molecular Function**	Log2FC	Average total spectra (quantitative value)		Average total unique peptides		
				Untreated	Salt	Untreated	Salt	
Up-regulated								
Uncharacterized GPI-anchored protein AT5G19240	Q84VZ5	-	1.51	2.33	6.67	1.33	3.33	
Nitrilase 2	P32962	Indole-3-acetic acid (IAA) biosynthesis	1.33	8.33	21	4.67	9.33	
Down-regulated								
ABC transporter C family member 3	Q9LK64	ABC-type glutathione S-conjugate transporter activity	ND in salt	5	0	3.67	0	
Callose synthase 3	Q9LXT9	(1->3)-beta-D-glucan biosynthetic process, cell wall organisation	ND in salt	6	0	2.67	0	

*Uniprot accession number; ** Information on molecular function obtained from TAIR (<https://www.arabidopsis.org/>); Benjamini-Hochberg adjusted p -value = 0.00155

Table 3.5: Plasma membrane proteins with altered abundance between lines in untreated conditions

Protein	Accession number*	Molecular Function**	Log ₂ FC	Average total spectra (quantitative value)		Average total unique peptides		
				No-0	35S:: <i>NIT2</i>	No-0	35S:: <i>NIT2</i>	
Higher abundance in 35S:: <i>NIT2</i> compared with No-0								
Fasciclin-like arabinogalactan protein 13	Q9FFH6	Plant-type cell wall organization or biogenesis, cell surface adhesion	3.70	1	13	1	8	
Mechanosensitive ion channel protein 1	Q8VZL4	Monoatomic ion transport, cellular response to oxidative stress	3.32	1	10	1	7	
Ammonium transporter 1	P54144	Ammonium transmembrane transport	3.0	0.67	5.33	0.67	2.67	
ABC transporter C family member 5	Q7GB25	Inositol hexakisphosphate transport, response to salt stress, stomatal opening	2.91	0.67	5	0.67	4	
Urea-proton symporter DUR3	F4KD71	Urea transmembrane transport, response to nitrogen starvation	2.70	0.67	4.33	0.67	3.33	
LysM domain receptor-like kinase 5 (LYK5)	O22808	Chitin binding, innate immune response	2.58	1.33	8	1.33	6	
Probable phospholipid-transporting ATPase 11 (ALA11)	Q9SAF5	Protein localization involved in auxin polar transport, PIN trafficking	2.58	4.67	17	4.67	13.33	
Putative phospholipid-transporting ATPase 9 (ALA9)	Q9SX33	Protein localization involved in auxin polar transport, PIN trafficking	2.46	1.33	7.33	1.33	6.33	
Protein EARLY-RESPONSIVE TO DEHYDRATION 7	O48832	Cell membrane lipid composition remodelling, response to water deprivation, response to salt stress	2.09	1.33	5.67	1.33	4.67	
ABC transporter C family member 4	Q7DM58	Stomatal aperture regulation, folic acid transmembrane transport, response to water deprivation	0.92	11.67	22	11	18.33	
ABC transporter G family member 36	Q9XIE2	Auxin precursor indole 3-butyric acid (IBA) efflux transport, auxin efflux transmembrane transporter activity, response to salt stress	0.40	92.67	124.33	50	58	
Nitrilase 2	P32962	Indole-3-acetic acid (IAA) biosynthesis	ND in No-0	0	8.33	0	4.67	
Phospholipid-transporting ATPase 10 (ALA10)	Q9LI83	Protein localization involved in auxin polar transport, PIN trafficking	ND in No-0	0	3.67	0	2.67	
ABC transporter C family member 3	Q9LK64	ABC-type glutathione S-conjugate transporter activity	ND in No-0	0	5	0	3.67	

Protein HYPER-SENSITIVITY-RELATED 4	Q8VZG2	Plant-type hypersensitive response, response to bacterium	ND in No-0	0	2.67	0	2
Clathrin light chain 3	F4J5M9	Clathrin heavy chain binding, clathrin-dependent endocytosis	ND in No-0	0	5.33	0	3.67
Protein ACCELERATED CELL DEATH 6	Q8LPS2	Regulates salicylic acid (SA) signalling leading to cell death, defense response to bacterium	ND in No-0	0	4	0	2.67
Protein PLANT CADMIUM RESISTANCE 1	Q9LQU2	Involved in glutathione-independent cadmium resistance	ND in No-0	0	7	0	1.67
Lower abundance in 35S::NIT2 compared with No-0							
Annexin D4 (ANN4)	Q9ZVJ6	Calcium ion binding, response to salt stress	-2.32	5	1	4.33	1
Coatomer subunit alpha-1	Q94A40	Member of the Coat Protein I (COPI) complex, retrograde vesicle-mediated transport	-1.97	15.67	4	14.33	4
Coatomer subunit beta-1	Q9SV21	Member of the Coat Protein I (COPI) complex, retrograde vesicle-mediated transport	-1.93	12.67	3.33	10.33	3
Coatomer subunit alpha-2	Q9SJT9	Member of the Coat Protein I (COPI) complex, retrograde vesicle-mediated transport	-1.54	20.33	7	17.33	6.33
Patellin-1 (PATL1)	Q56WK6	Membrane-trafficking, negatively regulates plant salt tolerance	-1.37	34.33	13	23.33	10.33
Dynamin-related protein 1E (DRP1E)	Q9FNX5	Microtubule binding, vesicle-mediated transport	-1.35	7.67	3	7	3
Tubulin alpha-5 chain	B9DHQ0	Structural constituent of cytoskeleton	-1.25	40	17	20.33	10.33
Patellin-2	Q56ZI2	Protein localization involved in auxin polar transport	-1.10	15.67	7.33	14.67	7
Tubulin beta-5 chain	P29513	Structural constituent of cytoskeleton	-0.9	52.67	31.67	33.33	23.67
Dynamin-related protein 1A (DRP1A)	P42697	Clathrin and microtubule binding, clathrin-dependent endocytosis	-0.53	18.33	12.67	14.67	10.33
Dynamin-2B (DRP2B)	Q9LQ55	Clathrin and microtubule binding, clathrin-dependent endocytosis	-0.42	27.33	15.67	22	14.67
Serine/threonine-protein phosphatase 2A (PP2AA1)	Q38845	Auxin polar transport, abscisic acid-activated signaling pathway	ND in 35S::NIT2	4.67	0	4	0
Pyridoxal 5'-phosphate synthase subunit PDX1.1	O80448	Vitamin B6 biosynthetic process	ND in 35S::NIT2	6.67	0	5	0
Pyridoxal 5'-phosphate synthase subunit PDX1.3	Q8L940	Vitamin B6 biosynthetic process, response to oxidative stress, response to salt stress	ND in 35S::NIT2	8.67	0	6	0
Non-classical arabinogalactan protein 31	Q9FZA2	May contribute to cell wall strengthening, response to jasmonic acid	ND in 35S::NIT2	7	0	4.33	0

*Uniprot accession number; ** Information on molecular function obtained from TAIR (<https://www.arabidopsis.org/>); Benjamini-Hochberg adjusted p -value = 0.00868

Table 3.6: Plasma membrane proteins with altered abundance between lines in salt-stress conditions

Protein	Accession number*	Molecular Function**	Log ₂ FC	Average total spectra (quantitative value)		Average total unique peptides	
				No-0	35S:: <i>NIT2</i>	No-0	35S:: <i>NIT2</i>
Higher abundance in 35S:: <i>NIT2</i> compared with No-0							
Nitrilase 2	P32962	Indole-3-acetic acid (IAA) biosynthesis	3.39	2	21	1.33	9.33
Urea-proton symporter DUR3	F4KD71	Urea transmembrane transport, response to nitrogen starvation	2.66	1	6.33	0.67	4.33
Probable LRR receptor-like serine/threonine-protein kinase AT5G10290	C0LGT1	Defense response to bacterium, innate immune response	2.17	0.67	4.67	0.67	4.33
ABC transporter G family member 36	Q9XIE2	Auxin precursor indole 3-butyric acid (IBA) efflux transport, auxin efflux transmembrane transporter activity, response to salt stress	1.34	47	119.33	29	51.33
Potassium transporter 8 (KUP8)	Q9M7J9	Potassium ion transport	1	1.33	2.67	1.33	1.33
Glycerophosphodiester phosphodiesterase GDPDL1	Q7Y208	Cell wall organisation, intracellular phosphate ion homeostasis	0.90	14.67	27.33	8.67	14.33
Plasma membrane H ⁺ -ATPase isoform 2 (AHA2)	P19456	Proton transmembrane transport, regulation of intracellular pH	0.24	98.67	116.33	46.67	48.33
Amino acid permease 6	P92934	Aspartate and tryptophan transmembrane transport	ND in No-0	0	3	0	2.33
Sugar transport protein 7	O04249	L-arabinose transmembrane transport	ND in No-0	0	2.67	0	1.67
Cellulose synthase-like protein G3 (CSLG3)	Q0WVN5	Cellulose biosynthesis, primary cell wall biogenesis	ND in No-0	0	3.67	0	3.33
Probable pectin methylesterase (CGR3)	Q0WPN7	Pectin biosynthesis	ND in No-0	0	3.67	0	2.67
Receptor-like protein kinase AT5G59670	Q9FN94	Defense response to bacterium, innate immune response	ND in No-0	0	5.67	0	5.33
Protein ACCELERATED CELL DEATH 6	Q8LPS2	Regulates salicylic acid (SA) signalling leading to cell death, defense response to bacterium	ND in No-0	0	5.67	0	3.67
Lower abundance in 35S:: <i>NIT2</i> compared with No-0							
Annexin D4 (ANN4)	Q9ZVJ6	Calcium ion binding, response to salt stress	-3.46	11	1	9	1
Dynamamin-related protein 1C (DRP1C)	Q8LF21	Microtubule binding, mitochondrion organization	-3.32	10	1	10	1

Coatomer subunit alpha-2	Q9SJT9	Member of the Coat Protein I (COPI) complex, retrograde vesicle-mediated transport	-2.46	18.33	3.33	15.67	3.33
Aquaporin TIP1-1	P25818	urea transmembrane transporter activity, water channel activity	-1.74	6.67	1.67	3.67	1.67
Protein EARLY-RESPONSIVE TO DEHYDRATION 7	O48832	Cell membrane lipid composition remodelling, response to water deprivation, response to salt stress	-1.4	9.67	3.67	7	2.33
Probable aquaporin TIP2-2	Q41975	Water transport	-0.95	10.33	5.33	6.67	3.33
Dynamin-related protein 1A (DRP1A)	P42697	Clathrin and microtubule binding, clathrin-dependent endocytosis	-0.93	13.33	7	9.67	6.33
ABC transporter G family member 22	Q93YS4	ATPase-coupled transmembrane transporter activity, response to water deprivation, transpiration	-0.77	19.33	11.33	13.33	9.33
Glyceraldehyde-3-phosphate dehydrogenase GAPC2	Q9FX54	Gluconeogenesis, glycolytic process	-0.59	24.67	16.33	14.33	11
Mechanosensitive ion channel protein 5	Q9LH74	Monoatomic ion transport, response to osmotic stress	ND in 35S::NIT2	3	0	2.67	0
Boron transporter 1	Q8VYR7	Efflux-type boron transporter for xylem loading	ND in 35S::NIT2	2.33	0	2.33	0
Syntaxin-122	Q9SVC2	SNARE binding during exocytosis, vesicle docking, vesicle fusion	ND in 35S::NIT2	4	0	2.33	0
NDR1/HIN1-like protein 1	Q9SRN0	Defence response to virus	ND in 35S::NIT2	3.67	0	3	0

*Uniprot accession number; ** Information on molecular function obtained from TAIR (<https://www.arabidopsis.org/>); Benjamini-Hochberg adjusted p -value = 0.00718

3.3.5. Identification of PM proteins differentially regulated between the 35S::*NIT2* line and No-0

Given the differences in the number of salt-responsive proteins observed between the 35S::*NIT2* line and No-0, it was likely that alterations in the PM proteome contribute towards the improved salt tolerance of the 35S::*NIT2* line. In order to further explore this idea, genotype-specific differences in PM proteomes were assessed both in untreated and saline conditions. The quantitative comparisons were performed as outlined in section 3.3.4., with the fold-change in abundance in the 35S::*NIT2* line calculated relative to No-0.

3.3.5.1. Genotype-specific differences in PM proteins in control conditions

The PM proteins that were differentially expressed between the 35S::*NIT2* line and No-0 in the untreated samples can be found in Table 3.5, ranked according to Log₂FC values. A total of 11 proteins were found to have significantly higher abundance in the 35S::*NIT2* line than in No-0, and a further seven proteins were only detected in the 35S::*NIT2* samples. Notably, this included several proteins involved in auxin-related processes. Nitrilase 2 was only detected in the 35S::*NIT2* line. Additionally, ALA9 and ALA11 had higher abundance in the 35S::*NIT2* line, and ALA10 was not detected in No-0. These three proteins function redundantly in PIN trafficking, thereby affecting polar auxin transport (PAT) (Zhang, Adamowski, et al., 2020). The ABCG36 transporter, which contributes to auxin efflux (Strader & Bartel, 2009), was also found to have slightly, yet significantly, higher abundance in the 35S::*NIT2* line. Several other transporters had higher abundance, including the Mechanosensitive ion channel protein 1 (MSL1), Ammonium transporter 1 (AMT1;1), Urea-proton symporter DUR3, and the ABCC4 transporter, the latter of which plays a role in stomatal aperture regulation in response to water deprivation. An interesting observation was that ERD7, which was significantly up-regulated in response to salt stress in No-0 but not salt-responsive in the 35S::*NIT2* line (see section 3.3.5), was found to have higher abundance in the 35S::*NIT2* line when compared to No-0 under control conditions. Other PM proteins with increased abundance included the LysM domain receptor-like kinase 5 (LYK5), which detects chitin leading to the initiation of innate immune responses, and FLA13, which is largely uncharacterised, but is thought to potentially play a role in cell wall organisation and biogenesis, or in cell surface adhesion.

A total of 11 proteins were found to have significantly lower abundance in the 35S::*NIT2* line than in No-0, and a further four proteins were not detected in the 35S::*NIT2* samples. The proteins whose abundance was found to decrease the most in the 35S::*NIT2* line was ANN4, which is thought to play a role in the salt and osmotic stress responses via calcium-dependent

signalling (Ma et al., 2019). Notably, eight of these proteins are involved in membrane-trafficking/endocytosis in some way, including three Coatamer subunit proteins, DRP2B, DRP1A and 1E (all of which function in clathrin and microtubule binding during clathrin-dependent endocytosis), PATL1, and PATL2. Additionally, the Tubulin alpha-5 chain and Tubulin beta-5 chain cytoskeleton structural proteins were also less abundant in the 35S::*NIT2* line. The Vitamin B6 biosynthesis proteins PDX1.1 and PDX1.3 were not detected in the 35S::*NIT2* line.

3.3.5.2. Genotype-specific differences in PM proteins in saline conditions

The PM proteins that were differentially expressed between the 35S::*NIT2* line and No-0 in the salt-stressed samples can be found in Table 3.6, ranked according to Log₂FC values. Here, seven proteins were found to have significantly higher abundance in the 35S::*NIT2* line than in No-0, and an additional six proteins were only detected in the 35S::*NIT2* samples. As was seen in the untreated comparison, Nitrilase 2 was more abundant in the 35S::*NIT2* line, and was the protein with the largest fold-change in abundance (Log₂FC = 3.39). The auxin efflux transporter ABCG36 was also found to have increased abundance, with the difference in expression between the two lines observed in saline conditions being greater than that observed in untreated conditions (Log₂FC = 1.34 as opposed to 0.4). Likewise, DUR3 was again found to have increased abundance in the 35S::*NIT2* line. The potassium transporter KUP8, was also found to have increased abundance in the 35S::*NIT2* line. The LRR RLK AT5G10290, which has also been implicated in the innate immune response, was also found to have higher abundance. Notably, a very small yet significant increase in levels of the PM H⁺-ATPase isoform 2 (AHA2) was observed in the 35S::*NIT2* line compared with No-0. The Glycerophosphodiester phosphodiesterase GDPDL1 protein, which plays a role in cell wall organisation, was also found to have higher abundance in the 35S::*NIT2* line. The proteins only detected in the 35S::*NIT2* line included two transporters (Amino acid permease 6, Sugar transport protein 7), a RLK involved in bacterial defence response, two proteins involved in cell wall modification (CSLG3 and CGR3), and ACCELERATED CELL DEATH 6 (ACD6), which has been shown to be involved in the regulation of salicylic acid signalling during innate immune responses as well as leaf senescence (Rate et al., 1999).

A total of nine proteins were found to have significantly lower abundance in the 35S::*NIT2* line than in No-0, and a further four proteins were not detected in the 35S::*NIT2* samples. ANN4 was again found to be the protein whose abundance decreased the most in the 35S::*NIT2* line. Additionally, a number of proteins involved in membrane trafficking/endocytosis had lower

abundance, as was observed in the untreated comparison, including DRP1A, DRP1C and Coatamer subunit alpha-2. Interestingly, ERD7 was found to have lower abundance in the 35S::*NIT2* line compared with No-0 in response to salt stress, whereas the opposite was observed in untreated conditions. Notably also, the tonoplast aquaporins TIP1-1 and TIP2-2 were present in lower levels in the 35S::*NIT2* line, along with the ABCG22 transporter, which plays a role in the regulation of transpiration in response to water deprivation (Kuromori, Sugimoto & Shinozaki, 2011). Two transporter proteins were not detected in the 35S::*NIT2* line: MSL5 and Boron Transporter 1.

A visual representation of the up- and down-regulated proteins in each of the four two-way comparisons can be found in the form of a basic heatmap in appendix chapter 7, Figure 7.2.

3.4. Discussion:

3.4.1. PM proteins were robustly detected in the 35S::*NIT2* and No-0 ePM samples

The previous PM proteomic studies conducted have identified a suite of high abundance PM proteins that are consistently, and robustly detected. Here I report the detection of a very similar set of PM proteins, most of which were detected in both No-0 and the 35S::*NIT2* line, providing confidence to the notion that PM proteins were successfully enriched in our samples. Other similar LC-MS/MS proteomic analyses performed on leaf PM protein samples by Bernfur et al. (2013) and Zarrini et al. (2012) identified a total of 41 and 177 PM proteins, respectively. The 288 PM proteins that were confidently detected in at least two biological replicates per sample group, and functionally assigned, in our analysis exceeds these numbers. Furthermore, the proportion of the total proteins identified in these two previous studies (703 and 900, respectively) that were annotated as membrane proteins was 26.7% and 33.8%, respectively, and in turn the proportion of these membrane proteins that were assigned as PM proteins was 21.8% and 58.2%, respectively. By comparison, the proportion of the total proteins identified across the four sample groups in our analysis that were annotated as membrane proteins ranged between 51.9% to 61%, and of these membrane proteins ~50% were annotated as PM proteins in all four of the sample groups. The protocol that I utilised was thus clearly successful in obtaining a high yield of membrane proteins, and ultimately as the proportion of PM proteins identified was comparable to previous studies, it was successful in enriching for PM proteins as well. Additionally, both of these previous studies utilised a Two-Phase aqueous system to obtain more pure PM fractions prior to MS analysis, whereas an enriched PM fraction was used

for MS analysis in this chapter. It would thus appear that it is unnecessary to use ultra-pure PM protein fractions in order to detect a large number of PM proteins, although the number of PM proteins detected in our analysis would presumably have been increased with a reduction in the amount of contaminating cytoplasmic and organelle membrane proteins. The 105 transporters detected here is more than is reported in previous studies. Notably eight of the eleven PM H⁺-ATPase isoforms, all of the PIPs (except for PIP2;4), and 14 ABC family transporters were detected.

It was hoped that some of the PM Na⁺ and K⁺ transporters/channels whose gene expression was measured in chapter 2 would be detected at the protein level. However, aside from NHX1 and TIP2;2 (which are both located on the tonoplast membrane), this was not the case. Consistent with the gene expression analysis, the levels of NHX1 protein were not salt-responsive in either No-0 or the 35S::*NIT2* line, and no difference was observed between the two lines in either conditions. *TIP2;2* RNA levels were significantly higher in No-0 than the 35S::*NIT2* line in control conditions, with no difference observed in saline conditions, whereas *TIP2;2* protein abundance was significantly higher in No-0 than the 35S::*NIT2* line in saline conditions, but not in the control. It was not unexpected that the other Na⁺ and K⁺ transporters/channels were not detected here, as they have not been detected in any of the previous PM proteomics studies discussed in section 3.1.2., presumably because they are present in relatively low abundance on the plasma membrane. A more targeted approach involving antibody pull-downs would likely be required in order to measure their abundance in the future. Nevertheless, some Na⁺ and K⁺ transporter proteins were detected in the ePM samples, including the Sodium/calcium exchanger NCL, as well as KUP3, 4, 5, 8, 12, and the two-pore potassium channel 1. Of particular interest were NCL and KUP8, which were found to be up-regulated in response to salt in No-0, and have higher abundance in the 35S::*NIT2* line than No-0 under saline conditions, respectively. These two transporters will be discussed further below.

3.4.2. Differences in the leaf PM proteome exist between the 35S::*NIT2* line and No-0 in both control and saline conditions

It is immediately apparent that there were significant differences in the PM proteomes of the two lines, with a number of transporters, RLKs/signalling proteins, cell membrane and cell wall-related proteins, and membrane trafficking proteins that had altered abundance between the two lines in both control and saline conditions. In general, several PM proteins were found to be salt-responsive in No-0, whereas almost no proteins were salt-responsive in the 35S::*NIT2*

line. This lack of changes in the PM proteome could suggest that the 35S::*NIT2* plants are not in a state of stress, or perhaps that their salt stress response is already up-regulated in control conditions. It is established that the 35S::*NIT2* plants are more salt-tolerant (Cackett et al., 2022), and in the previous chapter it was discussed how this can be explained, at least in part, by improved Na^+/K^+ homeostasis, and altered gene expression of several ion transporters/channels. Unfortunately, I could not detect any of these transporters in the proteomics analysis, but provided that the changes in gene expression of these transporters are reflected at the protein level, it is plausible that the improved ion homeostasis mechanism of the 35S::*NIT2* line is sufficient to mitigate the toxic effect of Na^+ on the plants, and thus large-scale changes in the PM proteome do not occur. Here I will discuss the potential contribution of notable PM proteins in the context of the salt stress response, and how the altered abundance of these proteins might contribute to the difference in salt tolerance between the two lines.

3.4.2.1. Salt-related proteins

A natural starting point is to consider changes in any proteins known to play a role in salt-tolerance, of which there were several. Perhaps most notable was the sodium/calcium exchanger NCL, which was the only transporter with confirmed Na^+ transport activity present in the list of differentially regulated proteins, where it was found to be up-regulated in No-0 in response to salt. NCL, which has been shown to be localised to both the plasma and tonoplast membranes, plays a role in the maintenance of Ca^{2+} homeostasis during the salt stress response (Wang, Li, et al., 2012). The $\text{Na}^+/\text{Ca}^{2+}$ antiport activity of NCL was demonstrated using an established fluorescence-based assay involving a CHO-K1 cell-based heterologous system, where Ca^{2+} transport in the presence of Na^+ was assessed based on changes in fluorescence. *NCL* is broadly expressed in the shoot, and its expression was shown, via RT-qPCR and GUS-staining, to be up-regulated in seedlings in response to treatment with 150 mM NaCl. Furthermore, *ncl* mutants were found to be less sensitive to salt stress than the wild-type or 35S::*NCL* overexpressing plants. The *ncl* mutants displayed altered Ca^{2+} homeostasis, with lower Na^+ and higher Ca^{2+} contents compared with the wild-type under saline conditions, as well as decreased net Ca^{2+} flux at the root surface, as measured using a non-invasive ion-selective microelectrode. Collectively, this data led the authors to propose that NCL functions in calcium-signalling in response to salt stress. There is also evidence implicating NCL with an indirect role in the negative regulation of auxin signalling (Li et al., 2016). *NCL* expression was decreased in seedlings in response to IAA, and *ncl* mutants displayed increased LR number, root elongation, and root-tip DR5::*GUS* expression (auxin-related phenotypes)

compared with the wild-type, with the opposite phenotypes observed in 35S::*NCL* lines. Interestingly, in contrast to the findings of Wang et al. (2012), these *ncl* mutants were more sensitive to salt stress, with increased Na⁺ and decreased Ca²⁺ ion accumulation, respectively. If a lack of NCL decreases salt-tolerance, it would make sense for the plant to up-regulate it in response to salt, as is observed here for No-0. It must be noted that these studies utilised different *ncl* T-DNA mutant lines (SALK vs SAIL), and the treatment length, as well as concentration of NaCl used was different. Nonetheless, it is clear that NCL plays a role in Ca²⁺ homeostasis/signalling during the salt stress response, as well as a potential role in the integration of auxin signalling. The finding that NCL is only salt-responsive in No-0 and not the 35S::*NIT2* line suggests a potential difference in Ca²⁺ signalling between the lines under saline conditions.

Calcium signalling is a crucial part of the plant salt stress response, regulating a diverse range of cellular processes over time, including rapid signalling responses following salt perception, integration and positive feedback with ROS signalling, and the regulation of Na⁺ efflux via the SOS pathway (van Zelm, Zhang & Testerink, 2020). It was thus interesting that two other proteins involved in Ca²⁺ signalling had altered abundance: ANN1 was down-regulated in response to salt in No-0, and ANN4 had lower abundance in the 35S::*NIT2* line compared with No-0 in both control and saline conditions. ANN1 has been shown to be essential for mediating the ROS-activated Ca²⁺ influx across the plasma membrane that occurs in response to increasing extracellular NaCl, with the resulting elevation in free cytosolic Ca²⁺ acting as a second messenger for adaptive signalling (Laohavisit et al., 2013). NaCl-induced influx of Ca²⁺ across the plasma membrane of root epidermal cells was abolished in *ann1* mutants, and consequently, both adaptive signalling and the production of secondary roots in response to salt was impaired. Despite the function of ANN1 in the salt stress response being characterised in Arabidopsis roots, ANN1 has been shown to function in the activation of calcium-dependent systemic defence in response to leaf herbivory (Malabarba et al., 2021), so it is possible that its reported role during salt stress also occurs in the leaves. Thus, the down-regulation of ANN1 in response to salt observed here would suggest that there is possibly a reduction in this ROS-mediated Ca²⁺ signalling in No-0 leaves. Like with NCL, the absence of ANN1 salt-responsiveness would suggest that this altered Ca²⁺ signalling does not occur in the 35S::*NIT2* line under saline conditions. In contrast with ANN1, ANN4 was not salt-responsive in either No-0 or the 35S::*NIT2* line, but rather had lower abundance in the 35S::*NIT2* line than in No-0 in both control and saline conditions. ANN4 also functions in transmembrane Ca²⁺ signalling that contributes to a number of stress responses, including oxidative, heat, plant immunity, and

most pertinently, salt stress (Liao, Zheng & Guo, 2017; Ma et al., 2019; Zhao et al., 2019). ANN4 has been shown to play a key role in regulating the calcium signal that activates the SOS pathway in response to salt stress, via interactions with the calcium censer SCaBP8 and the protein kinase SOS2 (Ma et al., 2019). The salt-induced increase in cytosolic Ca^{2+} was greatly diminished in *ann4* mutants, which were also found to be more salt-sensitive than the wild-type, with reduced germination. ANN4, SCaBP8 and SOS2 formed a negative feedback loop where SCaBP8 was shown to promote the interaction and subsequent phosphorylation of ANN4 by SOS2, which in turn increased the interaction between ANN4 and SCaBP8, thereby repressing ANN4 (Ma et al., 2019). The authors suggested that ANN4 likely functions as a Ca^{2+} transporter/channel, or channel cofactor, and acts in conjunction with SCaBP8 and SOS2 to create a fine-tuned Ca^{2+} signal to activate the SOS pathway for long term salt stress responses. However, more work needs to be done to determine what effect altered ANN4 expression has on Na^+ efflux transport via SOS1. Ultimately, the finding that ANN4 was decreased in abundance in the 35S::*NIT2* line in both control and saline conditions could suggest that there are constitutive alterations in the calcium-dependent SOS pathway signalling in this line.

Another protein that has been implicated in SOS regulation and the salt stress response that was found to have altered abundance between No-0 and the 35S::*NIT2* line was PATL1. PATL1 is a putative regulator of membrane trafficking, with it speculated to participate in clathrin-dependent endocytosis aiding in cell plate remodelling (Zhou et al., 2019). Interestingly, PATL1 has been shown to negatively modulate salt tolerance by affecting Na^+/H^+ antiport (Zhou et al., 2018). PATL1 was shown to directly interact with SOS1, and PATL1 T-DNA mutants with elevated PATL1 expression were found to be more salt sensitive with a concomitant decrease in Na^+/H^+ antiport activity, which together led the authors to hypothesise that PATL1-mediated membrane trafficking could regulate SOS1 localisation or cycling during salt stress. Unfortunately, I did not detect SOS1 protein in any of our samples, so I cannot begin to draw any comparisons here. Nonetheless, if PATL1 does negatively modulate salt tolerance, the finding that it was down-regulated in response to salt stress in No-0 makes sense. Furthermore, the observation that PATL1 was not salt-responsive in the 35S::*NIT2* line, but had decreased abundance in control conditions compared with No-0, suggests that the 35S::*NIT2* line is potentially predisposed to a better salt stress response due to lower PATL1 levels, and thus possibly increased SOS1 occupancy at the PM. While SOS1 protein was not detected here, in chapter 2, *SOS1* expression was shown to be significantly higher in the shoots

of the 35S::*NIT2* line compared with No-0 under saline conditions. Collectively, the finding of altered ANN4 and PATL1 abundance combined with increased *SOS1* expression strongly suggests that there is an alteration in the activity of the SOS pathway in the 35S::*NIT2* line under saline conditions.

The possibility that the 35S::*NIT2* line perhaps has PM protein alterations that predispose or prime it to have a better salt stress response is not only based on the differences in PATL1 levels. ERD7 was the protein with the highest degree of up-regulation in response to salt in No-0, but was found to be unresponsive in the 35S::*NIT2* line. Despite this, ERD7 levels were significantly higher in the 35S::*NIT2* line compared with No-0 in control conditions, suggesting that up-regulation in response to salt is unnecessary due to the pre-existing higher ERD7 abundance. ERD7 was first identified as one of 26 cDNAs that was expressed in response to dehydration stress, but it has subsequently been shown to be expressed in response to salt (100 mM NaCl), osmotic, and cold stress (Kiyosue, Yamaguchi-Shinozaki & Shinozaki, 1994; Kreps et al., 2002). Recently, ERD7 has been shown to play a role in remodelling cell membrane lipid composition during cold stress (Barajas-Lopez et al., 2021). In that study, the ERD7 protein was shown to accumulate in microsomal protein fractions from cold-stressed *Arabidopsis* seedlings. It was found to bind negatively charged phospholipids, and confocal microscopy revealed that it was localised in the vicinity of the cell/plasma membrane. Importantly, *erd7 edn2 edn1* (EDN 1 and 2 are close homologs) triple mutants were found to have reduced freezing tolerance and cold acclimation, concomitant with a reduction in membrane fluidity, leading the authors to conclude that ERD7 interacts with the phospholipids in cellular membranes to affect membrane fluidity during the response to cold stress. Notably, in this same study, ERD7 protein was also shown to accumulate in response to treatment with 100 mM NaCl for one hour, albeit to a lesser extent than in response to cold treatment. Our finding of increased ERD7 accumulation in response to salt stress in No-0 supports these results. Although speculative, it is plausible that ERD7 may play a similar role in plasma membrane lipid remodelling during salt stress. The pre-existing high ERD7 levels seen in control conditions could suggest that the PM of the 35S::*NIT2* line is predisposed or primed to respond to the negative effects of salinity in an ERD7-dependent manner. Furthermore, it was interesting that ERD7 levels were significantly lower in the 35S::*NIT2* line compared with No-0 under saline conditions. Perhaps the lower abundance of ERD7 under saline conditions suggests that the pre-existent higher levels observed in untreated conditions were sufficient to elicit an effect on the PM in the 35S::*NIT2* line, whereas in No-0, where ERD7 levels are very

low in untreated conditions, ERD7 has to be massively up-regulated in response to salt stress to protect the PM, the result being that there are higher levels in No-0 than the 35S::*NIT2* line. All said, the differences in ERD7 abundance between the two lines was very clear, and a further investigation of this protein, and its potential effect on the PM during salt stress, would be highly interesting.

3.4.2.2. Proteins involved in osmotic stress responses and water relations

It is well established that salinity imposes an osmotic stress on plants due to the high concentration of Na⁺ in the soil surrounding the roots causing an alteration in osmotic potential, and a reduction in water uptake (Munns & Tester, 2008). One of the most obvious osmotic stress response mechanisms employed by the plant upon exposure to salinity is to reduce stomatal aperture, thereby reducing stomatal conductance and water loss through transpiration. Multiple ABC transporter family proteins involved in stomatal regulation were found to have altered abundance across the different comparisons. The ABCC4 transporter (also known as multidrug resistance-associated protein 4 (MRP4)) was up-regulated in response to salt in No-0, but was not salt-responsive in the 35S::*NIT2* line. ABCC4 is highly expressed in guard cells and has been shown to play a role in the regulation of stomatal opening, with *mrp4* mutants found to have increased stomatal aperture, and consequently increased water loss during desiccation, compared with the wild-type (Klein et al., 2004). The increase in ABCC4 abundance observed in No-0 is thus presumably in an attempt to reduce stomatal aperture, and thereby limit water loss during salt stress. Interestingly, ABCC4, along with ABCC5/MRP5 which also plays a role in stomatal regulation (Klein et al., 2003), was found to have increased abundance in the 35S::*NIT2* line compared with No-0 in control conditions, despite not being salt-responsive, suggesting that the potential altered regulation of stomatal conductance via increased ABCC4 is constitutively present in the 35S::*NIT2* line. Likewise, stomatal conductance could also be affected in the 35S::*NIT2* line under saline conditions via a decrease in the levels of ABCG22 compared with No-0. The guard cell-expressed ABCG22 has also been shown to be required for stomatal regulation, with *abcg22* mutants found to have increased leaf water loss, and decreased temperatures, implying elevated stomatal transpiration, as well as decreased drought-tolerance compared with the wild-type (Kuromori, Sugimoto & Shinozaki, 2011). Decreased ABCG22 levels could suggest an increase in stomatal conductance in the 35S::*NIT2* line compared with No-0 under saline conditions. It was suggested in chapter 2 that the 35S::*NIT2* line may have altered stomatal conductance under saline conditions due to differences in the expression of *KAT1* and *GORK*, which co-

ordinately regulate stomatal closure through the control of guard cell K⁺ levels. The observation of decreased ABCG22 in the 35S::*NIT2* line could provide further evidence towards this idea. Furthermore, the increased ABCC4 and ABCC5 abundance could provide evidence for differences in stomatal conductance between the 35S::*NIT2* line and No-0 under control conditions as well. It must however be acknowledged that stomatal regulation involves a complex network of proteins that respond to many abiotic and biotic factors, so the difference in abundance of the ABC proteins mentioned here may not necessarily be sufficient to alter stomatal conductance.

While regulation of stomatal conductance in response to salinity controls water relations at the organ level, at the cellular level, this is achieved by aquaporins (PIPs and TIPs). While no PIPs were found to be differentially regulated, it was notable that the tonoplast-located, TIP1;1 and TIP2;2 both had reduced abundance in the 35S::*NIT2* line in saline conditions. TIP2;2, along with NHX1, is one of the few transporters/channels whose transcript levels were measured (see chapter 2) that was detected at the protein level. As previously discussed, reduced levels of *TIP2;2* expression have been linked with increased salinity tolerance (Boursiac et al., 2005; Feng, Xu, et al., 2018). In the transcriptomics analysis, *TIP2;2* expression was significantly lower in the shoots of the 35S::*NIT2* line compared with No-0 under control conditions, with no difference between the two lines in saline conditions. However, here I report that *TIP2;2* protein levels were significantly lower in the 35S::*NIT2* line compared with No-0 in saline conditions. This discrepancy is not surprising, as changes in transcription do not always reflect on the translational level. Furthermore, the period of salt treatment differed between the two analyses (7 days as opposed to 3 days). Nonetheless, it is plausible that the reduced *TIP2;2* protein abundance observed in the 35S::*NIT2* line under saline conditions may contribute to its improved salt-tolerance phenotype, as is observed with a reduction in *TIP2;2* abundance in the *tip2;2* mutant (Feng, Xu, et al., 2018).

3.4.2.3. Auxin-related proteins

Given the overall hypothesis that *NIT2* expression, and thus auxin biosynthesis is up-regulated to modulate growth during salt stress, it was both noteworthy and unsurprising that several PM proteins involved in auxin-related signalling, as well as biosynthesis were up-regulated in response to salt, as well as differentially abundant between the two lines. *NIT2* was differentially regulated in all four of the comparisons. In No-0, it was only detected in the salt-stressed samples, indicating that IAA biosynthesis is very likely increased in response to salt. In the 35S::*NIT2* line, *NIT2* was detected in both control and saline conditions, but was still

up-regulated in response to salt. This was interesting as it suggests that salt stress either induced a further increase in expression in the 35S::*NIT2* line where *NIT2* is already highly expressed, or an increase in translation from the existent *NIT2* transcripts. Additionally, this increased *NIT2* abundance could reflect the increased expression of the endogenous *NIT2* gene that is expected in response to salt (Cackett et al., 2022). As expected, *NIT2* was more abundant in the 35S::*NIT2* line than No-0 in both control and saline conditions, confirming that increased *NIT2* expression does result in meaningful increases in *NIT2* protein levels. This observation provides direct evidence that the increased IAA contents previously reported in the 35S::*NIT2* line compared with No-0 under saline conditions can be linked with increased *NIT2* protein levels (Cackett et al., 2022). Interestingly, the increased IAA levels in the 35S::*NIT2* line were not observed under control conditions, with it being hypothesised that excess IAA is degraded, whereas it is used for growth modulation during salt stress (Cackett et al., 2022). However, here *NIT2* abundance was also increased in the 35S::*NIT2* line compared with No-0 in control conditions, suggesting that IAA levels could also be increased in control conditions. It should be noted that the Cackett study assessed two-week-old seedlings germinated and grown in 100 mM NaCl, as opposed to here, where four-week-old plants were exposed to 75 mM NaCl for three days, so it is entirely possible that endogenous IAA levels under control and saline conditions could be different. It is not entirely clear whether *NIT2* is a plasma membrane associated protein, as its subcellular localisation has yet to be resolved, with it annotated as both plasma membrane and cytosol-located according to the Uniprot and TAIR databases. The *NIT1* and *NIT2* clones were first identified in the screening of a cDNA expression library with an antibody raised against purified plasma membrane samples, in which the conversion of IAN to IAA had been measured (Bartling et al., 1992). However, as *NIT2* encodes only a single cluster of hydrophobic amino acids, the authors suggested that the protein is likely localised to the cytosolic face of the PM from which it readily dissociates. The robust detection of *NIT2* in our ePM samples adds evidence to support that it is associated with the plasma membrane.

Another protein that was up-regulated in response to salt in No-0 and has been linked with auxin biosynthesis is *BGLU18*. Very recent evidence has suggested that *BGLU18* allows for the use of the glucosinolate, glucobrassicin (GB), as a source of auxin during drought stress in *Arabidopsis* (Hornbacher et al., 2022). Brassicales are capable of synthesising IAN from GB, a process which also involves the nitrile specifier proteins, *NSP1* and *NSP5*, which can subsequently be converted to IAA by *NIT2*. The authors found that the expression of *BGLU18*, *NSP1*, *NSP5*, and *NIT2*, as well as the GB, IAN, and IAA contents were all significantly

elevated in drought stressed plants compared with the control. Furthermore, GB accumulated in the leaves of drought stressed *bglu18* mutants, which the authors suggest was due to enhanced GB synthesis owing to a lack of breakdown products. Given that BGLU18 abundance was increased alongside NIT2 in No-0 plants in response to salt, it is possible that this pathway could be used for the synthesis of auxin by NIT2 during salt stress as well. This is predicated on the idea that GB levels would also be increased during salt stress as a source of IAN – a supposition that would need to be tested via measuring glucosinolate contents. Further credence to this idea is that *BGLU18* transcript abundance has also been found to be highly up-regulated in response to salt stress in two-week-old No-0 and Col-0 seedlings (Cackett, 2019). The observation that BGLU18 was not salt-responsive in the 35S::*NIT2* line as well was somewhat surprising. Given that increased conversion of IAN to IAA by NIT2 would deplete the endogenous IAN pool, it would make sense that BGLU18 would also be up-regulated to increase the conversion of GB to IAN. It is however possible that the constitutively higher NIT2 levels in this line somehow forms a feedback mechanism that perturbs BGLU18 up-regulation.

In addition to increases in the levels of auxin biosynthesis-related proteins in response to salt, several proteins involved in the regulation of PIN trafficking and PAT were also differentially regulated. One such protein was CPK29, which was found to be up-regulated in response to salt in No-0. CPK29 directly interprets Ca^{2+} signals to modulate the trafficking of PIN proteins thereby affecting PAT and subsequent signalling responses (Lee et al., 2021). CPK29 was shown to interact with and phosphorylate specific residues on the regulatory domain of PIN proteins, and when these residues were mutated, PIN-trafficking and polarity was compromised. Likewise, PIN trafficking was compromised in *cpk29* mutants, resulting in defects in PIN-mediated auxin distribution and auxin phenotypes, such as LR formation and hypocotyl gravitropism. Notably, in agreement with our finding of CPK29 up-regulation in response to salt, *CPK29* expression has been shown to be rapidly increased in 10-day-old seedlings when exposed to 150 mM NaCl (Latz et al., 2013). It is well established that Ca^{2+} signalling is increased in response to salt, so the finding that CPK29 abundance is also increased could suggest that it serves as a point of integration between Ca^{2+} and auxin signalling during salt stress. In other words, the higher abundance of CPK29 could allow better detection of salt-induced Ca^{2+} signals, resulting in increased PIN-phosphorylation and trafficking, causing altered PAT, and thereby affecting auxin-related growth processes.

Additionally, the abundance of the phospholipid transporting ATPases, ALA9, ALA10, and ALA11, which function redundantly with ALA3 in PIN trafficking and the regulation of PAT in Arabidopsis roots (Zhang, Adamowski, et al., 2020), was increased in the 35S::*NIT2* line compared with No-0 in control conditions. In this study by Zhang et al. (2020), *ala3* T-DNA mutants were shown to have many auxin-related developmental defects (such as delayed root gravitropic responses and shortened etiolated hypocotyls), as well as defects in asymmetric auxin distribution and PIN trafficking compared with the wild-type, leading the authors to propose that ALA3 must be crucial for these processes. The assertion that ALA9, ALA10 and ALA11 act redundantly with ALA3 was based on the analysis of *ala3/5/9*, *ala3/4/11*, *ala3/4/5/9/11*, and *ala3/4/5/9/10/11* triple, quintuple, and sextuple mutants, respectively. These mutants displayed more severe auxin-developmental phenotypes than the *ala3* mutant, and further defects in PIN polarity/trafficking. While Zhang et al. (2020) only visualised altered PIN trafficking in the roots of the *ala* mutants, the observation of altered shoot phenotypes in these lines strongly suggests that PIN trafficking was perturbed throughout the plant. The elevated levels of ALA9, 10, and 11 seen in the 35S::*NIT2* line could thus possibly suggest that it is predisposed towards altered auxin transport in the shoots via ALA-mediated changes in PIN trafficking. So, not only could auxin levels be increased in this line compared with No-0, but the ability to transport that auxin could be as well. As none of these ALA proteins were salt-responsive in either line, it would be expected that they would also have elevated levels in the 35S::*NIT2* line compared with No-0 in saline conditions. However, this was not the case, with the apparent increases in ALA9 and ALA11 abundance ($\text{Log}_2\text{FC} = 1.1$ and 1.34 , respectively) in the 35S::*NIT2* line not being significant. It would be very interesting to measure changes in auxin levels in the roots and shoots of these hydroponically grown No-0 and 35S::*NIT2* plants in control and saline conditions, to complement the auxin measurements from the seedlings of these lines (Cackett et al., 2022). Furthermore, it would be worthwhile to visualise auxin localisation in conjunction with PINs in the lines, so as to properly assess potential genotype-specific differences in PAT.

The final auxin-related protein that had altered abundance between the two lines was the ABCG36 transporter, which has been shown to play a role in auxin homeostasis by controlling the efflux of the IAA precursor Indole-3-butyric acid (Strader & Bartel, 2009). ABCG36 levels were increased in the 35S::*NIT2* line compared with No-0 in both control and saline conditions. Aside from a role in auxin homeostasis, ABCG36 has also been implicated in improving salt-tolerance. Transgenic Arabidopsis overexpressing ABCG36 were found to have significantly

increased shoot biomass and root length compared with the wild-type when grown under saline conditions (80 mM NaCl), with the opposite phenotype observed in *abcg36* mutants (Kim et al., 2010). Furthermore, the *ABCG36* overexpressing plants accumulated significantly less Na^+ in both the root and shoot than the wild-type. However, *ABCG36* has not been shown to possess Na^+ transport activity, so it is unclear exactly how it affects ion homeostasis. The authors suggested that it could potentially act as a regulator of another transporter/channel, as has been demonstrated for other ABC transporters (Matsuo et al., 2003). Nonetheless, as increased *ABCG36* expression improves salt-tolerance, it is thus plausible that the increased *ABCG36* abundance observed in saline conditions also contributes towards the improved salt-tolerance phenotype of the 35S::*NIT2* line. Furthermore, the increased *ABCG36* abundance observed in control conditions could predispose the 35S::*NIT2* line to an improved salt-stress response.

Overall, the finding that *NIT2* abundance was increased in No-0 in response to salt was particularly significant as it provides good evidence towards the overall hypothesis that auxin biosynthesis is up-regulated, via increased *NIT2*, to modulate growth during salt stress. Likewise, the up-regulation of *BGLU18* in No-0 provides further evidence for increased auxin biosynthesis in response to salt. Furthermore, the finding that proteins involved in the regulation of PIN trafficking (ALAs) had increased abundance in the 35S::*NIT2* line compared with No-0 in control conditions could suggest a possible constitutive alteration in PAT. Ultimately, the finding that auxin-related proteins have increased abundance in the 35S::*NIT2* line provides further evidence that *NIT2* functions in the synthesis of IAA (and is doing so in response to salt), as opposed to some other function.

3.4.2.4. Plasma membrane H^+ -ATPase

Central to the proposed hypothesis, of the induction of auxin-modulated growth in response to salt stress, is the activation of the PM H^+ -ATPase. The isoforms AHA1, 2, 3, 7, 9, and 11 were robustly detected in all of the samples and were among the most abundant of all of the proteins detected. Of these isoforms, AHA2 was the only one to have altered abundance in any of the comparisons. The finding that AHA2 levels were slightly increased in the 35S::*NIT2* line compared with No-0 under saline conditions compliments the observation of increased shoot AHA2 expression in the 35S::*NIT2* line reported in chapter 2. However, it must be noted that the increase in protein abundance ($\text{Log}_2\text{FC} = 0.24$) is far smaller than the observed increase in transcript abundance ($\text{Log}_2\text{FC} = 1.7$). Furthermore, no difference in AHA2 protein levels was observed between the two lines in untreated conditions, whereas AHA2 expression was significantly higher in the 35S::*NIT2* line than No-0 in both untreated and saline conditions. It

would thus appear that changes in AHA2 transcription in the shoots of the 35S::*NIT2* line compared with No-0 do not necessarily reflect at the protein level. However, given that AHA2 is such an abundant protein, even a small increase in protein levels in the 35S::*NIT2* line compared with No-0 could translate to a significant difference in the electrochemical potential generated across the PM in these lines. It must of course be noted that the activity of the PM H⁺-ATPase is largely regulated by PTMs (Falhof et al., 2016). To address this, a phosphoproteomic analysis on ePM samples isolated from the shoots of untreated and salt-stressed No-0 and 35S::*NIT2* plants would need to be conducted in order to assess any changes in PTMs of the PM H⁺-ATPase in response to salt, and between the two lines. This should be done in conjunction with established H⁺-ATPase activity assays (Larsson & Widell, 2000) on these same ePM fractions. Together, this would provide insight into potential salt-induced changes, as well as genotype-specific changes in PM H⁺-ATPase activity.

3.4.2.5. Transporters

Other than those already mentioned, a number of PM transporters with a wide range of purported transport activities were differentially regulated between the two plant lines. Of particular interest in the context of salt stress is the transport of potassium. Notably the high-affinity K⁺ transporter, KUP8 was found to have increased abundance in the 35S::*NIT2* line compared with No-0 under saline conditions. KUP8 is part of a family of 13 KT/KUPs, which have been implicated in K⁺ transport in tissues throughout the plant. Unfortunately, KUP8 is less well characterised than many of the other KT/KUPs, and has yet to be implicated in K⁺ homeostasis in response to salt stress, as is the case for family members such as KUP1 and KUP7 (Sun et al., 2015; Han et al., 2016). *KUP8* has been shown to be expressed in the root, leaves, siliques, and flowers (Ahn, Shin & Schachtman, 2004). KUP8 has been speculated to play a role in regulating K⁺ homeostasis during the plant response to heavy metals, as *kup8-2* mutants displayed increased heavy metal resistance, as measured by improved root and leaf fresh weights, and decreased lipid peroxidation compared with the wild-type when exposed to heavy metals (Sanz-Fernández et al., 2021). However, the authors did not suggest a mechanism whereby altered K⁺ transport mediated this resistance. The only other functional characterisation of KUP8 comes from a study looking at the effect of altered K⁺ homeostasis on cell growth and drought responses, where *kup2kup6kup8* triple mutants were found to display enhanced cell expansion, decreased stomatal closure, and decreased tolerance to water deficit stress, suggesting that KUP8, along with these other KUP transporters are important for osmotic adjustment during growth and drought responses (Osakabe et al., 2013). The scant

detail on the function of KUP8 makes it difficult to propose what affect increased abundance, and therefore potential altered K^+ transport, might have on salt-tolerance in the 35S::*NIT2* line.

It would also appear that nitrogen transport could possibly be altered in the 35S::*NIT2* line. The transport of ammonium and urea is potentially increased due to a higher abundance of AMT1;1 and DUR3, respectively, in the 35S::*NIT2* line compared with No-0 in control conditions, with the increased DUR3 levels also observed in saline conditions. In shoots, AMT1;1 functions in the uptake of NH_4^+ that is transported from the roots, as well as in the re-uptake of apoplastic NH_4^+ derived from photorespiration (Mayer & Ludewig, 2006). DUR3 is the main avenue of high-affinity urea uptake from the soil, but in the shoots, it plays a role in urea retranslocation in senescing leaves by retrieving urea from the apoplast (Kojima et al., 2007; Bohnert et al., 2015). The possible effect of increased ammonia and urea transport, particularly in control conditions, is unclear.

Another notable PM protein with putative ion transport activity that was found to be salt-responsive was the Mechanosensitive ion channel protein 6 (MSL6). MSL6 was up-regulated in response to salt in No-0. In general, MSLs allow cells to respond to mechanical stimuli, which in the case of plants include gravity, temperature, changes in turgor pressure, and physical touch (Haswell, 2007). Unfortunately, MSL6 is uncharacterised, so I can only speculate as to its function and why it would be up-regulated during salt stress, but a plausible explanation could be that it acts in some way to detect altered turgor pressure due to salt-induced changes in water content in the leaf. If this is indeed the case, it is interesting that MSL6 is not salt-responsive in the 35S::*NIT2* line.

3.4.2.6. Cell wall-related proteins

Salinity has a widespread effect on the biology of the cell wall, and several cell wall-modifying processes are activated in response to salt stress. As previously mentioned, salinity imposes an osmotic stress on the plant, which at the cellular level results in the extrusion of water, and consequently a reduction in turgor pressure (van Zelm, Zhang & Testerink, 2020). This in turn results in an alteration in cell wall integrity (CWI), and the cell wall must be actively modified and reinforced in order to adjust to the new turgor pressure that is established following osmotic adjustment (Colin et al., 2023). Furthermore, the ionic effects of salinity may also impact the mechanical property of the cell wall by affecting both cell wall components, and the proteins involved in cell wall modification. Salt stress is thought to cause a softening of the cell wall via affecting the pectin matrix in which the cellulose network is embedded (Feng, Kita, et al., 2018). The high concentration of Na^+ ions in the apoplast is suggested to displace pectin-bound

Ca²⁺ that is required for cross-linking (Manunza et al., 1998). In contrast, salt stress can also result in the strengthening of the cell wall due to the cross-linking of cell wall components by peroxidases which are activated by salt-stress induced ROS production (Tenhaken, 2015). Additionally, there is evidence that salt stress activates pectin methylesterases (PMEs), which convert secreted pectin into a de-methylesterified form that can be cross-linked, thereby affecting CWI (Gigli-Bisceglia et al., 2022). Finally, salt stress also results in the temporary removal of cellulose synthases from the plasma membrane, thereby affecting the synthesis of new cellulose microfibrils (Colin et al., 2023). Given the complex effects of salt stress on CWI, it was unsurprising that several PM proteins implicated in these processes were differentially regulated in the 35S::*NIT2* and No-0 samples. Notably, both the Cellulose-synthase like protein G3 and the pectin methylesterase CGR3 were absent in the salt-stressed No-0 samples, whereas their expression was maintained in the 35S::*NIT2* line. Additionally, the abundance of the Glycerophosphodiester phosphodiesterase, GDPDL1, was significantly increased in the 35S::*NIT2* line compared with No-0 under saline conditions. GDPDL1 (also known as SHAVEN3-LIKE 1 (SVL1)), along with other GPI-anchored Glycerophosphodiester Phosphodiesterase-Like Proteins, has been shown to play a role in cell wall organisation, with *svl1* mutants having several phenotypes associated with reduced cell wall rigidity (Hayashi et al., 2008). Furthermore, these *svl1* mutants had decreased crystalline cellulose accumulation, and reduced rates of methyl esterified pectin formation. GDPDL1 was thus proposed to function in cell wall organisation by playing a role in pectin network formation at the PM. The degree of pectin methylesterification has an effect on the plasticity of the cell wall, as well as cell to cell adhesion, thus pectin methylesterases, like CGR3, play an important role in the regulation of cell wall-stiffness and mechanical strength. In fact, *cgr3cgr2* double mutants, which have reduced methylesterification and altered cell wall pectin composition, display a number of negative growth phenotypes, including reductions in rosette size, root length, and hypocotyl length (with a concomitant reduction in root and hypocotyl cell length) (Kim et al., 2015). Interestingly, *CSLG3* has been proposed to contribute to increasing the cell wall cellulose content in order to enhance cell wall cadmium retention and reduce its toxicity (Xiao et al., 2020). *CSLG3* expression was found to be significantly increased in the cadmium tolerant Tor-1 Arabidopsis ecotype, which also displayed increased cell wall cellulose, and cellulose-bound cadmium. It is possible that increased *CSLG3* abundance in the 35S::*NIT2* line may result in increased cell wall cellulose content in response to salt stress. Likewise, the increased GDPDL1 and CGR3 levels may also affect cell wall organisation by their respective pectin-

modifying actions. Together, this could constitute a mechanism to increase CWI in response to salt stress, which may well contribute to the improved salt-tolerance of the 35S::*NIT2* line.

There is further evidence that the extracellular surface of the PM could be altered in the 35S::*NIT2* line, with FLA13 being found to be more abundant than in No-0 under control conditions. While the function of FLA13 is unknown, other FLAs have been implicated in cell wall biogenesis: FLA11 and FLA12 (which are also classified as Group A FLAs) have been found to affect secondary cell wall composition, thereby affecting the tensile strength of Arabidopsis stems (MacMillan et al., 2010). It is possible then that the increased FLA13 abundance in control conditions could indicate some pre-existing alteration in the cell wall in the 35S::*NIT2* line. Furthermore, FLAs have been shown to contribute towards salinity tolerance: the SOS5 locus, which was identified in a screen for mutants hypersensitive to salt stress, was found to encode FLA4, which was shown to be a cell surface adhesion protein required for normal cell expansion (Shi et al., 2003). Additionally, one of only two proteins found to be up-regulated in response to salt stress in the 35S::*NIT2* line was an uncharacterised GPI-anchored protein (AT5G19240). However, no information is available as to a potential function, so I can only speculate that increased abundance might result in some alteration of the extracellular space in response to salt.

3.4.2.7. Membrane trafficking proteins

There appear to be large-scale differences in membrane trafficking between the two lines. In No-0, several proteins involved in membrane trafficking were down-regulated in response to salt, including PATL1, which was discussed previously, as well as DRP2A and DRP2B, which function co-ordinately in multiple pathways of post-Golgi trafficking (Huang et al., 2015). DRP2A and DRP2B have been shown to interact with each other and co-localise to endocytic vesicle formation sites on the plasma membrane, and the clathrin-enriched trans-Golgi network. Additionally, their resident time on the plasma membrane has been shown to be increased in the presence of actin and microtubule polymerisation inhibitors, suggesting that they act in a cytoskeleton polymerisation-dependent manner. Arabidopsis encodes 16 Dynamin Related Proteins, all of which are large GTPases, that are classified into 6 sub-families based on homology and function (Hong et al., 2003). The DRP1 and DRP2 sub-families have both been implicated in clathrin-mediated endocytosis, where they mediate the release of clathrin-coated vesicles from the PM, as well as cell plate maturation during cytokinesis (Bednarek & Backues, 2010; Ekanayake et al., 2021). The down-regulation of PATL1, DRP2A and DRP2B suggests that certain endocytic/membrane trafficking processes are possibly reduced in

response to salt stress in No-0. Notably, no proteins involved in membrane trafficking were down-regulated in response to salt in the 35S::*NIT2* line. However, several such proteins were found to have decreased abundance in the 35S::*NIT2* line compared with No-0 in control conditions, including PATL1, PATL2, DRP2B, DRP1A, and DRP1E, as well as in saline conditions (DRP1A and DRP1C). This would suggest that instead of being down-regulated in response to salt, membrane trafficking processes co-ordinated by these proteins are potentially constitutively down-regulated in the 35S::*NIT2* line. It is well established that perturbations in endocytosis, as well as exocytosis, results in the modulation of PM protein levels, thereby affecting a wide range of physiological functions at, and associated with, the PM (Reynolds et al., 2018). The constitutively reduced plasma membrane trafficking that has been proposed in the 35S::*NIT2* line may help to explain the lack of salt-responsive PM proteome changes observed.

In addition to the changes in abundance of membrane trafficking proteins, there was also a salt-induced reduction of microtubule component proteins in No-0. This is interesting as salt stress has been shown to induce rapid and temporary microtubule depolymerisation in the roots of *Arabidopsis* seedlings (Wang, Li & Yuan, 2007). After time, microtubule reorganisation was observed, but seedlings in which microtubule reorganisation did not occur had decreased salt-tolerance. The reduction in microtubule component proteins in No-0 could have a possible effect on microtubule reorganisation, and consequently salt-tolerance.

3.4.2.8. Other PM proteins of interest

Another interesting genotype-specific difference was that ACD6 was only expressed in the 35S::*NIT2* line, both in the control and salt-stressed samples. ACD6 is an integral PM protein that acts as a positive regulator of defence responses to bacterial pathogens in conjunction with salicylic acid signalling (Rate et al., 1999). ACD6 along with salicylic acid form a positive feedback loop that regulates the levels of the FLS2 and CERK1 PAMP-receptors at the PM (Tateda, Zhang & Greenberg, 2015). Although completely incidental to this study, this could suggest that the defence response of the 35S::*NIT2* line is improved, or perhaps rather, given the absence of ACD6, that No-0 is more susceptible. However, very recently, *ACD6* expression has been shown to be increased in two-week-old *Arabidopsis* seedlings in response to a number of abiotic stresses, including salt (150 mM for 3 days) (Yang et al., 2022). The physiological implications of ACD6 being up-regulated in response to salt stress are unclear, however, it is noteworthy that I observed higher ACD6 protein levels in the 35S::*NIT2* line that displays increased salt-tolerance. It must be noted that ACD6 abundance was not salt-responsive in the

35S::*NIT2* line, but perhaps the constitutively higher ACD6 levels might also predispose the 35S::*NIT2* line towards improved salt-tolerance compared with No-0.

3.4.3. Limitations in the experimental design employed

Due to the technical challenges associated with plasma membrane protein purification/enrichment, as well as the large amount of processing time required per sample in the simplified enrichment protocol utilised, the total number of samples that I could reliably and accurately process was limited. As such, it was unfeasible to assess both the shoot and root PM proteomes of untreated and salt-stressed 35S::*NIT2* and No-0 plants, as was the case for the gene expression analysis in chapter 2, without compromising the quality of this experiment. As mentioned previously, there was a gap in the literature reporting on the salt-stress proteome, both in the shoot and the root. However, the decision to focus on the shoot as opposed to the root, was based on multiple factors; firstly, recent salt-stress proteomics analyses have focused on the root (Vialaret et al., 2014; Rodriguez et al., 2021), so reporting on the salt-stress proteome in the shoot, specifically, was more pertinent. Secondly, the observed salt-responsive increases in *NIT2* expression are greater in the shoot than the root, with the increased salt tolerance of the 35S::*NIT2* line also being more pronounced in the shoot tissue. Finally, I had initially utilised a different PM purification protocol that required 30g of tissue per sample, which was logistically an unfeasible amount of root tissue given the yields obtained from the hydroponics plant growth system, whereas the shoot yields were much higher.

One of the rationales for the proteomics analysis performed herein was to potentially try and corroborate the differences in ion transporter/channel gene expression between the 35S::*NIT2* line and No-0 genetic background under salt stress conditions that was presented in chapter 2. Clearly, the inability to obtain root PM proteomic data to corroborate the root gene expression data reveals a limitation in the experimental design of this chapter. Cross comparisons of the two shoot datasets were limited as well since most of the ion transporter/channel genes measured were not detected at the protein level, due to them being low abundance proteins, as discussed earlier. However, it must be stressed that corroborating this gene expression data was only part of the rationale for the PM proteomic analysis; the overall aim was to assess salt-responsive changes in the shoot PM proteomes of the two lines, as well as to identify any differences in the shoot PM proteome of the 35S::*NIT2* line that could help to explain its improved salinity tolerance, not just to assess differences in the abundance of ion transporters/channels. From the findings presented in this chapter, it can be seen that this general ‘discovery’ proteomic approach did identify several interesting salt-responsive shoot

PM proteins, some of which could well contribute to the increased salinity tolerance of the 35S::*NIT2* line. Furthermore, given the small amount of tissue required per sample (500-700 mg), the simplified enrichment protocol, and downstream proteomics approach employed here, is suitable to assess the root PM proteome. Differences between the salt-responsive PM proteomes of No-0 and 35S::*NIT2* roots can thus be assessed in the future to address the current gap in the data.

An important caveat that should be made is that while the more general ‘discovery’ proteomic approach adopted here serves very well as a preliminary investigation for the purpose of identifying salt-responsive PM proteins for further study, it did not conclusively show that the PM proteins of interest identified were involved in the salt stress response. In order to confirm whether the identified ‘salt-responsive’ proteins are in fact important contributors for salt-tolerance, further functional characterisation of mutant lines is required. Further study of the PM proteins of interest identified herein in the context of salt-tolerance thus represents important future work.

3.4.4. Summary

In this chapter, label free quantitative LC-MS/MS analysis was successfully employed to assess the PM proteome in enriched PM protein samples isolated from the shoots of 35S::*NIT2* and No-0 plants grown under saline and control conditions. A wide range of PM proteins involved in transport, signalling, membrane trafficking and structure, cell wall-related functions, and response to biotic/abiotic stresses were robustly detected across the sample groups. A total of 21 PM proteins were found to be salt-responsive in the No-0 background. Most notably, ERD7, and the sodium/calcium exchanger NCL, both of which play a role in the response to salt stress, as well as ABCC4, which regulates stomatal conductance, were up-regulated. Furthermore, NIT2 was only detected in the salt-stressed No-0 samples and not in the control No-0 samples, providing strong evidence towards the overall hypothesis that auxin biosynthesis is increased to modulate growth in saline conditions. In support of this, the protein kinase, CPK29, which is involved in the regulation of PAT, was also up-regulated. In contrast, just four PM proteins, including NIT2, were salt-responsive in the 35S::*NIT2* line, which is known to have improved salt-tolerance (Cackett et al., 2022). It was notable that a subset of PM proteins were salt-responsive in No-0 but not in the 35S::*NIT2* line, yet were found to have increased or decreased abundance in the 35S::*NIT2* line compared with No-0 in control conditions (ERD7, ABCC4, PATL1, and DRP2B). These proteins could be particularly important in pre-disposing the 35S::*NIT2* line to improved salt-tolerance (especially ERD7 and PATL1 which are involved in

the salt stress response), and thus would be interesting to study further. Furthermore, genotype-specific differences in several other PM proteins were observed in both control and saline conditions. Notably, auxin-related proteins displayed increased abundance in the 35S::*NIT2* line in both control (NIT2, ALA9, 10, and 11, and ABCG36) and saline conditions (NIT2 and ABCG36), confirming that increased *NIT2* mRNA levels result in a corresponding increase of NIT2 protein, thereby activating auxin-responsive processes. In support of our model, the increased AHA2 abundance in the 35S::*NIT2* line under saline conditions could contribute to the observed alterations in ion homeostasis in this line. Furthermore, the increased abundance of KUP8 in the 35S::*NIT2* line in saline conditions could also possibly contribute to this improved Na⁺/K⁺ homeostasis. Additionally, the increased abundance of cell wall-modifying proteins (CSLG3, CGR3, GDPDL1) in the 35S::*NIT2* line under both control and saline conditions potentially suggests a restructuring of the cell wall that could occur as a result of changes in cell expansion, supporting the hypothesis that cell wall loosening expansins are activated to modulate growth following salt-induced auxin-mediated activation of the PM H⁺-ATPase. While no further functional characterisation of the role of the identified candidate proteins in salt-tolerance was performed, the data generated in this discovery-based proteomics approach has generated findings that will lead to testable hypotheses in the future. Ultimately, the results presented here are the first to report on the leaf PM proteome of salt-stressed *Arabidopsis* plants. Additionally, I report genotype-specific differences in the leaf PM proteomes of No-0 and 35S::*NIT2* plants, and identify PM proteins that may potentially contribute towards the improved salt-tolerance phenotype of the 35S::*NIT2* line.

CHAPTER 4: ATTEMPTED GENERATION AND FUNCTIONAL CHARACTERISATION OF AN *EXPANSIN 11* MUTANT

4.1. Introduction:

4.1.1. Rationale for knocking down *Expansin 11* expression in the 35S::*NIT2* line

As explored in chapter 2, the increased salinity tolerance of the 35S::*NIT2* line can be explained by beneficial alterations in Na⁺ and K⁺ homeostasis, as well as the altered expression of a number of ion transporter/channel genes. Aside from the effect on ion homeostasis, the second component central to our hypothesis of how salt-induced IAA production, and hence activation of the PM H⁺-ATPase underlies the improved salinity tolerance of the 35S::*NIT2* line, is the effect that it has on acid or expansion growth. In this chapter, to try and delineate ion homeostasis from growth I decided to knockdown the expression of an effector protein acting downstream of the PM H⁺-ATPase, which is likely involved in growth modulation, but not ion homeostasis. The expansin proteins were an obvious target given their role in cell wall loosening following the activation of auxin-mediated expansion growth (Cosgrove, 2016). Interestingly, two α -expansins, *Expansin 5* and *Expansin 11* (*EXPA5* and *EXPA11*), were identified as being salt-responsive, along with *NIT2*, in a transcriptomics experiment that was conducted by our group to identify salt-specific differentially expressed genes in two-week-old Col-0 Arabidopsis seedlings (Cackett et al., 2022). Additionally, in a transcriptomics experiment conducted to identify DEGs between the 35S::*NIT2* line and its genetic background, No-0, *EXPA11* was found to be strongly up-regulated in the 35S::*NIT2* line, with the up-regulation in expression increased two-fold further in response to salt-treatment (Cackett, 2019). As the 35S::*NIT2* line has been shown to display improved salinity tolerance compared with No-0 (Cackett et al., 2022), and as *EXPA11* expression is greatly increased in this line, it is plausible that it acts to improve growth and the salinity tolerance of the line.

Thus, in order to assess the contribution of *EXPA11* to the growth and overall salinity tolerance of the 35S::*NIT2* line, I aimed to knock out/down its expression. This would hopefully help to delineate the contributions of ion homeostasis, and activation of expansion growth, to the salinity tolerance of the 35S::*NIT2* line. Importantly, it was necessary to generate this line myself, as not only did I want to knock out/down *EXPA11* expression in the 35S::*NIT2* line specifically, but the only purported *expa11* mutant line available was generated in the Landsberg erecta (Ler-0) genetic background, as opposed to that of No-0 for the 35S::*NIT2* line.

4.1.2. Gene-silencing via artificial microRNAs in plants

In order to knock-down the expression of *EXPA11* in the 35S::*NIT2* line, an RNA interference (RNAi) approach was employed. Several classical RNAi approaches have been adopted in recent years, which utilise the endogenous post-transcriptional gene silencing pathways present in plants. In short, gene silencing in plants involves the production of small RNAs (sRNAs) from longer RNA precursors by Dicer family proteins, which serve as the specificity component within the RNA-induced silencing complex (RISC), where they associate with ARGONAUTE (AGO) proteins to target and silence target transcripts through AGO-mediated endonucleolytic cleavage, or translational repression (Schwab et al., 2006, Carbonell, 2017). The sRNAs can be divided into two classes, small interfering RNAs (siRNAs) and microRNAs (miRNAs), which differ in their biosynthesis. In particular, siRNAs are formed from double-stranded RNA (dsRNA) pre-cursors, yielding multiple ~21bp dsRNA intermediates, of which either of the strands can be incorporated into the active RISC; whereas miRNAs are synthesised from a longer single-stranded hairpin RNA precursor yielding a single 19-24bp miRNA molecule following Dicer-mediated processing (Schwab et al., 2006; Ossowski, Schwab & Weigel, 2008). A number of the classical RNAi approaches, such as Transcriptional gene silencing (TGS), Virus-induced gene silencing (VIGS), and hairpin RNAi (hpRNAi), involve the production of siRNAs (Ossowski, Schwab & Weigel, 2008). However, despite their widespread use, these approaches are not ideal as siRNAs have been shown to have high levels of off-target effects on cellular transcripts that share sequence similarity with the siRNAs generated, and as such ‘second-generation RNAi’ technologies, like artificial microRNA (amiRNA) (and even more recently, synthetic trans-acting siRNAs (tasiRNAs)) have emerged with the aim of specific target transcript silencing, without off-target effects (Carbonell, 2017). Like endogenous plant siRNAs, plant miRNAs have perfect or near-perfect complementarity, however they have also been found to have endogenous target mRNAs with up to five mismatches. The miRNAs are thus capable of targeting specific endogenous transcripts without off-target effects despite not having perfect complementarity, whereas limited complementarity in siRNAs does result in off-target effects (Schwab et al., 2005). Importantly, the sequence parameters of plant miRNA:target recognition have been deduced (Schwab et al., 2005), which has allowed for the design of amiRNAs to knock down the expression of specific transcripts. The amiRNA technology utilises endogenous plant miRNA precursors, where the sequence of the miRNA-miRNA* duplex has been altered to target the particular transcript of interest (Ossowski, Schwab & Weigel, 2008). Thus amiRNA technology is an elegant RNAi approach in plants, as amiRNAs have not only been shown to display the same high level of

specificity as endogenous miRNAs (Schwab et al., 2005, 2006), but can also be expressed under the control of both tissue-specific, and constitutive promoters (Alvarez et al., 2006; Schwab et al., 2006). The main tool for the design of plant amiRNAs is the Web MicroRNA Designer, WMD3 (Ossowski, Schwab & Weigel, 2008; Schwab et al., 2010), which allows for the design of amiRNA sequences in more than 200 plant species. For a full explanation of the WMD3 tool and design process, refer to Ossowski et al. (2008), but in short, the tool generates 21-mer amiRNA and amiRNA* sequences that are optimised for both maximum specificity and effectiveness against a chosen target gene. Overlapping PCR is used to replace the miRNA sequence from a miRNA precursor with the designed amiRNA sequence. In the case of *A. thaliana*, the MIR319a precursor is used.

4.1.3. Chapter aims

The first aim was to validate the previous observation of increased *EXPA11* expression in the 35S::*NIT2* line under both control and saline conditions. Following this, the main aim of this chapter was to utilise a stable amiRNA approach to generate a 35S::*NIT2* line in which the expression of *EXPA11* was knocked down/out, and then to perform functional characterisation thereof under salinity stress conditions. Specifically, the functional characterisation involved several aims; 1) To determine if knocking down *EXPA11* expression affects the improved growth and survival phenotypes displayed by 35S::*NIT2* plants when exposed to salt stress at an early and later developmental stage. 2) To determine if knocking down *EXPA11* expression affects the improved root/shoot Na⁺ and K⁺ homeostasis displayed by the 35S::*NIT2* line. 3) To assess the effect of knocking down *EXPA11* expression on auxin-related growth phenotypes of untreated and salt-treated 35S::*NIT2* plants. Ultimately, the aim of this approach was to assess the contribution of *EXPA11* to growth and ion homeostasis of the 35S::*NIT2* line, so as to better understand the basis of its improved salt-tolerance.

4.2. Materials and methods:

4.2.1. Stock solutions, chemicals, enzymes, and vectors used

The 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) was obtained from Promega (Madison, Wisconsin, USA). The Zyppy Plasmid Miniprep Kit (catalogue no. D4036) was obtained from Zymo Research (Irvine, California, USA). The KAPA Taq PCR kit (catalogue no. KK1015), Kapa HiFi HotStart

ReadyMix PCR kit (catalogue no. KK2501), and the Super-Therm DNA Polymerase PCR kit (catalogue no. JMR-801) from Roche Molecular Systems Inc. (California, USA) were used. The pRS300 template vector (catalogue no. 22846) was obtained from Addgene (Massachusetts, USA). The Gateway™ pDONR™221 vector (catalogue no. 12536017), Gateway™ BP Clonase™ II Enzyme mix (catalogue no. 11789020), and Gateway™ LR Clonase™ II Enzyme mix (catalogue no. 11791020) used for Gateway® Cloning Technology were obtained from Invitrogen by Thermo Fisher Scientific (Massachusetts, USA). The Gateway™ pB2GW7 vector was obtained from the VIB-UGent Center for Plant Systems Biology (University of Gent, Belgium).

4.2.2. Primer design

Primers were designed using NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), and checked for self-complementarity using DNAMAN v 4.13 (Lynnon Biosoft, San Ramon, USA). A forward primer was designed to amplify the p35S sequence in the Gateway® expression vector pB2GW7 (sequence was obtained from the VIB-UGent Center for Plant Systems Biology website (<https://gatewayvectors.vib.be/>)). Similarly, forward and reverse primers were designed to amplify the BASTA resistance gene in the pB2GW7 expression vector. Primers to detect *EXPA11* (AT1G20190) expression via RT-qPCR were also designed.

4.2.3. DNA manipulations

4.2.3.1. Visualisation of DNA by gel electrophoresis

Prior to gel electrophoresis, PCR products were mixed with 6 x Orange G loading dye (10 mM Tris HCl pH 7.5, 60 mM Ethylenediaminetetraacetic acid (EDTA) pH 8.0 and 60% (v/v) glycerol) in a product to loading dye ratio of 5:1. Products were separated in 1% (w/v) agarose gels made up in 1 x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA pH 8.0), containing 0.16 µg.mL⁻¹ ethidium bromide (EtBr) and electrophoresed in 1 x TAE running buffer containing 0.16 µg.mL⁻¹ EtBr. Following electrophoresis, products were visualised under short wavelength (310 nm) with a 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. Product sizes were determined by comparison with either a 100 bp NEB Quick-Load® ladder, or a 1 kb NEB Quick-Load® ladder.

4.2.3.2. DNA purification from agarose gels

The PCR products that were excised from agarose gels were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's guidelines, except the DNA was eluted in 30 µL of nuclease free H₂O instead of 50 µL as instructed in the manual.

4.2.3.3. DNA sequencing and analysis

Capillary sequencing of PCR products and plasmid DNA 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, San Ramon, USA), and Chromas software (version 2.33, Technelysium, Brisbane, Australia). The TAIR database (<http://arabidopsis.org>) was used to obtain relevant information on gene and protein sequences for multiple sequence alignments.

4.2.3.4. Plasmid DNA purification

Plasmid DNA was purified from transformed *E. coli* cultures using the Zyppy Plasmid Miniprep Kit (Zymo Research, Irvine, California, USA) according to the manufacturer's protocol. In general, plasmid DNA was purified from 3 mL of bacterial culture.

4.2.3.5. DNA extraction from Arabidopsis tissue

Arabidopsis DNA was extracted using a modified version of the Edwards DNA extraction protocol (Edwards, Johnstone & Thompson, 1991). For plants grown in petri dishes, DNA was extracted from individual seedlings that had been grown for 10-14 days. For soil-grown plants, DNA was extracted from individual leaves harvested from four-week-old plants. Harvested seedlings or individual leaves were homogenised in microcentrifuge tubes along with 400 µL of DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8, 0.5% (w/v) Sodium dodecyl sulphate (SDS)). Samples were homogenised for 3 min using two small steel ball bearings (Bearing Man Group, Cape Town, South Africa) in a paint shaker (SO-10m shaker, Fluid Management, USA). The homogenised samples were incubated at 60°C for 10 min. Following this, 400 µL of chloroform was added and the samples were shaken briefly. The samples were then centrifuged at 11 000 x *g* for 10 min, and the aqueous phase was transferred into a new microcentrifuge tube. Subsequently, 1/10 of the volume of 3M Sodium acetate pH 5.2, along with twice the volume of ice-cold ethanol was added. The DNA was precipitated at -20°C for > 1 hr. Following this, the DNA was collected via centrifugation

at 11 000 x *g* for 10 min. The supernatant was discarded, and the pellets washed with 1 mL of 70% (v/v) ethanol. The samples were centrifuged again at 11 000 x *g* for 10 min, and the supernatant discarded. The DNA pellets were left to air dry and then re-suspended in 30 µL of Tris-EDTA pH 8. All DNA samples were then stored at -20 °C.

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

Multiple different PCR enzymes and kits were used depending on the purpose of the PCR as well as the primer pair used. The annealing temperature (*T*_a) used in these reactions varied according to the primer pair used (refer to Table 4.2). All PCR experiments were performed using the SimpliAmp™ Thermal Cycler (Applied Biosystems™, Foster City, USA). The specific information for each PCR kit used is summarised hereafter:

4.2.4.1. Kapa HiFi HotStart Readymix PCR Kit

The Kapa HiFi HotStart Readymix PCR kit (Kapa Biosystems, Cape Town, South Africa) contains a high-fidelity enzyme, so was used for all PCRs involved in the generation of the *EXPA11*amiRNA construct, and subsequent cloning procedures. For all PCRs with this kit, the reaction master mix was set up as per the manufacturer's guidelines. Unless otherwise specified, the following cycling parameters were used: 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).

4.2.4.2. Kapa Taq PCR Kit

The Kapa Taq PCR kit (Kapa Biosystems, Cape Town, South Africa) was used for PCRs not requiring high-fidelity amplification. It was used for PCRs to detect the *EXPA11*amiRNA construct in the pB2GW7 expression vector, as well as in PCRs using extracted plant DNA to confirm transgenic offspring. For all PCRs, the reaction master mix was set up as per the manufacturer's guidelines. The following cycling parameters were used: 1 x (95°C for 3 min), 35 x (95°C for 30 sec, 53°C for 30 sec, 72°C for 1 min) and 1 x (72°C for 3 min).

4.2.4.3. Super-Therm DNA Polymerase PCR Kit

The Super-Therm DNA Polymerase PCR kit (Roche Molecular Systems Inc. California, USA) was used for PCRs to detect the *EXPA11*amiRNA construct in the pDONR221 donor vector. For all PCRs, the reaction master mix was set up as per the manufacturer's guidelines. The following cycling parameters were used; 1 x (94°C for 5 min), 35 x (94°C for 15 sec, 55°C for 30 sec, 72°C for 30 sec) and 1 x (72°C for 10 min).

4.2.5. Generating the *EXPA11*amiRNA construct

The *EXPA11*-specific artificial microRNA (amiRNA) construct was generated using the protocol for the cloning of amiRNAs (Ossowski, Schwab & Weigel, 2008), in conjunction with the online WMD3 – Web MicroRNA Designer tool (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>).

4.2.5.1. amiRNA sequence generation and primer design

The WMD3 tool was used to generate a series of four oligonucleotide sequences (Oligo I, II, III, IV) that allow for the generation of a 21-mer amiRNA specific to *EXPA11*. These oligos, along with two additional generic oligonucleotides (Oligo A and B) allowed for the replacement of the 20-mer miR319a sequence of the pRS300 template vector, via overlapping PCR. The Oligo A and B sequences hybridise to the 5' and 3' ends of the miR319a sequence of the pRS300 vector, respectively. *attB1* and *attB2* sequences were added to the 5' flanks of Oligo A and B, respectively, allowing for cloning into the Gateway® donor vector pDONR221 and the downstream use of Gateway® cloning technology. See Table 4.1. for all six primer sequences.

4.2.5.2. amiRNA construct generation via overlapping PCR

Three initial PCRs were performed in order to separately generate the 5' arm, central loop, and 3' arm, and in the process replace the miR319a sequence with the *EXPA11*-specific amiRNA and amiRNA* sequences. The 5' arm reaction used the Oligo A-*attB1* and Oligo IV primers; the central loop reaction used the Oligo II and Oligo III primers; and the 3' arm reaction used the Oligo I and Oligo B-*attB2* primers. The pRS300 vector was used as template in all three of these PCR reactions. The recommended cycling parameters were used for all three PCR reactions: 1 x (95°C for 2 min), 40 x (95°C for 30 sec, 52°C for 30 sec, 72°C for 40 sec) 1 x (72°C for 7 min). The three fragments were purified as outlined in section 4.2.3.2. and used as template in a final reaction with Oligo A-*attB1* and Oligo B-*attB2* primers, yielding the full length *EXPA11*amiRNA construct. The following recommended cycling parameters were used: 1 x (95°C for 2 min), 25 x (95°C for 30 sec, 55°C for 30 sec, 72°C for 90 sec) 1 x (72°C for 7 min). The full length *EXPA11*amiRNA construct was sequence verified as outlined in section 4.2.3.3.

4.2.6. Gateway® Cloning Technology

Gateway® cloning technology was used to generate a vector that constitutively expresses the *EXPA11*-specific amiRNA (pB2GW7-*EXPA11*amiRNA)

4.2.6.1. Gateway® vectors used

The donor vector pDONR221 was used. This vector contains *attP* sites which allowed for the recombination of the *attB*-flanked *EXPA11*amiRNA construct via BP recombination reaction, and the generation of the entry clone pENTR221-*EXPA11*amiRNA. Additionally, pDONR221 has a Kanamycin resistance gene, allowing for selection and propagation of positive *E. coli* transformants. The *EXPA11*amiRNA construct was then transferred to the plant expression vector, pB2GW7 to allow for overexpression in plants (Karimi, Inzé & Depicker, 2002). The pB2GW7 vector contains *attR* sites which allowed for transfer of the *attL*-flanked *EXPA11*amiRNA construct from the pENTR221-*EXPA11*amiRNA entry clone into pB2GW7 via an LR recombination reaction. Additionally, pB2GW7 has the constitutive p35S promoter from the *Cauliflower Mosaic Virus (CaMV)*, which was used to drive overexpression of the *EXPA11*amiRNA construct. It also contains a Spectinomycin resistance gene, allowing for selection and propagation of positive *E. coli*, and *A. tumefaciens* transformants, as well as a BASTA resistance gene conferring resistance to glufosinate ammonium (GFSA), the active ingredient in the herbicide BASTA.

4.2.6.2. BP recombination reaction

The Gateway™ BP Clonase™ II enzyme mix was used in conjunction with the donor vector pDONR221 and the *EXPA11*amiRNA-*attB* fragment. The BP recombination reaction was performed as outlined in the Gateway® Technology manual. Briefly, 50 femtomoles of purified *EXPA11*amiRNA-*attB* fragment was mixed in a microcentrifuge tube, along with 150 ng of pDONR221 and TE buffer pH 8 (10 mM Tris-HCl pH 8, 1 mM EDTA) up to 8 µL. The BP Clonase™ II enzyme mix was thawed on ice for 2 min and then vortexed twice for 2 sec. Following this, 2 µL of BP Clonase™ II enzyme mix was added and then the mixture vortexed twice for 2 sec. The reaction mixture was then incubated at 25°C for 1 hr. Following this, 1 µL of 2 µg.µL⁻¹ Proteinase K solution was added, and then incubated in a water bath at 37°C for 10 min. The reaction mix was then used to transform chemically competent DH5α *E. coli*.

4.2.6.3. LR recombination reaction

The Gateway™ LR Clonase™ II enzyme mix was used in conjunction with the expression vector pB2GW7 and the sequence verified entry clone pENTR221-*EXPA11*amiRNA. The LR

recombination reaction was performed as outlined in the Gateway® Technology manual. 150 ng of pENTR221-*EXPA11*amiRNA was mixed in a microcentrifuge tube, along with 150 ng of pB2GW7 and TE buffer pH 8 up to 8 µL, at room temperature. The LR Clonase™ II enzyme mix was thawed on ice for 2 min and then vortexed twice for 2 sec. Following this, 2 µL of LR Clonase™ II enzyme mix was added and then the mixture vortexed twice for 2 sec. The reaction mixture was then incubated at 25°C for 1 hr. Following this, 1 µL of 2 µg.µL⁻¹ Proteinase K solution was added, and then incubated in a water bath at 37°C for 10 min. The reaction mix was then used to transform DH5α *E. coli*.

4.2.7. Bacteriological work in *E. coli*

4.2.7.1. *E. coli* growth

Escherichia coli (*E. coli*) strain DH5α was used throughout the cloning process. All *E. coli* was cultured in either Luria-Bertani (LB) broth (10 g NaCl, 10 g Peptone, 5 g Yeast extract, 1 L dH₂O) or on LB agar plates. *E. coli* transformed with pDONR221 or pB2GW7 was cultured using media supplemented with 50 µg.mL⁻¹ kanamycin or 100 µg.mL⁻¹ spectinomycin respectively. All cultures were grown at 37°C, with cultures grown in LB broth under constant shaking.

4.2.7.2. Transformation of chemically competent *E. coli*

All transformation of *E. coli* was done via heat shock. Prior to transformation, the BP or LR recombination reaction mixes (see section 4.2.6.) were briefly centrifuged. For each transformation, 3 µL of the reaction mix was added to a microcentrifuge and kept on ice. Competent *E. coli* DH5α was thawed on ice for 5 min, and 50 µL of *E. coli* cells was added to the microcentrifuge tubes containing the reaction mix and gently mixed. The tubes were incubated on ice for 30 min. Following this, the cells were heat-shocked at 42°C for 45 sec in a water bath, and then immediately incubated on ice for 2 min. Subsequently, 950 µL of room temperature LB broth was added, followed by an incubation at 37°C for 90 min under constant shaking. The cell pellets were collected by centrifugation at 1500 x *g* for 5 min. Following this, 900 µL of supernatant was discarded and the pellets were resuspended in the remaining 100 µL. Both 10 µL and 90 µL of the re-suspended mixture was plated onto LB agar plates (supplemented with the appropriate antibiotic) using sterile spread plating technique. The plates were incubated at 37°C overnight.

4.2.8. Bacteriological work in *A. tumefaciens*

4.2.8.1. *A. tumefaciens* growth

Agrobacterium tumefaciens (*A. tumefaciens*) strain GV3101 was used. All *A. tumefaciens* was cultured in either LB broth or on LB agar plates supplemented with 150 $\mu\text{g} \cdot \mu\text{L}^{-1}$ rifampicin and 15 $\mu\text{g} \cdot \mu\text{L}^{-1}$ gentamicin. The *A. tumefaciens* transformed with pB2GW7 was cultured using media additionally supplemented with 100 $\mu\text{g} \cdot \mu\text{L}^{-1}$ spectinomycin. All cultures were grown at 30°C, with cultures in LB broth grown under constant shaking.

4.2.8.2. Transformation of competent *A. tumefaciens*

Transformation of *A. tumefaciens* was performed via heat shock. Specifically, 5 μg of pB2GW7-*EXPA11*amiRNA plasmid DNA, or pB2GW7 (empty vector) plasmid DNA was added to 100 μL of frozen competent *A. tumefaciens* cells in a microcentrifuge tube. The tubes were incubated at 37°C for 5 min in a water bath, to allow for both the thawing and heat shock of the cells. Subsequently, 900 μL of room temperature LB broth was added, followed by an incubation at 30°C for 6 hr under constant shaking. The cell pellets were collected by centrifugation at 1500 x *g* for 5 min. After which, 900 μL of the supernatant was discarded and the pellets were resuspended in the remaining 100 μL . Both 10 μL and 90 μL of the re-suspended mixture were plated onto separate LB agar plates (supplemented with the appropriate antibiotics) using sterile spread plating technique. The plates were incubated at 30°C for 2-3 days until colonies appeared.

4.2.8.3. Preparation of *A. tumefaciens* cultures for floral dip transformation

A single colony of *A. tumefaciens* transformed with either pB2GW7-*EXPA11*amiRNA, or pB2GW7 empty vector, was inoculated into 5 mL of LB broth supplemented with the appropriate antibiotics. These were incubated at 30°C for 2 days under constant shaking. The whole 5 mL of the cultures was then inoculated into 500 mL of antibiotic-supplemented LB broth, and incubated at 30°C overnight under constant shaking. The cells were harvested by centrifugation at 3500 x *g* for 15 min at room temperature in an Avanti® J-E centrifuge (Beckman Coulter, Brea, USA). The cells were resuspended in a 5% (w/v) sucrose, 0.05% (v/v) Silwet L-77 surfactant solution.

4.2.8.4. Floral dip transformation of *A. thaliana*

The protocol used was based on the floral dip method (Clough & Bent, 1998). Arabidopsis plants in the bolting stage were used. Any siliques present were removed prior to the floral dip.

The aerial parts of the plants were submerged in the prepared *A. tumefaciens* solutions (see 4.2.8.3.) for 20 sec. Following draining of excess *A. tumefaciens* solution, the plants were placed horizontally on a tray and then covered with clingfilm to increase the relative humidity. The trays were placed in a controlled plant growth room overnight. The following day, the plants were placed upright and covered with Aracons/Aratubes (Arasystems, Ghent, Belgium) to prevent contact and cross fertilisation.

4.2.9. Arabidopsis seed stocks

Seeds for the *Nitrilase2* overexpressing plant line (35S::*NIT2*) were used for the floral dip transformation of *A. tumefaciens* transformed with pB2GW7-*EXPA11*amiRNA, as well as the floral dip transformation of *A. tumefaciens* transformed with pB2GW7 EV. These seeds were initially obtained from Bonnie Bartel (Rice University, Houston, Texas) (Normanly et al., 1997).

4.2.10. Arabidopsis growth conditions

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

4.2.11. Arabidopsis growth in soil

Arabidopsis seeds were grown on soil consisting of 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 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.

4.2.12. Selection of transgenic offspring by GFSA selection

Seeds from the first transgenic generation (T1), and the subsequent generations (T2 and T3) were screened on plates containing glufosinate ammonium (GFSA), the active ingredient in the herbicide, BASTA. Only transgenic plants containing the Bar gene within the T-DNA sequence that is integrated into their genome from the pB2GW7 vector would be resistant to GFSA and survive. Seeds from each line were sterilised as outlined in chapter 2, section 2.2.4 and grown on solid PN media (Plant Nutrient media (see chapter 2, section 2.2.5) with 7% (w/v) phyto

agar) supplemented with 10 $\mu\text{g.mL}^{-1}$ GFSA, or on PN solid media without GFSA (germination control). For each potential transgenic line that could contain either the 35S::*EXPA11*amiRNA construct or the pB2GW7 EV, 50 seedlings were grown per plate. The seedlings were grown under constant plant growth room conditions until GFSA-induced plant death could be observed (usually 7-10 days). Survival was scored visually by the development of true leaves and the absence of yellowing associated with GFSA-induced death. Survival ratios were calculated for each line for each transgenic generation (T1/2/3). One would expect 100% survival for the PN germination controls, and on GFSA-supplemented plates, one would expect 1%, 75%, and 100% survival in the T1, T2, and T3 generations respectively. Pearson's Chi-Square goodness of fit tests were utilised to determine whether the number of surviving T2 seedlings for each line was significantly different from the expected 3:1 survival to death ratio. Seedlings surviving GFSA treatment and displaying the expected segregation ratios were transferred to soil and grown to produce the next transgenic generation of seed. This process was repeated until homozygous transgenic lines were obtained (T3).

4.2.13. Confirmation of the presence of pB2GW7-*EXPA11*amiRNA or pB2GW7 EV in gDNA of transgenic offspring

For every transgenic generation (T1/2/3), gDNA was extracted from ten seedlings for each line using the Edwards DNA extraction protocol (see section 4.2.3.5.). For both pB2GW7-*EXPA11*amiRNA and pB2GW7 EV samples, PCR was used to detect the presence of the *bar* gene located within the pB2GW7 vector. For the pB2GW7-*EXPA11*amiRNA samples, the p35S-*EXPA11*amiRNA coding sequence was detected via PCR using p35S and Oligo B-*attB2* primers. All these primers can be found in Table 4.1/4.2).

4.2.14. RNA extraction, cDNA synthesis and gene expression analysis via RT-qPCR

RNA was extracted as outlined in chapter 2, section 2.2.8., except that when RNA was extracted from a small amount of tissue (seedlings or single leaves), ± 100 mg of tissue was homogenised for four minutes in 1,5 mL microcentrifuge tubes using three small steel ball bearings (Bearing Man Group, Cape Town, South Africa) in a paint shaker (SO-10m shaker, Fluid Management, USA). All cDNA was synthesised using either Oligo(dT) primers or random hexamer primers as outlined in chapter 2, section 2.2.8.4. Gene expression was analysed via RT-qPCR as described in chapter 2, section 2.2.9.

4.2.15. *EXPA11* expression analysis in pB2GW7 *EXPA11*amiRNA/EV T3 lines

For both the pB2GW7 *EXPA11*amiRNA and pB2GW7 EV, for each T3 line that had been confirmed as homozygous via GFSA selection, seedlings were transferred from the GFSA-supplemented PN media onto soil. For the genetic background, 35S::*NIT2*, seedlings were transferred from PN media plates without GFSA, that were grown at the same time as the pB2GW7 *EXPA11*amiRNA/EV T3 lines. Once the plants had bolted, four leaves were harvested per plant and pooled. This was done for three plants per line, giving three biological replicates. Additionally, for expression analysis under salt stress conditions, No-0, 35S::*NIT2*, and the pB2GW7 *EXPA11*amiRNA/EV T3 lines were grown hydroponically for three weeks on ¼ strength PN media, then treated with ¼ strength PN media supplemented with 75 mM NaCl for one additional week. The rosette tissue was harvested from three individual plants, giving three technical repeats. For both the soil-grown and salt-stressed plants, RNA was extracted and cDNA synthesised, and the expression of *EXPA11* was determined via RT-qPCR analysis. *EXPA11* expression was normalised to the reference gene *MON1*, and the normalised data was averaged and plotted along with the associated standard error. The *EXPA11* and *MON1* qPCR primers used can be found in Table 4.1.

4.2.16. Detection of *EXPA11*amiRNA construct expression in cDNA of pB2GW7 *EXPA11*amiRNA/EV T3 lines

Here cDNA was synthesised using random hexamer primers from the RNA extracted from 35S::*NIT2*, 35S::*EXPA11*amiRNA 3,1-1 T3, and pB2GW7 empty vector 2,1-2 T3 plants grown on soil under standard conditions (see section 4.2.15.). Three samples were screened for each of the lines via PCR using 5' Arm F and Loop R primers (see Table 4.1) and the KAPA Taq PCR Kit using the cycling parameters in section 4.2.4.2.

4.2.17. Salt stress tolerance phenotyping assays

4.2.17.1. Early development salt stress assay

Growth of the 35S::*EXPA11*amiRNA and pB2GW7 EV T3 lines was compared to that of the 35S::*NIT2*, and No-0 genetic backgrounds. Seeds were sterilised as outlined in chapter 2, section 2.2.4. and sown onto solid PN-media (see chapter 2, section 2.2.5.) with 7% (w/v) phyto agar in petri dishes. For salt-stress treatment, the media was supplemented with 75, 100, and 125 mM NaCl. For each line, 50 seedlings were grown per plate and four plates were set up for each treatment, giving four biological repeats. Following fourteen days, the number of surviving seedlings (as determined by the development of true leaves) as well as the total fresh

weight of each plate was measured. The data was captured in Excel, and the average fresh weight per plant was calculated by dividing the total fresh weight per plate by the number of surviving seedlings, and plotted along with the associated standard error.

4.2.17.2. Late development salt stress assay

Growth of the 35S::*EXPA11*amiRNA and pB2GW7 EV T3 lines was compared to that of the 35S::*NIT2*, and No-0 genetic backgrounds. Seeds were sterilised and grown hydroponically (see chapter 2, section 2.2.6.) in ¼ strength PN media for three weeks, followed by one more week in ¼ strength PN media supplemented with 0 or 75 mM NaCl. For both treatments, four plants of each genotype were grown in a single Araponics tank (Araponics, Liège, Belgium), giving four technical repeats. The root and shoot fresh weight of each plant was measured. The data was captured in Excel, and the average root/shoot fresh weight per plant was calculated and plotted along with the associated standard error.

4.2.18. Statistical analysis

All statistical analyses were performed using Statistica Software version 13.5 (Statsoft, Tulsa, USA). Where two-way analysis of variance (ANOVA) or General Linear Model (GLM) analysis was performed on the data, significance was determined using Fisher's least significant difference (LSD) post-hoc analysis. Where two-sample t-tests were conducted to determine statistically significant differences between sample groups, Bonferroni correction for multiple testing was performed. For all analyses performed, p -values $\leq 0,05$ were considered significant.

Table 4.1: Primers used in the generation of the *EXPA11* knock-down construct (*EXPA11*amiRNA construct), transgenic screening, and expression analysis

Primer name	Primer sequence (5' → 3')
Oligo A-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT CTGCAAGGCGATTAAGTTGGGTAAC
Oligo B-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT GCGGATAACAATTCACACAGGAAACAG
Oligo I	GATAACCGCAATAAAAAAGAGCGTTCTCTCTTTTGTATTCC
Oligo II	GAACGCTCTTTTATTGCGGTTATCAAAGAGAATCAATGA
Oligo III	GAACACTCTTTTATAGCGGTTTTCACAGGTCGTGATATG
Oligo IV	GAAAACCGCTATAAAAAAGAGTGTTCTACATATATATTCCT
p35S F	AATATCGGGAAACCTCCTCG
Bar F	AAGTCCAGCTGCCAGAAACC
Bar R	GAAGTACAGAAACCGCAACG
qEXPA11 F	TTGCGGTTGATGCGTTTAG
qEXPA11 R	TTGCGGTTGATGCGTTTAG
qMON1 F	CAGACAAGGCGATGGCGATA
qMON1 R	GCTTTCTCTCAAGGGTTTCTGGGT
5' Arm F	CGCTCGGACGCATATTACAC
Loop R	GACTCGGTATTTGGATGAATGAG
CCA1 promoter F	TGTAGTGAACCGCACGAGAA
CCA1 promoter R	CGTTAATCTCTCCCACCAGTTT

Note: The bold sequence in the Oligo A-attB1 and Oligo B-attB2 primers is the *attB1/attB2* sequence respectively

The bold sequence in the Oligo I/II/III/IV primers is the 21-mer sequence specific to *EXPA11*

Note: The *CCA1* promoter primers were obtained and/or adapted from (Gendron et al., 2012)

Table 4.2: Function of the different primer combinations in generation of *EXPA11*amiRNA construct, transgenic screening, and expression analysis

Primer combination	Product size (bp)	Function	PCR kit used and T_a
Oligo A-<i>att</i>B1 Oligo IV	301	Amplification of 5' arm	Kapa Hifi – 52°C
Oligo II Oligo III	171	Amplification of central loop	Kapa Hifi – 52°C
Oligo B-<i>att</i>B2 Oligo I	327	Amplification of 3' arm	Kapa Hifi – 52°C
Oligo A-<i>att</i>B1 Oligo B-<i>att</i>B2	759	Amplification of full-length <i>EXPA11</i> amiRNA construct Detection of <i>EXPA11</i> amiRNA construct in pDONR221 and pB2GW7	Kapa Hifi – 55°C Kapa Taq – 53°C
p35S F Oligo B-<i>att</i>B2	1182	Detection of <i>EXPA11</i> amiRNA construct in transgenic Arabidopsis lines	Kapa Taq – 53°C
Bar F Bar R	733	Detection of Bar gene in transgenic Arabidopsis lines	Kapa Taq – 57°C
q<i>EXPA11</i> F q<i>EXPA11</i> R	118	RT-qPCR	SYBR® FAST - 60°C
q<i>MON1</i> F q<i>MON1</i> R	240	RT-qPCR	SYBR® FAST - 60°C
5' Arm F Loop R	157	RT-PCR detection of <i>EXPA11</i> amiRNA expression in cDNA	Kapa Taq – 53°C
<i>CCA1</i> promoter F <i>CCA1</i> promoter R	122	RT-PCR amplification of gDNA-specific product in cDNA	Kapa Taq – 53°C

4.3. Results

4.3.1. Confirmation of *Expansin 11* expression in the *Nitrilase 2* overexpressing line

Previous transcriptomics experiments conducted by our group (Cackett, 2019) found *EXPA11* to be significantly up-regulated in two-week old 35S::*NIT2* seedlings compared with the genetic background, No-0, when both were germinated and grown under untreated conditions (~100-fold), and in the presence of 100 mM NaCl (~350-fold) (Figure 4.1 A). To validate this transcriptomics data, RT-qPCR was performed using cDNA synthesised from RNA extracted from 35S::*NIT2* and No-0 seedlings grown according to the same experimental design used in the transcriptomics experiment. Referring to Figure 4.1 B, it can be seen that *EXPA11* was again found to be significantly up-regulated in the 35S::*NIT2* line under both untreated conditions (~100-fold), and in response to 100 mM NaCl treatment (~200-fold) compared with No-0.

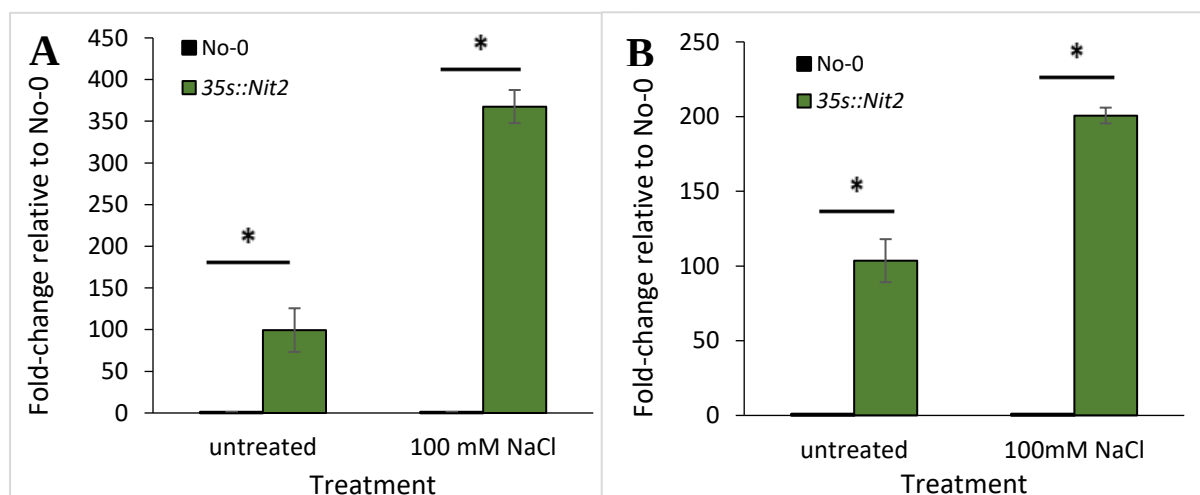


Figure 4.1: Validation of *EXPA11* expression in 35S::*NIT2* line via RT-qPCR analysis

Expression of *EXPA11* in two-week-old 35S::*NIT2* seedlings (green bars) relative to the No-0 genetic background (black bars) in untreated and 100 mM NaCl conditions in the Microarray (A) and RT-qPCR analysis (B). *EXPA11* expression was normalised to the reference gene *MON1*. The results are an average of three biological replicates. The black stars (*) denote a statistically significant difference between No-0 and 35S::*NIT2*, as determined by two-sample t-tests. Bonferroni correction for multiple testing was performed, and p -values ≤ 0.05 were considered significant. Error bars indicate standard error.

Additionally, I wanted to assess *EXPA11* expression in 35S::*NIT2* plants exposed to salt-stress at a later developmental stage, as, unlike in two-week-old seedlings, using older, larger plants allows for the measurement of expression separately in the root and the shoot. To this end, RT-qPCR was performed using cDNA synthesised from RNA extracted from 35S::*NIT2* and No-

0 plants grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 75 mM NaCl, or maintained on ¼ strength PN media (untreated control) for one week. Referring to Figure 4.2 A, no *EXPA11* expression was detected in No-0 shoots or roots under untreated conditions. In response to salt-treatment, very low levels of expression were detected in No-0 roots, while no expression was detected in the shoot. By contrast, *EXPA11* expression was detected in all tissues and treatments for the 35S::*NIT2* line, with greater expression observed in the roots than the shoots (~23-fold in both untreated, and 75 mM NaCl), and particularly high expression observed in the roots in response to salt-treatment. To assess the effect of salt-treatment on expression in the root and shoot of the 35S::*NIT2* line, *EXPA11* expression was determined relative to that of the untreated shoots (Figure 4.2 B). It was observed that relative *EXPA11* expression was significantly increased in response to salt-treatment in both the root and the shoot (~10-fold in both tissues).

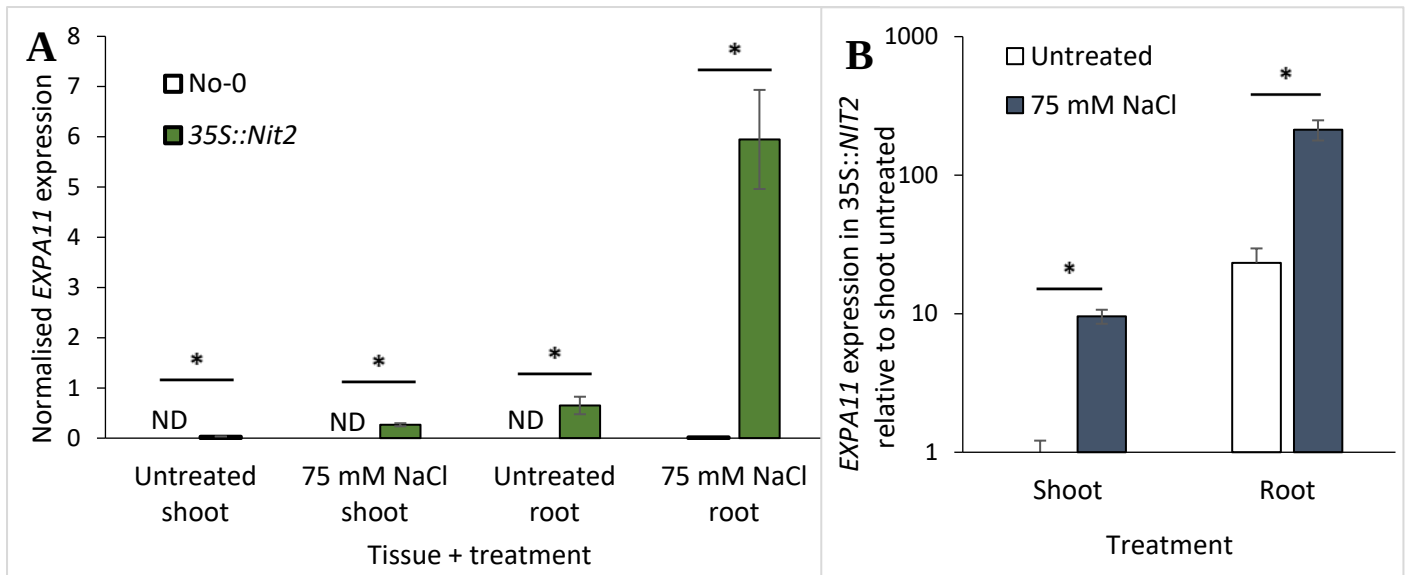


Figure 4.2: *EXPA11* expression in root and shoot tissue of 35S::*NIT2* and No-0 plants exposed to salt stress

EXPA11 expression was measured by RT-qPCR on cDNA synthesised from RNA extracted from No-0 and 35S::*NIT2* plants that were grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 75 mM NaCl, or maintained on ¼ strength PN media (untreated control) for one week. (A) Normalised *EXPA11* expression in root and shoot of No-0 and 35S::*NIT2* in untreated and 75 mM NaCl conditions. (B) Expression of *EXPA11* in 35S::*NIT2* root and shoot relative to the shoot untreated. Expression was normalised to the reference gene *MON1*. Root and shoot tissue from four plants for each treatment from both genotypes was harvested and pooled separately. Each treatment sample had three biological replicates (n = 3). Asterisks (*) denote a statistically significant difference between No-0 and 35S::*NIT2*, as determined by two-sample t-tests. Bonferroni correction for multiple testing was performed, and *p*-values ≤ 0,05 were considered significant. Error bars indicate standard error. ND = no detection.

4.3.2. Generation of an *Expansin 11* knockdown line

4.3.2.1. Generation of the *EXPA11*-knockdown vector

To knock down *EXPA11* expression in the 35S::*NIT2* line, an artificial microRNA approach was adopted, as described in Ossowski et al. (2008). The online WMD3 – Web MicroRNA Designer tool was used to generate a series of four oligonucleotides, which were used to replace the 20-mer Arabidopsis mi319a sequence contained within the pRS300 template plasmid with a 21-mer amiRNA sequence specific to *EXPA11* by overlapping PCR, as shown in Figure 4.3. Briefly, the 5' arm, central loop, and 3' arm fragments of the amiRNA were separately amplified using the pRS300 vector as template, and the respective primer combinations as outlined in Figure 4.3 A, B, and C, respectively. All three of these fragments were then used as the template in an overlapping PCR with primers designed to add *attB1* and *attB2* sites to the 5' and 3' ends of the construct, as shown in Figure 4.3 D. All three of the initial fragments, as well as the final *EXPA11*amiRNA fragment generated were sequence verified.

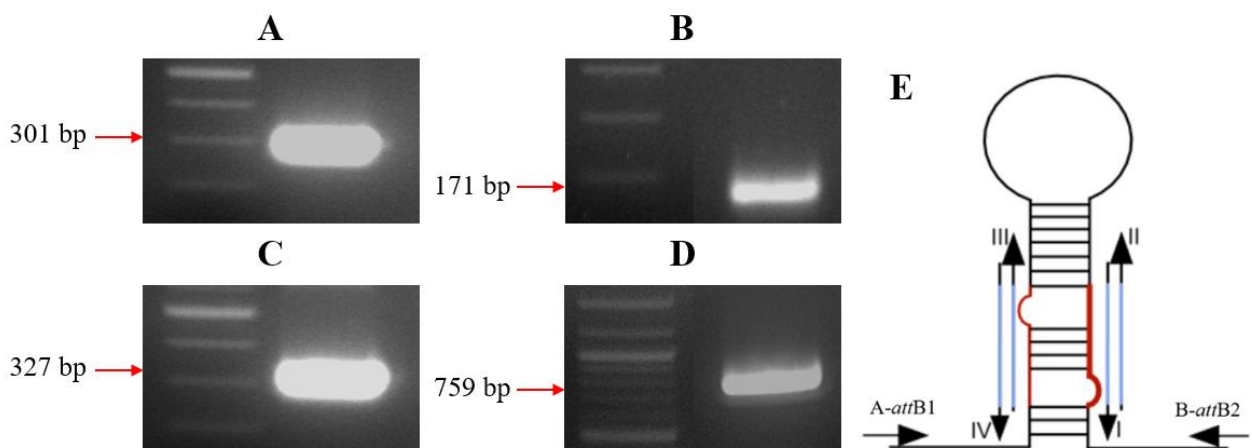


Figure 4.3: Construction of the *EXPA11*amiRNA

PCR was performed using (A) Oligo A-*attB1* and Oligo IV primers for the amplification of the 5' arm, (B) Oligo II and Oligo III primers for the amplification of the central loop, and (C) Oligo I and Oligo B-*attB2* primers for the amplification of the 3' arm, respectively. All three reactions used the pRS300 plasmid as template (Addgene catalogue # 22846). (D) The full length *EXPA11*amiRNA construct was generated via PCR using OligoA-*attB1* and OligoB-*attB2* primers and the simultaneous use of the purified products from (A), (B), and (C) as template. The MW marker included in all cases is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S). (E) Schematic of the primers used to generate the partial and full length *EXPA11*amiRNA construct (modified from Ossowski et al. 2008).

To express the *EXPA11*amiRNA in Arabidopsis, Gateway® Cloning technology was used to clone the *EXPA11*amiRNA fragment into pDONR221, and then subsequently into the pB2GW7 plant expression vector, where its expression is driven by the constitutive 35S promoter from the *Cauliflower mosaic virus* (CaMV p35S). Figure 4.4 shows the vector map of the pB2GW7-*EXPA11*amiRNA vector generated.

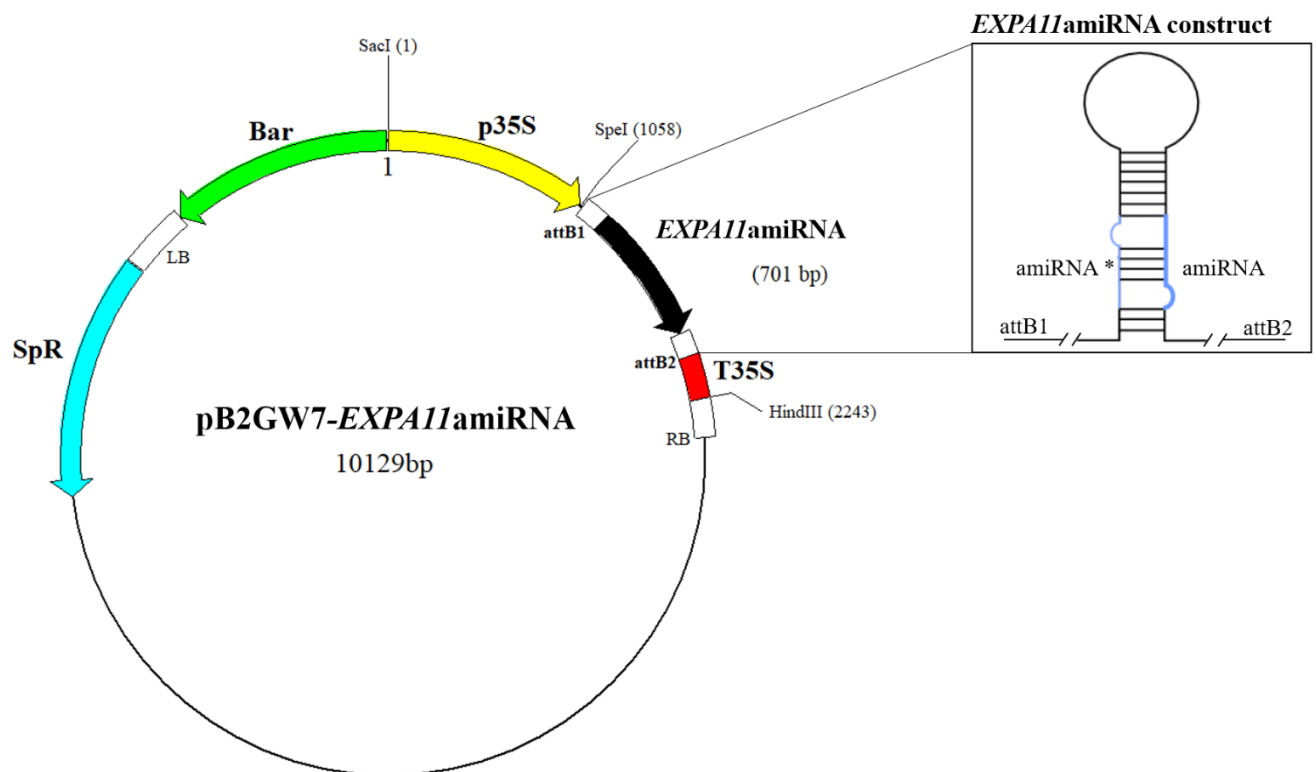


Figure 4.4: Map of the recombinant pB2GW7-*EXPA11*amiRNA generated via Gateway cloning

Expression of the *EXPA11*amiRNA sequence is driven by the constitutive 35S promoter from the *Cauliflower Mosaic Virus* (p35S) and terminated by the 35S terminator sequence (T35S). The spectinomycin resistance gene (SpR) allows for selection of positive *E. coli* and *A. tumefaciens* transformants. The *bar* gene (Bar) provides resistance to the herbicide Glufosinate ammonium, and allows for the selection of transgenic offspring in the next generation following the floral-dip transformation of 35S::*NIT2* Arabidopsis plants. The inset shows the *EXPA11* amiRNA hairpin loop structure that is produced.

4.3.2.2. Transformation of pB2GW7-EXPA11amiRNA into *E. coli* and *A. tumefaciens*

Following the Gateway® cloning reactions, the pB2GW7-EXPA11amiRNA vector was transformed into DH5α *E. coli*, and positive transformants were selected on spectinomycin-containing LB agar plates. A colony PCR performed on 10 discrete colonies confirmed the successful transformation into *E. coli*, as seen in Figure 4.5 A. Plasmid DNA isolated from a single positive *E. coli* transformant was sequence confirmed, and then used to transform *A. tumefaciens*. Positive transformants were selected on LB agar plates containing spectinomycin, rifampicin, and gentamicin. As seen in Figure 4.5 B, a colony PCR performed on 10 discrete colonies confirmed the successful transformation of *A. tumefaciens* with the pB2GW7-EXPA11amiRNA vector. A single positive transformant was then used to inoculate the liquid LB cultures that were used for the floral dip transformation of 35S::NIT2 Arabidopsis plants.

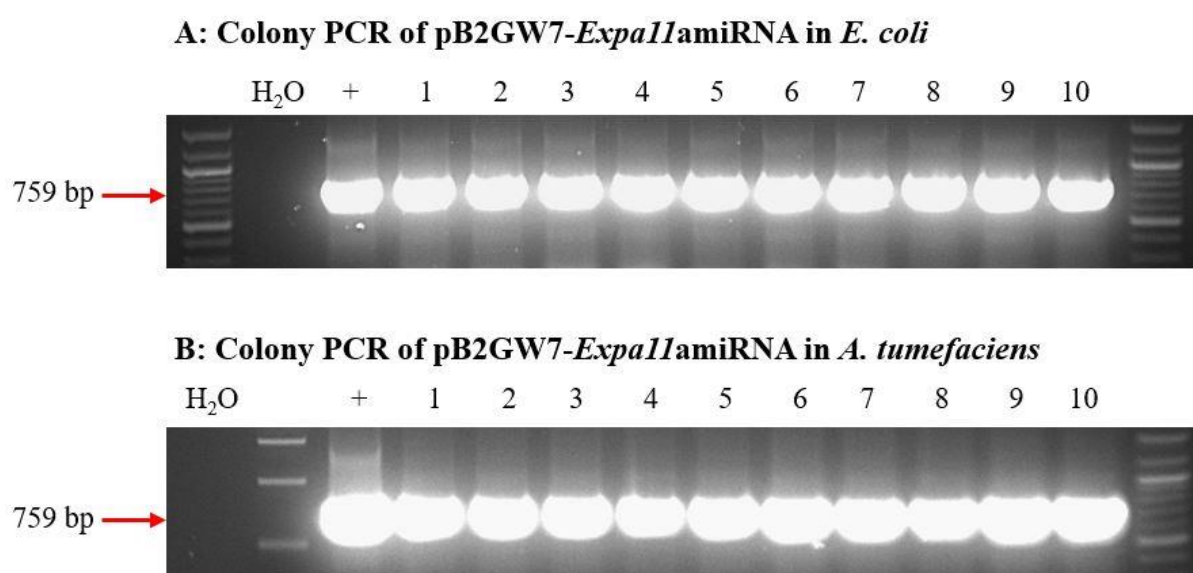


Figure 4.5: PCR confirmation of successful transformation of pB2GW7-EXPA11amiRNA into *E. coli* and *A. tumefaciens*

(A) Following transformation of *E. coli* with pB2GW7-EXPA11amiRNA, 10 discrete colonies surviving on LB spectinomycin (100 ug.mL⁻¹) plates were screened by colony PCR with OligoA/B-*att*B primers. The positive control used the EXPA11amiRNA PCR product as template. The MW marker included is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S). (B) Following transformation of *A. tumefaciens* with pB2GW7-EXPA11amiRNA, 10 discrete colonies surviving on antibiotic (100 ug.mL⁻¹ spectinomycin, 150 ug.mL⁻¹ rifampicin, 15 ug.mL⁻¹ gentamicin) plates were screened by colony PCR using OligoA/B-*att*B primers. The positive control used the EXPA11amiRNA PCR product as template. The MW markers included are the New England Biolabs (NEB) Quick-Load® 1 kb DNA ladder (Catalogue #NO468) and the Quick-Load® 100 bp DNA ladder (Catalogue #NO467S).

4.3.2.3. Homozygosity of the *EXPA11* amiRNA and empty vector control (EV) lines

In order to confirm the generation of stable transgenic *Arabidopsis* lines, I assessed the survival of the first, second, and third transgenic generations of the various 35S::*EXPA11*amiRNA, and pB2GW7 EV lines on Glufosinate ammonium-supplemented PN plates (GFSA). The aim was to select for homozygous lines with a single insertion of the vector DNA. For the first transgenic generation (T1), 100 seedlings from each of the 35S::*NIT2* plants that underwent *A. tumefaciens*-mediated floral dip transformation with the pB2GW7-*EXPA11*amiRNA or pB2GW7 empty vector were screened, and lines with one surviving seedling were taken forward (data not shown). In the second transgenic generation (T2), it is important to ensure that one has selected segregating transgenic lines containing one insertion of the vector DNA, which corresponds to a survival to death ratio of 3:1 on GFSA. Table 4.3 shows the results of the GFSA-screening of 50 seedlings of each of the T2 lines. The 35S::*EXPA11*amiRNA T2 lines ‘3,1’, ‘5,1’ and ‘14,2’, all had 40 surviving seedlings, which was revealed by Chi-Squared goodness of fit tests not to be significantly different from the anticipated 3:1 ratio ($p = 0.414$), and were thus taken forward. However, for the line ‘8,1’ all 50 seedlings survived, suggesting the possibility of a double insertion of the 35S::*EXPA11*amiRNA T-DNA. Given that multiple insertions of the 35S::*EXPA11*amiRNA construct would potentially increase the chances of knocking down *EXPA11* expression, this line was also taken forward. The EV T2 lines ‘2,1’, and ‘4,1’ had 35 and 40 seedlings surviving, respectively, both of which were revealed by Chi-Squared tests not to be significantly different from the anticipated 3:1 ratio ($p = 0.414$), thus these lines were taken forward. However, lines ‘7,1’ and ‘24,2’, had 48 and 50 surviving seedlings respectively, suggesting a possible double insertion of the pB2GW7 T-DNA, so these lines were abandoned from further analysis. Figure 4.6 shows a representative example of the 35S::*EXPA11*amiRNA T2 line ‘3,1’, and pB2GW7 EV T2 line ‘4,1’ displaying the expected 3:1 survival ratio on GFSA, as well as the untransformed 35S::*NIT2* genetic background displaying a zero percent survival. For all the T2 lines taken forward, six surviving individuals were transferred to soil.

In the third transgenic generation (T3), homozygous lines can be determined from a one hundred percent survival rate on GFSA, however, given that the surviving plants in the T2 are a 2:1 mix of heterozygotes to homozygotes, one would expect four out of the six surviving T2 plants for each line to be heterozygous. Appendix chapter 7, Table 7.4 shows the results of the GFSA-screening of the T3 offspring from all six of the surviving T2 plants for each line that were transferred to soil. For the 35S::*EXPA11*amiRNA T3 lines, ‘3,1’ and ‘5,1’ as well as the

EV T3 lines ‘2,1’ and ‘4,1’, two of the six lines screened had one hundred percent survival rate, indicating homozygosity, whereas the remaining four lines had the 3:1 survival ratio expected for heterozygotes. It is thus likely that all these T3 lines that had a one hundred percent survival rate were homozygous and contained a single insertion of the pB2GW7 T-DNA. The 35S::*EXPA11*amiRNA T3 line ‘8,1’, and the EV T3 line ‘24,2’ had four out of six, and five out of six, with one hundred percent survival, respectively, providing further evidence to the possibility of a double insertion, as discussed earlier. For the 35S::*EXPA11*amiRNA T3 line ‘14,2’, which displayed the anticipated 3:1 survival to death ratio associated with a single insertion in the T2, five out of the six lines screened displayed one hundred percent survival. However, given the small sample size of six, the expected probability ratios may not hold as expected, and thus is it not unlikely that five of the six lines screened could be homozygous with a single insertion.

While GFSA-selection identifies lines that have been successfully transformed with the T-DNA, it does not discriminate between the insertion of the empty vector or pB2GW7-*EXPA11*amiRNA. To this end, gDNA was isolated from both T2 and T3 35S::*EXPA11*amiRNA and EV lines, and PCR screening was performed (data for T2 not shown). Referring to Figure 4.7, the 35S::*EXPA11*amiRNA construct was detected in the gDNA from all eight of the individual plants tested from the various 35S::*EXPA11*amiRNA T3 lines. For the various EV T3 lines, the *bar* gene present in the pB2GW7 vector was detected in the gDNA of all eight of the individuals tested (Figure 4.8), and importantly, the 35S::*EXPA11*amiRNA construct was not detectable in these lines (Figure 4.9), confirming the insertion of the EV T-DNA. Considering the above, and as seen in Table 4.4, it was concluded that the 35S::*EXPA11*amiRNA T3 lines ‘3,1-1’ and ‘5,1-3’, as well as the pB2GW7 EV T3 lines ‘2,1-2’ and ‘4,1-4’ were homozygous, with a single insertion of the respective T-DNA, and thus suitable to be taken forward for *EXPA11* expression analysis. Furthermore, it was decided that the 35S::*EXPA11*amiRNA T3 line ‘8,1-2’, which was thought to contain a double insertion, would also be taken forward for gene expression analysis.

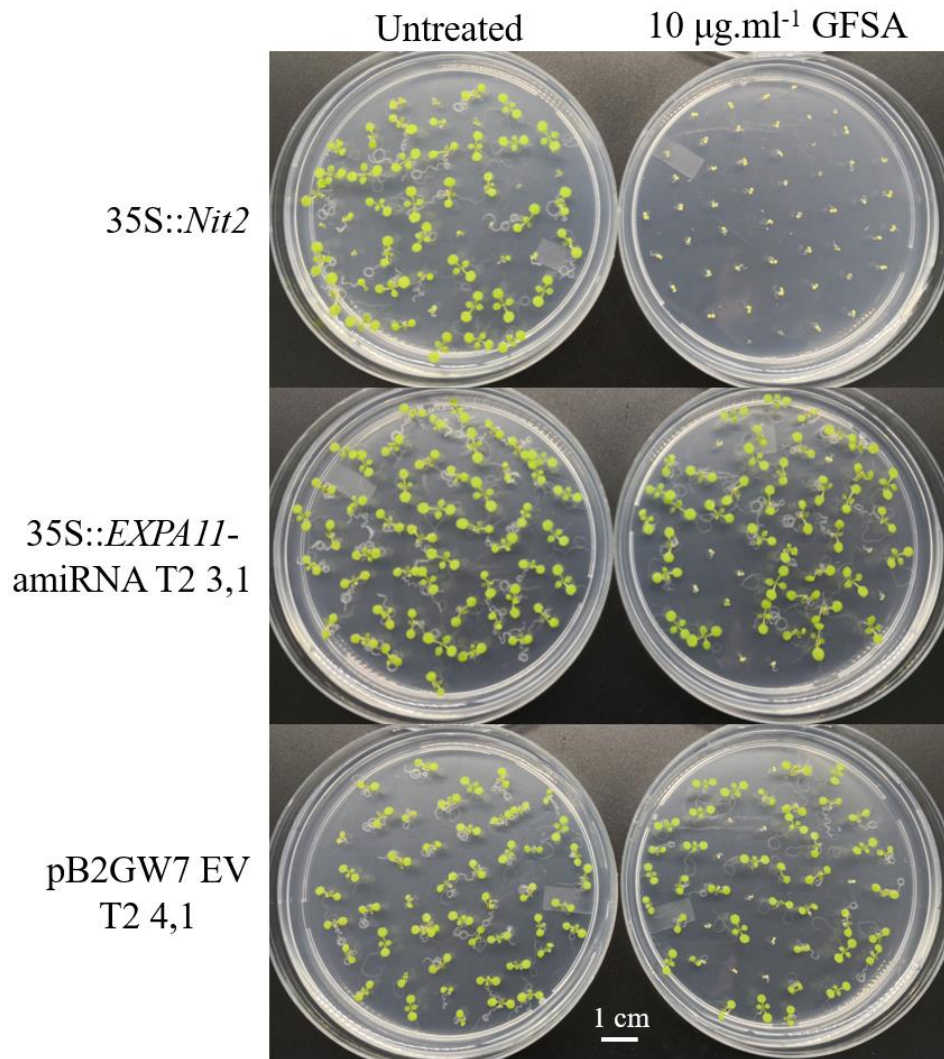


Figure 4.6: Glufosinate ammonium selection of T2 35S::EXPA11amiRNA and empty vector control lines

Seeds from the second transgenic generation (T2) of the 35S::EXPA11amiRNA and pB2GW7 empty vector lines were screened for survival on PN media supplemented with 10 µg.mL⁻¹ Glufosinate ammonium (GFSA), or on untreated PN media (germination control). Here, 50 seedlings were grown per plate for each T2 line screened. Seedlings were grown for nine days whereafter survival was scored in the GFSA-treated seedlings by the development of true leaves, as well as a lack of visual yellowing that is associated with GFSA-induced plant death. **(Top panel)** The genetic background, 35S::NIT2 was included as a positive control for plant death due to GFSA sensitivity. **(Middle panel)** An example of a 35S::EXPA11amiRNA T2 line, and **(Bottom panel)** an example of a pB2GW7 EV T2 line with the 3:1 survival ratio expected for segregating lines following GFSA treatment.

Table 4.3: GFSA-screening results of 35S::*EXPA11*amiRNA and empty vector control T2 lines

	Untreated		GFSA (10 µg.mL ⁻¹)	
Line	Germination (of 50)	Survival	Germination (of 50)	Survival
35S:: <i>NIT2</i>	49	49	48	0
amiRNA 3,1	50	50	50	40
amiRNA 5,1	50	50	50	40
amiRNA 8,1	50	50	50	50
amiRNA 14,2	50	50	50	40
EV 2,1	50	50	50	35
EV 4,1	50	50	50	40
EV 7,1	50	50	50	48
EV 24,2	50	50	50	50

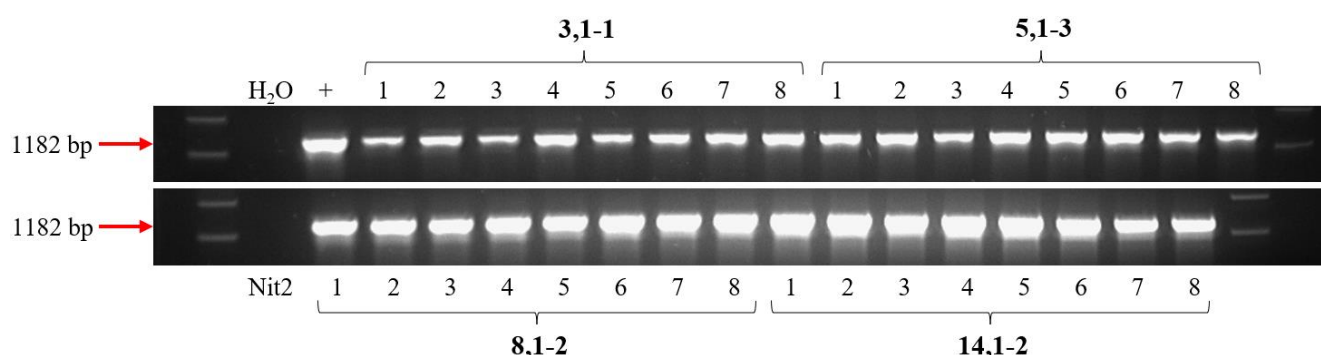


Figure 4.7: Presence of 35S::*EXPA11*amiRNA construct in T3 lines

For each third transgenic generation (T3) line, DNA extractions were performed on 8 seedlings. The PCR with p35S F and OligoB-*attB2* R primers was conducted to confirm the presence of the 35S::*EXPA11*amiRNA construct in the genomic DNA (gDNA) of the T3 lines. The T3 lines that were screened are '3,1-1', '5,1-3', '8,1-2' and '14,1-2'. The positive control used the gDNA from the second transgenic generation (T2) of the T3 line 8,1-3 as template. A 35S::*NIT2* ('*NIT2*') gDNA sample was used as a negative control. The MW marker included is the New England Biolabs (NEB) Quick-Load® 1 kb DNA ladder (Catalogue #NO468).

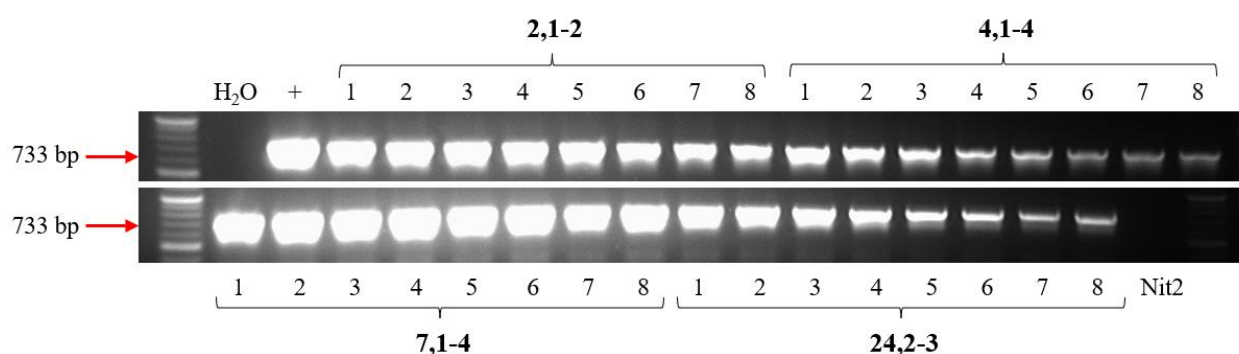


Figure 4.8: Presence of *bar* gene in T3 empty vector lines

For each third transgenic generation (T3) pB2GW7 empty vector (EV) line, DNA extractions were performed on 8 seedlings. The PCR with *bar* F and R primers was conducted to confirm the presence of the *bar* gene in the genomic DNA (gDNA) of the T3 lines. The T3 lines that were screened are '2,1-2', '4,1-4', '7,1-4' and '24,2-3'. The positive control used the gDNA from the 35S::*EXPA11* amiRNA T3 line 14,1-2 (see figure 1.5) as template. A 35S::*NIT2* ('NIT2') gDNA sample was used as a negative control. The MW marker included is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S).

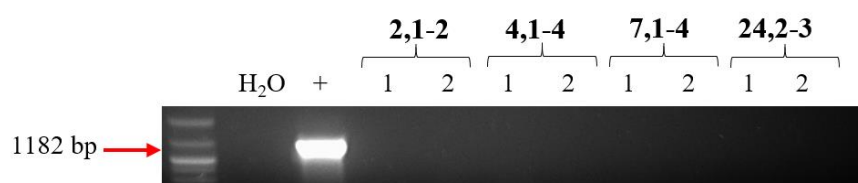


Figure 4.9: Absence of 35S::*EXPA11* amiRNA construct from T3 empty vector lines

The PCR with p35S F and OligoB-*attB2* R primers was conducted to confirm the presence of the 35S::*EXPA11*amiRNA construct in the genomic DNA (gDNA) of the T3 EV lines. Two gDNA samples from the four pB2GW7 empty vector (EV) lines from Figure 1.8 were screened. The positive control used the gDNA from the 35S::*EXPA11* amiRNA T3 line 14,1-2 (see Figure 1.7) as template. The MW marker included is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S).

Table 4.4: GFSA-screening results of 35S::*EXPA11*amiRNA and empty vector control T3 lines that were selected for *EXPA11* expression analysis

Line	Untreated		GFSA (10 µg.mL ⁻¹)	
	Germination (of 50)	Survival	Germination (of 50)	Survival
35S:: <i>NIT2</i>	48	48	47	0
amiRNA 3,1-1	50	50	50	50
amiRNA 5,1-3	50	50	50	50
amiRNA 8,1-2	50	50	50	50
EV 2,1-2	50	50	50	50
EV 4,1-4	50	50	50	50

4.3.2.4. Gene expression analysis of the *EXPA11*amiRNA and EV control lines

For all gene expression analysis experiments, expression of *EXPA11* in the 35S::*EXPA11*amiRNA and pB2GW7 EV T3 lines, was compared with the 35S::*NIT2* genetic background. Tissue from three plants grown on soil from each of the chosen T3 lines, as well as 35S::*NIT2*, was harvested for RNA extraction. Then cDNA was synthesised and RT-qPCR analysis was performed to measure *EXPA11* expression. Figure 4.10 shows that none of the homozygous 35S::*EXPA11*amiRNA T3 lines tested displayed the expected knockdown in expression compared with the 35S::*NIT2* control, with line ‘5,1-5’ actually having increased *EXPA11* expression, albeit not significantly so. Furthermore, although the pB2GW7 EV T3 line ‘4,1-4’ displayed the expected result of unaltered *EXPA11* expression, the line ‘2,1-2’ was found to have a greater than 2,5-fold increase in expression compared with the 35S::*NIT2* control. A second gene expression analysis was performed, in which the lines were grown hydroponically for three weeks on untreated media, and then one week on media supplemented with 75 mM NaCl. This experiment included both the 35S::*NIT2*, and No-0 (the genetic background of the 35S::*NIT2* line) lines as controls. The total rosette tissue from three plants of each of the T3 lines as well as the controls was harvested for RNA extraction, and subsequently RT-qPCR analysis was performed to measure *EXPA11* expression. Referring to Figure 4.11, it was found once again that none of the 35S::*EXPA11*amiRNA T3 lines tested displayed altered *EXPA11* expression compared with the 35S::*NIT2*. Likewise, the pB2GW7 EV T3 ‘2,1-2’ was found to display a greater than 2-fold increase in expression (although this was not significant). Consistent with the findings in section 4.3.1, the 35S::*NIT2* line was found to have greatly up-regulated *EXPA11* expression compared with its genetic background, No-0.

As no knockdown of *EXPA11* expression was detected in the 35S::*EXPA11*amiRNA T3 lines, it was necessary to confirm if the amiRNA construct was actually being expressed. Thus, cDNA was synthesised from the RNA samples of the 35S::*NIT2*, pB2GW7 EV T3 ‘2,1-2’, and 35S::*EXPA11*amiRNA T3 ‘3,1-1’ soil-grown plants, using random hexamer primers, as the amiRNA construct is expressed without a polyA tail so would not be present in the cDNA synthesised previously using OligodT primers. Figure 4.12 shows, as expected, that the *EXPA11*amiRNA construct was only expressed in the 35S::*EXPA11*amiRNA T3 ‘3,1-1’ line, and not in the EV or genetic background. Furthermore, to validate that detection of the *EXPA11*amiRNA construct was due to its expression and not amplification of any contaminating gDNA in the random hexamer cDNA samples, a PCR was performed using primers that would amplify a region of DNA that would only be present in gDNA. The

promoter region of a randomly chosen gene (*CCA1*) was identified as a suitable candidate, and Figure 4.13 shows, as anticipated, that there was no contaminating gDNA in the random hexamer-synthesised cDNA samples. Additionally, as seen in Figure 4.14, the full-length *EXPA11* coding DNA sequence was detectable in the cDNA synthesised with oligo dT primers from all of the soil-grown 35S::*EXPA11*amiRNA T3 lines tested, which stands in agreement with the qPCR results in Figure 4.10.

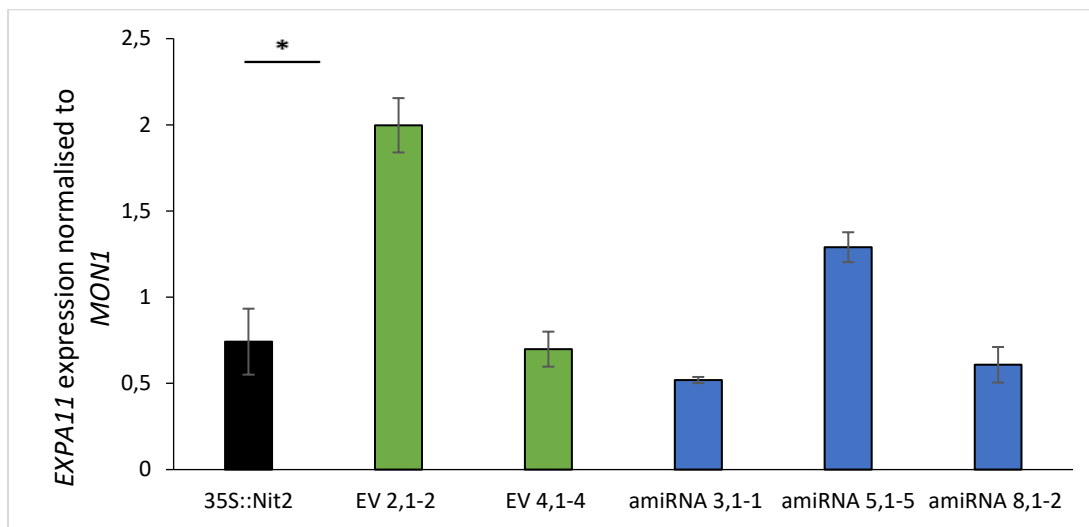


Figure 4.10: Expression of *EXPA11* in the 35S::*EXPA11* amiRNA and empty vector control T3 lines

An RT-qPCR experiment was conducted using *EXPA11* qPCR primers on cDNA synthesised from RNA extracted from 35S::*NIT2*, 35S::*EXPA11*amiRNA T3, and pB2GW7 empty vector control T3 plants grown on soil under standard conditions. The pB2GW7 empty vector T3 lines that were screened are ‘EV 2,1-2’ and ‘EV 4,1-4’, as represented by the green bars. The 35S::*EXPA11*amiRNA T3 lines that were screened are ‘amiRNA 3,1-1’, ‘5,1-5’ and ‘8,1-2’, as represented by the blue bars. *EXPA11* expression was normalised to the reference gene, *MON1*. The results are an average of three biological replicates, where each replicate was three leaves pooled per plant for RNA extraction and subsequent cDNA synthesis for each of the genotypes. The black stars (*) denote a statistically significant difference between 35S::*NIT2* (black bar) and the other genotypes tested, as determined by two-sample t-tests. Bonferroni correction for multiple testing was performed, and *p*-values ≤ 0.05 were considered significant. Error bars indicate standard error.

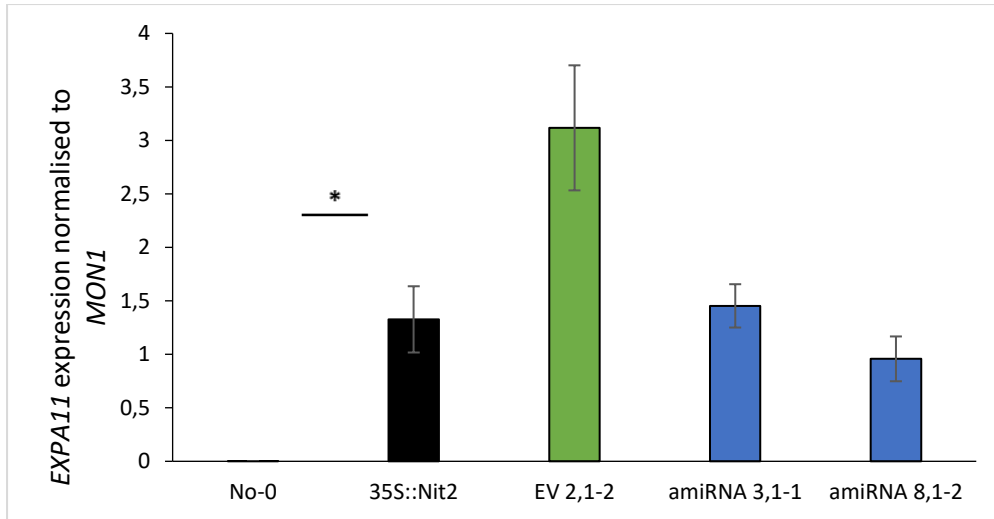


Figure 4.11: Expression of *EXPA11* in salt-stressed 35S::*EXPA11* amiRNA and empty vector T3 lines

An RT-qPCR experiment was conducted using *EXPA11* qPCR primers on cDNA synthesised from RNA extracted from No-0, 35S::*NIT2*, 35S::*EXPA11*amiRNA T3, and pB2GW7 empty vector control T3 plants grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 75 mM NaCl, or maintained on ¼ strength PN media (untreated control) for one additional week. The pB2GW7 empty vector T3 line that was screened is ‘EV 2,1-2’, as represented by the green bar. The 35S::*EXPA11*amiRNA T3 lines that were screened are ‘amiRNA 3,1-1’, and ‘8,1-2’, as represented by the blue bars. *EXPA11* expression was normalised to the reference gene, *MON1*. The results are an average of three biological replicates, where each replicate was the rosette tissue from one plant pooled for RNA extraction and subsequent cDNA synthesis for each of the genotypes. The black stars (*) denote a statistically significant difference between 35S::*NIT2* (black bar) and the other genotypes tested, as determined by two-sample t-tests. Bonferroni correction for multiple testing was performed, and p -values ≤ 0.05 were considered significant. Error bars indicate standard error.

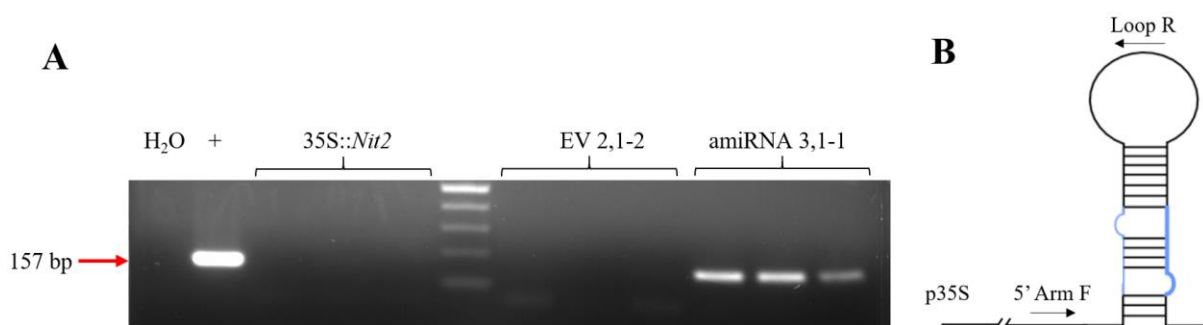


Figure 4.12: Expression of *EXPA11* amiRNA construct in 35S::*EXPA11*amiRNA and empty vector T3 lines

(A) RT-PCR was conducted with 5' Arm F and Loop R primers to detect the expression of the *EXPA11* amiRNA construct in cDNA synthesised, using random hexamer primers, from RNA extracted from 35S::*NIT2*, 35S::*EXPA11*amiRNA 3,1-1 T3, and pB2GW7 empty vector 2,1-2 T3 plants grown on soil under standard conditions. Three random hexamer cDNA samples were screened for each of the lines. The positive control used the *EXPA11*amiRNA fragment as template. The MW marker included is the New England Biolabs (NEB) Quick-

Load® 100 bp DNA ladder (Catalogue #NO467S). **(B)** Schematic of the region of the expressed *EXPA11* amiRNA construct that is amplified by the 5' Arm F and Loop R primers.

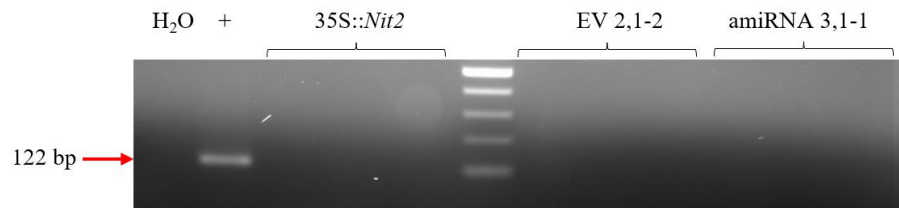


Figure 4.13: Absence of contaminating gDNA in the random hexamer cDNA of the 35S::*EXPA11*amiRNA and empty vector T3 lines

RT-PCR was conducted with *CCA1* promoter F and R primers to detect the presence of contaminating gDNA in the cDNA synthesised, using random hexamer primers, from RNA extracted from 35S::*NIT2*, 35S::*EXPA11*amiRNA 3,1-1 T3, and pB2GW7 empty vector 2,1-2 T3 plants grown on soil under standard conditions. Three random hexamer cDNA samples were screened for each of the lines. The positive control used Col-0 gDNA as template. The MW marker included is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S).

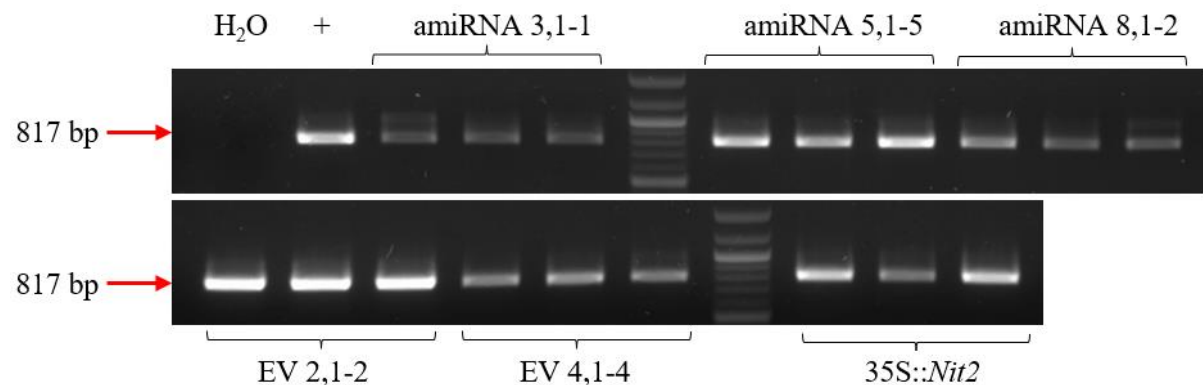


Figure 4.14: Detection of full-length *EXPA11* coding DNA sequence in the 35S::*EXPA11*amiRNA and empty vector T3 lines

The PCR was conducted with *EXPA11* F and R primers to detect the expression of the full-length *EXPA11* coding DNA sequence in cDNA synthesised using oligo dT primers from RNA extracted from 35S::*NIT2*, 35S::*EXPA11*amiRNA T3, and pB2GW7 empty vector control T3 plants grown on soil under standard conditions. Three cDNA samples were screened for each of the lines. The positive control used a 35S::*NIT2* cDNA sample, previously shown to have high levels of *EXPA11* expression, as template. The MW marker included is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S).

4.3.3. Phenotypic characterisation of the *EXPA11*amiRNA lines

While the 35S::*EXPA11*amiRNA lines tested did not display altered *EXPA11* expression compared with the 35S::*NIT2* genetic background, phenotypic characterisation of these lines, as well as the EV lines, was conducted. There is evidence to suggest that amiRNAs can act in a translational-inhibitory manner, as opposed to post-transcriptionally, as phenotypic effects have been observed without reductions in target RNA expression (Ossowski, Schwab & Weigel, 2008). The growth of the aforementioned lines was assessed in seedlings exposed to salt stress early in development, as well as in hydroponically grown plants exposed to salt stress at a later developmental stage. No-0, the genetic background of the 35S::*NIT2* line, was included in these experiments as a control to determine if the 35S::*NIT2* line displayed the increased salt-tolerant phenotype that has been previously observed; two-week-old 35S::*NIT2* seedlings have significantly greater fresh weight per plant than No-0 when grown on 75, 100, and 125 mM NaCl (early development) (Cackett et al., 2022), and two-week-old hydroponically grown 35S::*NIT2* plants have significantly greater relative root and shoot fresh weight than No-0 following treatment with 75 mM NaCl for one week (later developmental stage) (Cackett, 2019).

4.3.3.1. Growth of 35S::*EXPA11*amiRNA plants exposed to salt stress early in development

In order to assess whether the 35S::*EXPA11*amiRNA lines have altered growth in response to salt stress during early development, selected 35S::*EXPA11*amiRNA, empty vector, 35S::*NIT2*, and No-0 lines were germinated and grown for two weeks in petri dishes containing PN media (untreated control) or PN supplemented with 75, 100, and 125 mM NaCl. The average fresh weight of the surviving seedlings was measured. Looking at Figure 4.15 A, the average fresh weight of the surviving 35S::*EXPA11*amiRNA and empty vector T3 seedlings was lower than that of both No-0 and the 35S::*NIT2* line under control conditions, and in response to salt-treatment in all of the concentrations tested. For all five lines, a General Linear Model (GLM) analysis was performed to assess the effect of genotype and the effect of salt concentration on fresh weight, as well as to assess the interaction between genotype and salt treatment. This revealed that there was a significant effect of both genotype ($p < 0.001$) and salt concentration ($p < 0.001$) on seedling fresh weight as well as a significant interaction between genotype and treatment ($p < 0.001$). In this analysis, the average seedling fresh weight of No-0 and the 35S::*NIT2* was found not to be different from each other, but was significantly greater than that of the three transgenic lines at all treatment conditions (which were not

different from each other). However, when comparing only No-0 and the 35S::*NIT2* line using two sample T-Tests, the average fresh weight per seedling was significantly increased in No-0 ($p = 0.0027$), and significantly increased in the 35S::*NIT2* line ($p = 0.0196$), in untreated and 125 mM NaCl conditions, respectively, with no difference between the two lines in 75 and 100 mM NaCl. Given the lower fresh weight observed in untreated conditions in the 35S::*EXPA11*amiRNA and EV transgenic lines, the fresh weight of all the lines in each salt treatment was also calculated relative to their respective untreated fresh weight, (Figure 4.15 B). No major differences in relative fresh weight were observed between the transgenic lines and the 35S::*NIT2* line in the three NaCl concentrations, however all of these lines had higher relative fresh weight than No-0. Again, for all five lines, a GLM analysis was performed to assess the effects of genotype, and salt concentration on seedling relative fresh weight, and the interaction between these two effects. Here, it was found that there was a significant effect of both genotype ($p = 0.01$) and salt concentration ($p < 0.0001$) on the relative fresh weight, but there was no significant interaction between these two effects ($p = 0.598$). The relative fresh weight of the 35S::*NIT2* line, both 35S::*EXPA11*amiRNA lines, and the EV line was thus significantly greater than that of No-0 at all salt concentrations tested. This observation of significantly increased relative fresh weight in the 35S::*NIT2* line compared with No-0 in saline conditions is consistent with our previous findings.

While all the transgenic lines displayed lower average fresh weight than their genetic background, 35S::*NIT2*, in all of the treatments, there was no difference between the empty vector and 35S::*EXPA11*amiRNA lines. This suggests that the expression of the *EXPA11*amiRNA construct as opposed to expression of only the other genes present on the vector backbone, not only has no effect on *EXPA11* expression, but also has no effect on growth. Furthermore, it is notable that in the relative fresh weight measurements, all these transgenic lines generated in the 35S::*NIT2* genetic background were the same as the 35S::*NIT2* line in all three salt concentrations. Additionally, all these transgenic lines had significantly increased relative fresh weight compared with No-0, as expected. Based off this, there is little evidence to suggest that seedlings of the 35S::*EXPA11*amiRNA lines generated have an altered salt-stress phenotype when compared with the empty vector line, or the 35S::*NIT2* line.

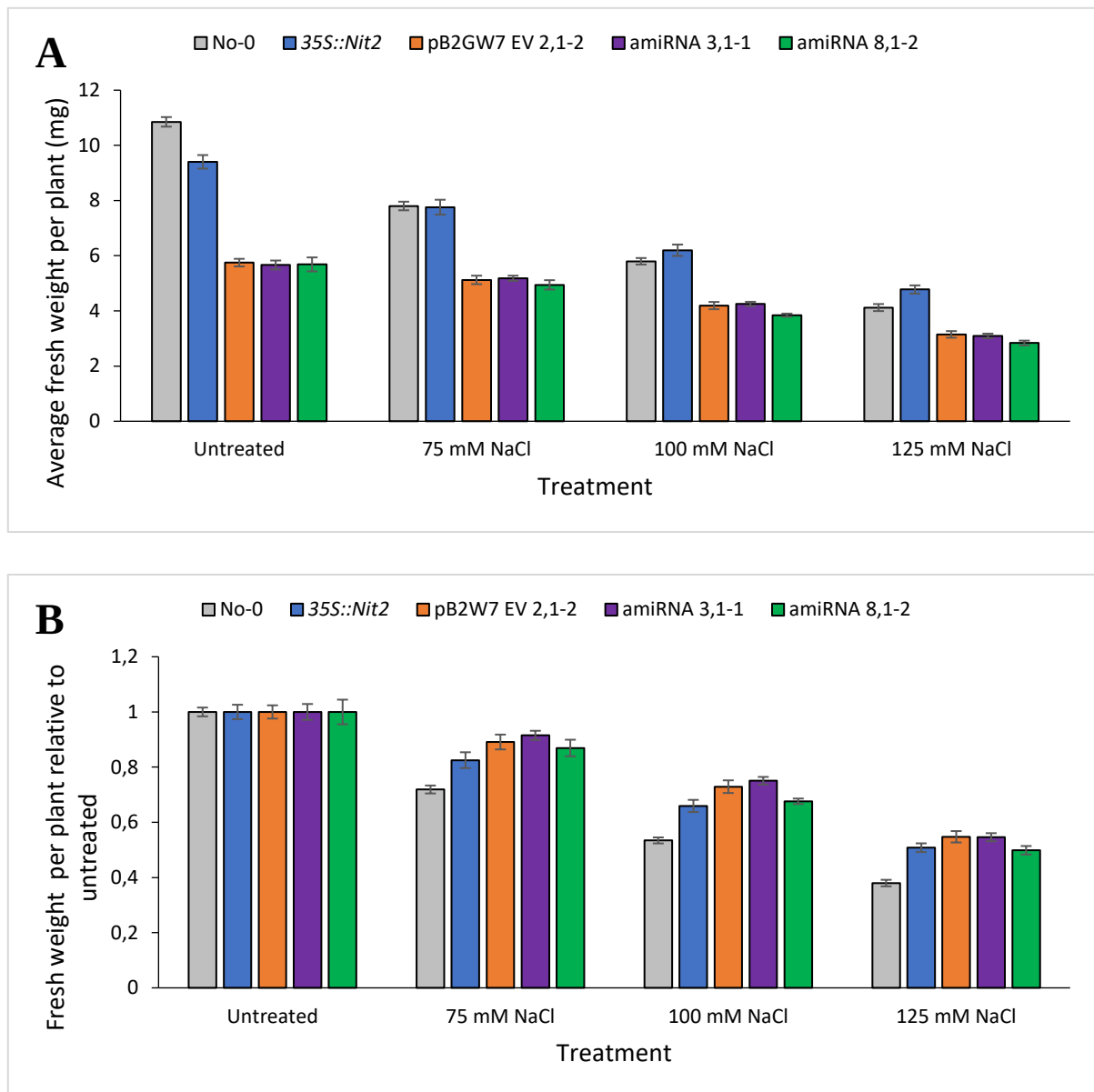


Figure 4.15: Fresh weight of 35S::EXPA11amiRNA and empty vector T3 seedlings exposed to salt stress

The 35S::NIT2, No-0, 35S::EXPA11amiRNA 3,1-1 and 8,1-2 T3, and pB2GW7 empty vector 2,1-2 T3 plants were germinated and grown for fourteen days in petri dishes containing PN-phyto agar (7% w/v) supplemented with 0, 75, 100, and 125 mM NaCl. Here, 50 seedlings were germinated per petri dish. Survival was scored by development of true leaves, and the number of surviving seedlings was counted per petri dish. The average fresh weight per plant was calculated by dividing the total fresh weight of each petri dish by the number of surviving seedlings. **(A)** Average fresh weight per plant of each of the lines in untreated and 75, 100, 125 mM NaCl conditions. **(B)** Fresh weight of each of the lines in each treatment relative to the untreated control. The results are an average of two independent biological experiments, each consisting of four technical replicates. General Linear Model analysis was employed to assess the effect of genotype, effect of salt concentration, and the interaction between genotype and treatment. Fisher LSD post-hoc analysis was utilised. $p \leq 0.05$ was considered significant. Error bars indicate standard error.

4.3.3.2. Growth of 35S::EXPA11amiRNA plants exposed to salt stress later in development

In order to determine whether the 35S::EXPA11amiRNA lines displayed an altered phenotype compared with the empty vector and 35S::NIT2 genetic background when exposed to salt stress later on in development, the aforementioned lines, along with No-0, were grown hydroponically for three weeks on 1/4 strength PN media, and then either maintained on untreated media, or grown in 1/4 strength PN supplemented with 75 mM NaCl for one week further. The average fresh weight of the roots and shoots was measured, and a two-way ANOVA was performed to assess the effect of genotype, the effect of salt treatment, and the interaction between genotype and treatment, for both datasets, respectively. For both the average root and shoot fresh weights, it was found that there was a significant effect of both genotype ($p = 0.007$ and $p < 0.001$, respectively) and salt treatment ($p < 0.001$ and $p < 0.0001$, respectively) as well as a significant interaction between these two effects ($p < 0.001$, respectively). Additionally, the root and shoot fresh weights of each line were calculated relative to their respective untreated control, and the same two-way ANOVA was performed on these datasets. As observed for the average fresh weight data, for both the root and shoot relative fresh weights, it was found that there was a significant effect of both genotype ($p = 0.001$ and $p < 0.001$, respectively) and salt treatment ($p = 0.015$ and $p < 0.0001$, respectively) as well as a significant interaction between these two effects ($p = 0.001$ and $p < 0.001$, respectively).

Referring to Figure 4.16 A, it can be seen that the two 35S::EXPA11amiRNA lines displayed no alteration in root fresh weight compared with the empty vector or 35S::NIT2 genetic background under either untreated or salt-stress conditions. Additionally, the root fresh weight of all these lines was unaffected by the salt-treatment. In contrast, while No-0 had a significantly higher root fresh weight compared with all the other lines under untreated conditions, it displayed a significant reduction in root fresh weight in response to salt-treatment. The negative effect of salt treatment on No-0 is further highlighted when considering the root fresh weight relative to the untreated (Figure 4.16 B), where a significant reduction is observed in No-0 in 75 mM NaCl, with no change in any of the other lines. Importantly, these phenotypic differences in root growth between No-0 and the 35S::NIT2 line are consistent with those previously reported. An almost identical pattern was observed in the shoot fresh weight measurements (Figure 4.16 C), where no difference in shoot fresh weight was observed between the 35S::EXPA11amiRNA, empty vector, or 35S::NIT2 lines in untreated conditions, or in response to salt-treatment. Unlike for the root fresh weight, all these lines did display a

slight decrease in shoot fresh weight under salt stress. As seen for the root data, No-0 displayed a significantly higher shoot fresh weight than the other lines under untreated conditions, and a significant, large reduction in shoot fresh weight in response to salt treatment. Furthermore, the relative reduction in shoot fresh weight in saline conditions in No-0 was also found to be significantly larger than the other lines (Figure 4.16 D), as previously reported. Overall, these results confirm that the 35S::*EXPA11*amiRNA lines also do not display an altered growth phenotype in response to salt stress at later stages of development compared with the empty vector line or the 35S::*NIT2* genetic background. Furthermore, the pre-established phenotypical differences between the 35S::*NIT2* line and No-0 under untreated and salt stress conditions were observed as expected.

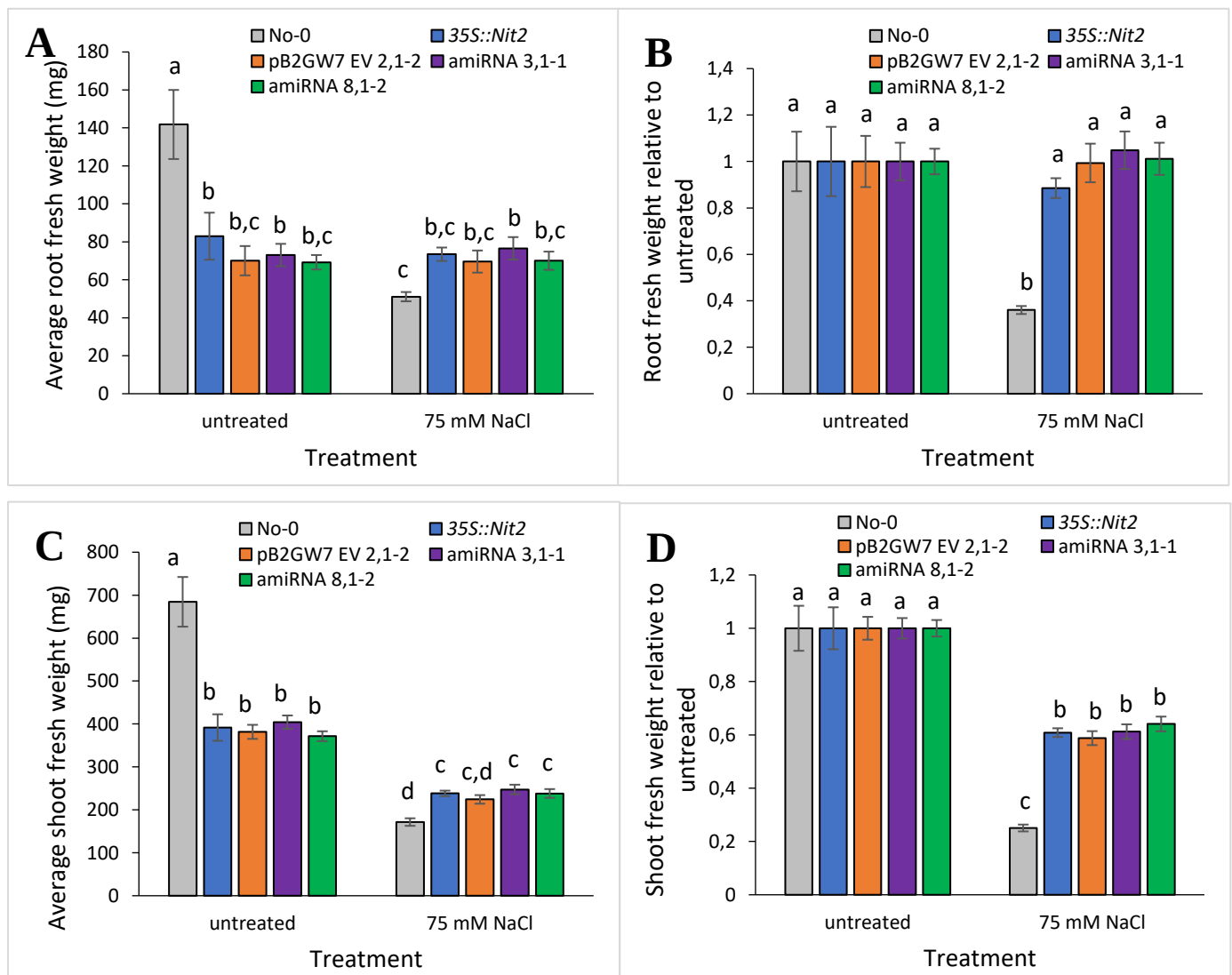


Figure 4.16: Root and shoot fresh weight of 35S::*EXPA11*amiRNA and empty vector T3 plants exposed to salt stress later in development

The 35S::*NIT2*, No-0, 35S::*EXPA11*amiRNA 3,1-1 and 8,1-2 T3, and pB2GW7 empty vector 2,1-2 T3 plants were grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 75 mM NaCl, or maintained on ¼ strength PN media (untreated control) for one additional week. Here, four plants from each line were germinated and grown in the same hydroponics box for each of the treatments to give one biological replicate. The average fresh weight of the root (**A**) and shoot (**C**) tissue of the four plants for both lines were calculated. Root (**B**) and shoot (**D**) fresh weight relative to their respective untreated controls was also calculated. The results are an average of three biological replicates. The letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a Two-way ANOVA. Error bars indicate standard error.

4.4. Discussion

4.4.1. *EXPA11* expression is strongly up-regulated in the 35S::*NIT2* line

The first aim of this chapter was to validate the findings of the previous transcriptomics experiment in which *EXPA11* expression was found to be massively increased in the two-week-old 35S::*NIT2* seedlings compared with No-0, both in control conditions, and when exposed to 100 mM NaCl (Cackett, 2019). I was indeed able to validate this result via RT-qPCR, where the 35S::*NIT2* line was found to have 100-fold and 200-fold increased expression relative to No-0 in control and salt stress conditions, respectively. The expression pattern in untreated conditions was almost identical to that seen in the transcriptomics experiment, however, the extent of up-regulation in response to salt treatment was not as pronounced in the qPCR data as was seen in the transcriptomics experiment (2-fold as opposed to 3.5-fold more than in untreated conditions). This is not unexpected though, as there is inherent variability between microarrays and RT-qPCR as techniques for measuring gene expression – different probes for the same gene on the microarray slide will give different intensity values, and different qPCR primer sets for the same gene can report different expression in the same cDNA. As such, one would not expect the reported fold-changes in expression between the two techniques to be identical. Nonetheless, it is clear that *EXPA11* is very highly expressed during early seedling development in the 35S::*NIT2* line. Interestingly, it would appear that *EXPA11* is likely more important for root growth later on in development in the 35S::*NIT2* line than it is in the shoot, with much higher expression detected in the roots than the shoots of the four-week-old plants. Notably, as was seen in seedlings, *EXPA11* expression was increased in response to salt-stress, with the same magnitude (10-fold) observed in the shoot and the root. *EXPA11* expression was

conspicuously absent in No-0 (apart from very low levels in salt-stressed roots). This would suggest that *EXPA11* is possibly not crucial for growth and development in older No-0 plants, but when it is highly expressed, it confers some form of growth advantage under saline conditions, as is seen in the 35S::*NIT2* line. Interestingly, the finding that *EXPA11* expression is much higher in the roots than the shoots of the older salt-stressed 35S::*NIT2* plants stands in agreement with the root and shoot fresh weight data, where root growth was maintained under saline conditions, whereas the shoot fresh weight decreased (Figure 4.16). This observation is clearly a correlation, and not necessarily the cause, but it could suggest that tissues with higher *EXPA11* expression maintain growth better under saline conditions, which is in line with our hypothesis that it plays a role in growth modulation in the 35S::*NIT2* line during salt stress.

4.4.2. The amiRNA approach failed to knock-down *EXPA11* expression

While homozygous 35S::*EXPA11*amiRNA transgenic lines were successfully created (see Table 4.4 and Figure 4.7), gene expression analysis revealed that there was no knock-down in expression in these lines compared with the 35S::*NIT2* genetic background, under both untreated (Figure 4.10) and salt-stress conditions (Figure 4.11). This was obviously a disappointing finding, as this amiRNA approach has been widely used to knock-down expression of genes in *Arabidopsis*, and can be expected to result in around a 75% reduction in target gene expression (Ossowski, Schwab & Weigel, 2008). For example, using the same amiRNA approach, Spartz et al. (2014) were able to knock down the expression of multiple *PP2C.D* family members with a single amiRNA construct, with the knock-down in expression varying from ~60-90%. Just as in our study, they utilised the online WMD3 tool to design their amiRNA, which was generated using the mi319a precursor, and expressed under the control of the constitutive 35S promoter of the pMDC44 plant expression vector (Spartz et al., 2014). The possibility that I did not observe a reduction in *EXPA11* expression due to the *EXPA11*amiRNA construct not being expressed was eliminated, as PCR analysis using specially designed primers was able to detect the construct in only the 35S::*EXPA11*amiRNA T3 line, and not in the empty vector T3 or 35S::*NIT2* (Figure 4.12). Importantly, this analysis utilised cDNA synthesised with random hexamer primers as template, as the *EXPA11*amiRNA construct would not be expressed with a polyA tail, making the normal oligo dT primers used for cDNA synthesis unsuitable. Additionally, I was able to show that there was no contaminating gDNA present in the random hexamer cDNA of the 35S::*EXPA11*amiRNA T3 line (Figure 4.13), which might have confounded the evidence for the detection of the *EXPA11*amiRNA construct. There is evidence to suggest that amiRNAs can act in a translational-inhibitory manner, as

opposed to post-transcriptionally, as phenotypic effects have been observed without reductions in target RNA expression (Ossowski, Schwab & Weigel, 2008). However, given that our 35S::*EXPA11*amiRNA T3 lines do not display an altered phenotype compared with the empty vector 35S::*NIT2*, under untreated conditions or when exposed to salt stress (Figure 4.15, and 4.16), it seems unlikely that this is the case. In order to eliminate the possibility of a translational-inhibitory role for the *EXPA11*amiRNA, I would need to measure the levels of *EXPA11* protein expression in the 35S::*EXPA11*amiRNA T3, empty vector T3 and 35S::*NIT2* lines. Unfortunately, there is not currently an available antibody against *EXPA11*, making this unfeasible, but it does represent a possible future avenue of research.

In hindsight, there are some changes that could have been made to our approach in order to increase the likelihood of generating a successful *EXPA11* amiRNA knock-down line; Firstly, while I chose the first set of sequences generated that satisfied all the required design criteria, it could perhaps have been prudent to design multiple different amiRNA and amiRNA* sequences using the WMD3 tool. The use of additional amiRNAs designed to target various points of the *EXPA11* transcript would likely increase the chances of this approach working. However, upon review of recent papers that have utilised the WMD3 tool to design amiRNAs targeting various Arabidopsis genes, it would not appear that the respective authors chose to design more than one amiRNA sequence (Andrés et al., 2020; López-Márquez et al., 2021; Ma et al., 2021). Furthermore, utilising multiple amiRNAs would involve exponentially more work, both in the cloning process, and especially in the subsequent screening of transgenic lines. Given that the WMD3 tool and this amiRNA system have been utilised successfully in the knock-down of many genes, there is little reason to doubt the validity of the amiRNA and amiRNA* sequences that I chose from the list of generated sequences. A second consideration would be to screen more T1, T2, and T3 lines. Having a larger pool of transgenic homozygous plants could well increase the chances of finding a line in which *EXPA11* expression was knocked-down. Unfortunately, of the 25 35S::*NIT2* plants that were floral-dipped with *Agrobacterium* transformed with pB2GW7-*EXPA11*amiRNA, only seven T1 lines were identified in the subsequent GFSA-screening. As such, I would need to repeat the floral dip transformation in order to increase the number of available T1 (and thus T2, and T3) lines that can be screened. However, given the possibility that the amiRNA designed does not work, this strategy might not be worthwhile considering the amount of work involved in screening additional lines.

It is also possible that issues arose from the use of the constitutive CaMV 35S promoter to drive the expression of the *EXPA11*amiRNA construct in a genetic background in which *NIT2* expression is also constitutively driven by the CaMV 35S promoter. There is evidence to suggest that the expression of multiple transgenes via 35S promoters can result in gene silencing via *Trans*-inactivation (Daxinger et al., 2007). Gene-silencing of a variety of diverse transgenic constructs, using the 35S promoter, was observed in SALK- and GABI-generated T-DNA tagging mutant Arabidopsis lines, in which the T-DNA insertion contains the 35S promoter, but this silencing was not observed in SAIL- lines, where the T-DNA insertion lacks the 35S promoter (Daxinger et al., 2007). The authors asserted that gene silencing was mediated by the 35S promoter homology between the transgene construct and the T-DNA. I cannot preclude the possibility that similar silencing of the 35S::*EXPA11*amiRNA construct may have occurred in our lines due to the presence of the 35S promoter driving *NIT2* expression. It is thus possible that the use of an alternative constitutive promoter to drive *EXPA11*amiRNA expression would yield a different result. A number of alternative constitutive viral promoters capable of driving transgene expression ubiquitously throughout Arabidopsis plants, to a similar level as the CaMV p35S, have been identified, including the *Soybean chlorotic mottle virus* NCR promoter, the *Figwort mosaic virus* 34S promoter, and very recently, the *Paper mulberry vein banding virus* promoter (PmVP), which has been shown to have 1.5-fold increased activity compared to the CaMV p35S in transgenic *N. benthamiana* (Sanger, Daubert & Goodman, 1990; Shirasawa-Seo et al., 2002; Smith et al., 2022). Additionally, several native Arabidopsis promoters capable of driving ubiquitous expression have been identified as suitable alternatives to CaMV p35S; the β -carotene hydroxylase 1 promoter (pAtBch1), has been shown to drive two-fold higher GUS expression than the CaMV p35S throughout the plant, and the *Arabidopsis translationally controlled tumour protein* promoter (pAtTCTP) has been found to drive ~55% of the GUS activity of the CaMV p35S (Liang et al., 2009; Han et al., 2015). The use of an alternative viral or native constitutive promoter to drive expression of the *EXPA11*amiRNA construct is an avenue that could be explored in the future.

4.4.3. 35S::*EXPA11*amiRNA lines do not display an altered salt-stress phenotype

The 35S::*EXPA11*amiRNA lines generated do not appear to have any meaningful difference in phenotype compared with the empty vector or 35S::*NIT2* genetic background when exposed to salt stress during early development, or when exposed later in development. While the average seedling fresh weight of the 35S::*EXPA11*amiRNA and empty vector lines was significantly lower than the 35S::*NIT2* and No-0 lines, under both control and salt-stress conditions, the

relative fresh weight data shows that these transgenic lines are no different from the 35S::*NIT2* line, and also display the improved growth phenotype compared with No-0 in saline conditions (Figure 4.15). The results of the later development hydroponics salt-stress assays (Figure 4.16) conclusively show that there is no difference in the root or shoot fresh weight of the 35S::*EXPA11*amiRNA and empty vector lines compared with the 35S::*NIT2* under either untreated or salt-stress conditions. It is interesting that the reduction in size seen in the seedlings of the transgenic lines compared with No-0 and the 35S::*NIT2* line, was not observed in the older hydroponically grown plants. The reason for this discrepancy is unclear, but based off observation, these hydroponics assays are a very robust method of determining any sort of salt-related growth phenotypes, and likely represent a more physiologically relevant growth system than the media plates used to look at seedling development. Notably, the phenotypic differences between the 35S::*NIT2* and No-0 lines in response to salt stress at a later developmental stage were observed as reported previously (Cackett, 2019). Collectively, these data strongly suggest that the 35S::*EXPA11*amiRNA lines generated do not have an altered salt stress phenotype compared with the 35S::*NIT2* genetic background. The finding that *EXPA11* expression was increased in response to salt in the 35S::*NIT2* line in both seedlings and older plants (Figure 4.1 and 4.2, respectively), provided limited preliminary evidence to support the hypothesis that *EXPA11* could be an effector that contributes towards the increased salt tolerance of this line. As such, it was also hypothesised that reducing *EXPA11* expression might decrease salt tolerance. Given that the 35S::*EXPA11*amiRNA lines generated did not display reduced *EXPA11* expression compared with the 35S::*NIT2* genetic background, it was indeed unsurprising that they were not different from the 35S::*NIT2* genetic background in terms of their salt tolerance.

4.4.4. Alternate approaches for generating an *EXPA11* knock-down line

As mentioned previously, the first consideration I took was to look for an *expa11* mutant line in the literature. I identified a potential candidate: the Cold Spring Harbor Laboratory Genetrap line, CSHL_GT21784, which is purported to have a T-DNA insertion in the 5' UTR of *EXPA11*. However, I was unable to verify the presence of the T-DNA insertion in the genomic DNA of the individuals that I tested (data not shown), and thus abandoned further use of this line. In any case, I wanted to knock down the expression of *EXPA11* in the 35S::*NIT2* line specifically, as I aimed to assess the potential role that *EXPA11* might play downstream of auxin activation in response to salt stress in the 35S::*NIT2* line, and thus if *EXPA11* potentially contributes to the increased salinity tolerance that the 35S::*NIT2* displays. Additionally, as

previously mentioned, this purported T-DNA insertion line was generated in the Landsberg erecta (Ler-0) genetic background, and not in No-0, as is the case for the 35S::*NIT2* line. Consequently, I elected to adopt the targeted amiRNA approach. While this amiRNA approach has been shown to be a robust method of knocking-down gene expression in Arabidopsis, there are alternate RNAi strategies that could be employed. In particular, the CRISPR/Cas9 single guide RNA system has emerged as a highly successful method for targeted gene knockout in Arabidopsis and other plants (Feng et al., 2013; Jiang et al., 2013). Furthermore, CRISPR/Cas9-mediated gene transformation has been shown to be heritable in Arabidopsis, and simple PCR-based assays have been developed to identify transformants without the need for selection markers at the target locus (Feng et al., 2014; Miki et al., 2018). Very recently, the CRISPR/Cas9 system has been successfully used to generate homozygous *atexpa4* and *atexpb5* Arabidopsis knockout mutants (Liu et al., 2021). Aiming to assess the contribution of these expansins to pollen tube growth, the authors generated *atexpa4* and *atexpb5* knockout mutant lines using CRISPR/Cas9, and then crossed them to yield *atexpa4atexpb5* double mutants. Subsequent comparison of the single knockouts, double knockout, and the WT revealed that EXPA4 and EXPB5 were redundantly required for pollen tube growth. This study elegantly showed that CRISPR/Cas9 technology represents a highly promising, future approach to knockout the expression of *EXPA11*, and potentially other genes of interest.

4.4.5. Summary

In this chapter, I successfully validated previous transcriptomics findings from our group showing that *EXPA11* is massively up-regulated 35S::*NIT2* seedlings compared with their genetic background, No-0, both under control and saline conditions. Furthermore, I provide the first characterisation of *EXPA11* expression in the root and shoot of 35S::*NIT2* and No-0 plants when exposed to salt stress at a later developmental stage; *EXPA11* expression was found to be conspicuously absent in both the root and shoot of No-0 plants, where it was only detected at very low levels in salt-stressed roots. By contrast, *EXPA11* expression was detected in both the root and the shoot of the 35S::*NIT2* plants, where it was up-regulated in response to salt-treatment in both tissues. Overall *EXPA11* expression was much higher in the roots, suggesting that its contribution towards growth during salt stress in the 35S::*NIT2* line is perhaps more significant there than in the shoot. Unfortunately, the amiRNA approach adopted proved to be unsuccessful in knocking down *EXPA11* expression in the 35S::*NIT2* line; while stable transgenic lines expressing the *EXPA11*amiRNA construct in the 35S::*NIT2* genetic background were created, no knock-down in expression was detected, and subsequent analyses

of these transgenic lines showed that they had no meaningful phenotypic differences when compared with the 35S::*NIT2* line under either control or saline conditions. As such, no further analyses were performed, and I was unable to complete the aim of assessing the effect of knocking down *EXPA11* expression on the growth and/or ion homeostasis of the 35S::*NIT2* line. The use of CRISPR/Cas9 technology is suggested as a promising new tool that could be utilised to achieve this aim.

CHAPTER 5: GENERATION AND PHENOTYPIC CHARACTERISATION OF AN *EXPANSIN 11* OVEREXPRESSING LINE

Note: The leaf cell size analysis herein, including epoxy resin embedding, light microscopy, and subsequent data analysis, was performed by Lara Reale, Martina Cerri, and Ele Cinti at the Department of Agricultural, Food and Environment Sciences at the University of Perugia. I provided them with fixed whole plants. The ICP-OES analysis herein was conducted by the analytics laboratory in the Chemical Engineering Department at the University of Cape Town. I provided them with air-dried plant samples.

5.1. Introduction:

5.1.1. Previous characterisation of Arabidopsis Expansin 11

To date, EXPA11, and its role during Arabidopsis growth and development, remains largely uncharacterised. There are 26 α -expansins identified in Arabidopsis, all of which are thought to modify the mechanical extensibility of cell walls, and thus play a role in a wide number of plant growth processes (Lee, Choi and Kende, 2001, Li et al., 2002). Sequence-based phylogenetic analyses have revealed the existence of ten Arabidopsis EXPA clades, with EXPA11 identified as the only member of clade V (Sampedro et al., 2005). In the literature, EXPA11 has mostly been identified as a gene of interest in large-scale gene expression experiments designed to identify DEGs during ethylene (De Paepe et al., 2004) and abscisic acid (Ko, Yang & Han, 2006) signalling responses, as well as in response to pathogens (Brodersen et al., 2006; Kempema et al., 2007). Additionally, EXPA11 was identified in a proteomics experiment seeking to understand cell wall protein dynamics during cell elongation (Irshad et al., 2008). However, in all these cases, EXPA11 was identified as part of a suite of other cell wall-modifying genes or proteins, and its specific role in these responses was not investigated further.

Of note, there have been several studies implicating EXPA11 in hypocotyl development. In an investigation of the role of the Sucrose non-fermenting 1 related protein kinase (SnRK1) energy-signalling hub in sucrose-induced hypocotyl elongation, *EXPA11* transcript abundance was found to increase in constant darkness conditions, which favour hypocotyl elongation, and decrease in response to treatment with 1-N-naphthylphthalamic acid (NPA), which inhibits polar auxin transport and subsequent hypocotyl elongation (Simon et al., 2017). Additionally, hypocotyl elongation was found to be induced in response to sucrose, concomitant with an

increase in *EXPA11* expression. Collectively, this suggests a role for *EXPA11* in both auxin- and sucrose-induced hypocotyl elongation. Further evidence for a role in hypocotyl elongation comes from a study of a *mur3-3* mutant, which has galactose-deficient xyloglucan in its cell walls resulting in severely shortened dark-grown hypocotyls (Xu et al., 2017). In this study, *EXPA11* was identified as one of several cell wall-related genes with decreased expression in the *mur3-3* mutant compared to wild-type plants. Furthermore, complementation tests revealed that over-expression of *EXPA11* in the *mur3-3* mutant partially restored the shortened hypocotyl phenotype. Based off these studies, *EXPA11* is proposed to have a positive role in promoting hypocotyl elongation.

Aside from a purported role in hypocotyl cell elongation during early development, very recent evidence has also linked reduced *EXPA11* expression with the inhibition of cell elongation in the leaves of adult plants following inflorescence development (Hou et al., 2022). In this study, characterising the role of the bHLH transcription factor, *HLH4*, in the regulation of cell elongation, *EXPA11* (and *EXPA8*) expression was found to be significantly reduced in the leaves of *HLH4*-overexpressing plants which displayed a wide range of dwarf phenotypes, including a reduction in leaf size, and leaf mesophyll cell size. Furthermore, using a transient LUC reporter system, the authors showed that *HLH4* inhibits the transcriptional activity of the *EXPA11* (and *EXPA8*) promoter(s). Expression of the auxin-signalling transcription factors *indole-3-acetic acid 7* and *17* (also known as *AUXIN RESISTANT 2* and *3*, respectively) was also significantly decreased in the *HLH4*-overexpressing plants, and the same inhibitory effect of *HLH4* was observed on their respective promoters. This study suggests that *EXPA11* promotes leaf expansion, where it acts downstream of, or in parallel with, a signalling cascade involving auxin.

While the studies mentioned above provide some insight into the role of *EXPA11* in cell wall-modification, the promotion of cell elongation, and its role in auxin-induced growth responses, much more work is required to understand the extent of its involvement in plant responses to the environment. In particular, despite the growing body of evidence that expansins are involved in the plant response to salinity stress, *EXPA11* remains unstudied in this context. The aim of this chapter was to address this gap in the literature and provide insight into its potential role in growth-modulation during salinity stress.

5.1.2. Chapter aims

The overall aim was to generate and functionally characterise plant lines stably overexpressing *EXPA11*. Specifically, the functional characterisation involved several aims; 1) To determine if overexpression of *EXPA11* affects the growth and survival of plants exposed to salt stress at an early and later developmental stage. 2) To assess the effect of *EXPA11* overexpression on the leaf growth and leaf cell size of untreated and salt-treated plants. 3) To assess if the *EXPA11* overexpressing line displays alterations in a known auxin-related growth phenotype, hypocotyl elongation, during salt stress. 4) To determine if Na⁺ and K⁺ root/shoot homeostasis is altered in the *EXPA11* overexpressing line.

5.2. Materials and methods:

5.2.1. Stock solutions, chemicals, enzymes, and vectors used

The 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) was obtained from Promega (Madison, Wisconsin, USA). The Zyppy Plasmid Miniprep Kit (catalogue no. D4036) was obtained from Zymo Research (Irvine, California, USA). The KAPA Taq PCR kit (catalogue no. KK1015), KAPA HiFi HotStart ReadyMix PCR kit (catalogue no. KK2501), and the Super-Therm DNA Polymerase PCR kit (catalogue no. JMR-801) were purchased from Roche Molecular Systems Inc. (California, USA). The Gateway™ pDONR™221 vector (catalogue no. 12536017), Gateway™ BP Clonase™ II Enzyme mix (catalogue no. 11789020), and Gateway™ LR Clonase™ II Enzyme mix (catalogue no. 11791020) were obtained from Invitrogen by Thermo Fisher Scientific (Massachusetts, USA). The Gateway™ pB2GW7 vector was obtained from the VIB-UGent Center for Plant Systems Biology (University of Gent, Belgium).

5.2.2. Primer design

The coding DNA sequence (CDS) sequence of *EXPA11* (AT1G20190) was obtained from The Arabidopsis Information Resource, TAIR (<https://www.arabidopsis.org/>). Primers were designed using NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and checked for self-complementarity using DNAMAN v 4.13 (Lynnon Biosoft, San Ramon, USA). Forward and reverse primers were designed to amplify the entire CDS of *EXPA11*. These forward and reverse primers included the *attB1* and *attB2* sequences, respectively, on their 5' flank, to allow for the downstream cloning into the Gateway® donor vector pDONR221.

5.2.3. DNA manipulations

All DNA visualisation by gel electrophoresis, DNA purification from agarose gels, DNA sequencing and analysis, plasmid DNA purification, and DNA extraction from Arabidopsis plants was performed exactly as outlined in the respective sub-sections of chapter 4, section 4.2.3.

5.2.4. DNA and cDNA amplification by PCR

Multiple different PCR enzymes and kits were used depending on the purpose of the PCR as well as the particular primer pair used. All PCR experiments were performed using the SimpliAmp™ Thermal Cycler (Applied Biosystems™, Foster City, USA). In general, the same PCR strategy and enzymes that are outlined in chapter 4, section 4.2.4. were used here; The KAPA HiFi HotStart ReadyMix PCR kit was used for the high-fidelity amplification of the *attB*-flanked *EXPA11* CDS that was cloned into the pDONR221 donor vector. All subsequent PCRs not requiring high-fidelity amplification, such as those performed to detect the *EXPA11* CDS in the pDONR221 donor vector and pB2GW7 expression vector, as well as PCRs performed on extracted plant DNA to confirm transgenic offspring, utilised the KAPA Taq PCR kit or Super-Therm DNA Polymerase PCR kit. The primers used in the various PCRs can be found in Table 5.1. The enzyme kit and annealing temperature (T_a) used in these reactions can be found in Table 5.2.

5.2.5. Gateway® Cloning Technology

Gateway® cloning technology was used to generate a vector constitutively expressing the *EXPA11* CDS (pB2GW7-*EXPA11*). The donor vector, pDONR221, and expression vector, pB2GW7, were used as before. Refer to chapter 4, section 4.2.6.1. for information on these vectors. The BP and LR recombination reactions were performed as outlined in chapter 4, sections 4.2.6.2. and 4.2.6.3., respectively.

5.2.6. Bacteriological work in *E. coli*

All bacteriological work in *E. coli* was performed as outlined in chapter 4, section 4.2.7. *E. coli* DH5 α was transformed with pENTR221-*EXPA11*, and pB2GW7-*EXPA11*, respectively, at the different stages of the Gateway® cloning process.

5.2.7. Bacteriological work in *A. tumefaciens*

All bacteriological work in *A. tumefaciens*, as well as the subsequent floral dip transformation of *A. thaliana* was performed as outlined in chapter 4, section 4.2.8. *A. tumefaciens* was transformed with pB2GW7-*EXPA11* for use in the floral dip transformation.

5.2.8. Arabidopsis seed stocks

Seeds for the wild type, No-0, were used for the floral dip transformation of *A. tumefaciens* transformed with pB2GW7-*EXPA11*. These seeds were initially obtained from Bonnie Bartel (Rice University, Houston, Texas) (Normanly et al., 1997). Seeds for a homozygous transgenic pB2GW7 empty vector No-0 line were obtained from Nina Esterhuysen (University of Cape Town, South Africa).

5.2.9. Arabidopsis growth conditions

Arabidopsis plant growth was carried out under standard conditions in a plant growth room (100 μM photons.m⁻².s⁻¹, 16-h light/8-h dark, 22°C, 50 – 60% relative humidity).

5.2.10. Arabidopsis growth in soil

Plant growth in soil was performed as outlined in chapter 4, section 4.2.11.

5.2.11. Selection of transgenic offspring by GFSA selection

Selection of transgenic lines was performed as outlined in chapter 4, section 4.2.12.

5.2.12. Confirmation of the presence of pB2GW7-*EXPA11* in gDNA of transgenic offspring

For every transgenic generation (T1, T2 or T3), gDNA was extracted from ten seedlings for each line using the Edwards DNA extraction protocol (see chapter 4, section 4.2.3.5.). For each sample, PCR was used to detect the presence of both the Bar gene located within the T-DNA insert, as well as the p35S-*EXPA11* sequence. Refer to Table 5.1/5.2 for the primer sequences and enzymes used for the different PCR reactions.

5.2.13. RNA extraction, cDNA synthesis and gene expression analysis via RT-qPCR

RNA was extracted, and cDNA synthesised using Oligo(dT) primers as outlined in chapter 2, section 2.2.8. Gene expression was analysed via RT-qPCR as described in chapter 2, section 2.2.9.

5.2.14. *EXPA11* expression analysis in 35S::*EXPA11* T3 lines

For each T3 line that had been confirmed as homozygous by GFSA selection, seedlings were transferred from the plate containing GFSA-supplemented PN media onto soil. For the genetic background, No-0, seedlings were transferred from PN media plates without GFSA, that were grown at the same time as the 35S::*EXPA11* T3 lines. Tissue was harvested from four-week-old plants. Four leaves were harvested per plant and pooled. This was repeated for three plants per line, giving three biological replicates. The RNA was extracted and cDNA synthesised, and the expression of *EXPA11* was determined via RT-qPCR analysis. *EXPA11* expression was normalised to the reference gene *MON1*, and the normalised data was averaged and plotted in Excel along with the associated standard error. The qPCR primers used can be found in Table 5.1.

5.2.15. Salt stress tolerance phenotyping assays

5.2.15.1. Early development salt stress assay

Growth of the 35S::*EXPA11* lines was compared to that of the No-0 pB2GW7 empty vector (EV) line. Seeds were sterilised as outlined in chapter 2, section 2.2.4. and sown onto solid PN-media (see chapter 2, section 2.2.5.) with 7% (w/v) phyto agar in petri dishes. The media was either supplemented with 0, 50, 75, 100, or 125 mM NaCl, or the iso-osmolar concentration of sorbitol (100, 150, 200, 250 mM). For each line, 50 seedlings were grown per plate and four plates were set up for each treatment, giving four replicates. After eleven days, the number of surviving seedlings (as determined by the development of true leaves) as well as the total fresh weight of each plate was measured. The data was captured in Excel, and the average survival as well as average fresh weight per plant was calculated and plotted along with the associated standard error. The experiment was repeated several times to give biological repeats.

5.2.15.2. Late development salt stress assay

Growth of the 35S::*EXPA11* lines was compared to that of the No-0 pB2GW7 empty vector (EV) line. Seeds were sterilised and grown hydroponically (see chapter 2, section 2.2.6.) in ¼ strength PN media for three weeks, followed by one more week in ¼ strength PN media supplemented with 0, 50, 75, or 100 mM NaCl. For each treatment, approximately eight plants of both genotypes were grown in a single Araponics tank (Araponics, Liège, Belgium), giving eight technical repeats. The root and shoot fresh weight of each plant was measured. The data was captured in Excel, and the average root and shoot fresh weight per plant, as well as the

average root:shoot ratio, was calculated, and plotted along with the associated standard error. The experiment was repeated several times to give biological repeats.

5.2.16. Leaf morphology analysis

The 35S::*EXPA11* and pB2GW7 EV Arabidopsis plants were grown hydroponically for three weeks in ¼ strength PN media, followed either by treatment with ¼ strength PN media supplemented with 75 mM NaCl for three days, or maintenance in ¼ strength PN media for three days (control).

5.2.16.1. Leaf area measurements

For leaf surface area measurements, the first four emerged rosette leaves, from four individual plants, were removed at the base of the leaf and flattened onto white paper with clear tape. The leaves were photographed, and ImageJ software (publicly available from <https://imagej.nih.gov/ij/>) was used to measure the entire surface area of the leaf (both the lamina and petiole). The data was captured in Excel, where average leaf surface was calculated and plotted along with the associated standard error.

5.2.16.2. Cell size analysis via light microscopy

For the cell size analysis, whole plants were placed in 50 mL falcon tubes and fixed in 0.075M sodium cacodylate buffer pH 7.2 (SCB) with 5% (w/v) glutaraldehyde at 4 °C overnight. The fixed plants were then sent to Lara Reale and Martina Cerri at the Department of Agricultural, Food and Environmental Sciences at the University of Perugia for epoxy resin embedding and subsequent microscopy analysis. Here, the fixative was removed, and the fixed plants were washed five times for 6 min in SCB. The rosette leaves were removed, and the rest of the plant was discarded. The leaves were then post-fixed in a solution of 1% (w/v) osmium tetroxide in SCB for 1.5 h at 4 °C. The washes in SCB were repeated as before. Following this, the leaves were dehydrated in increasing concentrations of ethanol as follows: two times 10 min washes in 25%, 50%, 75%, 85%, 95% (v/v) ethanol, respectively, followed by a final seven washes in 100% ethanol for 7 min each. Once complete, the dehydrated leaves were impregnated with epoxy resin (Epon 812, 2-dodecenylsuccinic anhydride and methyl nadic anhydride mixture) as follows: the leaves were washed three times for 7 min in a solution of 100% ethanol and propylene oxide in a 1:1 ratio, followed by a 7 min incubation in propylene oxide. The leaves were then incubated overnight at room temperature in a solution of propylene oxide and resin in a ratio of 3:1, 1:1, and finally 1:3, over three consecutive days, respectively. Finally, the impregnated leaves were embedded in resin and heated at increasing temperatures of 30, 45,

and 60 °C for 12 h, respectively. Tangential semithin sections (1-2 µm) of the leaves were obtained with an ultramicrotome (OmU2; Reichert, Heidelberg, Germany) equipped with a glass blade. The semithin sections were stained with 0.1% (w/v) toluidine blue and observed under a light microscope (BX53; Olympus, Tokyo, Japan). CellSens software (Olympus, Tokyo, Japan) was used to measure the area of the epidermal and palisade mesophyll cells in the respective semithin leaf sections. At least 50 cells were measured across the first three emerged rosette leaves from each plant, and three plants were analysed for each treatment from both lines. In addition, cell size measurements were also performed on fixed rosette leaves, prior to the epoxy resin embedding process. Here, the same light microscope and imaging software was used to measure epidermal cell area from the first three emerged rosette leaves from each plant, and again three plants were analysed for each treatment from both lines. The epidermal cell size measurements from the two analyses were combined.

5.2.17. Hypocotyl elongation assay to assess auxin-related growth phenotyping

Growth of the 35S::*EXPA11* lines was compared to that of the No-0 pB2GW7 empty vector (EV) line. Seeds were sterilised and sown onto 20 mL of PN-phyto agar (7% w/v) in 100 mL magenta jars. The media was supplemented with either 0 or 75 mM NaCl. Then 30 seeds were sown per jar, in triplicate, giving three biological repeats. The jars were wrapped in aluminium foil to ensure complete darkness, then placed under standard growth room conditions for five days. The etiolated seedlings were removed from the jars and photographed. ImageJ software (publicly available from <https://imagej.nih.gov/ij/>) was used to measure the hypocotyl length and the data was captured in Excel, where average hypocotyl length was calculated and plotted along with the associated standard error.

5.2.18. Measurement of ion content in salt-stressed EV and 35S::*EXPA11* plants via ICP-OES

5.2.18.1. Experimental design

The 35S::*EXPA11* and pB2GW7 EV Arabidopsis plants were grown hydroponically for three weeks in ¼ strength PN media, followed either by treatment with ¼ strength PN media supplemented with 75 mM NaCl for one week, or maintenance in ¼ strength PN media for one week (control). Root and shoot tissue from four plants per treatment or control was harvested and pooled from both genotypes. The entire experiment was repeated six times to give six biological replicates (i.e., 48 samples in total).

5.2.18.2. Sample preparation

Intact plants were removed from the Araponics system and the roots were immediately rinsed three times with dH₂O to remove surface NaCl. Root and shoot tissue from untreated (control) and 75 mM NaCl treated plants was harvested separately. Tissue from four plants was pooled to constitute one sample. All samples were collected into envelopes and placed in an oven at 60°C until completely dried. The samples were then submitted to the Analytical Laboratory, Department of Chemical Engineering (University of Cape Town, South Africa). Here, the samples were ashed, weighed into Teflon containers, and acid digested as follows: 10 mL of 55% H₂O HNO₃ was added to the samples and then heated under pressure at 200°C for 30 minutes using a Mars 6 Microwave digester (CEM Corporation, Charlotte, NC, USA). All digested samples were then filtered using a 0.2 µm filter. Subsequently, 1mL of each sample was diluted to 100 mL using 2% H₂O HNO₃.

5.2.18.3. ICP-OES analysis

Sodium, Potassium, Magnesium, and Calcium ion content was measured in the root and shoot tissue of the untreated and 75 mM NaCl treated plants using a Varian ES 730 Inductive Coupled Plasma – Optical Emission (ICP-OES) Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The ICP-OES spectrophotometer was calibrated using multi-element standards containing the ions of interest.

5.2.18.4. ICP-OES data processing

Average ion contents in µg.g⁻¹ dry weight (DW) were determined from the six repeats for each treatment for both genotypes, as well as the standard error surrounding these averages. Graphs comparing the contents of the various ions between the two genotypes in the root and shoot tissue were generated in Excel for both untreated and 75 mM NaCl treated plants. Graphs of the potassium ion/sodium ion content were also generated.

5.2.19. Statistical analysis

All statistical analyses were performed using Statistica Software version 13.5 (Statsoft, Tulsa, USA). Where one-way analysis of variance (ANOVA) analyses were performed on the data, significance was determined using Duncan's multiple range test post-hoc analysis. Where two-sample t-tests were conducted to determine statistically significant differences between sample groups, Bonferroni correction for multiple testing was performed. For all analyses performed, p -values $\leq 0,05$ were considered significant.

Table 5.1: Primers used in the generation of the *EXPA11* over-expressor vector (pB2GW7-*EXPA11*)

Primer name	Primer sequence (5' → 3')
<i>EXPA11</i> - attB1 F	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATGTCAAAGTCTCTAGCTGGATTG
<i>EXPA11</i> - attB2 R	GGGGACCACTTTGTACAAGAAAGCTGGGTTT AAAACTGAACGTTGGAAGAATA
p35S F	AATATCGGGAAACCTCCTCG
Bar F	AAGTCCAGCTGCCAGAAACC
Bar R	GAACTGACAGAACCGCAACG
q <i>EXPA11</i> F	TTGCGGTTGATGCGTTTAG
q <i>EXPA11</i> R	GCCGAGTAAAGATCTCCGTAAC
qMON1 F	CAGACAAGGCGATGGCGATA
qMON1 R	GCTTTCTCTCAAGGGTTTCTGGGT

Note: The bold sequence in the *EXPA11*- attB1 F/*EXPA11*- attB2 R primers is the attB1/attB2 sequence respectively

Table 5.2: Function of the different primer combinations

Primer combination	Product size (bp)	Function	PCR kit used and T _a
<i>EXPA11</i> - attB1 F <i>EXPA11</i> - attB2 R	817	Amplification of full length <i>EXPA11</i> CDS Detection <i>EXPA11</i> CDS in pDONR221 donor vector	KAPA Hifi – 65°C Super-therm – 54°C
p35S F <i>EXPA11</i> - attB2 R	1240	Detection of <i>EXPA11</i> CDS in pB2GW7 expression vector Detection of transgenic Arabidopsis lines	KAPA Taq – 53°C
Bar F Bar R	733	Detection of Bar gene in transgenic Arabidopsis lines	KAPA Taq – 57°C
q <i>EXPA11</i> F q <i>EXPA11</i> R	118	RT-qPCR	SYBR® FAST - 60°C

5.3. Results

5.3.1. Generation of an *Expansin 11* overexpressing line

5.3.1.1 Generation of the *EXPA11*-overexpression vector

To generate an Arabidopsis line overexpressing *EXPA11*, a vector containing the constitutive *CaMV* 35S promoter was used to drive expression of the *EXPA11* coding DNA sequence (CDS). The full-length *EXPA11* CDS was amplified from a 100 mM NaCl-treated 35S::*NIT2* cDNA sample in which *EXPA11* was known to be highly expressed (see chapter 4, Figure 4.1). Gateway® cloning technology was used to clone the *EXPA11* CDS into pDONR221, and subsequently into the pB2GW7 plant expression vector, where its expression is driven by the *CaMV* 35S promoter. Refer to appendix chapter 7, Figure 7.3 for a map of the pB2GW7-*EXPA11* vector generated. Sequence verification of the *EXPA11* CDS was conducted at each step throughout the cloning process.

5.3.1.2. Transformation of pB2GW7-*EXPA11* into *E. coli* and *A. tumefaciens*

Following the Gateway® cloning reactions, the pB2GW7-*EXPA11* vector was transformed into *E. coli* DH5 α , and positive transformants were selected on spectinomycin-containing LB agar plates. A colony PCR performed on 10 discrete colonies confirmed the successful transformation into *E. coli* (data not shown), and subsequently a PCR performed on the plasmid DNA preparations from two of these positive transformants once more confirmed the presence of the *EXPA11* CDS (Appendix chapter 7, Figure 7.4 A). Plasmid DNA isolated from a single positive *E. coli* transformant was used to transform *A. tumefaciens*, and positive transformants were selected on LB agar plates containing spectinomycin, rifampicin, and gentamicin. As seen in appendix chapter 7, Figure 7.4 B, a colony PCR performed confirmed the successful transformation of three distinct *A. tumefaciens* colonies with the pB2GW7-*EXPA11* vector. The negative results could have occurred due to several reasons: they could indicate that either no DNA was released from the *A. tumefaciens* cells during the colony PCR process, or perhaps no cells were transferred into the requisite PCR tube for that colony. Additionally, it is possible that the colony was transformed with empty vector, which would still allow growth on selective media, but give a negative result in the PCR. A single positive transformant was then used to inoculate the liquid LB cultures that were used for the floral dip transformation of Arabidopsis No-0 plants.

5.3.1.3. *Homozygosity of the EXPA11 overexpressing lines*

Initially the first, second, and third transgenic generations of the 35S::*EXPA11* lines were screened for survival on GFSA-supplemented PN plates. The aim was to select for homozygous lines with a single insertion of the vector DNA. For the first transgenic generation (T1), 100 seedlings from each of the No-0 plants that underwent *A.tumefaciens*-mediated floral dip transformation with the pB2GW7-*EXPA11* vector were screened, and lines with one or two surviving seedlings were taken forward (data not shown). In the second transgenic generation (T2) segregating transgenic lines mostly likely to contain one insertion of the vector DNA were selected based on their survival on GFSA. A single T-DNA insert would segregate 1 homozygous: 2 heterozygous: 1 wild-type, giving an expected survival rate of 75% (3:1). Appendix chapter 7, Table 7.5 shows the results of the GFSA-screening of 50 seedlings of each of the T2 lines. The 35S::*EXPA11* T2 lines highlighted in bold were taken forward as they had survival to death ratios closest to 3:1. For all the T2 lines taken forward, five surviving individuals were transferred to soil. In the third transgenic generation (T3), homozygous lines were expected to have 100% survival following GFSA-treatment, whereas heterozygotes were expected to still segregate 3:1 for Bar resistance. Appendix chapter 7, Table 7.6 shows the results of the GFSA-screening of the T3 offspring from all five of the surviving T2 plants for each line that was transferred to soil. For many of the 35S::*EXPA11* T3 lines, one or two of the five individuals screened had one hundred percent survival rate, indicating homozygosity, whereas the remaining lines had the 3:1 survival ratio expected for heterozygotes. It is thus likely that all these T3 lines that had a one hundred percent survival rate were homozygous and contained a single insertion of the pB2GW7 vector DNA. As such, the T3 lines ‘2,2-4’, ‘7,1-3’, ‘9,2-5’, ‘18,1-2’, ‘20,1-3’, and ‘21,1-3’ were selected, and a preliminary gene expression analysis was performed (see appendix chapter 7, Figure 7.5) to identify the lines with the highest levels of *EXPA11* expression. From this, the T3 lines ‘7,1-3’, ‘18,1-2’, and ‘20,1-3’ were selected and additional GFSA-screening was performed in triplicate. All three replicates for each of these three T3 lines displayed one hundred percent survival (data not shown). While GFSA-selection identifies lines that have been successfully transformed with the vector DNA, it does not discriminate between the insertion of the empty pB2GW7 vector or pB2GW7-*EXPA11*. To this end, gDNA was isolated from the 35S::*EXPA11* lines, and PCR screening was performed. Referring to appendix chapter 7, Figure 7.6, the 35S::*EXPA11* construct was detected in the gDNA from all ten of the individual plants tested from the chosen 35S::*EXPA11* T3 lines. Based off all of the above, it was concluded that the 35S::*EXPA11* T3 lines ‘7,1-3’,

‘18,1-2’, and ‘20,1-3’ were homozygous, with a single insertion of the *EXPA11*-overexpression construct DNA, and thus suitable to be taken forward for gene expression analysis.

5.3.1.4. Gene expression analysis of the chosen 35S::*EXPA11* lines

For all gene expression analysis experiments, expression of *EXPA11* in the 35S::*EXPA11* T3 lines, was compared with the genetic background, No-0. Additionally, *EXPA11* expression was also measured in the 35S::*NIT2* line. For each of the chosen lines, four leaves were harvested and pooled from one four-week-old plant that was grown on soil. This was repeated for three plants per line, giving three biological replicates. The RNA was extracted, cDNA synthesised, and the expression of *EXPA11* was measured via RT-qPCR analysis, and normalised to the expression of *MON1* (Hong et al., 2010). Referring to Figure 5.1, all three of the 35S::*EXPA11* T3 lines tested had significantly higher *EXPA11* expression than the genetic background, with the highest level of expression observed in the lines ‘7,1-3’ and ‘18,1-2’. The 35S::*NIT2* line also had significantly higher *EXPA11* expression than No-0, as expected.

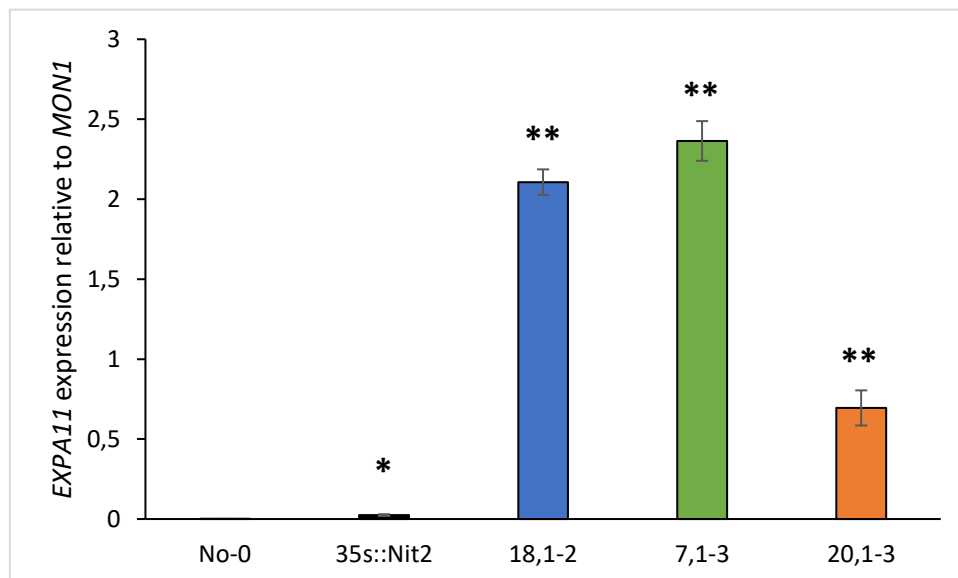


Figure 5.1: Expression of *EXPA11* in the 35S::*EXPA11* T3 lines

An RT-qPCR experiment was conducted using *EXPA11* qPCR primers on cDNA synthesised from RNA extracted from No-0, 35S::*NIT2*, and 35S::*EXPA11* T3 plants grown on soil under standard conditions. The 35S::*EXPA11* T3 lines that were screened are ‘7,1-3’, ‘18,1-2’, and ‘20,1-3’. *EXPA11* expression was normalised to the reference gene *MON1*. The results are an average of three biological replicates, where each replicate was four leaves pooled per plant for each of the genotypes. The black stars (*) denote a statistically significant difference between No-0 and the other genotypes tested, as determined by two-sample t-tests. Bonferroni correction for multiple testing was performed, and p -values ≤ 0.05 were considered significant (* for p -values ≤ 0.05 , *** for p -values < 0.005). Error bars indicate standard error.

5.3.2. Phenotypic characterisation of the *Expansin 11* overexpressing lines

The three 35S::*EXPA11* lines tested all displayed significantly increased *EXPA11* expression compared with the No-0 genetic background, thus phenotypic characterisation of these lines was conducted. The survival and fresh weight of the 35S::*EXPA11* lines was assessed in seedlings exposed to salt stress early in development, as well as in hydroponically grown plants exposed to salt stress at a later developmental stage. Additionally, some auxin-related growth phenotyping was conducted in the form of hypocotyl elongation assays. The phenotype of the 35S::*EXPA11* line was compared with a pB2GW7 empty vector (EV) line in the No-0 background that was previously generated by our research group (Nina Foreman, unpublished). To eliminate the possibility that any altered phenotype observed in the 35S::*EXPA11* line was due to the location of the vector DNA insertion, as opposed to the expression of the 35S::*EXPA11* construct, all assays were performed using the two 35S::*EXPA11* lines with the highest level of *EXPA11* expression, '7,1-3' and '18,1-2'.

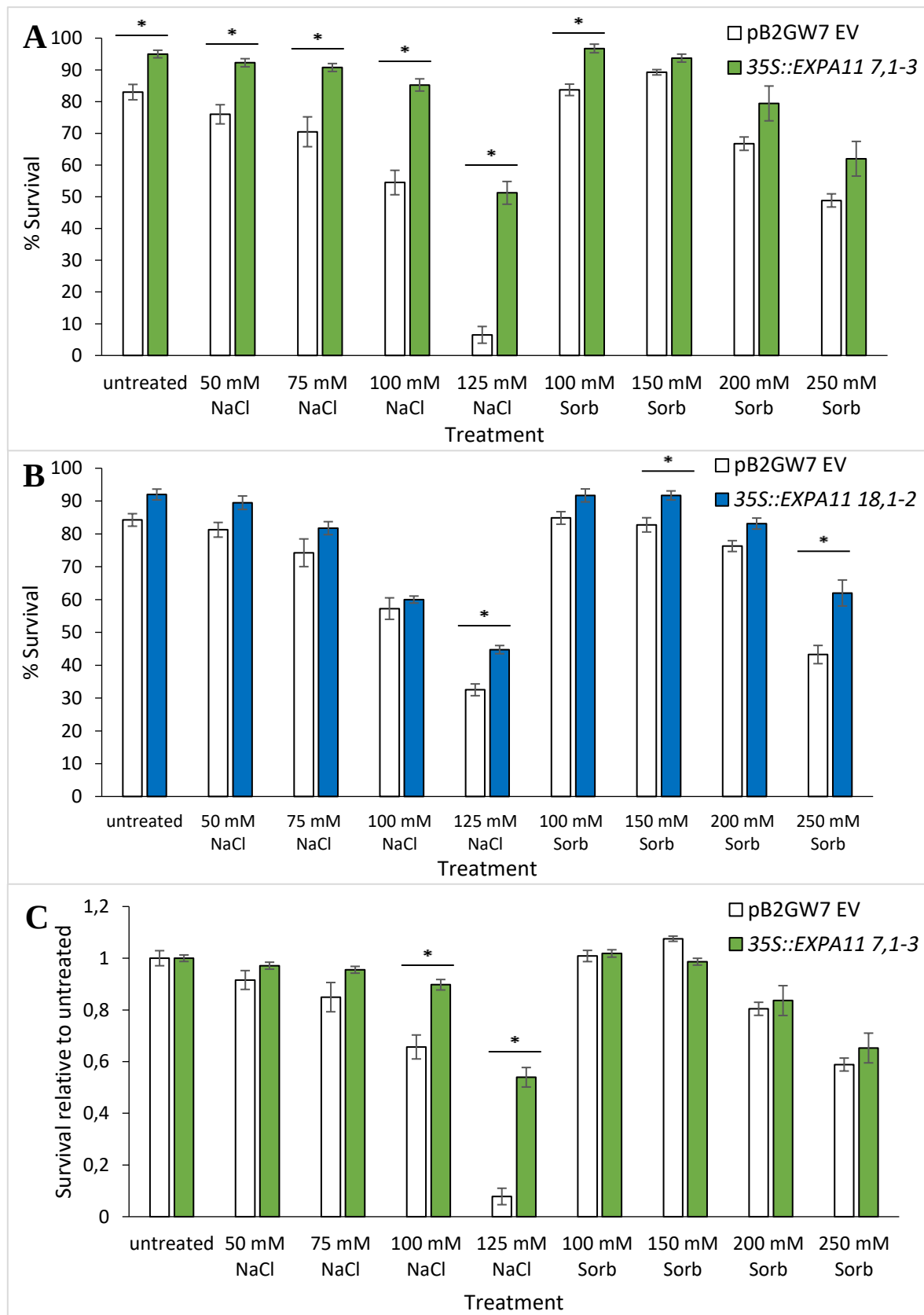
5.3.2.1. Survival and growth of 35S::*EXPA11* plants exposed to salt stress early in development

To determine if overexpression of *EXPA11* impacted the salt-tolerance of plants during early development, 35S::*EXPA11* and pB2GW7 EV plants were germinated and grown for eleven days on PN media (control) or PN media supplemented with increasing concentrations of NaCl, or the iso-osmolar concentrations of sorbitol. Two phenotypes were used to assess salt-tolerance: survival and growth (fresh weight yield). For each treatment, the number of surviving seedlings was counted and the fresh weight per plant was recorded. The average percentage (%) survival, survival relative to the untreated control, average fresh weight per plant, and fresh weight per plant relative to the untreated control was then compared between the 35S::*EXPA11* and pB2GW7 EV lines for each of the treatments. Referring to Figure 5.2 A, the 35S::*EXPA11* line 7,1-3 displayed a significantly improved % survival compared with the empty vector in control conditions as well as in all of the NaCl concentrations tested. Furthermore, the line displayed an improved % survival in response to the lowest sorbitol concentration (100 mM). As there was a difference in survival under control conditions, it was important to look also at the survival relative to the untreated control. Referring to Figure 5.2 C, there was an improved relative survival in the 35S::*EXPA11* line 7,1-3 at the two highest salt concentrations. The 35S::*EXPA11* line 18,1-2 only displayed significantly improved % survival in response to the highest NaCl concentration (125 mM), as well as in the 150 and 250 mM sorbitol treatments (Figure 5.2 B), and when considering the relative survival data this

increase for the 35S::*EXPA11* line 18,1-2 was only seen at 125 mM NaCl (Figure 5.2 D). Although the findings were slightly different for the two 35S::*EXPA11* lines, it would appear that *EXPA11* overexpression causes an increased survival in response to both salt (particularly at higher concentrations) and sorbitol treatment, as well as in control conditions.

Figure 5.3 A shows that the 35S::*EXPA11* line 7,1-3 displayed a significantly increased average fresh weight per plant when compared with the empty vector in untreated conditions, as well as in all of the NaCl and sorbitol treatments. Likewise, the 35S::*EXPA11* line 18,1-2 also displayed a significantly higher fresh weight in untreated conditions, as well as in response to 100 mM NaCl, and 100, 200 and 250 mM sorbitol (Figure 5.3 B). The 18,1-2 line appeared to have improved fresh weight at the other NaCl concentrations as well, however these differences were not found to be significant. This data would suggest that the average fresh weight per plant is improved under control conditions, and in plants grown on salt and sorbitol, in lines overexpressing *EXPA11*. As there was increased fresh weight observed in both 35S::*EXPA11* lines in control conditions, it was important to assess whether the improved seedling size observed in saline conditions was a salt-specific phenotype, or rather due to these lines growing larger in all conditions. Referring to Figure 5.3 D, it can be seen that there was no difference in fresh weight relative to the untreated control at any of the salt concentrations tested for the 35S::*EXPA11* line 18,1-2 when compared with the EV, whereas for the 35S::*EXPA11* line 7,1-3 (Figure 5.3 C) there was an improvement in relative fresh weight, but only at the highest salt concentrations. The difference in overall fresh weight incorporation that is observed between the two sets of experiments (i.e. between the 35S::*EXPA11* 7,1-3 and 18,1-2 datasets) is due to fluctuations in growth temperature - when the experiments for the 35S::*EXPA11* 7,1-3 line (Figure 5.3 A) were conducted, the temperature was slightly warmer, thus growth was increased. Importantly, all experiments involving the 35S::*EXPA11* 7,1-3 line and 35S::*EXPA11* 18,1-2 line were performed in identical growth temperatures, respectively. Furthermore, in both cases, the EV control was grown along with these lines. Despite the overall difference in fresh weight between the two datasets, the trend of increased fresh weight for each of the two 35S::*EXPA11* lines compared with their respective EV control under all treatment conditions was consistent across the two experimental sets. Overall, these results suggest that the overexpression of *EXPA11* may promote the survival of seedlings germinating and growing under salt (particularly at higher salt concentrations) and sorbitol stress. Furthermore, it would appear that the growth (fresh weight yield) of these 35S::*EXPA11* seedlings is improved under all conditions, and not just when exposed to salt. Collectively, this

would suggest that overexpression of *EXPA11* might not have the specific effect of improving the salinity tolerance of seedlings exposed to salt stress during early development, but rather that it improves early developmental growth in germinating seedlings in general.



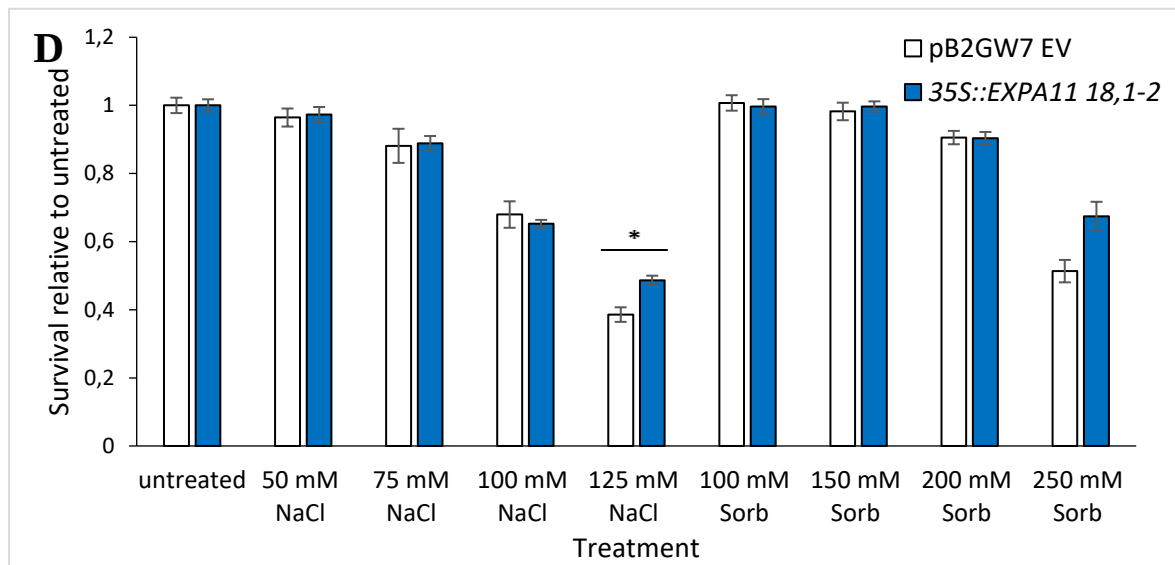
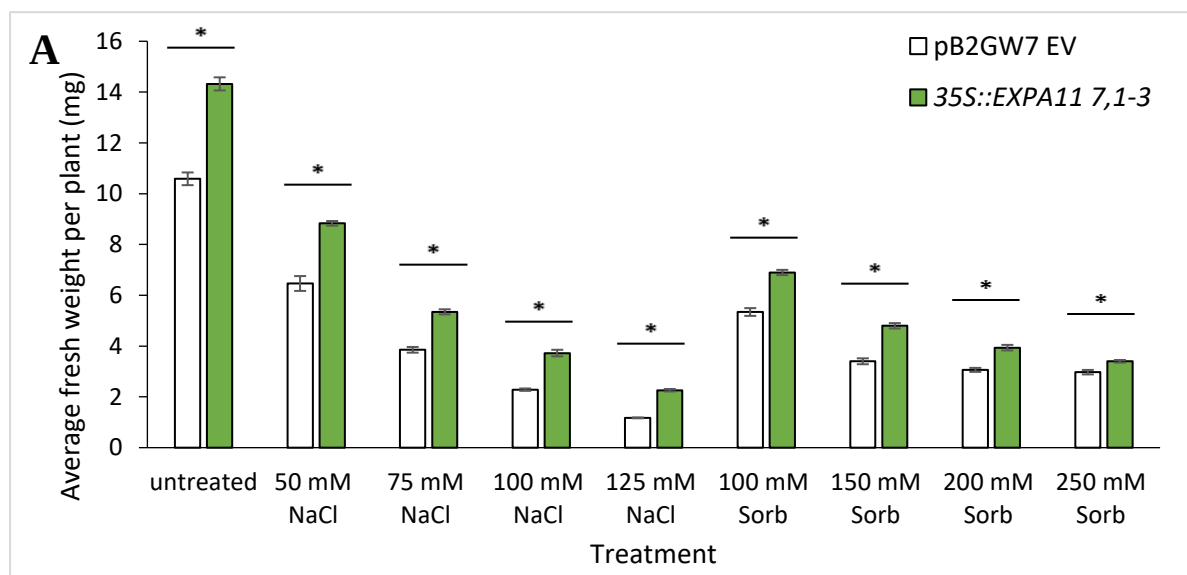


Figure 5.2: Survival of 35S::EXPA11 and No-0 EV plants exposed to salt stress early in development

The No-0 empty vector (EV) and 35S::EXPA11 lines were germinated and grown for eleven days in petri dishes containing PN-phyto agar (7% w/v) supplemented with the indicated concentrations of NaCl and sorbitol (Sorb). Here, 50 seedlings were germinated per petri dish. Survival was scored by development of true leaves, and the number of surviving seedlings was counted per petri dish and percentage survival calculated. (A) The average % survival of No-0 EV and 35S::EXPA11 line T4 7,1-3. (B) The average % survival of No-0 EV and 35S::EXPA11 line T4 18,1-2. (C) The survival relative to the untreated control for No-0 EV and 35S::EXPA11 line T4 7,1-3. (D) The survival relative to the untreated control for No-0 EV and 35S::EXPA11 line T4 18,1-2. The results are an average of two independent biological experiments, each with four technical repeats for each treatment per line. The black stars (*) denote a statistically significant difference between No-0 EV and 35S::EXPA11, as determined by two-sample t-tests. Bonferroni correction for multiple testing was performed, and p -values $\leq 0,05$ were considered significant. Error bars indicate standard error.



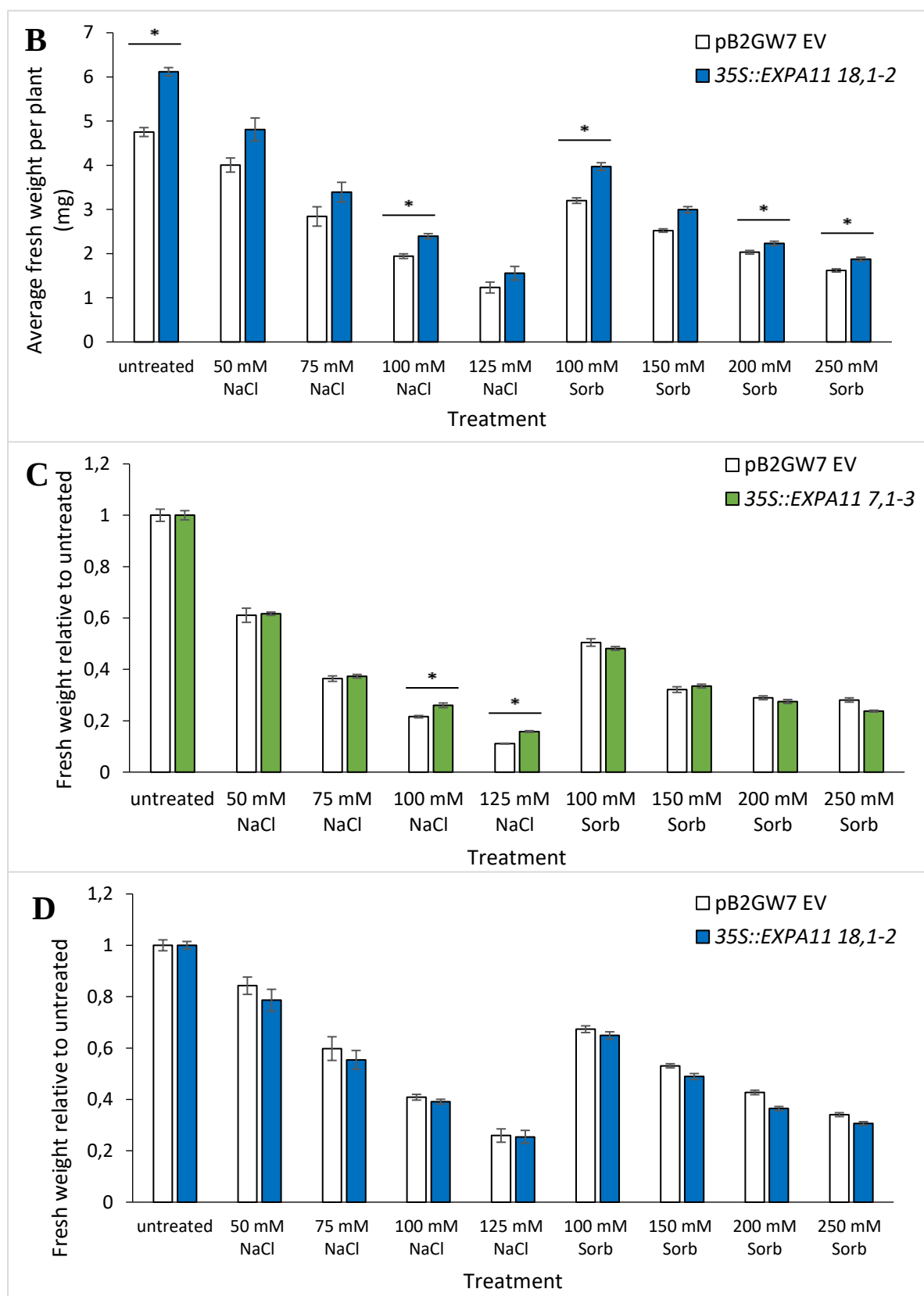


Figure 5.3: Average fresh weight of 35S::*EXPA11* and No-0 EV plants exposed to salt stress early in development

The No-0 EV and 35S::*EXPA11* lines were germinated and grown for eleven days in petri dishes containing PN-phyto agar (7% w/v) supplemented with the indicated concentrations of NaCl and sorbitol (Sorb). Here, 50 seedlings were germinated per petri dish. Average fresh weight per plant was calculated by dividing the total fresh weight of each petri dish by the number of surviving seedlings. **(A)** Average fresh weight per plant (mg) of No-0 EV and 35S::*EXPA11* line T4 7,1-3. **(B)** Average fresh weight per plant (mg) of No-0 EV and 35S::*EXPA11* line T4 18,1-2. **(C)** Fresh weight relative to the untreated control for No-0 EV and 35S::*EXPA11* line T4 7,1-3. **(D)** Fresh weight relative to the untreated control for No-0 EV and 35S::*EXPA11* line T4 18,1-2. The results are an average of two independent biological experiments, each with four technical repeats for each treatment per line. The black stars (*) denote a statistically significant difference between No-0 EV and 35S::*EXPA11*, as determined by two-sample t-tests. Bonferroni correction for multiple testing was performed, and p -values $\leq 0,05$ were considered significant. Error bars indicate standard error.

5.3.2.2. Growth of 35S::*EXPA11* plants exposed to salt stress later in development

The salt-tolerance of plants varies across the different developmental stages, with particular stages such as germination, early seedling development or flowering showing increased sensitivity (Singh, Kota & Flowers, 2021). However, improved germination is not necessarily a good indicator of improved salt-tolerance as a plant might germinate under saline conditions but not be salt-tolerant at a later developmental stage. Therefore, to assess the effect of exposure to salt stress at a later developmental stage, the 35S::*EXPA11* lines and No-0 empty vector (EV) plants were grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 50, 75, or 100 mM NaCl, or maintained on ¼ strength PN media (untreated control) for one additional week. The average root and shoot fresh weight was measured and compared between the lines. Referring to Figure 5.4 B, it was found that the 35S::*EXPA11* 18,1-2 line had a significantly larger root fresh weight than the empty vector when exposed to 75 mM NaCl, but no differences were observed for the other two NaCl concentrations or the untreated control. An almost identical pattern was observed for the 35S::*EXPA11* 7,1-3 line (Figure 5.4 A), however, the increase in root fresh weight in the 75 mM NaCl treatment was not found to be significant ($p = 0.0542$). When looking at the shoot fresh weight data, the 35S::*EXPA11* 7,1-3 line had significantly improved growth compared with the empty vector in the 75 mM and 100 mM NaCl treatments, whereas the 35S::*EXPA11* 18,1-2 line was significantly larger in all three NaCl treatments (Figure 5.4 C and D, respectively). For both lines, no significant difference in fresh weight was observed in control conditions. The different finding between the two 35S::*EXPA11* lines at 50 mM NaCl can likely

be attributed to a difference in the shoot growth of the empty vector line in these two assays. This variation in EV growth between the assays could be attributed to the fact that they were performed at different times, with different batches of EV seeds. Figure 5.5 shows an example of the hydroponically grown 35S::*EXPA11* and EV plants in control and saline conditions. In agreement with the shoot fresh weight data, it can clearly be seen that the shoot growth of the 35S::*EXPA11* plants is greater than that of the EV in all three NaCl concentrations, without an obvious difference in control conditions.

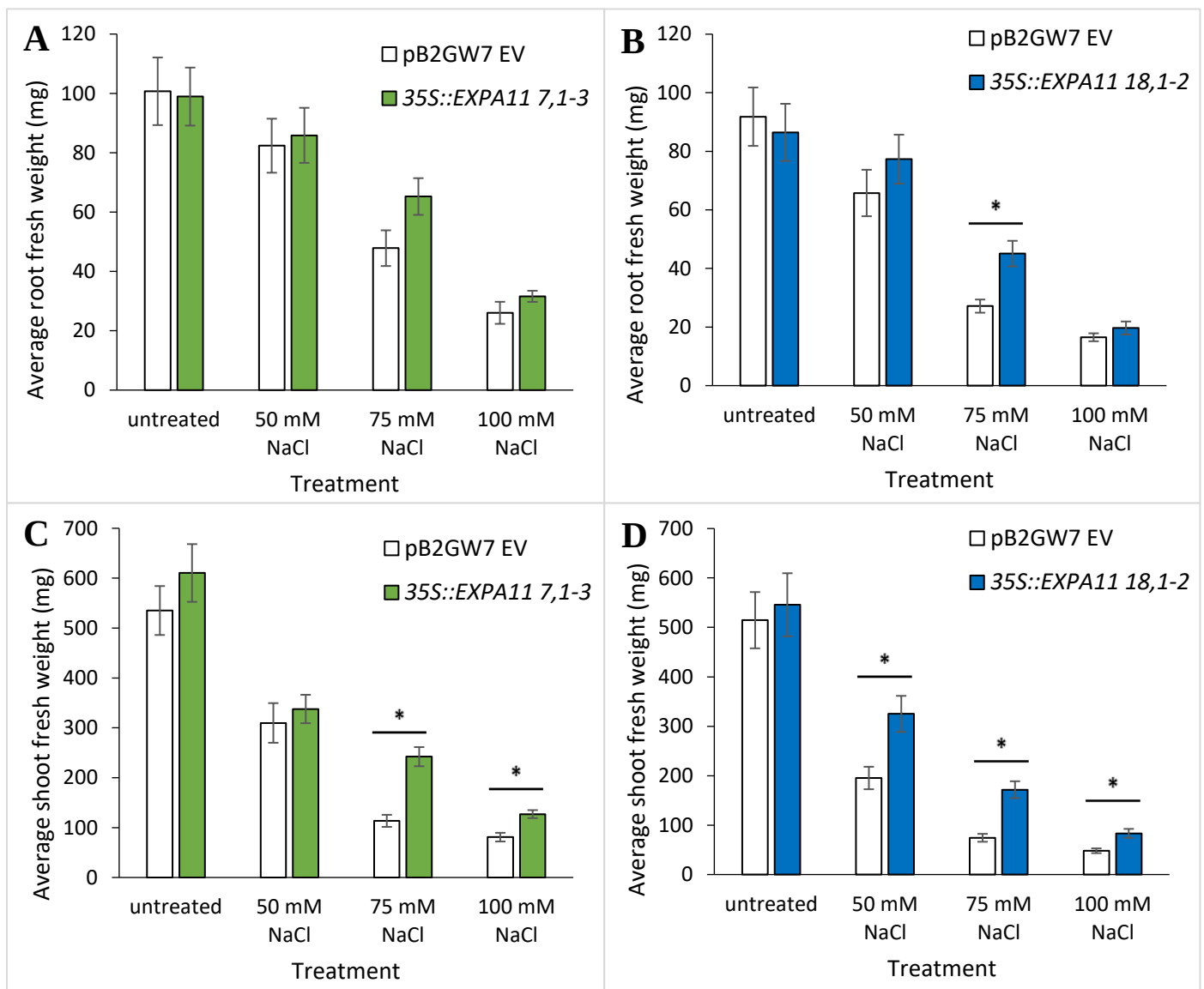


Figure 5.4: Average root and shoot fresh weight of 35S::*EXPA11* and No-0 EV plants exposed to salt stress later in development

The No-0 empty vector (EV) and 35S::*EXPA11* plants were grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 50, 75, or 100 mM NaCl, or

maintained on ¼ strength PN media (untreated control) for one additional week. Here, eight plants from both lines were germinated and grown in the same hydroponics box for each of the treatments. The experiment was repeated twice for both 35S::*EXPA11* lines. The average fresh weight of the root and shoot tissue for both lines was calculated. Average root fresh weight (mg) of No-0 EV and 35S::*EXPA11* line T4 (A) 7,1-3 and (B) 18,1-2 plants. Average shoot fresh weight (mg) of No-0 EV and 35S::*EXPA11* line T4 (C) 7,1-3 and (D) 18,1-2 plants. The black stars (*) denote a statistically significant difference between No-0 EV and 35S::*EXPA11*, as determined by two-sample t-tests. Bonferroni correction for multiple testing was performed, and p -values $\leq 0,05$ were considered significant. Error bars indicate standard error.

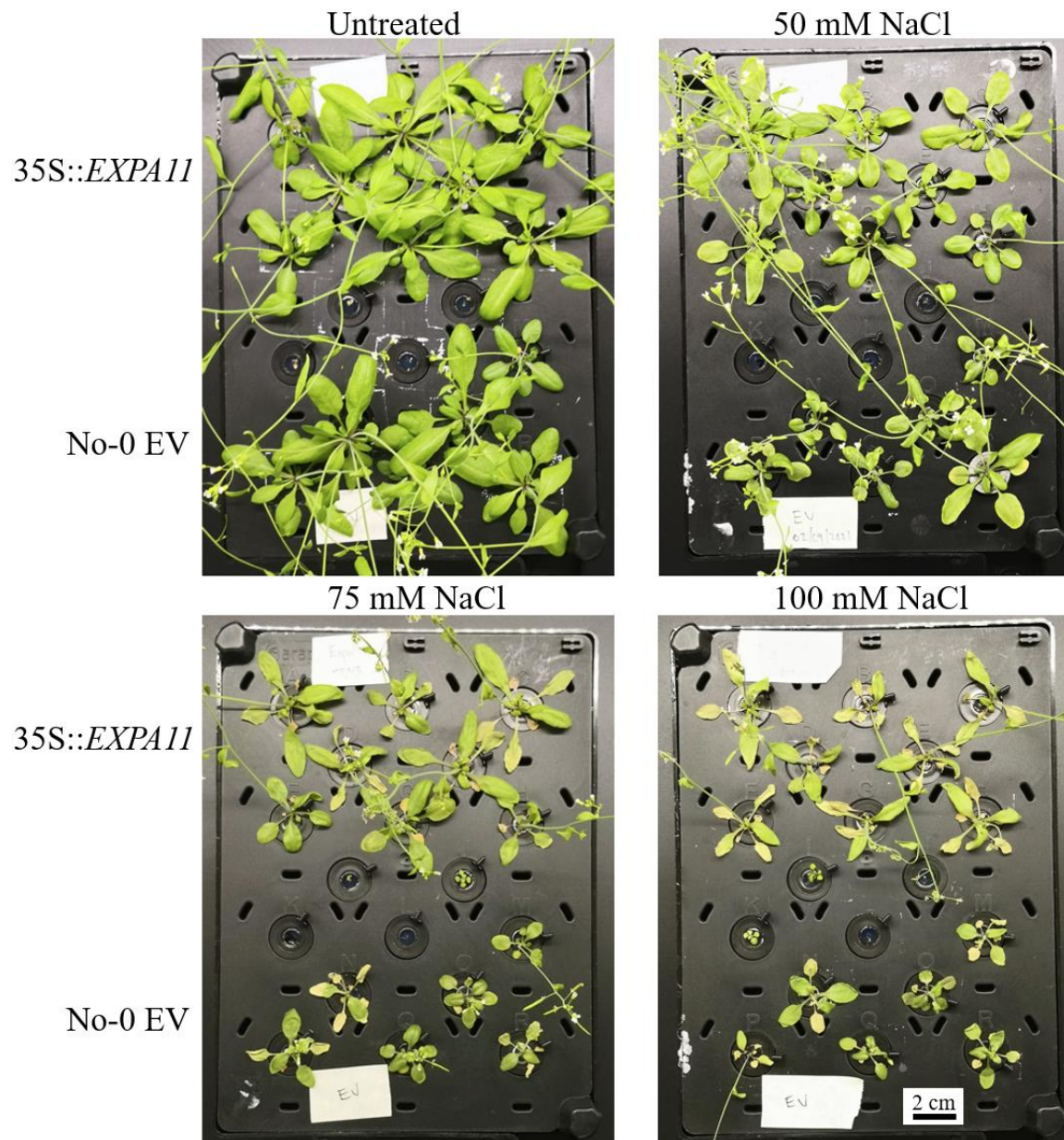
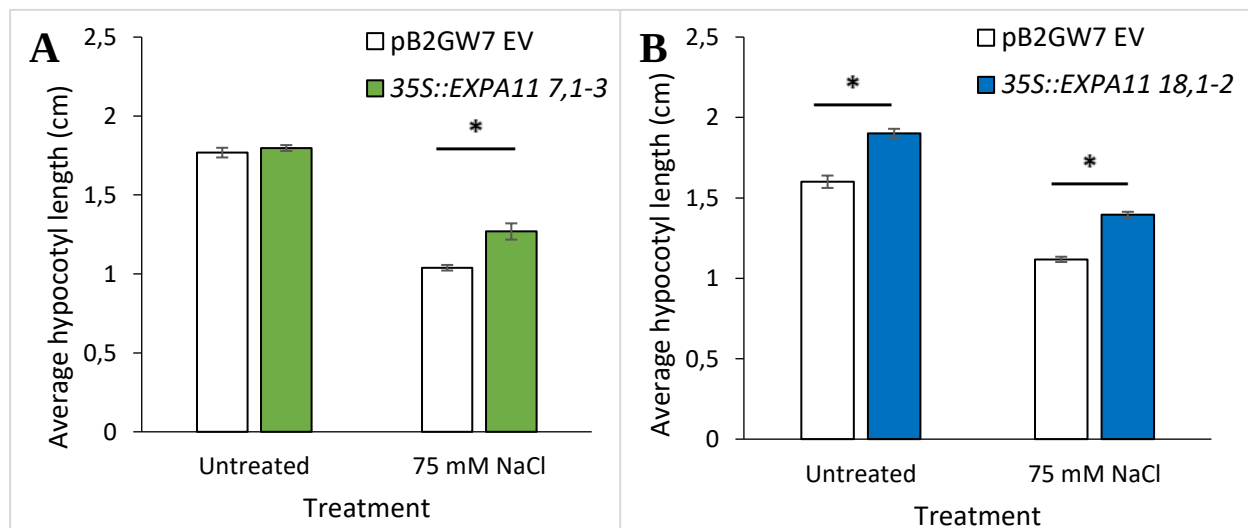


Figure 5.5: 35S::*EXPA11* and No-0 EV plants grown hydroponically in control and saline conditions

The No-0 empty vector (EV) and 35S::*EXPA11* plants were grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 50, 75, or 100 mM NaCl, or maintained on ¼ strength PN media (untreated control) for one additional week.

5.3.2.3. 35S::EXPA11 auxin-related growth phenotyping

Seedling hypocotyl elongation is a useful proxy for assessing auxin-mediated elongation growth in plants (Zhao et al., 2002). Salt-treatment has been shown to have a negative impact on hypocotyl elongation in dark-grown *Arabidopsis* seedlings (Cackett, 2019). However, the 35S::NIT2 line has been found to display a less severe inhibition of elongation in response to salt stress, with it being suggested that an increased IAA production in this line underlies the maintenance of hypocotyl growth under saline conditions (Cackett, 2019). Given our hypothesis that *EXPA11* acts as part of the auxin-induced activation (and modulation) of expansion growth in response to salt stress, it followed to assess potential auxin-related growth phenotypes in the 35S::*EXPA11* lines. As *EXPA11* has largely been implicated in hypocotyl elongation in the literature, this was an obvious auxin-related phenotype to assess. Therefore, the hypocotyl length of five-day-old No-0 EV and 35S::*EXPA11* dark-grown seedlings was measured under control and 75 mM NaCl conditions. As previously observed, treatment with 75 mM NaCl resulted in a reduction in average hypocotyl length compared with the control in the No-0 EV, and both of the 35S::*EXPA11* lines, 7,1-3 and 18,1-2 (Figure 5.6 A and B, respectively). Both of the 35S::*EXPA11* lines were found to have a significantly increased average hypocotyl length compared with the No-0 EV in response to salt treatment. However, in untreated conditions, only the 35S::*EXPA11* line 18,1-2 displayed increased hypocotyl elongation, whereas no difference was observed for the line 7,1-3. When looking at the hypocotyl lengths of the lines relative to their untreated control (Figure 5.6 C and D), the hypocotyls were significantly longer in the 35S::*EXPA11* lines when grown in salt. The improved hypocotyl growth phenotype suggests that the overexpression of *EXPA11* may improve the early growth and development of seedlings under saline conditions, as was observed in the early development salt stress assays.



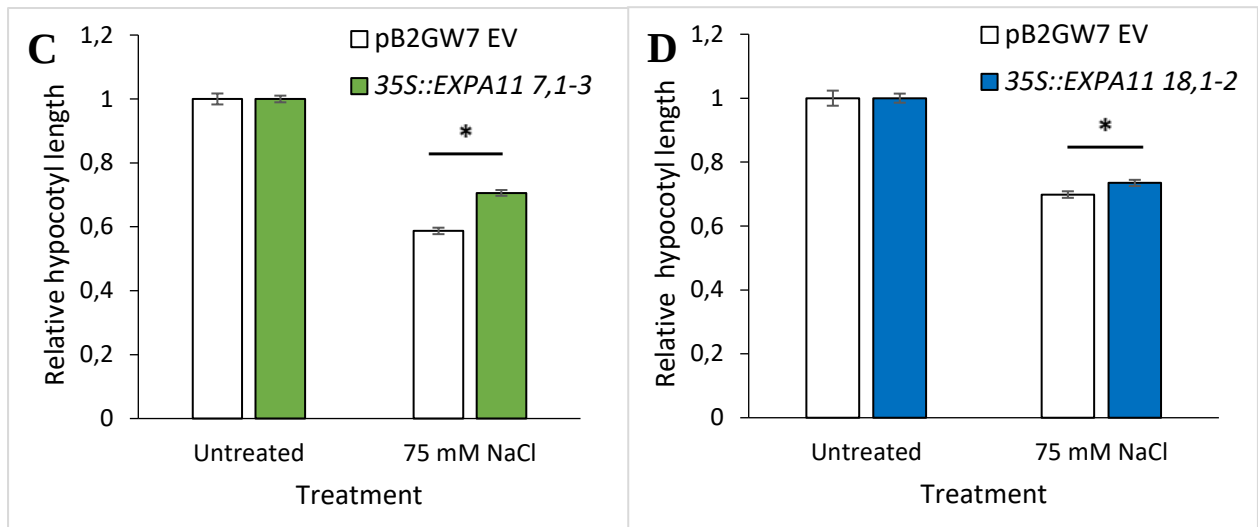


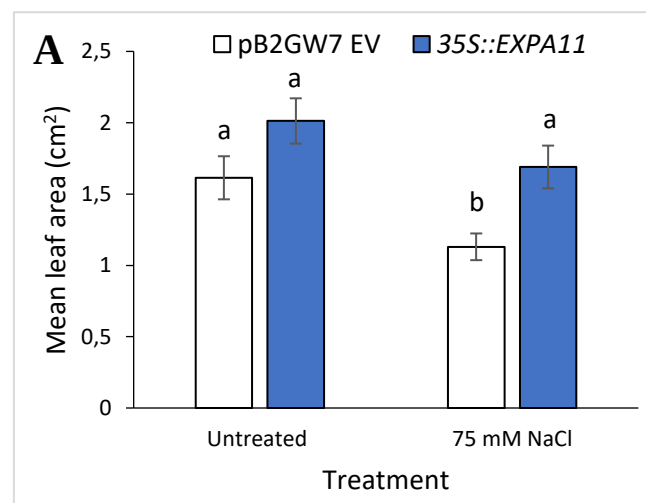
Figure 5.6: Average and relative hypocotyl lengths of dark-grown 35S::EXPA11 and No-0 EV under untreated and salt stress conditions

Both 35S::EXPA11 and No-0 EV seeds were sown on PN-phyto agar (7% w/v) (control) and PN-phyto agar supplemented with 75 mM NaCl. The seedlings were grown in complete darkness for five days, after which the hypocotyl length of each seedling was measured. Average hypocotyl lengths of No-0 EV and 35S::EXPA11 line T4 7,1-3 (A) and 35S::EXPA11 line T4 18,1-2 (B). The hypocotyl lengths were calculated relative to the untreated for both the No-0 EV and 35S::EXPA11 line T4 7,1-3 (C) and 35S::EXPA11 line T4 18,1-2 (D). The results are an average of six technical repeats, in each of which 20 hypocotyls were measured per line for each treatment. The black stars (*) denote a statistically significant difference between No-0 EV and 35S::EXPA11, as determined by two-sample t-tests. Bonferroni correction for multiple testing was performed, and p -values $\leq 0,05$ were considered significant. Error bars indicate standard error.

5.3.2.4. Leaf area and cell size measurements in 35S::EXPA11 plants exposed to salt stress

Given the role of several expansins in leaf development, as well as the link between reduced EXPA11 expression and the inhibition of leaf cell elongation (Hou et al., 2022), it followed naturally to assess whether the overexpression of EXPA11 had any effect on leaf size and morphology. To do this, 35S::EXPA11 line T4 18,1-2 and No-0 EV plants were grown hydroponically for three weeks on $\frac{1}{4}$ strength PN media, then, either treated with $\frac{1}{4}$ strength PN media supplemented with 75 mM NaCl, or maintained on $\frac{1}{4}$ strength PN media (untreated control) for three days. The average surface area (lamina and petiole) of the emerged rosette leaves was measured and compared between the two lines. Referring to Figure 5.7 A, it was found that leaf surface area was significantly increased in the 35S::EXPA11 line compared with the empty vector line following growth in saline conditions, with no significant difference observed between the two lines in untreated conditions. Exposure to salt stress resulted in a significant decrease in leaf area in the empty vector line, whereas the leaf growth observed in

untreated conditions was maintained in the 35S::*EXPA11* line when grown under saline conditions. To further characterise the effect of *EXPA11* overexpression on leaf morphology, light microscopy was performed to measure the size of the epidermal and palisade mesophyll cells of the leaves of 35S::*EXPA11* and empty vector plants grown exactly as described for the leaf area measurements. This was done by Lara Reale, Martina Cerri, and Ele Cinti at the Department of Agricultural, Food and Environment Sciences at the University of Perugia. Figure 5.7 B shows an example of a toluidine blue-stained semithin section from a resin-embedded leaf under the light microscope, with both the epidermal and palisade mesophyll cell layers clearly visible. When grown in control conditions, leaves of the 35S::*EXPA11* line had significantly greater epidermal and palisade cell size than the empty vector (Figure 5.7 C and D, respectively). However, both epidermal and palisade cell size was significantly decreased in response to salt stress in the 35S::*EXPA11* line, whereas no effect was observed in the empty vector line. Consequently, unlike in control conditions, no difference in epidermal cell size was observed between the two lines in salt-stressed leaves, and the palisade cells were in fact smaller in the 35S::*EXPA11* line than in the empty vector line. It should be noted that we are still actively generating data around these leaf cell size analyses, and Figure 5.7 B is a preliminary example of the microscopy imaging of leaf semithin sections performed during the analysis.



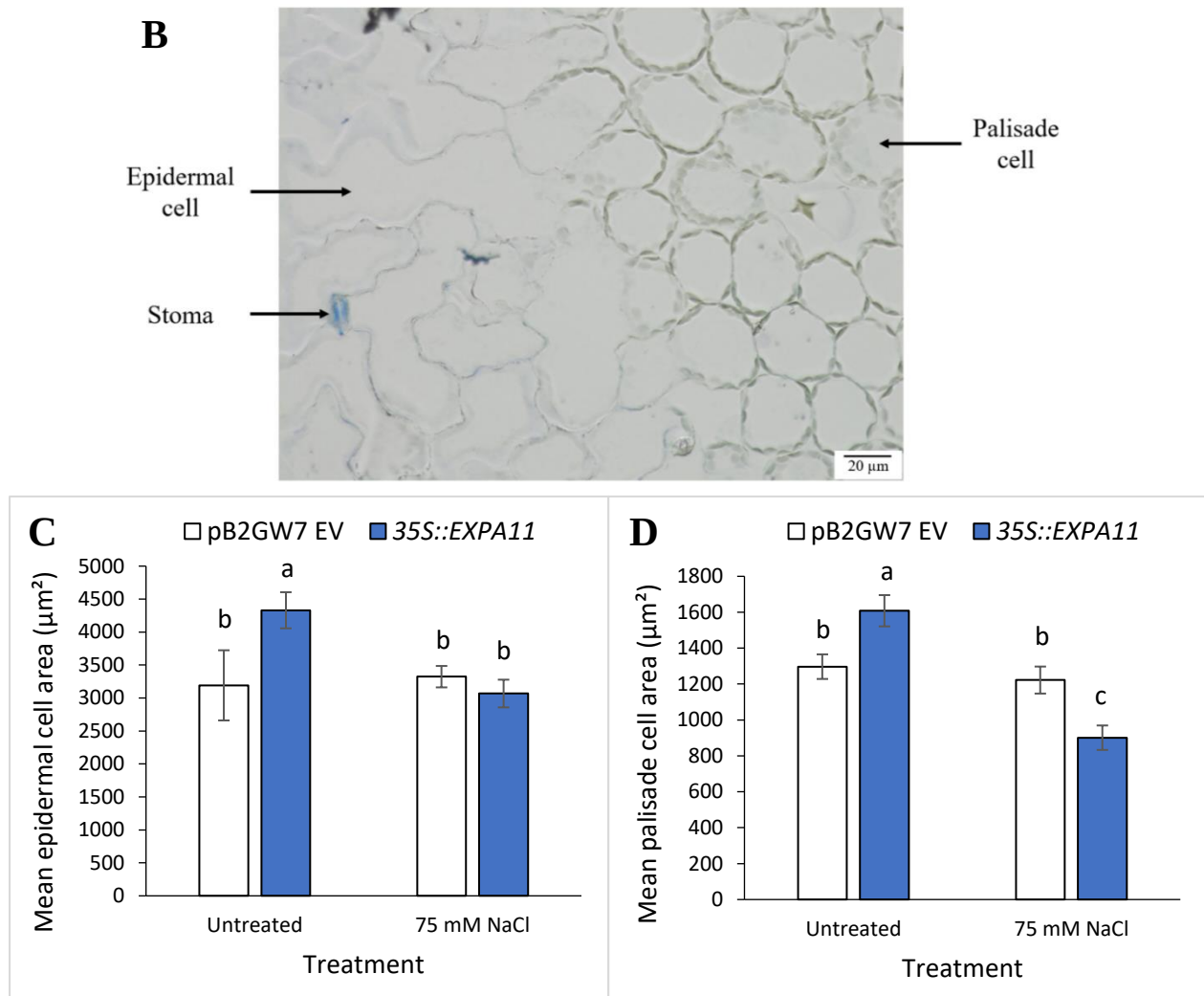


Figure 5.7: Leaf area, epidermal cell area, and palisade mesophyll cell area of 35S::EXPA11 and No-0 EV plants exposed to salt stress

The No-0 empty vector (EV) and 35S::EXPA11 line T4 18,1-2 plants were grown hydroponically for three weeks on $\frac{1}{4}$ strength PN media, then, either treated with $\frac{1}{4}$ strength PN media supplemented with 75 mM NaCl, or maintained on $\frac{1}{4}$ strength PN media (untreated control) for three days. For the leaf area, the first four emerged rosette leaves from four individual plants for each line and treatment were measured. The cell area measurements were performed on the first three rosette leaves from three individual plants for each line and treatment. **(A)** Mean rosette leaf surface area (cm^2) ($n = 16$). **(B)** Example of light microscope analysis of semithin leaf section showing epidermal and palisade cells. The semithin section is stained with toluidine blue. **(C)** Mean leaf epidermal cell area (μm^2). For the EV untreated and 75 mM NaCl $n = 50$ and 83 , respectively, and for the 35S::EXPA11 untreated and 75 mM NaCl $n = 75$ and 67 , respectively. **(D)** Mean leaf palisade mesophyll cell area (μm^2). For the EV untreated and 75 mM NaCl $n = 40$ and 30 , respectively, and for both the 35S::EXPA11 untreated and 75 mM NaCl $n = 30$. Letters on the graphs indicate significant differences ($p \leq 0.05$) in mean values as determined by Duncan's multiple range test post-hoc analysis following a one-way ANOVA. Error bars indicate standard error.

5.3.2.5. Na^+ and K^+ ion contents in 35S::*EXPA11* and EV roots and shoots under control and saline conditions

As discussed in chapter 2, the 35S::*NIT2* line was found to have significantly altered root/shoot Na^+ and K^+ ion partitioning compared with the wild-type – a finding that helps explain its improved salt-tolerance. Given the hypothesised role of *EXPA11* as a potential downstream effector in the auxin-mediated growth activation caused by the over-expression of *NIT2*, and the observation of improved growth of the 35S::*EXPA11* line under both control and saline conditions, it followed to assess whether there were any alterations in ion partitioning that occur in the 35S::*EXPA11* compared with its genetic background. The Na^+ and K^+ content was thus measured in the roots and shoots of 35S::*EXPA11* and pB2GW7 EV plants, following exposure to salt stress, or maintenance under control conditions, via ICP-OES. This analysis was performed by the analytics laboratory at the UCT Chemical Engineering Department. The plant lines were grown hydroponically for three weeks on ¼ strength plant nutrient (PN) media, then treated for one week with ¼ strength PN media supplemented with 75 mM NaCl or maintained on ¼ strength PN media (control), for one week further. The root and shoot tissue was harvested, air-dried, then ashed and acid-digested prior to analysis via ICP-OES. Tissue from four plants was pooled to give a single sample, and six biological replicates were used.

No differences in Na^+ or K^+ partitioning was observed in the root or shoot between the two lines under either untreated or saline conditions (Figure 5.8). As expected, in untreated conditions, very low levels of Na^+ were detected in the tissues of both lines (Figure 5.8 A). Following salt-treatment, the Na^+ content was significantly increased in both lines, more so in the shoots than in the roots (~3-fold) (Figure 5.8 B). Salt-treatment resulted in a significant decrease in K^+ content in the root and the shoot compared with the levels observed under untreated conditions (Figure 5.8 C and D), with a larger reduction observed in the root. Ultimately, no difference in the Na^+/K^+ ratio was observed between the two lines in either tissue under saline conditions (Figure 5.8 F), thus the overexpression of *EXPA11* has no effect on ion homeostasis.

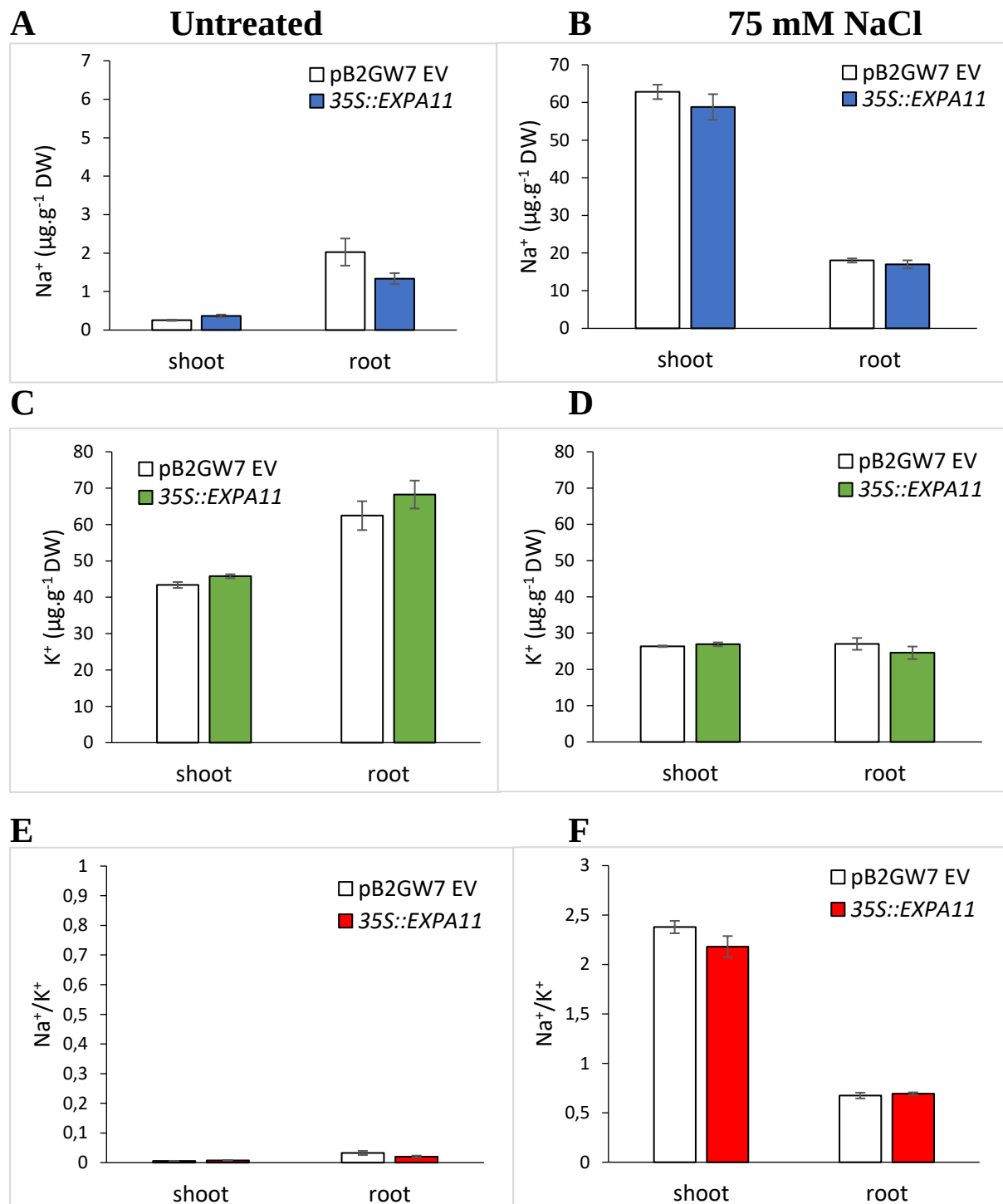


Figure 5.8: Na^+ and K^+ content in root and shoot tissue of 35S::EXPA11 and pB2GW7 EV plants exposed to salt stress

The pB2GW7 EV and 35S::EXPA11 plants were grown hydroponically for three weeks on $\frac{1}{4}$ strength PN media, then, either treated with $\frac{1}{4}$ strength PN media supplemented with 75 mM NaCl, or maintained on $\frac{1}{4}$ strength PN media (untreated control) for one additional week. Root and shoot tissue were harvested, oven dried, and then ashed and acid-digested. The ion content (expressed in $\mu\text{g}\cdot\text{g}^{-1}$ dry weight (DW)) was determined via ICP-OES analysis. **(A)** Na^+ and **(C)** K^+ content, and **(E)** Na^+/K^+ in roots and shoots of pB2GW7 EV and 35S::EXPA11

grown in untreated conditions. **(B)** Na⁺ and **(D)** K⁺ content, and **(F)** Na⁺/K⁺ in roots and shoots of pB2GW7 EV and 35S::*EXPA11* plants following 75 mM NaCl treatment. The results are an average of six biological repeats (n=6). Error bars indicate standard error.

5.4. Discussion

5.4.1. *EXPA11* overexpressing lines were successfully generated

The Gateway® technology cloning strategy and subsequent GFSA-screening of *Agrobacterium*-transformed plants proved to be successful in generating homozygous transgenic lines that stably expressed the *EXPA11* coding DNA sequence under the control of the 35S promoter. Furthermore, several of these 35S::*EXPA11* lines were found to have massively increased (10 000 to 35 000-fold) *EXPA11* expression when compared with the genetic background, No-0.

5.4.2. The *EXPA11* overexpressing lines have improved growth in saline conditions without a growth penalty under control conditions

Both of the 35S::*EXPA11* lines tested had improved growth when compared with the empty vector genetic background when exposed to salt during early development (Figures 5.2 and 5.3), and when exposed at a later developmental stage (Figure 5.4 and 5.5). In the early development salt stress assays, the 35S::*EXPA11* survived better than the EV line, both under saline conditions, as well as when exposed to the osmotic stress conditions imposed by sorbitol (Figure 5.2). However, this improved survival in response to salt treatment was more pronounced in the one 35S::*EXPA11* line. The average fresh weight of the seedlings for both of the 35S::*EXPA11* lines was greater than the EV under control, saline, and osmotic stress conditions, suggesting that the overexpression of *EXPA11* leads to improved growth in general during early development, and importantly, confers a growth advantage under saline and osmotic stress conditions. I am not the first to report that *AtEXPA* over-expression confers an advantage during Arabidopsis early seedling development under salinity and osmotic stress; Yan et al. (2014) showed that 35S::*EXPA2* lines display increased germination compared with the WT under 200 mM NaCl, and 200 or 400 mM mannitol stresses. Additionally, transgenic Arabidopsis lines over-expressing the rose α -expansin, *RhEXPA4*, had improved germination compared with the EV and WT under 100 mM NaCl conditions (Lü et al., 2013).

As previously mentioned, one of the more robustly reported roles for *EXPA11* in growth modulation is during hypocotyl elongation (Simon et al., 2017; Xu et al., 2017). The finding

that the 35S::*EXPA11* lines have significantly increased dark-grown hypocotyl lengths compared with the EV background not only provides further evidence towards this purported role, but is the first linking *EXPA11* expression with increased hypocotyl elongation under saline conditions. Additionally, our findings that hypocotyl elongation in both the 35S::*EXPA11* and EV lines was reduced in response to salt treatment is consistent with previous reports of an inhibitory effect of salinity on dark-grown hypocotyl elongation (Cackett, 2019). Hypocotyl emergence is the earliest shoot developmental stage following germination, so the finding that hypocotyl elongation was increased in the 35S::*EXPA11* lines under saline conditions suggests that the improved shoot growth phenotype of these lines observed later on in mature plants may be established already very early on during development. Interestingly, over-expression of *expansin-like A 2 (EXLA2)* has also been shown to increase dark-grown hypocotyl length (Boron et al., 2015). Similarly to the 35S::*EXPA11* lines generated in this chapter, *EXLA2* expression was driven by the *CaMV* 35S promoter. Notably, the observed increases in length of three- and seven-day-old hypocotyls of the 35S::*EXLA2* lines compared with their wild-type were very similar to those I report for the 35S::*EXPA11* lines and the EV here. This suggests that *EXPA11* is not the only contributor to hypocotyl elongation, but perhaps that it is particularly important for hypocotyl growth, and seedling establishment, in response to salinity. This possible role could be more firmly established by the phenotyping of an *expa11* mutant under saline conditions in the future.

When exposed to salt stress at a later developmental stage, the 35S::*EXPA11* lines had greater shoot fresh weight than the EV. In contrast, it would not appear that *EXPA11* over-expression influenced root growth in these older plants, as an improved root fresh weight was only observed in one salt concentration for one of the 35S::*EXPA11* lines (Figure 5.4) – likely an anomalous result. Interestingly, the improved growth of the 35S::*EXPA11* seedlings compared with the EV that was observed under control conditions was not seen in these larger, hydroponically grown plants. In agreement with the improved shoot fresh weight data, and clearly increased shoot size, the 35S::*EXPA11* line 18,1-2 also displayed increased rosette leaf surface area compared with the EV following exposure to 75 mM NaCl, with no significant difference observed in control conditions. The findings presented here are not the first to report on *EXPA* over-expression leading to increased leaf growth in *Arabidopsis*; over-expression of *EXPA10* has been shown to increase both leaf blade and petiole length (Cho & Cosgrove, 2000). It must be noted that the period of salt treatment was shorter in the leaf area assays than the shoot fresh weight assays (three days as opposed to seven days), and leaf area measurements

were only conducted on plants exposed to 75 mM NaCl, as opposed to the broader range of salt concentrations used in the shoot fresh weight assays. Nonetheless, there is little reason to believe that leaf area would not also be increased in the 35S::*EXPA11* line at these other salt concentrations given the clear differences in shoot growth observed (Figure 5.5). Additionally, the increase in leaf size as a result of *EXPA11* overexpression is in agreement to what was observed in the *HLH4*-overexpressing plants in which *EXPA11* expression was reduced with a concomitant reduction leaf size (Hou et al., 2022). Collectively, the findings of increased fresh weight, and larger leaf area strongly suggest that over-expression of *EXPA11* confers improved shoot growth in older plants under saline conditions.

While over-expression of *EXPA11* results in improved shoot growth in response to salt stress, it was also noteworthy that the 35S::*EXPA11* seedlings had improved germination and fresh weight when treated with sorbitol. It would thus also be worthwhile to assess the osmotic stress tolerance of these lines later in development as well. However, our group has found that it is not possible to treat plants with sorbitol using the hydroponics system employed for the salt treatments as contamination of the sorbitol-supplemented PN media occurs. An alternative approach could be to impose drought/water deficit stress on the plants grown in soil. This type of assay would, however, require significant optimisation as there are several factors that are difficult to control, such as variation in the amount of soil each plant is grown in, as well differences in drainage between plants. Nonetheless, further osmotic stress phenotyping of older 35S::*EXPA11* plants is something worth pursuing in the future.

A notable observation was that both of the 35S::*EXPA11* lines displayed improved growth compared with the EV in control conditions in the seedlings, whereas only one line was improved in the hypocotyl elongation assays, and neither were different from the EV in the hydroponically grown plants. This can likely be attributed to differences in the sampling times employed in these assays, and whether or not the time periods used allow for further growth capacity. The hydroponically grown plants were sampled after four weeks, by which time they had reached maturity and started bolting. It is plausible that upon reaching maturity, the 35S::*EXPA11* plants ceased growing slightly earlier than the EV plants, thus allowing for the EV plants to grow to the same final size in control conditions. However, in saline conditions, when the 35S::*EXPA11* plants have not reached their final size, they continue growing for the entire period, thus their improved growth phenotype compared with the EV is apparent. Likewise, for the hypocotyl elongation assays, while an elongation period of five days has been shown to be suitable for assessing genotype-specific growth differences (Zhao et al., 2002;

Cackett, 2019), it is the maximum time period that should be employed as, after which, the hypocotyls become too long and start to fall over whereafter their growth is reduced. It is possible that this was the case for the one 35S::*EXPA11* line, and as such the EV had grown to the same size by the end of the five day period. By contrast, the seedlings were sampled after eleven days growing on agar plates, a time point at which there are no limiting growth constraints. Thus the 35S::*EXPA11* lines were still actively growing in control conditions, and the improved growth phenotype compared with the EV was observed as it is in saline conditions. It is possible that the improved growth phenotype of the 35S::*EXPA11* lines would be more readily observable if sampling was performed at more time points (as opposed to just a single point), allowing for an assessment of growth rates over time. However, given that a destructive sampling approach is employed in our assays (Negrão, Schmöckel & Tester, 2017), this would require a very large number of plants to be grown, especially for the hydroponics assays where multiple NaCl concentrations were assessed. In the future, it would be prudent to perform such assays where the growth of the 35S::*EXPA11* and EV lines are compared over time. There are non-destructive sampling approaches involving image acquisition technology that are currently in use to accurately record changes in plant growth in response to salinity at regular time intervals, allowing for high throughput phenotyping (Berger, de Regt & Tester, 2012; Negrão, Schmöckel & Tester, 2017). Such phenomics approaches could be employed to further assess the growth of the 35S::*EXPA11* lines in saline conditions.

Given the increased shoot growth of the 35S::*EXPA11* line during salinity stress, the differences in leaf cellular morphology observed between the two lines were unexpected. In the EV control line, exposure to salt stress resulted in a reduction in leaf size, but there was no difference in either the epidermal or palisade mesophyll cell size compared with control conditions. This would suggest that exposure to salinity caused an inhibition of cell division, and thus there were fewer cells present in the salt-stressed EV leaves. It is well established that exposure to salt stress induces a rapid growth reduction, and there is evidence that growth arrest is, at least in part, achieved through modulation of the cell cycle. Rapid growth reduction in *Arabidopsis* roots was attributed to a transient inhibition of the cell cycle, resulting in a decrease in cell production in the meristem (West, Inzé & Beemster, 2004). Here, a decrease in cyclin-dependent kinase (CDK) activity and cyclin B 1;2::*GUS* promoter activity led the authors to suggest that cell cycle arrest occurred at the G2/M transition. Additionally, inhibition of root growth in salt-stressed maize seedlings was attributed to S-phase cell cycle arrest, with an observed reduction in cyclinB1 and A1 (CYCB1 and CYCA1) protein levels, as well as

decreased transcription of *CDKA*, *CDKB*, *CYCA*-family, and *CYCB*-family genes (Kamal et al., 2021). It is very plausible that the growth reduction due to inhibited cell division observed in salt-stressed EV leaves could be as a result of a similar mechanism involving cell cycle arrest.

In control conditions, the 35S::*EXPA11* line had larger epidermal and palisade mesophyll cells than the EV, however no difference in leaf area was observed. This is in direct contrast to the *EXPA10*-overexpressing plants previously mentioned, where increased leaf length was explained by an increase in petiole cell length (Cho & Cosgrove, 2000). It had been anticipated that improved growth would also be correlated with increased cell size in the 35S::*EXPA11* line. Perhaps an even more unexpected observation was that both epidermal and mesophyll cell size was decreased in response to salt stress in the 35S::*EXPA11* line, but was unaffected in the empty vector line, despite the 35S::*EXPA11* having significantly increased leaf size. Presumably, an explanation for this might be that instead of an increase in cell size, there is an increase in the number of (smaller) cells in the leaves of the 35S::*EXPA11* line. Unfortunately, cell number was not counted in our analyses, so the microscopy analysis of the embedded semithin leaf sections would have to be repeated to ascertain whether this is the case or not. Plant growth is achieved by cell growth/expansion, and cell division which leads to increased cell numbers. The balance between cell growth and the cell cycle integrates a complex, and poorly understood, network of feedback mechanisms involving various hormones, cell-signalling pathways, and cell wall functions (reviewed in Sablowski and Carnier Dornelas, 2014). An interesting phenomenon observed in plants is that of ‘compensation’ whereby the cell number and cell size in a particular organ are often balanced to maintain the overall size of that organ (Tsukaya, 2002). Alterations in *EXPA* expression regulate leaf growth in a complex manner by affecting both cell size and number. Furthermore, these affects can be different in a tissue-specific or temporal manner. There are several examples of increased *EXPA* expression improving growth through increased cell size: In the *EXPA10*-overexpressing plants, no change in petiole cell number was observed, so the increase in leaf length was solely attributed to an increase in cell length (Cho & Cosgrove, 2000). Likewise, increases in leaf, and petal limb size were attributed to increased cell expansion, and not changes in cell number, in transgenic aspen overexpressing *PttEXPA1*, and transgenic petunia plants overexpressing *PhEXPA1*, respectively (Gray-Mitsumune et al., 2007; Zenoni et al., 2011). There is also evidence that altered expansin expression affects leaf growth via changes in cell division: transgenic *Arabidopsis* plants where *EXPA1*, 3, 5, and 10 were knocked down

using an inducible amiRNA system displayed reduced laminal area while having fewer, but larger lamina epidermal and mesophyll cells (Goh et al., 2012). However, the authors did not comment on the mechanism whereby reduced *EXPA* expression could cause alterations in cell cycle signalling. Furthermore, the degree of the increase in *EXPA* gene expression can have opposite effects on growth in the same tissue, as was shown in transgenic Arabidopsis plants in which the cucumber *CsEXPA1* was inducibly-expressed (Goh et al., 2014). Upon moderate induction of *CsEXPA1* expression, lamina area as well as petiole length were increased, however upon high *CsEXPA1* expression induction, a large reduction in petiole length was observed concomitant with a decrease in both petiole cell length and number. Interestingly, while the lamina area was unaffected in the highly induced plants, fewer, larger lamina mesophyll/epidermal cells were present. This study speaks to the complex effect of *EXPA* expression on cell expansion and cell proliferation. It is clear that overexpression of *EXPA11* results in larger epidermal and mesophyll cells in control conditions, however, the observation that total leaf area is not significantly different in the 35S::*EXPA11* line compared with the EV line in control conditions can likely be explained by the process of compensation – the increase in cell size caused a reduction in cell division to yield leaves of the same size with fewer, larger cells. It must be noted that there is still a lot to be understood about compensation and the underlying signalling/feedback mechanism between cell expansion and division.

It is also clear that overexpression of *EXPA11* results in improved leaf growth under saline conditions. What is unclear though, is why an increased number of smaller cells would be the mechanism whereby this is achieved. Turgor-driven cell expansion is thought to be a cyclical process involving four steps (Wolf, Hématy & Höfte, 2012). Firstly, as has been extensively discussed earlier, PM H⁺-ATPase-driven apoplast acidification triggers the uptake of water into the cell, increasing turgor pressure, as well as activating expansin proteins which loosen the cell wall. Additionally, modification of the pectin matrix in which the cellulose microfibrils are embedded occurs, which further increases cell wall extensibility. The increased turgor and loosened cell wall result in an expansion in cell size. Thirdly, the increase in cell expansion is thought to activate a mechanosensing feedback mechanism that results in crosslinking, and dehydration of the cell wall to prevent further expansion. Here, it is thought that plasma membrane stretching caused by increased turgor opens stretch-activated calcium channels resulting in increased cytosolic Ca²⁺ levels that reduce growth via multiple mechanisms: it inhibits the PM H⁺-ATPase resulting in apoplast alkalisation and subsequently the inhibition of expansins, and the activation of pectin methylesterases which cross-link pectin.

Additionally, it activates the release of superoxide into the cell wall via the PM NADPH-oxidase, and the resulting H_2O_2 that is produced cross-links components in the cell wall, stopping extension. Finally, new cell wall material, such as cellulose microfibrils, is secreted to increase the strength of the cell wall. Given that cell-wall loosening by expansins forms a small part of a greater mechanism to promote expansion growth, it is possible that one of these other processes might be limiting cell expansion under salinity stress in the 35S::*EXPA11* line, despite its increased cell expansion phenotype under control conditions. Salt stress is known to elicit a wide number of changes in cell wall structure and biology (reviewed extensively in Colin et al. (2023)), many of which directly impact the four-step cell expansion process mentioned above. An obvious negative effect of salinity on cell expansion is that it lowers the osmotic potential of the plant, thereby reducing cellular water uptake and turgor pressure (van Zelm, Zhang & Testerink, 2020). The possibility thus exists that the turgor pressure is insufficient to drive cell growth, despite the increased *EXPA11* expression, and presumably increased cell wall loosening, in the 35S::*EXPA11* line. Additionally, salt-induced ROS production can result in the remodelling of the cell wall through the activation of peroxidases (Tenhaken, 2015). Peroxidase activity cross-links various cell wall components, resulting in local cell wall stiffening that is proposed to strengthen the cell wall to better withstand the osmotic changes imposed by salinity. Perhaps then increased ROS production under saline conditions has rendered the cell walls too rigid, preventing cell expansion despite increased *EXPA11* expression. Another possible explanation for decreased cell size in the 35S::*EXPA11* line is that PM H^+ -ATPase activity is decreased under saline conditions, so despite the potentially increased *EXPA11* present, the apoplastic pH is not low enough for maximal expansin activity. Salinity has been shown to induce apoplastic alkalinization in the leaves of maize and *Vicia faba* (Pitann, Schubert & Mühling, 2009; Pitann et al., 2011), lending some credence to this suggestion.

Despite the unexpected changes in leaf cell morphology observed, it would indeed appear that over-expression of *EXPA11* confers improved shoot growth in older plants under saline conditions. There is evidence in the literature linking larger plant size with improved salinity tolerance; in a study investigating natural variation in the effect of salinity on rosette size in *Arabidopsis* accessions, improved salt-tolerance was positively correlated with larger plant size (Julkowska et al., 2016). Furthermore, accessions with larger rosette size tended to exhibit less cellular damage, as measured by electrolyte leakage, under salinity stress. The authors proposed that the larger shoot volume effectively dilutes the influx of Na^+ into the shoot,

thereby preventing Na⁺ accumulation to the toxic levels that result in severe growth reduction. However, it must be noted that Na⁺ contents were not measured across the different ecotypes in this study, so this hypothesis should be considered with some degree of caution.

5.4.3. Overexpression of *EXPA11* has no effect on Na⁺ and K⁺ ion homeostasis

There is some evidence in the literature to suggest that overexpression of expansins can lead to a beneficial effect on ion homeostasis within the context of salinity tolerance; a transgenic rice overexpressing *OsEXPA7* was shown to have increased salinity tolerance, as measured by improved growth and survival, and improved Na⁺/K⁺ ratios in the root and shoot, due to decreased Na⁺ accumulation and increased K⁺ retention (Jadamba et al., 2020). The decreased Na⁺ accumulation was attributed to significantly higher *OsSOS1* expression, and therefore Na⁺ extrusion. Additionally, *OsNHX4* expression was increased in the roots, suggesting improved vacuolar Na⁺ sequestration. Considering this, I was interested to determine whether overexpression of *EXPA11* could have an effect on ion homeostasis, especially considering that there is increased *EXPA11* expression in the 35S::*NIT2* line, which has been shown to have improved ion homeostasis. However, the results of the ICP-OES analysis show that the overexpression of *EXPA11* has no effect on the root/shoot partitioning of Na⁺ or K⁺ in either control or saline conditions. The observation that Na⁺ accumulation was far greater in the shoot than the root in the pB2GW7 EV and 35S::*EXPA11* lines is consistent with what was observed for their genetic background, No-0 (see chapter 2, Figure 2.1 B). Clearly the improved growth of the 35S::*EXPA11* line under saline conditions cannot be attributed to an ability to better maintain Na⁺ within the root and thereby limit its accumulation to toxic levels within the aerial tissues, as has been postulated for the 35S::*NIT2* line. Furthermore, salt stress resulted in the anticipated reduction in root K⁺ content that occurs due to K⁺ efflux via the outward-rectifying K⁺ channel, GORK, in response to membrane depolarisation due to the increase in root Na⁺ uptake (Shabala & Cuin, 2007; Demidchik, 2014). The reduced root and shoot K⁺ observed in response to salt stress in the pB2GW7 EV and 35S::*EXPA11* lines is also consistent with the reduction observed in their genetic background, No-0 (see chapter 2, Figure 2.1 D). Thus, the pattern of root/shoot Na⁺ and K⁺ partitioning under both control and saline conditions is the same for No-0, the pB2GW7 EV, and 35S::*EXPA11* lines. Thus, while the overexpression of *EXPA11* may improve growth under saline conditions, this clearly cannot be attributed to an alteration in ion homeostasis. This is an interesting result as it suggests that salt-tolerance can be improved through increasing growth only, without affecting ion homeostasis. The observation that Na⁺ accumulation was the same between the two lines provides evidence

against the hypothesis proposed by Julkowska et al. (2016), suggesting that larger plant size and shoot volume effectively dilutes Na^+ to reduce its toxic effect. Larger plant size must improve salinity tolerance through some other unknown mechanism.

5.4.4. Summary

In summary, several novel transgenic *EXPA11* overexpressing lines were generated, that were found to have altered phenotypes compared with their EV genetic background. In particular, overexpression of *EXPA11* conferred improved shoot growth under saline conditions, which could be observed at multiple developmental stages. The finding that the 35S::*EXPA11* lines had improved etiolated hypocotyl elongation under saline conditions suggested that the improved shoot growth phenotype was established from the earliest point of shoot development. Additionally, it not only provided further evidence towards the purported role of *EXPA11* in hypocotyl elongation but was the first to suggest a role for *EXPA11* in early seedling establishment under saline conditions. Overexpression of *EXPA11* also altered leaf cell morphology, albeit in an unexpected way: the increased cell sizes observed in control conditions in the 35S::*EXPA11* line did not translate to increased leaf size compared with the EV, whereas in saline conditions, the 35S::*EXPA11* line had increased leaf size, but smaller cells. This finding highlighted the complexity underlying the balance between cell expansion and division during plant growth, and the need to better understand these mechanisms, particularly with the added effect of salinity. Based off the results in this chapter, I propose that overexpression of *EXPA11* improves salinity tolerance by increasing shoot growth, and importantly, without causing a growth penalty under control conditions.

CHAPTER 6: GENERAL DISCUSSION

6.1. Contribution to the field

The overall aim of this thesis was to test various aspects of our proposed molecular model whereby auxin modulates *Arabidopsis* growth in response to salt stress (refer again to Figure 1.4). In particular, I focused on further characterising the increased salinity tolerance of the 35S::*NIT2* line, by assessing salt-responsive alterations in ion homeostasis, the expression of ion transporters/channels, and changes in the shoot PM proteome. Additionally, to assess the contribution of *EXPA11* as a growth effector to improve salinity tolerance that acts downstream of auxin-induced PM H⁺-ATPase activation, I attempted to knock down *EXPA11* expression in the 35S::*NIT2* line, so as to delineate its contribution to ion homeostasis and expansion growth in this line. Finally, I generated and characterised *EXPA11*-overexpressing lines to determine if improved plant salt-tolerance can be achieved by altering expansion growth only, without affecting ion homeostasis.

6.1.1. Increased auxin biosynthesis in the 35S::*NIT2* line improves ion homeostasis in response to salt stress

The ICP-OES analysis revealed that the 35S::*NIT2* line had an improved ability to restrict Na⁺ to the root, thereby limiting its translocation to and accumulation in the shoot, as well as improved root K⁺ retention compared with the wild-type under saline conditions (Figure 2.1 B and D). Collectively, this resulted in improved K⁺/Na⁺ homeostasis, as seen by increased K⁺/Na⁺ ratios, in both the root and the shoot, which is established to be a key indicator of salt-tolerance (Figure 2.1 F). It should be noted that ICP-OES only allowed for the quantification of Na⁺ and K⁺ at the whole tissue level (root or shoot). As I do not know where these ions are located at a tissue-specific, cell type-specific, or sub-cellular level, the understanding of the alterations in Na⁺/K⁺ homeostasis between the two lines is therefore somewhat limited. Nonetheless, these alterations might be explained, at least in part, by differences in the expression of several Na⁺ and K⁺ transporters/channel genes between the 35S::*NIT2* line and No-0 (Figure 2.2), which led to the proposed model for improved ion homeostasis in the 35S::*NIT2* line under saline conditions (Figure 2.5). The key components of this model were: improved xylem Na⁺ retrieval due to higher root *HKT1;1* expression thereby limiting root to shoot Na⁺ transport; a potential restriction in root Na⁺ uptake due to reduced *GLR2.3* and *CHX17* expression; and enhanced Na⁺ vacuolar sequestration in the shoots due to increased *NHX3* expression. Additionally, the model included components of K⁺ homeostasis, such as improved root K⁺ uptake due to increased *AKT1* expression, and improved xylem K⁺ loading

in the root and unloading in the shoot via increased *SKOR* and *KUP1* expression, respectively. It must be acknowledged that the analysis of transporter/channel gene expression was by no means exhaustive, and it is very likely that there are other transporters/channels that are differentially expressed between the two lines in saline conditions that could also contribute to the alterations in ion homeostasis.

Obviously it is critical to confirm that the differential mRNA levels of these transporters/channels between No-0 and 35S::*NIT2* plants is also displayed at the protein level. Unfortunately, none of these transporters/channels were detected in the proteomic analysis of the shoot ePM proteins of 35S::*NIT2* and No-0 plants, presumably as they are low abundance proteins. A more targeted approach involving antibody pull-downs would likely be required to measure their abundance in the future. It could also be possible that an in-gel tryptic digest prior to mass spectrometry might not be the best way of detecting integral PM proteins like these transporters. Separating hydrophobic membrane proteins via PAGE can be difficult (hence the use of the Bis-Tris PAGE protocol employed here). Additionally, chymotrypsin has been shown to be a more effective protease than trypsin for the digestion and subsequent identification of peptides from the hydrophobic transmembrane domains of membrane proteins (Giannone et al., 2015). Thus, it could be worthwhile to instead analyse these PM protein samples by in-solution chymotrypsin digestion. Of those that were detected, NHX1 protein abundance was consistent with *NHX1* transcript levels (Figure 2.2 D), with no difference observed between the two lines in either control or saline conditions. However, TIP2;2 protein levels were lower in the 35S::*NIT2* line than in No-0 under saline conditions with no difference between the two lines in control conditions (Table 3.6), as opposed to *TIP2;2* mRNA levels, which were significantly lower in the 35S::*NIT2* line in control conditions with no difference between the two lines in saline conditions (Figure 2.2 N). Furthermore, AHA2 protein levels were increased in the shoots of the 35S::*NIT2* line compared with No-0 under saline conditions (Table 3.6), complementing what was observed for mRNA abundance (Figure 2.3 A), although no difference was observed between the two lines under control conditions as was the case for *AHA2* transcript levels. The proteomics analysis also revealed the largely uncharacterised KUP8 as a potential contributor to altered shoot K⁺ homeostasis, as its abundance was significantly increased in the 35S::*NIT2* line compared with No-0 under saline conditions (Table 3.6). Although not detected in the proteomics analysis, *KUP1* expression was also higher in the 35S::*NIT2* line under saline conditions (Figure 2.2 G). Together, this indicates that altered K⁺ transport via HAK/KUP/KT transporters might be an important mechanism for improved K⁺ homeostasis in the shoots of the 35S::*NIT2* line during salt stress. It must also be

noted that a matched proteomic analysis (as well as a targeted antibody pull-down approach) of the root ePM proteins of 35S::*NIT2* and No-0 plants is required in order to complement the root transporter/channel expression data, as was attempted for the shoot.

Altered K⁺ and Na⁺ transport is not only determined by changes in the protein levels and activity of ion transporters/channels but also by the electrochemical potential across the PM which is generated by the PM H⁺-ATPase and powers ion/H⁺ antiport and symport, as well as affecting the open state probability of voltage dependent ion channels. Our findings of increased *AHA2* expression and protein abundance in the shoots of the 35S::*NIT2* line compared with No-0 under saline conditions could possibly suggest that PM potential to power K⁺ and Na⁺ transport is increased, however, this needs to be directly measured in the future.

6.1.2. The 35S::*NIT2* line has an altered shoot PM proteome that may pre-dispose it to improved salt-tolerance

As the plasma membrane forms the barrier between the cell and the high concentrations of Na⁺ in the external environment under saline conditions, it contains a number of proteins functioning in Na⁺ sensing and signalling, ion transport, growth modulation, and salt stress responses. The results of the label free quantitative LC-MS/MS analysis of ePM samples from the 35S::*NIT2* line and No-0 are the first to report on the salt-stress PM proteome of *Arabidopsis* leaves. Importantly, the proportion of PM proteins that were identified in the ePM samples was similar to that which has been reported in previous studies (Zarrini et al., 2012; Bernfur et al., 2013), indicating that successful enrichment occurred. PM proteins involved in a broad range of processes were salt-responsive in No-0 (Table 3.3). Perhaps most notably, *NIT2* was only detected in response to salt, providing very good evidence to support the overall hypothesis that auxin synthesis is up-regulated to modulate growth under saline conditions. Further evidence to support this was that *BGLU18*, which converts GB to IAN, thus providing the substrate that is converted to IAA by *NIT2* (Hornbacher et al., 2022), and *CPK29*, which functions in PIN trafficking to affect PAT (Lee et al., 2021), were also up-regulated in response to salt. As expected, several proteins with an established role in the response to salt or osmotic stress were also differentially regulated (*ERD7*, *NCL*, *ABCC4*, *PATL1*, *ANN1*). By contrast, the more salt-tolerant 35S::*NIT2* line had just four salt-responsive PM proteins, one of which was *NIT2* (Table 3.4). The comparison of the PM proteomes between the two lines revealed genotype-specific differences under both control and saline conditions (Tables 3.5 and 3.6, respectively). As expected, *NIT2* protein levels were constitutively increased in the 35S::*NIT2* line, and auxin-related proteins also had increased abundance (*ALA9*, 10, and 11, and

ABCG36), providing further evidence that auxin-responsive processes are activated in this line even in the absence of salt. Furthermore, several of the notable salt-responsive proteins in No-0 were already found to have altered levels in the 35S::*NIT2* in control conditions, and were not differentially regulated in response to salt (ERD7, PATL1, ABCC4, DRP2B). I propose that these could be particularly important contributors to the increased salt-tolerance of the 35S::*NIT2* line.

The increased levels of several ABC family transporters involved in the regulation of stomatal conductance (ABCC4/ABCC5 and ABCG22 in control and saline conditions, respectively) suggests that stomatal aperture may be altered in the 35S::*NIT2* line compared with No-0. This complements the finding that the expression of the *KAT1* and *GORK* K⁺ channels, which function in the regulation of guard cell K⁺ levels and thus regulation of stomatal opening (Nakamura et al., 1995; Ache et al., 2000), also differed in the shoots between these two lines. While rapid stomatal closure upon exposure to salinity is one of the key osmotic responses implemented to limit water loss via transpiration, the resulting reduction in CO₂ assimilation severely limits photosynthesis and thus growth (James et al., 2008; Tilbrook & Roy, 2014). An improvement in stomatal conductance, as is suggested for the 35S::*NIT2* line, would thus be beneficial for growth under saline conditions as it would allow for the maintenance of photosynthetic carbohydrate production – this has been observed in a study of 50 wheat genotypes where increased stomatal conductance was strongly correlated with growth rate under salt stress (James et al., 2008). However, this would likely come at the cost of increased water loss. The question then remains whether maintenance of growth or reduction in water loss is more beneficial overall to salt-tolerance. Auxin is thought to play a positive role in stomatal opening, as it causes guard cell PM H⁺-ATPase activation that hyperpolarises the PM, facilitating K⁺ influx via *KAT1*, resulting in increased guard cell turgor that causes stomatal opening (Nakamura et al., 1995; Acharya & Assmann, 2009). It must also be noted that auxin plays an inhibitory role in stomatal differentiation and patterning, a process which involves coordinated signalling between almost all of the plant phytohormones (Wei et al., 2021). Presumably then increased auxin in the 35S::*NIT2* line may increase stomatal conductance, while reducing stomatal number. To further explore this hypothesis, it would be worthwhile to measure differences in stomatal number, aperture, water use efficiency, and leaf water loss between the 35S::*NIT2* line and No-0 under control and saline conditions.

6.1.3. The nature of the improved salt-tolerance of the 35S::*NIT2* line

It is apparent from both the ion transporter/channel expression and PM proteomics analyses that the salt stress response of the 35S::*NIT2* line is altered compared with No-0. The data presents several, not mutually exclusive, possibilities as to the nature of this improved salt-tolerance. The lack of salt-responsive PM proteins in the 35S::*NIT2* line, and the observation that a subset of the salt-responsive proteins in No-0 had altered abundance in the 35S::*NIT2* line in control conditions (ERD7, PATL1, ABCC4, DRP2B), or altered abundance in both control and saline conditions (ABCG36, ANN4, DUR3), could suggest that the processes that these proteins regulate or are involved in are constitutively activated in the 35S::*NIT2* line. The idea that some aspects of the salt stress response are constitutively active in the 35S::*NIT2* line could possibly explain the growth penalty that is observed in mature plants under normal growth conditions (Figure 4.16). It is well established that, while increasing resistance to pathogens, constitutive defence activation often leads to a reduction in plant growth in the absence of pathogenic threat – this is referred to as the growth-defence trade-off (Albrecht & Argueso, 2016). For example, while mutation in the negative regulator of immunity, *constitutive expresser of PR genes 30 (cpr30)*, leads to greatly enhanced resistance to the bacterial pathogen *Pseudomonas syringae*, the constitutive activation of defence responses leads to autoimmune phenotypes, including dwarfism (Gou et al., 2009). While not as extreme, it is possible that a similar growth trade-off is seen in the 35S::*NIT2* line due to the constitutive activation of certain salt stress responses.

Alternatively, the observation that some ion transporters/channels had a mis-regulated expression pattern in the 35S::*NIT2* line (SOS1, NHX3, KUP1, GORK, SKOR), where there was no difference in expression between the two lines in control conditions, but the down-regulation of these transporters/channels that was observed in No-0 in response to salt did not occur in 35S::*NIT2* plants, may indicate that the response of this line to salt stress is dampened. I propose that the maintained expression of these genes, along with the other ion transporters/channels that are differentially expressed between the two lines, contributes significantly to the improvement of Na⁺/K⁺ homeostasis in the 35S::*NIT2* line. While the genetic basis of the mis-regulation of these transporter genes is unclear, it is possible that they are co-regulated in response to the elevated auxin levels in the 35S::*NIT2* line – a theory that should be tested in the future. The idea that the salt stress response is dampened in the 35S::*NIT2* line could be explained by the hypothesised increase in PM H⁺-ATPase activation, and the resulting hyperpolarisation of the PM, which would presumably increase the threshold for tolerance before Na⁺ starts to be taken up and depolarisation-induced K⁺ efflux occurs. This

is supported by the observation that less total Na^+ is taken up and more total K^+ is retained in the 35S::*NIT2* line compared with No-0 under saline conditions (Figure 2.1 B and D). Furthermore, it might explain why differential expression of the five ‘mis-regulated’ transporters/channels was not observed, as the 35S::*NIT2* plants are not stressed to the same extent as No-0 plants are at this specific salt concentration (75 mM NaCl). However, PM H^+ -ATPase activity needs to be measured in the 35S::*NIT2* line and No-0 in both control and saline conditions to provide proper evidence for this idea. In this scenario, the growth penalty seen in the 35S::*NIT2* line could possibly be explained by the huge ATP cost that would be required for the constant increased activity of the PM H^+ -ATPase.

Currently, there is insufficient evidence to suggest that the increased salt-tolerance of the 35S::*NIT2* line is specifically caused by constitutive activation of some aspects of the salt stress response, or rather that it is due to an attenuated response, or some combination of the two mechanisms.

An additional consideration is that while manipulation of genes high up in regulatory networks can be a highly successful method to increase stress tolerance, the complex nature of, and considerable cross-talk between, these regulatory pathways can lead to many undesired pleiotropic effects under normal conditions, including growth handicap (Cabello, Lodeyro & Zurbriggen, 2014). The over-expression of *NIT2*, and consequently the increase in auxin biosynthesis, is presumably one such example, given that auxin is involved in the regulation of almost all aspects of plant growth and development through its role in controlling cell expansion, division, and differentiation (Du, Spalding & Gray, 2020). It has been suggested that manipulation of direct-action ‘effector’ genes, which operate at the end of these regulatory pathways, is a more targeted approach to improve responses to specific stresses (Cabello, Lodeyro & Zurbriggen, 2014). From the results presented in this thesis, I suggest that EXPA11 is one such effector for improving growth under saline conditions. Similarly, it could also be worthwhile to target an effector that likely only alters ion homeostasis, for example KUP8, or one of the other Na^+/K^+ transporters/channels that had altered expression in the 35S::*NIT2* line. This would hopefully provide further insight into whether the contribution of altered ion homeostasis or expansion growth is more, or equally, important in the auxin-mediated growth modulation during salt stress, and thus which one of these aspects is better to target for improving plant salt-tolerance.

6.1.4. EXPA11 plays an important role in growth modulation under saline conditions

A key component of the proposed model of auxin-mediated growth modulation in response to salinity is the activation of expansins. In particular, EXPA11 appears to be central to this altered growth. *EXPA11* expression has been shown to be up-regulated in response to salt in two-week-old seedlings (Cackett et al., 2022), as well as being massively increased in 35S::*NIT2* seedlings compared with No-0, with a further up-regulation observed in response to salt (Cackett, 2019) – a finding which was validated in this thesis (Figure 4.1). In older plants, *EXPA11* expression was found to be much higher in the roots of the 35S::*NIT2* line than in the shoots (Figure 4.2), which notably did not display a reduction in growth upon salt-treatment (Figure 4.16 A), as opposed to that which was observed in the shoots (Figure 4.16 C). Furthermore, *EXPA11* expression was conspicuously absent in No-0 shoots, and was only detected in salt-stressed roots (at a much lower level than the 35S::*NIT2* line), both of which tissues displayed a significantly greater growth reduction compared with the 35S::*NIT2* line upon salt-treatment. These findings correlating endogenous *EXPA11* expression with improved growth suggest that EXPA11 may play an important role in growth modulation in response to salt stress, particularly so in the 35S::*NIT2* line. Unfortunately, the failure to knockdown *EXPA11* in the 35S::*NIT2* line made it difficult to further characterise this contribution, so it is presently unclear whether EXPA11 is a direct effector of NIT2 in response to salt, and if improved growth is necessarily a mechanism of salt-tolerance rather than just something that occurs in the 35S::*NIT2* line as a side-effect of the improved salt-tolerance conferred by improved ion homeostasis.

The possible enhancement of cell wall-loosening due to increased *EXPA11* expression, is not the only evidence that the cell wall may be altered in the 35S::*NIT2* line compared with No-0 under saline conditions. The proteomics analysis revealed multiple PM proteins involved in processes contributing to CWI that had altered abundance in the 35S::*NIT2* line following salt stress. In particular, GDPDL1 had increased abundance compared with No-0, and CSLG3 and CGR3 were only detected in the 35S::*NIT2* line (Table 3.6). The increased levels of these proteins suggests a possible mechanism to enhance CWI, thereby contributing to the improved salt-tolerance of the 35S::*NIT2* line. Additionally, it could also suggest that they contribute to the synthesis of the new cell wall following auxin and EXPA11-mediated expansion growth in the 35S::*NIT2* line under saline conditions. In order to further assess the role of EXPA11, it would be prudent to look at the cell expansion phenotypes of the 35S::*NIT2* line under control and saline conditions, as was done for the 35S::*EXPA11* line.

While endogenous *EXPA11* expression seems likely to contribute more to root growth in older plants, the overexpression of *EXPA11* appears to have a larger effect on shoot growth (although it must be noted that the nature of the plate and hydroponics assays employed make it difficult to assess changes in root system architecture). The generated 35S::*EXPA11* lines showed an improved shoot growth phenotype under saline conditions compared with the EV genetic background at multiple stages of development: they had increased hypocotyl elongation (Figure 5.6), the earliest stage of shoot development, as well as increased rosette fresh weight, leaf area, and overall shoot size in adult plants (Figures 5.4 C and D, 5.7 A, and 5.5). Additionally, two-week-old 35S::*EXPA11* seedlings were larger than the EV when grown under both salt and osmotic stress conditions, as well as in the control (Figure 5.3). In terms of testing our overall proposed model, it perhaps would have been informative to include the 35S::*NIT2* line in all of the phenotypic assays assessing the generated 35S::*EXPA11* lines; however, this was not logistically possible given the sheer number of total plants that would need to be grown, and the labour-intensive nature of the early development salt stress plate assays, and even more so, the hydroponics assays. Furthermore, given that the degree of *EXPA11* up-regulation was much higher in the generated 35S::*EXPA11* lines than what was observed in the 35S::*NIT2* line (Figure 5.1), it could be argued that a direct comparison of the effect of increased *EXPA11* expression on phenotype between them may not be appropriate. Importantly, over-expression of *EXPA11* improved shoot growth under saline conditions without causing a growth penalty under control conditions, unlike the 35S::*NIT2* line. A reduction in shoot growth is one of the main effects that long-time exposure to salt stress has on plant physiology, as the rate of leaves dying due to toxic Na⁺ accumulation begins to exceed the rate of new leaf growth, resulting in insufficient photosynthetic capacity to supply the necessary carbohydrates for growth maintenance (Munns & Tester, 2008). The ability to sustain/improve leaf growth under saline conditions, as was observed in the 35S::*EXPA11* lines, is thus a highly desirable trait for the generation of plants with improved salt-tolerance, particularly given that shoot growth typically determines crop yields and therefore is most relevant in an agricultural context. The findings here strongly suggest that *EXPA11* is a gene that could be used to engineer salt tolerance without causing an adverse effect on normal plant growth.

6.2. Future work

The main aspect of our proposed model, whereby auxin biosynthesis is up-regulated to modulate growth during salt stress, that has not been properly addressed in this thesis is whether

the increase in auxin in response to salinity results in an increase in the activity of the PM H⁺-ATPase. In order to address this, several experiments should be performed. A phosphoproteomic analysis should be conducted on ePM samples isolated from control and salt-stressed 35S::*NIT2* and No-0 plants grown according to the same experimental design as was used in chapter 3. This would allow for the identification of important salinity-induced phosphorylation events that occur to the PM H⁺-ATPase (as well as other PM proteins), such as the phosphorylation of Thr-947. Additionally, it would provide information on whether the increased auxin biosynthesis in the 35S::*NIT2* line results in increased post-translational activation of the PM H⁺-ATPase. Alternatively, a more targeted approach could be adopted using the available AHA2 antibody to pull-down the PM H⁺-ATPase, followed by digestion and MS analysis to detect whether it is phosphorylated based off peptide fragment mass shifts. Such evidence for auxin-induced PM H⁺-ATPase post-translational activation should be considered in conjunction with physiological measurements of both outputs of PM H⁺-ATPase activity, namely H⁺ pumping and ATP hydrolysis, which can be measured in PM vesicles isolated from control and salt-stressed 35S::*NIT2* and No-0 plants using established protocols, such as Larsson & Widell (2000). It would also be prudent to measure root and shoot apoplast acidification *in planta* in response to salt stress in the 35S::*NIT2* line and No-0, as this would provide a good indication of whether increased PM H⁺-ATPase activity could lead to expansin-mediated acid growth modulation in these tissues. This could be done using the fluorescent pH indicator 8-hydroxypyrene-1,3,6-trisulfonic acid tri-sodium salt (HPTS), as was elegantly demonstrated by Barbez *et al.* (2017), who were able to measure concentration-dependent auxin-regulated changes in apoplastic pH at a cellular resolution in Arabidopsis roots. Crude attempts were made to measure both the acidification of apoplastic fluids extracted from leaves, as well as the acidification of media surrounding the roots, of 35S::*NIT2* and No-0 plants grown under control and saline conditions (data not shown), however these techniques did not yield quantitative results, so potential differences in PM H⁺-ATPase activity could not be inferred.

Given that the PM proteomics analysis of the shoots of control and salt-stressed 35S::*NIT2* and No-0 plants revealed several interesting salt-responsive PM proteins and genotype-specific differences, it would also be worthwhile to perform a matched proteomic analysis on root PM samples in order to further characterise the salt-stress PM proteome and identify root PM proteins that could contribute to the improved salt-tolerance phenotype of the 35S::*NIT2* line. This will be particularly important considering that, as it is the point of entry for Na⁺ into the plant, the response of the root plays a large role in the overall response of the plant. It would

also be prudent to confirm some of the proteomics data presented here using Western blot analysis to detect/quantify some of the proteins of interest for which antibodies are available. Furthermore, it is necessary to perform further functional characterisation of the most noteworthy ‘salt-responsive’ PM proteins to confirm whether they are involved in salt-tolerance – this should be done via performing salt stress assays with publicly available T-DNA mutants for these top candidates. Together, these experiments would greatly increase the confidence in our proteomics data. Additionally, in order to measure the levels of some of the ion transporter/channel proteins that were not detected in the proteomics analysis (but whose gene expression was measured), a targeted antibody approach, on both shoot and root ePM samples from control and salt-stressed 35S::*NIT2* and No-0 plants, could be utilised.

I have demonstrated that improved ion homeostasis is likely a major contributing factor towards the improved salt-tolerance of the 35S::*NIT2* line, with observed differences in ion contents, and altered expression of various Na^+/K^+ transporters/channels at the protein and transcript level. However, it is important to link the altered ion homeostasis under salt stress conditions with the action of these transporters/channels to determine their individual contributions to the transport of Na^+ and/or K^+ in the roots and shoots of the 35S::*NIT2* line and No-0. This could be addressed through *in planta* ion flux measurements using the non-invasive microelectrode ion flux measurements (MIFE™) technique, which allows for the concurrent high temporal and spatial resolution measurement of multiple different ions, such as H^+ , K^+ and Na^+ (Shabala et al., 2013). Additionally, the contribution of individual transporters/channels to overall Na^+ and K^+ flux in the 35S::*NIT2* line could be determined by MIFE™ analysis in conjunction with various channel inhibitors or blockers.

One of the major aims of this thesis that I was unable to address was the delineation of the contribution of altered ion homeostasis and *EXPA11*-mediated expansion growth to the improved salt-tolerance of the 35S::*NIT2* line. I suggest that the use of CRISPR/Cas9 technology to generate *EXPA11*-knockout 35S::*NIT2* lines, and subsequent characterisation of these lines should allow for this particular aim to be achieved. Additionally, an *EXPA11*-knockout mutant within the No-0 wild-type background should be generated and characterised, to complement the characterisation of *EXPA11*-overexpression and further the understanding of the role of *EXPA11* in growth modulation under saline conditions.

The characterisation of the 35S::*EXPA11* lines focused mainly on shoot development phenotypes under saline conditions, but as endogenous *EXPA11* expression was shown to be particularly high in the roots of the 35S::*NIT2* line, it would be prudent also to assess root development phenotypes (PR elongation, LR emergence, number and elongation, halotropic

responses, cell size/morphology analysis) in these lines. Additionally, the improved growth and survival of the 35S::*EXPA11* seedlings when grown on sorbitol suggests that *EXPA11* overexpression not only contributes towards salt stress tolerance, but potentially also to osmotic stress tolerance. Further characterisation of these 35S::*EXPA11* lines under osmotic stress conditions is thus suggested.

The cell size analysis of 35S::*EXPA11* leaves revealed that increased *EXPA11* expression resulted in altered cell sizes compared with the EV background in both control and saline conditions. However, given the unexpected observation of larger cells in the 35S::*EXPA11* line without altered leaf area under control conditions, yet smaller cell size and increased leaf area under saline conditions, more work needs to be done to understand how the modulation of *EXPA11* expression affects cell size and number. To address this, transcriptomics experiments could be performed on the 35S::*EXPA11* plants to measure the expression of genes involved in cell growth, division, and the regulation of the cell cycle. Additionally, considering the role of EXPAs in affecting cell wall dynamics and the large-scale affect that salinity has on the cell wall (see (Colin et al., 2023)), potential changes in cell wall structure, such as cellulose and pectin contents, extensibility, and the degree of cross-linking could be analysed in these lines (methods to determine these and other cell wall parameters are widely discussed in Cosgrove, (2022)). It would also be worthwhile to assess changes in cell size in the roots of these lines to complement the shoot data, as well as in the leaves and roots of the 35S::*NIT2* line, to determine if higher endogenous *EXPA11* expression causes similar phenotypes as when it is overexpressed.

6.3. Conclusion

Several aspects of the proposed model whereby auxin biosynthesis is up-regulated to modulate Arabidopsis growth in response to salinity were further characterised in this thesis. The previously observed improved salt-tolerance phenotype of the auxin-overproducing 35S::*NIT2* line could be attributed to beneficial alterations in Na^+/K^+ homeostasis caused by altered expression of several ion transporters/channels, supporting the idea that increased auxin-induced PM H^+ -ATPase activation powers improved ion homeostasis during salt stress. Measurement of salt-responsive changes in PM H^+ -ATPase activity is clearly needed in order to fully support our proposed model. Additionally, the 35S::*NIT2* line was found to have an altered shoot PM proteome that may pre-dispose it towards increased salt-tolerance, with a subset of PM proteins likely particularly important in this regard. This data provides novel

information on salt-responsive changes in the plasma membrane proteome of *Arabidopsis* shoots. The increased abundance of NIT2 observed in No-0 provided strong evidence to support the hypothesis that auxin biosynthesis is up-regulated in response to salt stress. Additionally, EXPA11 was identified as a growth effector acting downstream of the hypothesised PM H⁺-ATPase activation, with greatly increased expression observed in the 35S::NIT2 line compared with No-0, and a further up-regulation in response to salt. The novel lines overexpressing *EXPA11* displayed improved shoot growth under saline conditions at multiple developmental stages, and importantly no growth penalty was observed in normal conditions, strongly suggesting that EXPA11 is a good effector to target for improving salt-tolerance.

CHAPTER 7: APPENDIX

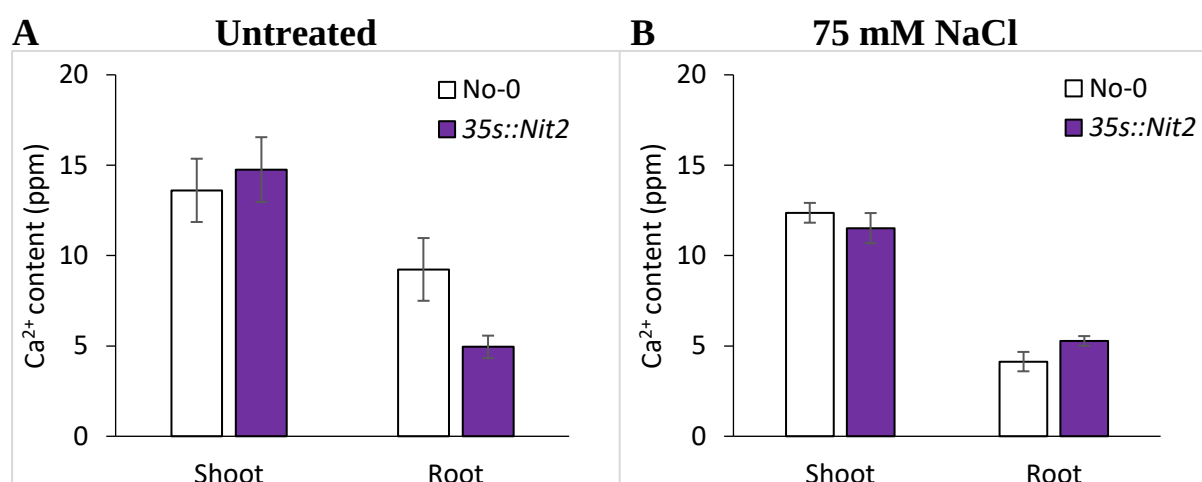


Figure 7.1: Ca^{2+} content in root and shoot tissue of 35S::NIT2 and No-0 plants exposed to salt stress

The No-0 and 35S::NIT2 plants were grown hydroponically for three weeks on ¼ strength PN media, then, either treated with ¼ strength PN media supplemented with 75 mM NaCl, or maintained on ¼ strength PN media (untreated control) for one additional week. Root and shoot tissue were harvested, oven dried, and then ashed and acid-digested. The ion content (expressed in $\mu\text{g}\cdot\text{g}^{-1}$ dry weight (DW)) was determined via ICP-OES analysis. **(A)** Ca^{2+} content in roots and shoots of No-0 and 35S::NIT2 plants grown in untreated conditions. **(B)** Ca^{2+} content in roots and shoots of No-0 and 35S::NIT2 plants following 75 mM NaCl treatment. The results are an average of six biological repeats ($n=6$). Asterisks (*) denote a statistically significant difference between No-0 and the 35S::NIT2, as determined by independent T-tests (* $p \leq 0.05$, ** $p < 0.005$, *** $p < 0.0005$). Bonferroni correction for multiple testing was performed. Error bars indicate standard error.

Table 7.1: Number of transcription factor binding sites identified by FIMO analysis in the -1000 bp upstream promoter sequences of the Na^+/K^+ transporter genes with ‘mis-regulated expression’ in the 35S::NIT2 line

Transcription Factor types	HKT1;1	SKOR	AKT1	SOS1	KUP1	NHX3	GORK	KAT1
MYBs	4	23	9	5	8	23	12	5
MYCs	0	0	0	4	0	6	0	2
WRKYs	0	2	31	2	5	4	7	23
bZIPs	6	0	0	2	0	13	5	4
bHLHs	3	8	0	5	0	13	0	8
NACs	46	9	40	2	2	6	7	15
ARFs	3	0	0	1	1	1	2	0

Note: Find Individual Motif Occurrences analysis was performed using the FIMO tool of MEME Suite version 5.4.1 software (<https://meme-suite.org/meme/tools/sea>) (Grant, Bailey & Noble, 2011). The promoter sequences were searched using the JASPAR CORE Position Frequency Matrix (PFM) non-redundant plant motif sequences obtained from JASPAR (<https://jaspar.genereg.net/downloads/>). The Araport11 loci – 1000 bp upstream promoter sequences were obtained using the Sequence Bulk Download tool on The Arabidopsis Information Resource (TAIR) (<https://www.arabidopsis.org/tools/bulk/sequences/index.jsp>).

Table 7.2: Binding sites of Transcription factors previously linked with salt stress in the -1000bp upstream promoter sequences of the all the Na⁺/K⁺ transporter genes with mis-regulation of expression in response to salt-treatment in the 35S::*NIT2* line as identified by MEME Suite FIMO analysis

motif_id	motif_alt_id	Promoter sequence	start	stop	strand	score	p-value	q-value	matched sequence
MA1179.1	MYB1	AT5G46240_KAT1	124	142	-	13.1184	0,00002	0.253	AATGCGGTTATAATTGACA
MA1179.1	MYB1	AT5G55470_NHX3	67	85	-	10.9605	9.13e-05	0.596	ATAGCCGTTTCTACACTAA
MA0574.1	MYB15	AT2G01980_SOS1	94	106	+	8.29268	3.26e-05	0.71	CGACGGTTGGTCG
MA0574.1	MYB15	AT3G02850_SKOR	195	207	+	4.41463	8.83e-05	0.959	CAAAGGTCGGTTA
MA1768.1	MYB30	AT4G10310_HKT1;1	358	372	+	12.7541	2.07e-05	0.368	CTTTTAACCACTACC
MA1771.1	MYB41	AT3G02850_SKOR	266	280	+	13.4754	1.52e-05	0.273	ACTTCTGACCTAACT
MA1771.1	MYB41	AT5G37500_GORK	476	490	-	10.8033	8.77e-05	0.525	AAAAATCACCTAATT
MA2027.1	MYB44	AT2G26650_AKT1	608	619	-	11.4286	5.48e-05	1	TAAACTGTCATA
MA2028.1	MYB49	AT3G02850_SKOR	269	283	+	12.7763	2.19e-05	0.207	TCTGACCTAACTGAA
MA2028.1	MYB49	AT5G37500_GORK	473	487	-	12.6447	2.39e-05	0.207	AATCACCTAATTTGC
MA2028.1	MYB49	AT5G27150_NHX1	510	524	+	11.5132	4.78e-05	0.276	TATAACCTAATTTAT
MA2028.1	MYB49	AT2G26650_AKT1	729	743	+	10.3158	9.32e-05	0.323	GATCACCTAAAATAT
MA1394.2	MYB73	AT2G26650_AKT1	608	619	-	11.6452	5.17e-05	0.968	TAAACTGTCATA
MA1777.1	MYB74	AT5G55470_NHX3	671	684	+	12.1148	4.5e-05	0.804	CTTCACCAAATAAT
MA1306.1	WRKY11	AT5G46240_KAT1	660	673	-	11.3939	3.92e-05	0.463	TGTTTTGACTTTGG
MA1306.1	WRKY11	AT2G26650_AKT1	802	815	-	10.1818	6.43e-05	0.463	TTGTTTGACTTCTA
MA1299.1	WRKY17	AT5G46240_KAT1	660	673	+	13.3947	1.41e-05	0.294	CCAAAGTCAAAACA
MA1299.1	WRKY17	AT2G26650_AKT1	802	815	+	10.0921	6.52e-05	0.456	TAGAAGTCAAACAA
MA1081.2	WRKY25	AT2G26650_AKT1	874	889	+	13.1639	2.03e-05	0.198	TTTTTGGTCAACTCTT
MA1081.2	WRKY25	AT2G30070_KUP1	938	953	+	10.9836	7.64e-05	0.497	GTGTTTGTCAACGAAA
MA1301.1	WRKY33	AT5G46240_KAT1	661	671	+	13.3485	2.09e-05	0.316	CAAAGTCAAAA
MA1301.1	WRKY33	AT2G26650_AKT1	803	813	+	10.6667	9.68e-05	0.631	AGAAGTCAAAC
MA1296.1	WRKY46	AT3G02850_SKOR	184	194	-	10.6939	5.26e-05	0.592	CTTTGGCTGTT
MA1296.1	WRKY46	AT2G26650_AKT1	803	813	-	10.602	5.43e-05	0.592	GTTTGACTTCT
MA1788.1	NAC054	AT4G10310_HKT1;1	194	212	+	12.1967	2.04e-05	0.195	CTTCTTCAACACGGTTTAA

MA1788.1	NAC054	AT4G10310_HKT1;1	680	698	+	10.6066	3.94e-05	0.195	CTTAGGATATAAGTAACAT
MA1788.1	NAC054	AT3G02850_SKOR	900	918	+	9.37705	6.16e-05	0.244	CTTTTACACTACGTTATCT
MA0937.1	NAC055	AT5G46240_KAT1	402	409	-	11.7317	5.43e-05	0.349	ATACGTAA
MA0937.1	NAC055	AT3G02850_SKOR	765	772	+	14.0569	1.79e-05	0.345	ACACGTAA
MA1044.1	NAC92	AT2G30070_KUP1	626	637	-	10.9016	6.54e-05	1	TAACAAGACAAC

Note: Find Individual Motif Occurrences analysis was performed using the FIMO tool of MEME Suite version 5.4.1 software (<https://meme-suite.org/meme/tools/sea>) (Grant, Bailey & Noble, 2011). The promoter sequences were searched using the JASPAR CORE Position Frequency Matrix (PFM) non-redundant plant motif sequences obtained from JASPAR (<https://jaspar.genereg.net/downloads/>). The Araport11 loci – 1000 bp upstream promoter sequences were obtained using the Sequence Bulk Download tool on The Arabidopsis Information Resource (TAIR) (<https://www.arabidopsis.org/tools/bulk/sequences/index.jsp>). *P*-values associated with the motif occurrences indicate the probability of a random sequence of the same length as the motif matching that position of the sequence with as good or better a score. *Q*-values indicate the false discovery rate if the occurrence is accepted as significant.

References: MYB1 (Wang, Tohge, et al., 2015), MYB15, WRKY17, WRKY25, WRKY33 (Jiang & Deyholos, 2006b), MYB49 (Zhang, Wang, et al., 2020), MYB73 (Kim et al., 2013), MYB74 (Xu et al., 2015), WRKY11 (Ali et al., 2018), WRKY46 (Ding et al., 2015), NAC055 (Tran et al., 2004), NAC092 (Balazadeh et al., 2010)

Table 7.3: Plasma membrane proteins identified via LC-MS/MS in the various 35S::NIT2 and No-0 untreated and salt-stressed ePM protein samples

Note: the proteins are arranged according to functional categories. Accession numbers are for the Uniprot Arabidopsis database. '1, 2, 3' designate the biological replicate, 'ND' indicates the protein was not detected in any of the replicates for that sample.

Protein name	Accession number	No-0 untreated	No-0 salt	35S::NIT2 untreated	35S::NIT2 salt
Transporters (105)					
Plasma membrane H ⁺ -ATPase 1	P20649	1,2,3	1,2,3	1,2,3	1,2,3
Plasma membrane H ⁺ -ATPase 2	P19456	1,2,3	1,2,3	1,2,3	1,2,3
Plasma membrane H ⁺ -ATPase 3	P20431	1,2,3	1,2,3	1,2,3	1,2,3
Plasma membrane H ⁺ -ATPase 6	Q9SH76	1,3	ND	1,2	ND
Plasma membrane H ⁺ -ATPase 7	Q9LY32	1,3	1,2,3	1,2,3	1,2,3
Plasma membrane H ⁺ -ATPase 8	Q9M2A0	3	ND	1,2	1,2,3
Plasma membrane H ⁺ -ATPase 9	Q42556	1,2,3	1,2,3	2,3	1,2,3
Plasma membrane H ⁺ -ATPase 11	Q9LV11	1,2,3	1,2,3	1,2,3	1,2,3
Calcium-transporting ATPase 1	Q37145	ND	1,2,3	1,2,3	1,2,3
Calcium-transporting ATPase 2	O81108	1,2,3	1,2,3	1,2,3	1,2,3
Calcium-transporting ATPase 4	O22218	1,2,3	1,2,3	1,2,3	1,2,3
Calcium-transporting ATPase 8	Q9LF79	1,2,3	1,2,3	1,2,3	1,2,3
Calcium-transporting ATPase 10	Q9SZR1	1,2,3	1,2,3	1,2	1,2,3
Calcium-transporting ATPase 11	Q9SZR1	1,2,3	1,2	1,2,3	1,2,3
Aquaporin PIP1-1	P61837	1,2,3	2,3	ND	ND
Aquaporin PIP1-2	Q06611	1,2,3	1,2,3	1,2,3	1,2,3
Aquaporin PIP1-3	Q08733	1,2,3	3	1,2,3	1,2,3
Aquaporin PIP1-4	Q39196	1,2,3	1,2,3	1,2,3	ND
Aquaporin PIP1-5	Q8LAA6	1,2,3	ND	1,2	ND
Aquaporin PIP2-1	P43286	1,2,3	1,2,3	1,2	1,2,3
Aquaporin PIP2-2	P43287	1,2,3	3	3	1,2
Aquaporin PIP2-3	P30302	1,2,3	1,2	1,3	1,2
Aquaporin PIP2-5	Q9SV31	1,3	1,2	1,2	ND
Aquaporin PIP2-6	Q9ZV07	1,2,3	2,3	1,2,3	2,3
Aquaporin PIP2-7	P93004	1,2,3	1,2,3	1,2,3	1,2,3
Aquaporin PIP2-8	Q9ZVX8	1,2,3	ND	1,3	1,2
ABC transporter B family member 1	Q9ZR72	1,2,3	ND	1,2,3	1,2,3
ABC transporter B family member 4	O80725	1,2,3	ND	1,2,3	1,2,3
ABC transporter B family member 6	Q8LPT1	1,2,3	1,2,3	1,2,3	1,2,3
ABC transporter B family member 19	Q9LJX0	1,2,3	1,2,3	1,2,3	1,2,3
ABC transporter B family member 20	Q9M3B9	1,2,3	1,2,3	1,2,3	1,2,3
ABC transporter B family member 21	Q9M1Q9	1,2,3	1,2,3	1,2,3	1,2,3
ABC transporter C family member 4	Q7DM58	1,2,3	1,2,3	1,2,3	1,2,3
ABC transporter C family member 5	Q7GB25	ND	ND	1,2	ND
ABC transporter C family member 8	Q8LGU1	ND	1,2,3	ND	ND
ABC transporter C family member 14	Q9LZJ5	1,2,3	1,2,3	ND	1,2,3
ABC transporter G family member 11	Q8RXN0	1,2,3	1,2,3	1,2	1,2,3
ABC transporter G family member 12	Q9C8K2	1,2,3	2,3	1,2	1,2,3
ABC transporter G family member 32	O81016	1,2,3	1,2,3	ND	1,2,3
ABC transporter G family member 36	Q9XIE2	1,2,3	1,2,3	1,2,3	1,2,3
Sugar transporter ERD6	O04036	1,2,3	1,2,3	1,2,3	1,2,3
Sugar transporter ERD6-like 17	Q94CI7	ND	1,2	1,2,3	1,2
Bidirectional sugar transporter SWEET11	Q9SMM5	1,2,3	1,2	1,3	1,2
Bidirectional sugar transporter SWEET12	O82587	1,2,3	3	1,2,3	2,3
Sugar transport protein 1	P23586	1,2,3	1,2,3	1,2,3	1,2,3
Sugar transport protein 7	O04249	ND	1,2,3	ND	ND
Sugar transport protein 13	Q94AZ2	1,2,3	1,2,3	1,2	1,3

Sucrose transport protein 1	Q39232	1,2,3	1,2,3	ND	1,2,3
Monosaccharide-sensing protein 1	Q96290	ND	ND	1,3	1,3
Monosaccharide-sensing protein 2	Q8LPQ8	1,2,3	2	1,3	2,3
Polyol transporter 5	Q8VZ80	1,2,3	1,2,3	1,2,3	1,2,3
Inositol transporter 4	O23492	1,2,3	ND	2,3	ND
Oligopeptide transporter 3	O23482	1,2,3	1,2,3	1,2,3	1,2,3
Oligopeptide transporter 7	O82485	1,2,3	2,3	1,2,3	2,3
Equilibrative nucleotide transporter 1	Q8VXY7	ND	1,2,3	1,2,3	ND
Urea-proton symporter DUR3	F4KD71	ND	1	2,3	1,2,3
Sulfate transporter 2.2	P92946	ND	3	1,2	1,2
Sulfate transporter 3.1	Q9SV13	ND	3	ND	1,2,3
Probable sulfate transporter 3.3	Q9SXS2	1	1	3	1,2,3
Probable purine permease 18	Q9C508	3	3	1,2	ND
Cation-chloride cotransporter 1	Q2UVJ5	1,2,3	1,2,3	2,3	ND
Potassium uptake transporter 3	Q9LD18	1,2,3	1,2,3	1,2,3	1,2,3
Potassium uptake transporter 4	Q9FE38	1,2,3	1,2,3	1,2,3	1,2,3
Potassium uptake transporter 5	Q8LPL8	1,2,3	1,2,3	1,2,3	1,2,3
Potassium uptake transporter 8	Q9M7J9	1,2,3	ND	ND	1,2,3
Potassium uptake transporter 12	O80739	2,3	ND	2	ND
Two-pore potassium channel 1	Q8LBL	1,3	1,2,3	1,2,3	ND
Protein NRT1/ PTR FAMILY 1.2	Q9M817	1,2,3	1,2,3	1,2,3	2,3
Protein NRT1/ PTR FAMILY 2.10	Q944G5	1,2,3	1,2,3	1,2,3	1,2,3
Protein NRT1/ PTR FAMILY 2.11	Q9LV10	1,2	ND	3	1,2
Protein NRT1/ PTR FAMILY 2.13	Q8RX77	ND	2	1	1
Protein NRT1/ PTR FAMILY 6.4	Q9LVE0	ND	1,2,3	1,2	1,3
Protein NRT1/ PTR FAMILY 8.1	Q9M390	2	1,2,3	1,2,3	1,2
Protein NRT1/ PTR FAMILY 8.3	P46032	ND	1,2,3	ND	ND
Copper transporter 6	Q8GWP3	1	1	1,2	1,2
Sodium/calcium exchanger NCL	Q8L636	1,2,3	1,2,3	1,2,3	1,2,3
Cyclic nucleotide-gated ion channel 1	O65717	1,2,3	ND	ND	ND
Ammonium transporter 1 member 1	P54144	1,3	ND	2,3	2,3
Ammonium transporter 2	Q9M6N7	ND	ND	ND	1,3
Nucleobase-ascorbate transporter 12	Q3E7D0	1	3	1	1,2
Amino acid permease 4	Q9FN04	1,2,3	2,3	1,2	1,2
Amino acid permease 6	P92934	ND	ND	2,3	1,2,3
Mechanosensitive ion channel protein 5	Q9LH74	ND	1,2,3	ND	ND
Mechanosensitive ion channel protein 6	Q9SYM1	1	2,3	1,3	1
Calcium permeable stress-gated cation channel 1	Q5XEZ5	1,2,3	2,3	ND	ND
Protein OSCA1 (CSCL5)	Q9XEA1	ND	ND	1,2,3	1,2,3
CSC1-like protein ERD4	Q9C8G5	1,2,3	1,2,3	1,2,3	1,2,3
Boron transporter 1	Q8VYR7	ND	1,2,3	ND	ND
Lysine histidine transporter 1	Q9FKS8	ND	1	1,2,3	1,2,3
Probable polyamine transporter	Q9C6S5	ND	ND	1,2,3	ND
WAT1-related protein WTR8	Q9LPF1	1	1,3	1	ND
Phospholipid-transporting ATPase 1	P98204	1,2,3	ND	1,2,3	1,2,3
Phospholipid-transporting ATPase 2	P98205	ND	2,3	1,2,3	ND
Phospholipid-transporting ATPase 3	Q9XIE6	ND	ND	ND	1,2,3
Phospholipid-transporting ATPase 9	Q9SX33	ND	ND	1,2,3	1,2,3
Phospholipid-transporting ATPase 10	Q9LI83	ND	ND	2,3	ND
Phospholipid-transporting ATPase 11	Q9SAF5	1,2,3	1,3	2,3	1,2,3
ALA-interacting subunit 1	Q9LTW0	ND	1,2	2,3	3
Auxin transporter protein 1 (AUX1)	Q96247	ND	2,3	ND	ND
Auxin transporter-like protein 1 (LAX1)	Q9LFB2	1,2,3	1,2,3	1,2,3	1,2,3
Auxin efflux carrier component 3 (PIN3)	Q9S7Z8	1,2,3	1,2,3	1,2,3	1,2,3
Auxin efflux carrier component 4 (PIN4)	Q8RWZ6	1,2,3	1,2,3	1,2,3	1,2,3
Auxin efflux carrier component 7 (PIN7)	Q940Y5	1,2,3	1,2,3	1,2,3	1,2,3

Receptor Kinases (62)					
Leucine Rich Repeat Protein 1	Q9FMD7	1,2,3	1,2,3	1,2,3	1,2,3
Kinase 7	Q9M8T0	1,2,3	1,2,3	1,2,3	1,2,3
Receptor-Like Kinase 1	Q9LP77	1,2,3	1,2,3	1,2,3	1,2,3
Probable inactive receptor kinase AT2G26730	O48788	1,2,3	1,2,3	1,2,3	1,2,3
Receptor-Like Kinase 902	Q9LVI6	1,2,3	1,2,3	1,2	1,2,3
Probable inactive receptor kinase At3g08680	Q9C9Y8	1,2,3	1,2,3	1,2	1,2,3
Cysteine-rich receptor-like protein kinase 2	Q9CAL3	1,2,3	1,2,3	1,2,3	1,2,3
Cysteine-rich receptor-like protein kinase 3	Q9CAL2	1,2,3	3	3	1,3
Cysteine-rich receptor-like protein kinase 8	O65468	1,2,3	1,2,3	ND	ND
Cysteine-rich receptor-like protein kinase 10	Q8GYA4	1,2,3	1,2,3	1,2,3	1,2,3
Cysteine-rich receptor-like protein kinase 13	Q0PW40	1,3	1	ND	ND
Cysteine-rich receptor-like protein kinase 17	Q8L710	1,2,3	1,2,3	1,2	2,3
Cysteine-rich receptor-like protein kinase 29	Q8S9L6	1,3	ND	ND	ND
Cysteine-rich receptor-like protein kinase 40	Q9SYS3	1,2,3	1,2	3	1,2
Chitin elicitor receptor kinase 1	A8R7E6	1,2,3	1,2,3	1,2,3	1,2,3
LysM RLK1-Interacting Kinase 1	C0LGN2	1,2,3	1,2,3	1,2,3	1,2,3
LysM domain receptor-like kinase 3	F4IB81	1,3	3	1	ND
NEMATODE-INDUCED LRR-RLK 2	C0LGG8	1,2,3	1,2,3	1,2,3	1,2,3
FERONIA Receptor-like protein kinase	Q9SCZ4	1,2,3	1,2,3	1,2,3	1,2,3
Brassinosteroid Insensitive 1 (BRI1)	O22476	1,3	ND	1,2	1,2,3
Brassinosteroid Insensitive 1-associated receptor kinase 1 (BAK1)	Q94F62	1,2,3	1,2,3	1,2,3	1,2,3
BAK1-Interacting Receptor-Like Kinase 1 (BIR1)	Q9ASS4	1,2,3	1,2,3	1,2,3	1,2,3
BAK1-Interacting Receptor-Like Kinase 2 (BIR2)	Q9LSI9	ND	1,2,3	ND	ND
BAK1-Interacting Receptor-Like Kinase 3 (BIR3)	O04567	1,2,3	1,2,3	1,2,3	1,2
SUPPRESSOR OF BIR1 1 (SOBIR1)	Q9SKB2	1,2	1	1,2	1,2,3
MEDOS 1	Q9FID9	1,2,3	1,2,3	1,2,3	1,2,3
Transmembrane Kinase 1	P43298	1,2,3	3	1,2,3	1,2,3
Transmembrane Kinase 3	Q9SIT1	ND	2,3	1,2,3	ND
Transmembrane Kinase 4	Q9LK43	1,2,3	1,2,3	1,2,3	1,2,3
BARELY ANY MERISTEM 1	O49545	1,2,3	1,2,3	1,2	1,2
Sucrose-Induced Receptor Kinase 1	Q0WR59	1,2,3	ND	1,2	2,3
MDIS1-interacting receptor like kinase 2	Q8VZG8	1,2,3	1,2,3	1,2,3	1,2,3
Receptor-like cytoplasmic kinase 1	Q9LUT0	1,2,3	1,2	1,2	1,2
Cytosolic ABA Receptor Kinase 3	O8071	1,2,3	1,2,3	1,3	1,2,3
Proline-rich receptor-like protein kinase PERK1	Q9LV48	1,2,3	1,2,3	1,2	1,2,3
Proline-rich receptor-like protein kinase PERK15	Q9C821	1,2	3	1,2	1,2,3
STRESS INDUCED FACTOR 4	C0LGG3	1,2,3	ND	ND	ND
Inflorescence Meristem Receptor-Like Kinase 2	Q9SCT4	1,2,3	2,3	1,3	1,2,3
HDS-ASSOCIATED RLK1	C0LGD7	1,2,3	2,3	1,2,3	2,3
FLAGELLIN-SENSITIVE 2	Q9FL28	1,3	ND	ND	ND
L-type lectin-domain containing receptor kinase S.1	Q9M9E0	1,2	2,3	3	1
Receptor-like protein kinase HERK 1	Q9LX66	ND	1,2,3	1,2,3	1,2,3

Receptor-like protein kinase THESEUS 1	Q9LK35	1,2	ND	1,2,3	ND
L-type lectin-domain containing receptor kinase IV.1	O80939	1,2,3	1,2,3	1,2,3	1,2,3
Calmodulin-binding receptor-like cytoplasmic kinase 3	Q9ASQ5	1,3	1,3	ND	ND
AT2G23200 Probable receptor-like protein kinase	O22187	1,3	1,2	3	1,2
AT1G56140 Probable LRR receptor-like serine/threonine-protein kinase	C0LGH3	1,3	ND	1,3	3
G-type lectin S-receptor-like serine/threonine-protein kinase At1g11410	Q9LPZ3	3	2,3	1,3	1,2
Probable LRR receptor-like serine/threonine-protein kinase PAM74	C0LGW2	ND	1,2	3	3
AT2G19130 G-type lectin S-receptor-like serine/threonine-protein kinase	O64477	1	1,2,3	1,2	1,2
Receptor-like protein 51	Q9SN38	1,2,3	1,2	1,3	1,2,3
Receptor-like protein 54	F4KHA2	1,3	1,2,3	1	1,2
STRUBBELIG-Receptor Family 6	Q9C8M9	1,2,3	1,2,3	1,2,3	1,2,3
STRUBBELIG-Receptor Family 7	Q9LUL4	1,2,3	1,2,3	1,2,3	1,2,3
G-type lectin S-receptor-like serine/threonine-protein kinase SD1-1	O81833	ND	ND	2,3	ND
Receptor-like protein kinase At5g59670	Q9FN94	ND	1	2,3	ND
Wall-associated receptor kinase 1	Q39191	ND	ND	2,3	ND
Probable serine/threonine-protein kinase PBL19	Q9LTC0	ND	ND	1,2	ND
Receptor-like serine/threonine-protein kinase SD1-8	O81905	ND	ND	2,3	ND
Leaf Rust 10 Disease-Resistance Locus Receptor-Like Protein Kinase-like 1.4	F4HQ17	ND	ND	2,3	ND
AT5G10290 Probable LRR receptor-like serine/threonine-protein kinase	C0LGT1	1	3	2	1,2
Receptor-like protein kinase ANXUR2	Q3E8W4	ND	ND	ND	1,2
Other signalling-related (54)					
Phototropin-1	O48963	1,2,3	1,2	1,2,3	1,2,3
Phototropin-2	P93025	1,2,3	1,2,3	1,2,3	1,2,3
Synaptotagmin-1	Q9SKR2	1,2,3	1,2,3	1,2,3	1,2,3
Serine/threonine-protein kinase BSK2	Q9LS26	1,2,3	1,2,3	1,2,3	1,2,3
Serine/threonine-protein kinase BSK3	Q8W4L3	1,2,3	1,2,3	1,2,3	1,2,3
Serine/threonine-protein kinase BSK5	Q9FIL1	1,2,3	1,3	1,2	2
Serine/threonine-protein kinase BSK6	Q9M324	1,2,3	1,2,3	1,2,3	1,2,3
Serine/threonine-protein kinase BSK7	F4I3M3	1,2,3	1,2,3	1,2,3	1,2,3
Serine/threonine-protein kinase BSK8	Q9FHD7	1,2,3	1,2,3	1,2,3	1,2,3
Calcium-dependent protein kinase 3	Q42479	1,2,3	1,2,3	1,2	1,2,3
Calcium-dependent protein kinase 5	Q38871	1,2,3	ND	ND	ND
Calcium-dependent protein kinase 6	Q38872	ND	1,2	3	ND
Calcium-dependent protein kinase 7	Q38873	ND	ND	3	2
Calcium-dependent protein kinase 8	Q42438	1,2	ND	3	1,2,3
Calcium-dependent protein kinase 9	Q38868	1,2,3	3	1,2	1,2
Calcium-dependent protein kinase 13	Q8W4I7	1,2,3	1,2,3	1,2	1,2
Calcium-dependent protein kinase 21	Q9ZSA2	1,2,3	1,2,3	1,2,3	1,2,3
Calcium-dependent protein kinase 29	Q8RWL2	1,3	1,2,3	1,2,3	1,2
Calcium-dependent protein kinase 32	Q6NLQ6	1,2,3	1,2,3	1,2	1,2
Auxin-induced in root cultures protein 12	Q94BT2	1,2,3	1,2,3	1,2	1,2
Phosphoinositide phospholipase C 1	Q39032	1,3	1,3	1,2,3	3
COP1-interactive protein 1	F4JZY1	1,2,3	1,2,3	1,2,3	1,2,3
Plasma membrane-associated cation-binding protein 1	Q96262	1,2,3	1,2	1,2,3	1,2
Protein BONZAI 1	Q941L3	1,2,3	1,2,3	1,2,3	1,2,3

Protein BONZAI 2	Q5S1W2	1,2,3	1,2,3	1,2,3	1,2,3
Calmodulin-2	P0DH97	1,2,3	1,3	1,2,3	1,2,3
Remorin 1.4	Q9FFA5	1,2	2,3	1,2,3	1,2
BTB/POZ domain-containing protein SR1IP1	Q66GP0	1,2,3	1,2,3	1,2,3	1,2,3
Disease resistance protein RPP8	Q8W4J9	1,2,3	2,3	1,2,3	1,2,3
PTI1-like tyrosine-protein kinase 1	Q8H1G6	1,2,3	1,2	1,2,3	1,2
PTI1-like tyrosine-protein kinase 3	B9DFG5	1,2,3	1,2,3	1,2	2,3
Membrane steroid-binding protein 1	Q9XFM6	1,2,3	1,2	1,2	1,2
Membrane steroid-binding protein 2	Q9M2Z4	1,2,3	1,2,3	1,2	1,2
Plant intracellular Ras-group-related LRR protein 4	Q9SVW8	1,2,3	1,2,3	1,2,3	1,2,3
Ras-related protein RABA1a	P28185	1,2,3	1,2,3	1,2	1,2,3
Ras-related protein RABA1b	Q39222	1,2,3	1,2	1	1,2
Ras-related protein RABA1c	Q9FK68	1,2	1,2	3	1,2
Ras-related protein RABA1d	Q9SN35	1,2,3	1,2,3	1,2	3
Ras-related protein RABA1f	Q9FJH0	1,2	3	1,2	ND
Ras-related protein RABA2a	O04486	1,2,3	1,2	3	1,2
Ras-related protein RABA4a	Q9FJN8	1,2,3	1,2	1,2	1,2
Ras-related protein RABA4b	Q9SMQ6	1,2,3	2	1,3	1,2
Ras-related protein RABA5c	P28187	1,2,3	1,2	1,2	1,2
Ras-related protein RABB1c	P92963	1,2,3	1,2	1,2	2,3
Ras-related protein RABC1	O23657	1,2	2	1,3	1,2
Ras-related protein RABD2a	P28188	1,2	ND	2,3	1,2
Ras-related protein RABD2b	Q9FPJ4	1,2,3	ND	1,2	1,2,3
Ras-related protein RABE1a	O24466	1,2,3	1,2	1,2	1,2
Ras-related protein RABE1c	P28186	1,2,3	3	1,2,3	3
Ras-related protein RABG3b	O04157	1,2,3	1,2	1,2	1,2
Ras-related protein RABG3a	Q948K8	1,2,3	1,2	1,2	1,2
Ras-related protein RABG3e	Q9XI98	1,3	3	1,2	3
Ras-related protein RABG3f	Q9LS94	1,2,3	1,2	3	1,2,3
Ras-related protein RABH1b	O80501	1,2,3	1,2	1,2	1,2,3
Membrane Trafficking and Structure (27)					
Clathrin heavy chain 1	Q0WNJ6	1,2,3	2,3	1,2,3	1,2,3
Clathrin heavy chain 2	Q0WLB5	1,2,3	1,2,3	1,2,3	1,2,3
Clathrin light chain 1	Q9SKU1	1,2,3	1,2,3	1,2,3	1,2,3
Clathrin light chain 2	O04209	1,2,3	2,3	1,2,3	1,2,3
Clathrin light chain 3	F4J5M9	ND	1,2,3	1,2,3	1,2,3
Putative clathrin assembly protein CAP1	Q8S9J8	1,2,3	1,2,3	1,2,3	1,2,3
Putative clathrin assembly protein CAP8	Q8LBH2	2,3	2,3	2,3	ND
Patellin-1	Q56WK6	1,2,3	1,2	1,2,3	1,2,3
Patellin-2	Q56ZI2	1,2,3	1,2,3	1,2,3	1,2,3
Syntaxin-71	Q9SF29	1,2,3	1,2,3	1,2,3	1,2,3
Syntaxin-121	Q9ZSD4	1,2,3	1,2,3	1,2	1,2
Syntaxin-122	Q9SVC2	1,2,3	1,2	1,2,3	3
Syntaxin-132	Q8VZU2	1,2,3	1,2,3	1,2,3	1,2,3
Dynamin-related protein 1A	P42697	1,2,3	1,2,3	1,2,3	1,2,3
Dynamin-related protein 1C	Q8LF21	1,2,3	1,2	3	3
Dynamin-related protein 1E	Q9FNX5	1,2,3	1,2,3	1,2	1,2
Dynamin-2A	Q9SE83	1,2,3	1,2	1,2,3	1,2,3
Dynamin-2B	Q9LQ55	1,2,3	1,2,3	1,2,3	1,2,3
Flotillin-like protein 1	Q501E6	ND	1	2	1,2,3
Kinesin-like protein KIN-14A	Q9LX99	1,2,3	1,2,3	1,2,3	1,2
Fasciclin-like arabinogalactan protein 1	Q9FM65	1,2,3	1,2,3	1,2,3	1,2,3
Fasciclin-like arabinogalactan protein 2	Q9SU13	1,2,3	1,2,3	1,2,3	1,2,3
Fasciclin-like arabinogalactan protein 7	Q9SJ81	1,2,3	1,2,3	1,2,3	1,2,3
Fasciclin-like arabinogalactan protein 8	O22126	1,2,3	1,2,3	1,2,3	1,2,3
Fasciclin-like arabinogalactan protein 9	Q9ZWA8	1,2,3	1,2,3	1,2,3	1,3

Fasciclin-like arabinogalactan protein 13	Q9FFH6	1,2,3	1,2	1,2,3	1,2,3
Non-classical arabinogalactan protein 31	Q9FZA2	1,2,3	1,2	3	1,2,3
Cell wall-related (17)					
Callose synthase 2	Q9SL03	1,2,3	1,2,3	1,2,3	1,2,3
Callose synthase 3	Q9LXT9	1,2,3	1,2,3	1,2,3	1,2
Callose synthase 9	Q9SFU6	1,2,3	1,2,3	ND	1,2,3
Callose synthase 10	Q9SJM0	1,2,3	1,2,3	1,2,3	1,2,3
Callose synthase 11	Q9S9U0	1,3	2,3	1	1,2,3
Callose synthase 12	Q9ZT82	1,2,3	1,2,3	1,2,3	1,2,3
Cellulose synthase A catalytic subunit 1	O48946	1,2,3	3	1,2	1,2
Cellulose synthase A catalytic subunit 3	Q941L0	1,2,3	2,3	1,2,3	1,3
Cellulose synthase A catalytic subunit 4	Q84JA6	1,2,3	3	1	ND
Cellulose synthase A catalytic subunit 6	Q94JQ6	1,3	1,2	1	1
Cellulose synthase-like protein B4	O80891	1,2,3	1,2,3	1,2,3	1,2
Cellulose synthase-like protein G3	Q0WVN5	ND	1	1	1,2,3
Pectinesterase 1	Q43867	1,2,3	1,2,3	1,2,3	1,2,3
Probable pectin methylesterase CGR2	Q9M2Y6	1,2,3	1,2,3	1,2,3	1,2,3
Glycosyltransferase-like KOBITO 1	Q9C9Z9	1,2,3	1,2,3	1,2,3	1,2,3
Xyloglucan endotransglucosylase/ hydrolase protein 4	Q39099	ND	2,3	ND	ND
Endoglucanase 25	Q38890	1,2,3	1,2	1,2,3	1,2,3
Other (23)					
Early nodulin-like protein 2	Q9T076	1,2,3	1,2,3	1,2,3	1,2,3
Annexin D1	Q9SYT0	1,2,3	3	1,2,3	1,2,3
Annexin D4	Q9ZVJ6	1,2,3	2,3	1	1
Hypersensitive-induced response protein 2	Q9CAR7	ND	1,2,3	ND	ND
Hypersensitive-induced response protein 3	Q9SRH6	ND	1,2,3	ND	ND
Hypersensitive-induced response protein 4	Q9FHM7	1,2,3	1,2,3	1,2,3	1,2,3
Dehydrin ERD14	P42763	1,2,3	1,2,3	1,2	1,2
Early-Responsive To Dehydration 7	O48832	ND	1,2,3	1,2,3	2,3
Respiratory burst oxidase homolog protein D	Q9FIJ0	1,3	1,2,3	1,2,3	1,2,3
Protein ACCELERATED CELL DEATH 6	Q8LPS2	ND	1	2,3	1,2,3
Heat shock protein 90-3	P51818	1,2,3	1,2	1,2,3	1,2
MLO-like protein 1	O49621	1,2,3	1,2,3	1,2,3	1,2,3
Phosphoinositide phosphatase SAC7	Q9C5G5	1,2,3	1,2,3	1,2,3	1,2,3
NDR1/HIN1-like protein 1	Q9SRN0	1,2,3	ND	1,2,3	1,2,3
NDR1/HIN1-like protein 2	Q9SRN1	1,2,3	1,2,3	1,3	3
NDR1/HIN1-like protein 3	Q9FNH6	ND	ND	1,2,3	ND
Non-specific lipid transfer protein GPI- anchored 1	Q9C7F7	2,3	3	1	ND
Glycerophosphodiester phosphodiesterase GDPDL1	Q7Y208	1,2,3	1,2	1,2,3	1,2,3
Glycerophosphodiester phosphodiesterase GDPDL3	Q9SZ11	1,2,3	1,2,3	1,3	1,2,3
Uncharacterized GPI-anchored protein 2 At5g19240	Q84VZ5	1,2,3	1,2,3	2	1,2,3
Uncharacterized GPI-anchored protein 4 At3g06035	Q84MC0	1,2,3	1,2	1,2	1,2
PLANT CADMIUM RESISTANCE 1	Q9LQU2	ND	1	2,3	1,2,3
Uncharacterized membrane protein At1g16860	Q9FZ45	1,2,3	1,2,3	1,2,3	1,2,3

Protein	Accession	No-0 salt vs ctrl	35S:: <i>NIT2</i> salt vs ctrl	35S:: <i>NIT2</i> ctrl vs No-0 ctrl	35S:: <i>NIT2</i> salt vs No-0 salt
Sodium/calcium exchanger NCL	Q8L636				
ABC transporter C family member 3	Q9LK64				
ABC transporter C family member 4	Q7DM58				
ABC transporter C family member 5	Q7GB25				
ABC transporter G family member 22	Q93YS4				
ABC transporter G family member 36	Q9XIE2				
Mechanosensitive ion channel protein 1	Q8VZL4				
Mechanosensitive ion channel protein 5	Q9LH74				
Mechanosensitive ion channel protein 6	Q9SYM1				
Protein NRT1/ PTR FAMILY 1.2 (NPF1.2) / Nitrate transporter 1.11	Q9M817				
Sulfate transporter 4.1	Q9FY46				
Ammonium transporter 1	P54144				
Urea-proton symporter DUR3	F4KD71				
Potassium transporter 8 (KUP8)	Q9M7J9				
Amino acid permease 6	P92934				
Sugar transport protein 7	O04249				
Boron transporter 1	Q8VYR7				
Aquaporin TIP1-1	P25818				
Probable aquaporin TIP2-2	Q41975				
Plasma membrane H ⁺ -ATPase isoform 2 (AHA2)	P19456				
Putative phospholipid-transporting ATPase 9 (ALA9)	Q9SX33				
Phospholipid-transporting ATPase 10 (ALA10)	Q9LI83				
Probable phospholipid-transporting ATPase 11 (ALA11)	Q9SAF5				
Serine/threonine-protein phosphatase 2A (PP2AA1)	Q38845				
Calcium-dependent protein kinase 29	Q8RWL2				
G-type lectin S-receptor-like serine/threonine-protein kinase AT2G19130	O64477				
LysM domain receptor-like kinase 5 (LYK5)	O22808				
Receptor-like protein kinase AT5G59670	Q9FN94				
Probable LRR receptor-like serine/threonine-protein kinase AT5G10290	C0LGT1				
Nitrilase 2	P32962				
Beta-D-glucopyranosyl abscisate beta-glucosidase (BGLU18)	Q9SE50				
Pyridoxal 5'-phosphate synthase subunit PDX1.1	O80448				
Pyridoxal 5'-phosphate synthase subunit PDX1.3	Q8L940				
EARLY-RESPONSIVE TO DEHYDRATION 7	O48832				
Cellulose synthase-like protein G3	Q0WVN5				

Callose synthase 3	Q9LXT9				
Glycerophosphodiester phosphodiesterase GDPDL1	Q7Y208				
Probable pectin methylesterase (CGR3)	Q0WPN7				
Uncharacterized GPI-anchored protein AT5G19240	Q84VZ5				
Fasciclin-like arabinogalactan protein 13	Q9FFH6				
Non-classical arabinogalactan protein 31	Q9FZA2				
Protein HYPER-SENSITIVITY-RELATED 4	Q8VZG2				
Protein ACCELERATED CELL DEATH 6	Q8LPS2				
Protein PLANT CADMIUM RESISTANCE 1	Q9LQU2				
NDR1/HIN1-like protein 1	Q9SRN0				
Annexin D1	Q9SYT0				
Annexin D4	Q9ZVJ6				
Patellin-1	Q56WK6				
Patellin-2	Q56ZI2				
Dynamamin-2A	Q9SE83				
Dynamamin-2B	Q9LQ55				
Dynamamin-related protein 1A	P42697				
Dynamamin-related protein 1C	Q8LF21				
Dynamamin-related protein 1E	Q9FNX5				
Tubulin alpha-4 chain	Q0WV25				
Tubulin alpha-5 chain	B9DHQ0				
Tubulin beta-5 chain	P29513				
Tubulin beta-9 chain	P29517				
Coatomer subunit alpha-1	Q94A40				
Coatomer subunit alpha-2	Q9SJT9				
Coatomer subunit beta-1	Q9SV21				

Figure 7.2: Basic heatmap showing differentially regulated proteins in the four two-way comparisons of the No-0 and 35S::*NIT2* control and salt-stressed shoot ePM sample groups

Key: Up-regulated Down-regulated ND in untreated ND in salt ND in No-0 ND in 35S::*NIT2* No significant difference

Note: ctrl = untreated control

Table 7.4: GFSA-screening results of 35S::*EXPA11*amiRNA and empty vector control T3 lines

Line	Untreated		GFSA (10 µg.ml ⁻¹)	
	Germination (of 50)	Survival	Germination (of 50)	Survival
35S:: <i>NIT2</i>	48	48	47	0
amiRNA 3,1-1	50	50	50	50
amiRNA 3,1-2	50	50	50	38
amiRNA 3,1-3	50	50	50	38
amiRNA 3,1-4	50	50	50	50
amiRNA 3,1-5	50	50	50	37
amiRNA 3,1-6	50	50	50	36
amiRNA 5,1-1	50	50	50	38
amiRNA 5,1-2	50	50	50	42
amiRNA 5,1-3	50	50	50	50
amiRNA 5,1-4	50	50	50	36
amiRNA 5,1-5	50	50	50	50
amiRNA 5,1-6	50	50	50	36
amiRNA 8,1-1	50	50	50	50
amiRNA 8,1-2	50	50	50	50
amiRNA 8,1-3	50	50	50	50
amiRNA 8,1-4	50	50	50	35
amiRNA 8,1-5	50	50	50	50
amiRNA 8,1-6	50	50	50	50
amiRNA 14,2-1	50	50	50	39
amiRNA 14,2-2	50	50	48	48
amiRNA 14,2-3	50	50	50	50
amiRNA 14,2-4	50	50	50	50
amiRNA 14,2-5	50	50	50	50
amiRNA 14,2-6	50	50	50	50
EV 2,1-1	50	50	50	39
EV 2,1-2	50	50	50	50
EV 2,1-3	50	50	50	37
EV 2,1-4	50	50	50	37
EV 2,1-5	50	50	50	50
EV 2,1-6	50	50	50	29
EV 4,1-1	50	50	50	37
EV 4,1-2	50	50	50	50
EV 4,1-3	50	50	50	32
EV 4,1-4	50	50	50	50
EV 4,1-5	50	50	50	36
EV 4,1-6	50	50	50	36
EV 7,1-1	50	50	50	44
EV 7,1-2	50	50	50	40
EV 7,1-3	50	50	50	50
EV 7,1-4	50	50	50	50
EV 7,1-5	50	50	50	37
EV 7,1-6	50	50	50	50
EV 24,2-1	50	50	50	35
EV 24,2-2	50	50	50	50
EV 24,2-3	50	50	50	50

EV 24,2-4	50	50	50	50
EV 24,2-5	50	50	50	50
EV 24,2-6	50	50	50	50

Note: 35S::*NIT2* is the positive control for plant death due to GFSA treatment

Note: The lines in **bold** were taken forward for gene expression analysis and phenotyping

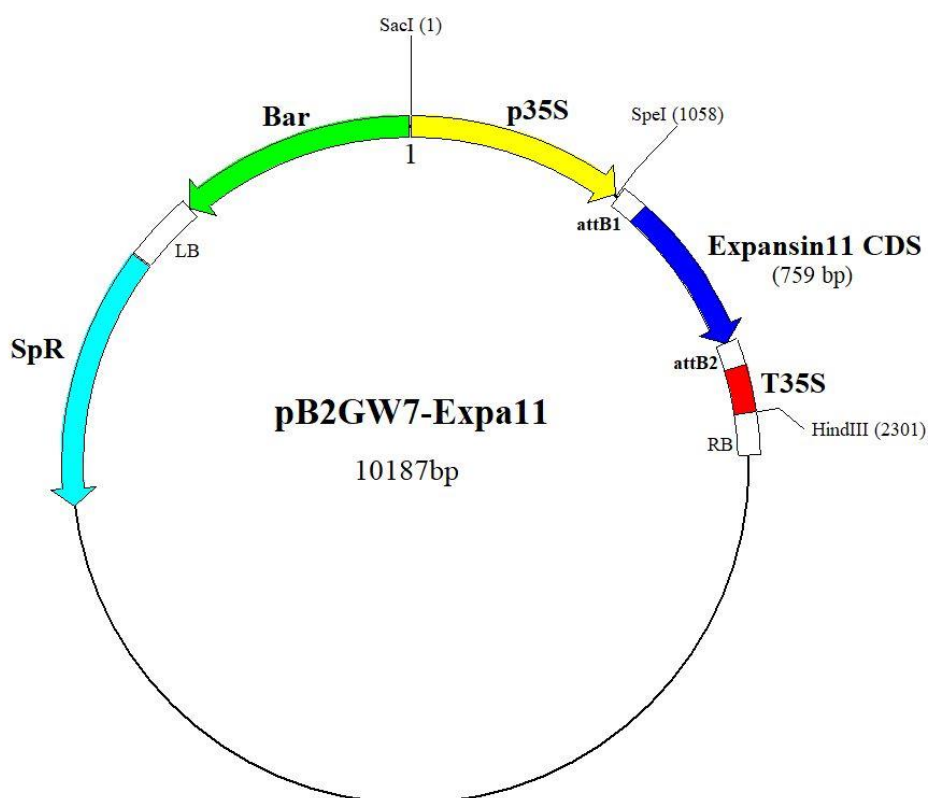


Figure 7.3: Map of the recombinant pB2GW7-*EXPA11* generated via Gateway cloning

Expression of the *EXPA11* coding DNA sequence (CDS) is driven by the constitutive 35S promoter from Cauliflower Mosaic Virus (**p35S**) and terminated by the 35S terminator sequence (**T35S**). The spectinomycin resistance gene (**SpR**) allows for selection of positive *E. coli* and *A. tumefaciens* transformants. The Bar gene (**Bar**) provides resistance to Glufosinate ammonium, the active ingredient in the herbicide, BASTA, and allows for the selection of transgenic plants.

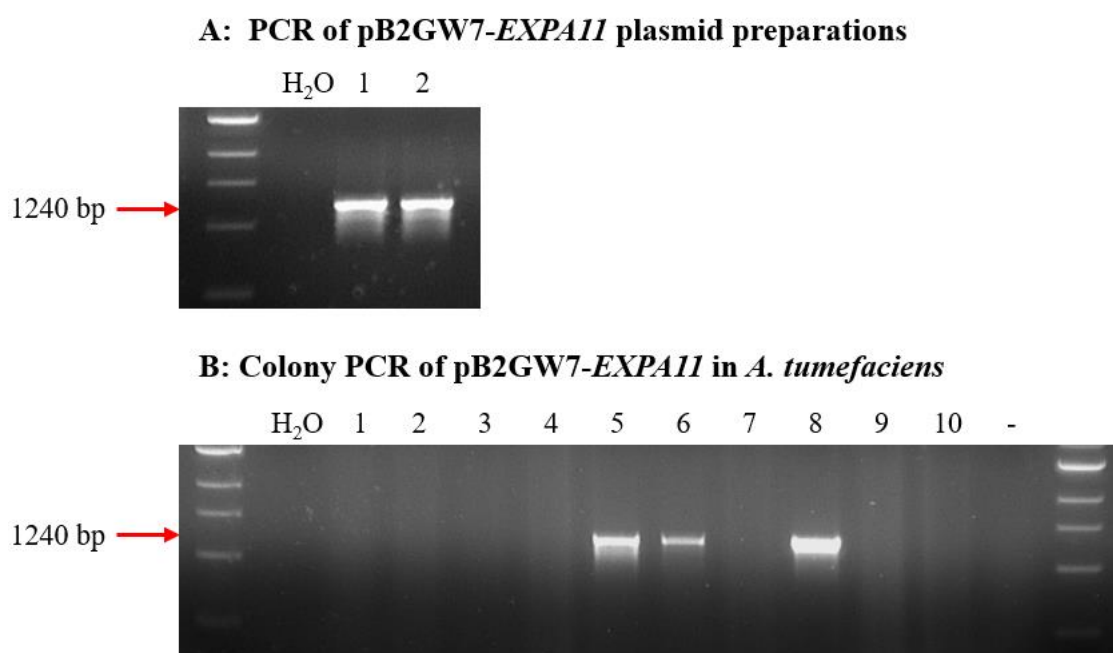


Figure 7.4: Transformation of pB2GW7-*EXPA11* in *E. coli* and *A. tumefaciens*

(A) *E. coli* was transformed with pB2GW7-*EXPA11* and plasmid DNA was subsequently extracted from colonies that survived spectinomycin screening. PCR was then performed using p35S F and *EXPA11-attB2* R primers. (B) Following transformation of *A. tumefaciens* with pB2GW7-*EXPA11* plasmid DNA, 10 discrete colonies surviving on spectinomycin plates were screened by colony PCR using p35S F and *EXPA11-attB2* R primers. The MW marker included in both (A) and (B) was the NEB Quick-Load® 1 kb DNA ladder (Catalogue #NO468).

Table 7.5: GFSA-screening results of 35S::*EXPA11* T2 lines

Line	Untreated		GFSA (10 µg.mL ⁻¹)	
	Germination (of 50)	Survival	Germination (of 50)	Survival
No-0	50	50	49	0
2,1	50	50	50	43
2,2	50	50	50	38
2,3	50	50	50	48
4,1	50	40	50	29
6,1	50	50	49	37
6,2	50	50	49	36
7,1	50	50	50	37
7,2	50	50	50	38
9,1	50	41	50	30
9,2	50	49	49	43
10,1	50	50	50	50
10,2	50	50	50	37
11,1	48	47	49	7
11,2	50	50	50	43
15,1	49	49	50	38
15,2	50	48	50	36

17,1	50	45	50	12
18,1	50	50	50	40
20,1	49	49	49	42
21,1	50	50	50	39
21,2	50	50	49	44
22,1	50	50	50	50
22,2	50	47	50	0
22,3	50	46	50	0
23,1	49	49	50	47

Note: The lines in **bold** were taken forward; No-0 is the positive control for plant death due to GFSA treatment

Table 7.6: GFSA-screening results of 35S::EXPA11 T3 lines

Line	GFSA (10 µg.ml ⁻¹)		Untreated	
	Germination (of 50)	Survival	Germination (of 50)	Survival
No-0	50	0	48	48
2,2-1	50	37		
2,2-2	50	34		
2,2-3	50	40		
2,2-4	50	50		
2,2-5	50	39		
4,1-1	50	50		
4,1-2	50	50		
4,1-3	50	37		
4,1-4	50	50		
4,1-5	50	35		
6,1-1	50	46		
6,1-2	50	43		
6,1-3	50	45		
6,1-4	50	24		
6,1-5	50	41		
7,1-1	50	39		
7,1-2	50	40		
7,1-3	50	50		
7,1-4	50	36		
7,1-5	50	40		
9,2-1	50	46		
9,2-2	50	40		
9,2-3	50	46		
9,2-4	50	48		
9,2-5	50	50		
10,2-1	50	35		
10,2-2	50	49		
10,2-3	50	39		
10,2-4	50	45		
10,2-5	50	50		
11,2-1	50	50		
11,2-2	50	47		

11,2-3	50	50
11,2-4	50	50
11,2-5	50	47
15,1-1	50	44
15,1-2	50	37
15,1-3	50	42
15,1-4	50	46
15,1-5	50	38
18,1-1	50	50
18,1-2	50	50
18,1-3	50	36
18,1-4	50	41
18,1-5	50	43
20,1-1	50	42
20,1-2	50	50
20,1-3	50	50
20,1-4	50	43
20,1-5	50	39
21,1-1	50	37
21,1-2	50	39
21,1-3	50	50
21,1-4	50	41
21,1-5	50	40
23,1-1	50	47
23,1-2	50	43
23,1-3	50	39
23,1-4	50	44

Note: No-0 is the positive control for plant death due to GFSA treatment

Note: The lines in **bold** were taken forward for gene expression analysis and phenotyping

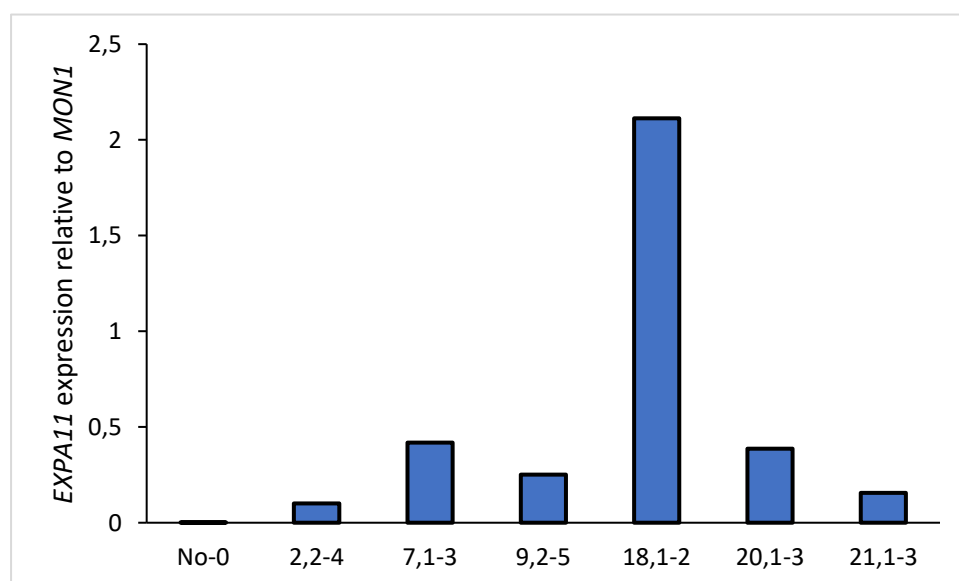


Figure 7.5: Initial analysis of *EXPA11* expression in the 35S::*EXPA11* T3 lines

An RT-qPCR experiment was conducted using *EXPA11* qPCR primers on cDNA synthesised from RNA extracted from No-0 and 35S::*EXPA11* T3 plants grown on soil under standard conditions. Four leaves were pooled per plant for RNA extraction for each of the genotypes. The 35S::*EXPA11* T3 lines that were screened are ‘2,2-4’, ‘7,1-3’, ‘9,2-5’, ‘18,1-2’, ‘20,1-3’, and ‘21,1-3’. *EXPA11* expression was normalised to the reference gene *MON1*.

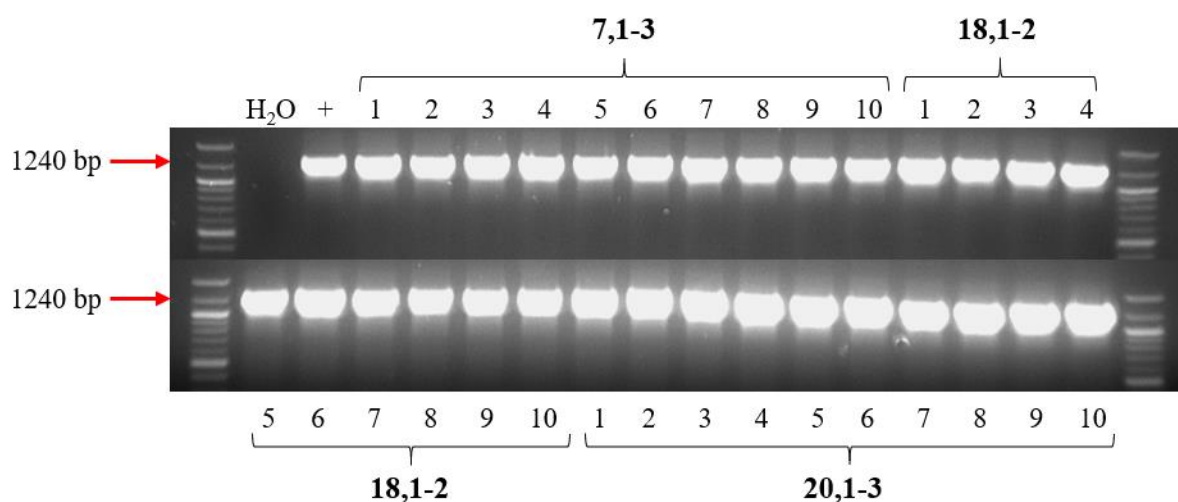


Figure 7.6: Presence of 35S::*EXPA11* construct in T3 lines

For each third transgenic generation (T3) line, DNA extractions were performed on 10 seedlings. The PCR with p35S F and *EXPA11-attB2* R primers was conducted to confirm the presence of the 35S::*EXPA11* construct in the genomic DNA (gDNA) of the T3 lines. The T3 lines that were screened are ‘7,1-3’, ‘18,1-2’, and ‘20,1-3’. The positive control used the gDNA from the second transgenic generation (T2) of the T3 line 18,1-2 as template. The MW marker included is the New England Biolabs (NEB) Quick-Load® 100 bp DNA ladder (Catalogue #NO467S).

CHAPTER 8: REFERENCES

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