An investigation into halotolerance mechanisms in *Arabidopsis thaliana*

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Abstract

Soil salinity is one of the most significant abiotic stresses that can reduce agricultural productivity. Consequently there is much interest in the mechanisms halophytes employ to tolerate high salt environments. The model organism *Arabidopsis* is salt sensitive questioning the suitability of this plant for salt tolerance studies. A salt tolerant *Arabidopsis* HHS (Habituated to High Salt) cell line was, however, established. Wild type *Arabidopsis* cell cultures cannot tolerate 70mM NaCl, but the HHS cell line was adapted from the wild type cell lines by sub-culturing into progressively higher levels of NaCl, The generation of the HHS cell line suggests that although *Arabidopsis* is generally considered to be salt sensitive, it does have the genetic potential to be salt tolerant. These cell cultures are useful for salt tolerance studies as to survive in high salinity, unlike the intact root system of a plant, each cell in culture must be expressing the required traits for salt tolerance. Measurements of total ion content of the cell lines indicate that the HHS *Arabidopsis* cells do not accumulate Na^+; instead mechanisms for either Na exclusion, or Na efflux must function.

Arabidopsis activation tagged lines were used in large scale gain-of-function screens to identify single sequences that allow seedlings to survive better under NaCl stress. 23 000 lines were screened on MS media with reduced K^+ (200μM) and Ca^{2+} (530μM) concentrations, supplemented with 0.75% sucrose and 80mM NaCl. It is well established that the toxic effects of Na^+ can be ameliorated by high external K^+ and Ca^{2+} concentrations. Under these conditions wild type seedling growth was impaired. Therefore, any mutants that were more efficient at K^+ uptake, preventing Na^+ uptake, Na^+ efflux, or show an increased sensitivity to the Ca^{2+} response flourished and were easily identified in the screen. Primary screening isolated 631 putative salt tolerant

mutants. Secondary screening under identical conditions isolated 50 mutants displaying a strong phenotype, 36 a weak phenotype and 109 were found to be false positive.

When compared with wild type, the activation tagged mutant JP5 was more salt tolerant. The JP5 mutant contained a single insertion of the pSKI015 activation tag vector located downstream from the *AtMYB64* gene. Expression of *AtMYB64* was up regulated in the JP5 mutant compared with wild type. Homozygous lines reported to carry a T-DNA insertion in the first intron of *AtMYB64* (a putative *AtMYB64* knockout) was hypersensitive to salt. Overexpression of *AtMYB64* conferred salt tolerance to transgenic Arabidopsis lines. These results suggest *AtMYB64* is a transcription factor that activates salt tolerance mechanisms in *Arabidopsis*.

Declaration

I declare that this thesis for the degree of doctor of philosophy has been composed entirely by myself and the work presented herein was performed by myself unless stated otherwise.

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Abbreviations

ABA	abscisic acid
ABRE	ABA responsive elements
ATP	adenosine triphosphate
ATHK1	*Arabidopsis thaliana* histidine kinase 1
BCECF	2',7'-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein
bp	base pair
BSA	bovine serum albumin
BTP	BIS-TRIS propane
cADPR	cyclic ADP-ribose
CBL1	calcineurin B-like protein
CaMV35S	cauliflower mosaic virus 35S promoter
CDPK	calcium-dependent protein kinases
COD	choline-oxidising enzyme *Arthrobacter globiformis*
COX	choline-oxidising enzyme *A. panescens*
CTAB	hexadecyltrimethylammonium bromide
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DRE/CRT	dehydration-responsive elements/C-Repeat
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Ena1	sodium transport ATPase 1
ER	endoplasmic reticulum
HKT1	K^+ transporter 1
HHS	habituated to high salt
IAA	3-indoleacetic acid
ICP-OES	inductively coupled plasma optical emission spectroscopy
IP3	inositol 1,4,5-triphophate
KDa	KiloDalton
MAPK	mitogen-activated protein kinase
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
MS	Murashigge-Skoog medium
MSMO	Murashigge-Skoog medium with minimal organics
MYB	MYB transcription factor
NAADP	nicotinic acid adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adeninde dinucleotide phosphate
PBFI	1,3-benzenedicarboxylic acid, 4,4'-[1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diylbis(5-methoxy-6,2-benzofurandiyl)] bis
PCI	phenol: chloroform: isoamylalcohol 25:24:1
PCR	polymerase chain reaction

PEG	polyethylene glycol
PEP	phosphoenolpyruvate
ProDH	proline dehydrogenase
PMF	proton motive force
PMSF	phenylmethanesulfonyl fluoride
PPFD	photosynthetic photon flux density
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
rpm	revolutions per minute
SBFI	1,3-benzenedicarboxylic acid, 4,4′-[1,4,10-trioxa-7,13-diazacyclopentadecane-7,13-diylbis(5-methoxy-6,2-benzofurandiyl)]bis-tetra ammonium salt
SDS	sodium dodecyl sulphate
SOS	salt overly sensitive
SPQ	6-methoxy-*N*-(3-sulfopropyl)quinolinium
TAIL-PCR	thermal asymmetric interlaced polymerase chain reaction
Tris	tris (hydroxymethyl)methylglycine
w/v	weight per volume (expressed as percentage)
v/v	volume per volume (expressed as a percentage)

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Chapter 1: Introduction

1.1 Salinisation: a Global Problem.

The earth's population is increasing in number at an alarming rate. By the year 2050, the human population is estimated to rise from 6.1 billion (mid-2001) to 9.3 billion, an increase of approximately 50% (http://www.unfpa.org/swp/2001/). Nine out of ten of these births are expected to occur in developing countries where grain self-sufficiency fell from 96% in 1967-71 to 88% in 1993-95 (Munns *et al.* 1995). With the projected increase in population coupled to increased urbanisation in developing countries, world agriculture is faced with an enormous challenge simply to maintain, let alone increase, present levels of food production.

One of the major factors affecting crop yield is soil salinisation which can take one of two forms; primary salinity is due to natural causes, while secondary salinity is due to human impact on the environment. Secondary salinisation occurs most often as a consequence of over-irrigation, when salt dissolved in irrigation water is left in the soil following evapotranspiration. It has been estimated that 20% of irrigated land suffers from this problem, which if left untreated can render the land useless. Irrigation is an essential aid to agriculture, approximately 15% of cultivated land in the world is irrigated, but as irrigated land is estimated to be at least twice as productive as rain-fed land; it produces one-third of the world's food (Munns *et al.* 2000).

Soil salinisation can also be caused by saline groundwater reaching the land surface, a problem aggravated by the clearing of native deep-rooted perennial woody vegetation and its replacement with shallow-rooted annual crops. As well as causing soil salinisation, raised saline groundwater tables may discharge saline water directly into rivers, streams and lakes, sources of water for irrigation. The problem of soil

salinisation is particularly severe in India, Pakistan, Russia, Australia, China's north plain, Afghanistan's Helmud Valley, California's San Joaquin Valley, the Valley of Mexico, and the Tigris and Euphrates River basin (The Food and Agriculture Organisation of the United Nations http://www.fao.org/ag/AGL/agll/spush/topic2.htm). Approximately 50% of the world's land is 'perennial desert or drylands' (United Nations Development Programme http://www.undp.org/drylands/what-are.htm). Productivity of these areas could be increased by irrigation. It has been estimated that between 1960 and 1980, expansion of irrigation has accounted for more than 50% increase in global food production (Greenway & Munns 1980) and so irrigation of these drylands could be a way to increase food production for a growing population. The associated problem of salinisation, however, questions the sustainability of irrigating drylands. Increasing salt tolerance of plants, by either traditional breeding or genetic manipulation technologies, together with altering farming practices to reduce occurrence of salinisation, could increase the sustainability of irrigation.

1.2 Plants Differ in their Ability to Tolerate Salt.

Plants vary in their ability to cope with salt stress, and can be classed, generally, as halophytes or glycophytes. Halophytes are tolerant of high concentrations of NaCl; some can complete their life cycle in salt concentrations as high as 560mM NaCl (seawater). Generally, halophytes have not evolved cellular metabolic processes that function in high salinity but instead have the ability to tolerate high salt concentrations because of cellular compartmentalisation of toxic ions, specialised anatomical and morphological adaptations, or avoidance mechanisms (Yeo 1998). Many halophytes are able to grow perfectly well in low or non-saline environments, and are termed facultative halophytes; others cannot and are termed obligate halophytes. Glycophytes

cannot complete their life cycle in more than 100mM NaCl. Although these classifications exist, there is in reality a continuous spectrum of tolerance that exists with true halophytes and very salt sensitive glycophytes at each extreme. In the middle of the spectrum there are the mesophytes, which show some degree of tolerance to salt. Most crops are glycophytes (Table 1.1), thus excess salinity in soil has a devastating effect on crop yield and quality.

1.3 Effects of Salinity on Plants.

Salinity simultaneously affects several aspects of plant physiology, from the cellular to the whole plant level. Salt stress results from a number of detrimental processes including the toxic action of Na^+ and Cl^- ions, the impairment of mineral nutrition, a modification in the water status of the plant tissues, and secondary stresses such as an oxidative stress linked to the production of toxic reactive oxygen intermediates.

Within hours of severe salt stress, the growth rate of shoots, and to a lesser degree root tissue, is reduced in salt sensitive plants. Similar changes are observed when KCl, mannitol or PEG are applied showing that the initial responses are not salt specific but occur as a consequence of the osmolarity of the external solution (Yeo *et al.* 1991). Often this initial decrease in growth rate upon exposure to salinity is similar for species that have different tolerance to salt. For example, bread wheat is considered more salt tolerant than durum wheat; however, short periods of salt treatment have a similar effect on the growth rates of both species (Munns *et al.* 1995). This initial decrease in growth rate is followed by a gradual recovery to a new, often reduced, rate of growth. This second phase of growth reduction results from the toxic action of Na^+ and Cl^- ions (Munns 2002). The tolerance of plants to salinity is often related to the concentration of

Tolerant	Moderately Tolerant	Moderately Sensitive	Sensitive
Barley (grain)	Soybean	Corn	Carrot
Cotton	Barley	Rice	Onion
Sugar beet	Wheat	Sugarcane	Soybean
	Rye	Potato	Lentil
	Peanut	Maize	Chickpea
		Rapeseed	Broad bean
		Tomato	Pepper
		Cabbage	

Table 1.1 Relative Salt Tolerance of Some Important Agricultural Crops.

Soil salinity is a major abiotic stress reducing agriculture production worldwide. Plants differ in their ability to tolerate high salinity and many important crops are susceptible to high NaCl levels in the soil. Information source: http://www.ussl.ars.usda.gov/salt_tol_db.htm

Na^+ in the shoot. Sodium specific damage is associated with the accumulation of Na^+ in leaf tissues and results in the necrosis of older leaves. Growth and yield reductions occur as a result of the shortening of the lifetime of individual leaves, thus reducing net productivity and crop yield (Munns 2002).

1.4 Physiological Strategies for Resistance to Salinity.

Three key strategies are found in salt resistant plants that confer some measure of tolerance. These are:

1.Tolerance and/or avoidance of desiccation (osmoregulation)

2.Maintainence of a low cytoplasmic Cl^- concentration

3.Maintainence of a high cytoplasmic K^+/Na^+ ratio

1.4.1 Tolerance and/or Avoidance of Desiccation.

In saline habitats the chemical potential of the soil water initially establishes a water potential imbalance between the apoplast and symplast that leads to turgor decrease which, if severe enough, can cause growth reduction. Halophytes can make osmotic adjustments to maintain a positive turgor pressure by the accumulation of charged ions in the vacuole. To maintain equal solute potentials in the vacuole and cytoplasm, however, solutes whose accumulation is not detrimental to cellular metabolism must accumulate in the cytoplasm. Compatible solutes are non-toxic compounds that can stabilise proteins and cellular structures, and can increase the osmotic pressure of the cell. Proline, glycine betaine, and polyhydric alcohols such as sorbitol and mannitol are examples of compatible solutes which are highly soluble and do not interfere with cellular metabolism, even at high concentrations. Glycine betaine is believed to function as an osmoprotectant, for example, by stabilizing the quaternary structures of proteins

and highly ordered state of membranes, at high concentrations of salts or at extreme temperatures, whereas proline stabilises subcellular structures and serves as a free-radical scavenger (Chen & Murata 2002). Many studies have investigated the effect of over expressing compatible solutes on transgenic plants ability to tolerate abiotic stress (reviewed by Chen & Murata 2002; Sakamoto & Murata 2002).

In *Arabidopsis*, proline levels were increased in transgenic plants in which antisense cDNA was used to decrease ProDH expression (Nanjo *et al.* 1999). The *AtProDH* gene encodes the gene for proline dehydrogenase, an enzyme that catalyzes the degradation of proline. These transgenic plants accumulated higher levels of proline compared with wild type and displayed increased tolerance to salt (600mM NaCl), albeit for a short duration (0.5hr) and freezing stress (¯7 °C for two days).

Arabidopsis is a non-accumulator of glycine betaine but transgenic plants engineered to synthesise glycine betaine by the overexpression of choline-oxidising enzymes, for example COD (from the soil bacteria *Arthrobacter globiformis*) exhibit improved salt tolerance (Hayashi *et al.* 1998). In another study *A. panescens* COX (homologous to the *Arthrobacter globiformis* COD gene) was used to transform *Brassica napus*, *Arabidopsis* and tobacco. Moderate improvements in tolerance to drought, salinity and freezing were reported but the tolerance was variable between the species (Huang *et al.* 2000).

Over expression of compatible solutes offers protection against some of the toxic effects of Na^+ and Cl^- but at a considerable metabolic cost, and significant amounts of Na^+ would still need to be compartmentalised. The over production of these solutes, therefore, must be accompanied by up regulation of Na^+ transporters, either on the plasma membrane to efflux Na^+ out of the cell, or the tonoplast membrane to sequester Na^+ into the vacuole (Tester & Davenport 2003).

1.4.2 Maintenance of a Low Cytoplasmic Cl^- Concentration.

In plants chloride has two main roles: one as a counter anion for cation transport by maintaining an electrical charge balance for the uptake of essential cations (Ca^+, K^+, Mg^+, NH^{4+}): the second as a major osmotically active solute and is involved in both turgor and osmoregulation. Chloride is a micronutrient essential for healthy plant growth. A minimal requirement for crop growth of 1 g kg^{-1} dry weight has been suggested, a quantity that can generally be supplied by rainfall (White & Broadley 2001). Chloride deficient plants are rarely found in agriculture or nature, however excessive accumulation of Cl^- in plant tissue can be detrimental to crop growth by interfering with the ability of plants to acquire or assimilate other essential nutrient ions.

1.4.3.Maintenance of High Cytoplasmic K^+/Na^+ Ratios.

Potassium has a particularly important role in plant growth and development and a high cytosolic K^+/Na^+ ratio is important for maintaining cellular metabolism. Essential physiological functions of K+ in plant cells include enzyme activation, osmoregulation, control of membrane potential, and opening and closing of stomatal pores. Due to the similarities in the physicochemical properties between Na^+ and K^+, Na^+ exerts its toxic effect by competing with K^+, initially at transport sites for K^+ uptake into the cell resulting in K^+ deficiencies, and then at the intracellular level competing for binding sites essential for cellular growth. Unlike glycophytes, all salt-resistant plants are capable of demonstrating a strong preference for K^+ over Na^+ accumulation at the root / soil boundary. This is not only true for salt 'excluders', but also for salt 'includers' growing in brackish conditions. Any one, or combination, of three mechanisms may

account for this. When compared with glycophytes, salt-resistant plants may show: (1), reduced levels of Na^+ uptake: (2), better discrimination for K^+ acquisition: (3), enhanced Na^+ efflux (Fig 1.1). Unfortunately, it is still unclear which mechanism(s) operate(s) in salt tolerant plants.

1.4.3.1 Reduced Sodium Uptake.

In plants, high cytoplasmic sodium concentrations are toxic, and Na^+ influx into root cells has been shown to occur by channels and transporters for essential ions such as K^+ and Ca^{2+} but which are also Na^+ permeable. The LCT1 transporter, for example, was originally cloned from wheat by complementation of yeast, where LCT1 was found to restore yeast growth at micromolar K^+ concentrations (Schachtman *et al.* 1997). The LCT1 transporter functions as a non selective cation permeable transporter mediating not only K^+ influx but also Rb^+, Cd^{2+}, Na^+ and Ca^{2+} transport (Clemens *et al.* 1998; Schachtman *et al.* 1997). Studies have shown LCT1 expression renders yeast more NaCl sensitive (Amtmann *et al.* 2001), and it is thought that this transporter may play a significant role as a pathway for Na^+ uptake in plants under high salt conditions. The plasma membrane of salt tolerant plants may contain fewer non-selective cation transporters and channels compared with salt sensitive plants, or have other mechanisms of restricting sodium influx either into the root cells or into the xylem stream.

1.4.3.2 Better Discrimination for K^+ Uptake.

In plants there are two mechanisms of K^+ uptake. The first mechanism has a high affinity for K^+ (km of 10-30 μM) to allow uptake at low K^+ concentrations (micromolar range). The second is a low affinity mechanism that mediates K^+ uptake at

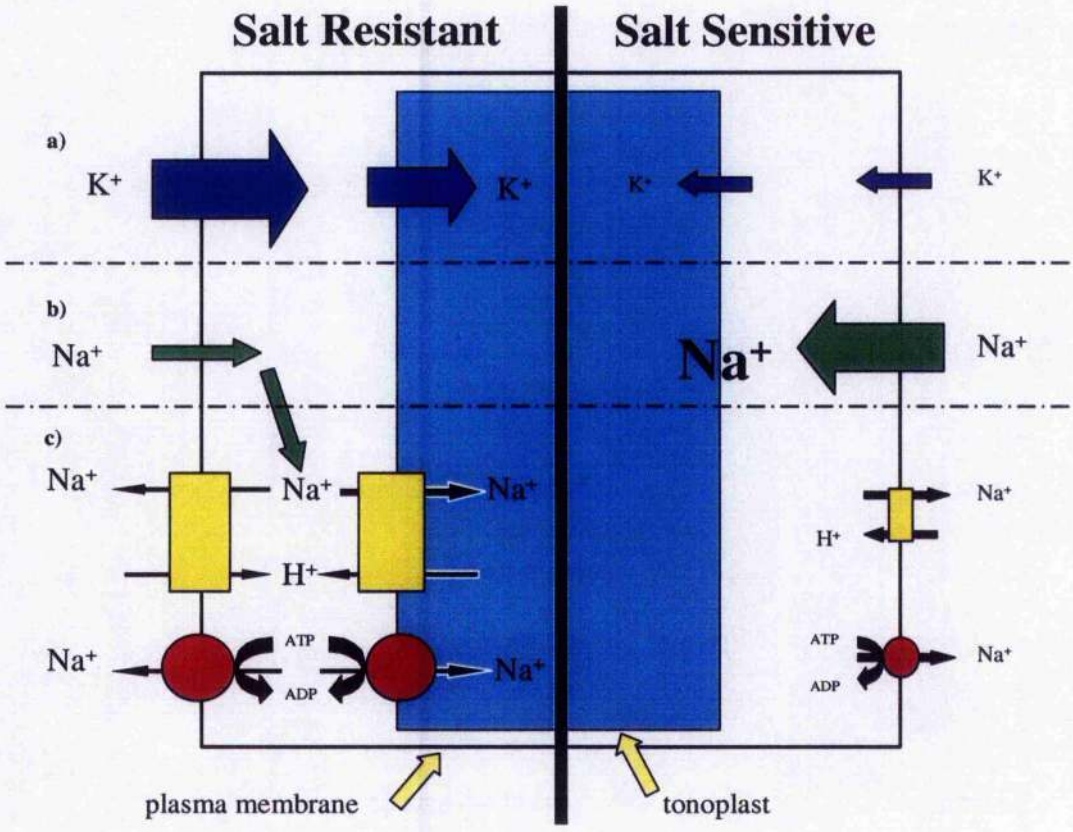


Figure 1.1 Possible Mechanisms of Maintaining Ion Balance Across the Plasma Membrane of Salt-Resistant Plant Cells.

When compared with salt-sensitive plants, salt-resistant plants are believed to show increased levels of **(a)** potassium uptake, **(b)** decreased levels of sodium uptake, and **(c)** more efficient methods of sodium efflux by Na^+-ATPases (red) or Na^+/H^+ antiporters (yellow).

Na^+, the major toxic cation found in saline soils, has a similar physiochemical structure to K^+ and competes for uptake, interfering with K^+ nutrition. The mechanism for Na^+ uptake into plant cells is unknown, but has been assumed to occur through the K^+ pathway(s) or non-specific cation channels such as LCT1. Na^+ efflux might occur through a Na^+-ATPase. To date, no Na^+-ATPase has been identified in higher plants but have important roles in salt tolerance of *S. cerevisiae* (*ENA1-4* system) and the moss *Physcomitrella patens*. The Na^+ / H^+ antiporter SOS1 is located on the plasma membrane and transports Na^+ out of the cell. NHX1 is a Na^+/H^+ antiporter transporter located on the tonoplast membrane and transports Na^+ out of the cytoplasm into the vacuole.

high external K^+ concentrations (millimolar range). Sodium most likely occurs via this route.

A high affinity mechanism for K^+ uptake, HKT1, was originally identified by complementation of a yeast strain defective in K^+ uptake with wheat root cDNA (Schachtman & Schroeder 1994). Detailed analyses of HKT1 expressed in yeast and *Xenopus* oocytes demonstrated two transport modes of HKT1, a saturable high-affinity K^+-Na^+ symporter and a low affinity Na^+ transport (Rubio *et al.* 1995b). High affinity K^+ uptake is stimulated by micromolar extracellular Na^+ concentrations. However, at high toxic extracellular Na^+ concentrations, K^+ uptake mediated by HKT1 is blocked and selective, low affinity, channel-like Na^+ uptake occurs (Gassmann *et al.* 1996; Rubio *et al.* 1995a). HKT1 homologs have been isolated or detected from many plant species, including *Arabidopsis* (Uozumi *et al.* 2000), rice (Fairbairn *et al.* 2000) and the halophyte *Mesembryanthemum* (Sul *et al.* 2003). The *Arabidopsis* genome includes only one HKT1 gene, *AtHKT1*. AtHKT1 has been shown to mediate only Na^+ in Xenopus oocytes and is not significantly permeable to K^+. Expression of *AtHKT1* has been shown to occur only in phloem tissues and is suggested to function by recirculating Na^+ from the shoots to the roots by the phloem sap (Berthomieu *et al.* 2003). T-DNA insertions in *AtHKT1* suppressed the salt hypersensitivity and K^+-deficient phenotype of the salt overly sensitive 3 (sos3) mutant (Rus *et al.* 2001). Antisense expression of wheat HKT1 in transgenic wheat was shown to significantly reduce ^{22}Na uptake (Laurie *et al.* 2002). These results suggest suppression of HKT1 expression can improve salt tolerance.

Another class of high affinity K^+ carriers, the KUP/HAK family, was identified on the basis of homology to known genes from bacteria and fungi, and has been shown to

mediate low affinity Na^+ influx at high extracellular Na^+ concentrations (Santa-Maria *et al.* 1997).

1.4.3.3 Sodium Efflux.

Sodium efflux from root cells can prevent Na^+ accumulating to toxic levels within the plant. Efflux of sodium from cells may occur by a Na^+-ATPase, or a Na^+/H^+ antiporter mechanism, or both.

In the yeast *Saccharomyces cerevisiae* ENA1, a Na^+-ATPase mediates Na^+ efflux across the plasma membrane and functions in addition to the Na^+/H^+ antiporter system (Haro *et al.* 1991). To date, no Na^+ -ATPases have been identified in higher plants, although *ENA1* homologs have been described in the *Physcomitrella patens*. It has been suggested these pumps existed in primitive land plants but these genes may have been lost during the evolution of higher plants (Benito & Rodriguez-Navarro 2003).

To function, the Na^+/H^+ antiporter requires the establishment of a proton motive force (pmf), which in plants and fungi is generated by the plasma membrane P-type H^+-ATPase, and the tonoplast V-type H^+-ATPase and H^+-PPase. These H^+ pumps generate an electrochemical potential difference of protons that drives the H^+ coupled Na^+ transport mechanism of the antiporter.

In *Arabidopsis*, Na^+ efflux across the plasma membrane is catalysed by the Na^+/H^+ antiporter SOS1 and has been demonstrated to play a crucial role in sodium extrusion from root epidermal cells under salinity (Shi *et al.* 2000). The SOS1 protein has 12 transmembrane domains in the N-terminal half and a long hydrophilic C-terminal tail (Shi *et al.* 2002) and has been demonstrated to exchange Na^+ specifically, and cannot transport Li^+ or K^+ (Qiu *et al.* 2002). The transcript level of *SOS1* has been shown to be upregulated by NaCl stress but not by drought, cold, or abscisic acid (Shi *et al.* 2000). In transgenic *Arabidopsis* plants, over expression of *SOS1* has been demonstrated to confer

salt tolerance whereas SOS1 deficient *mutants* display hypersensitivity to salt stress and accumulate more Na^+ in shoots than wild type plants (Shi *et al.* 2003).

1.4.3.4 Cellular Ion Compartmentation.

Since the sensitivity to salt of cytosolic enzymes is similar in both glycophytes and halophytes, any excess Na^+ and Cl^- ions must be sequestered in the vacuole, and compatible solutes (section 1.4.1) function to balance the solute potential of the cytoplasm. Thus vacuolar compartmentation of Na^+ and Cl^- can be an essential mechanism for survival in saline conditions. (Binzel *et al.* 1998) demonstrated that tobacco cells adapted to and growing in 428 mM NaCl accumulated 780 mM Na^+ and 624 mM Cl^- in the vacuole, whereas cytoplasmic levels of both ions were below 100 mM. This compartmentation of ions was not observed in non-adapted tobacco cells.

Sodium sequestration into the vacuole depends on expression and activity of Na^+/H^+ antiporters as well as on V-type H^+-ATPases and H^+-PPases. These phosphatases generate the necessary proton gradient required for activity of Na^+/H^+ antiporters. In *Arabidopsis*, transgenic plants over expressing the vaculolar H^+-PPase, *AVP1,* displayed enhanced salt tolerance that was correlated with the increased ion content of the plants (Gaxiola *et al.* 2001).

In *Arabidopsis*, the *AtNHX* family of Na^+/H^+ antiporters are localised on the tonoplast membrane and function in Na^+ sequestration in the vacuole (Blumwald 2000). The steady state level of *AtNHX1* transcript is upregulated by treatment with NaCl and ABA. This upregulation by NaCl is reduced in the ABA-insensitive mutant *(abi1)* and ABA deficient mutants *aba2-1 and aba3-1*, but not in *sos1*, *sos2* or *sos3* mutants (Shi & Zhu 2002). This suggests that an SOS-independent, ABA-dependent pathway regulates the expression of the AtNHX1 Na^+/H^+ antiporter in response to salt stress.

Over expression of the *Arabidopsis* NHX1 considerably increased salt tolerance of *Arabidopsis* (Apse *et al.* 1999) and subsequently has been successfully used to create salt tolerant transgenic lines of tomato (Zhang & Blumwald 2001) and *Brassica napus* (Zhang *et al.* 2001). Tomato is a highly salt-sensitive crop (Table 1.1) but transgenic plants over expressing *AtNHX1* can tolerate very high concentrations of salt (200mM NaCl). When subjected to high salt levels these transgenic tomato plants produced fruit of comparable yield and quality with control plants grown under non-stress conditions, and although the leaves of the transgenic plants accumulated high sodium concentrations, the tomato fruits displayed very low sodium content (Zhang & Blumwald 2001). *B. napus* is also a salt sensitive crop (Table 1.1). Transgenic plants over expressing *AtNHX1*, however, were able to grow, flower, and produce seeds in the presence of 200mM NaCl. Growth of transgenic plants over expressing *AtNHX1* was only marginally affected by high salinity (200mM NaCl) despite accumulation of Na^+ to almost 6% of their dry weight. Seed yield and seed oil quality were not affected by high salt concentrations (Zhang *et al.* 2001).

1.5 Regulation of Salt Tolerance Mechanisms.

Each of the processes that can affect plant salt tolerance described previously must be regulated in response to plant stress. First, salt stress must be 'sensed', which is often followed by the generation of secondary messengers that can initiate a transduction pathway that finally targets proteins directly involved in stress tolerance or transcription factors controlling stress specific genes (Fig 1.2).

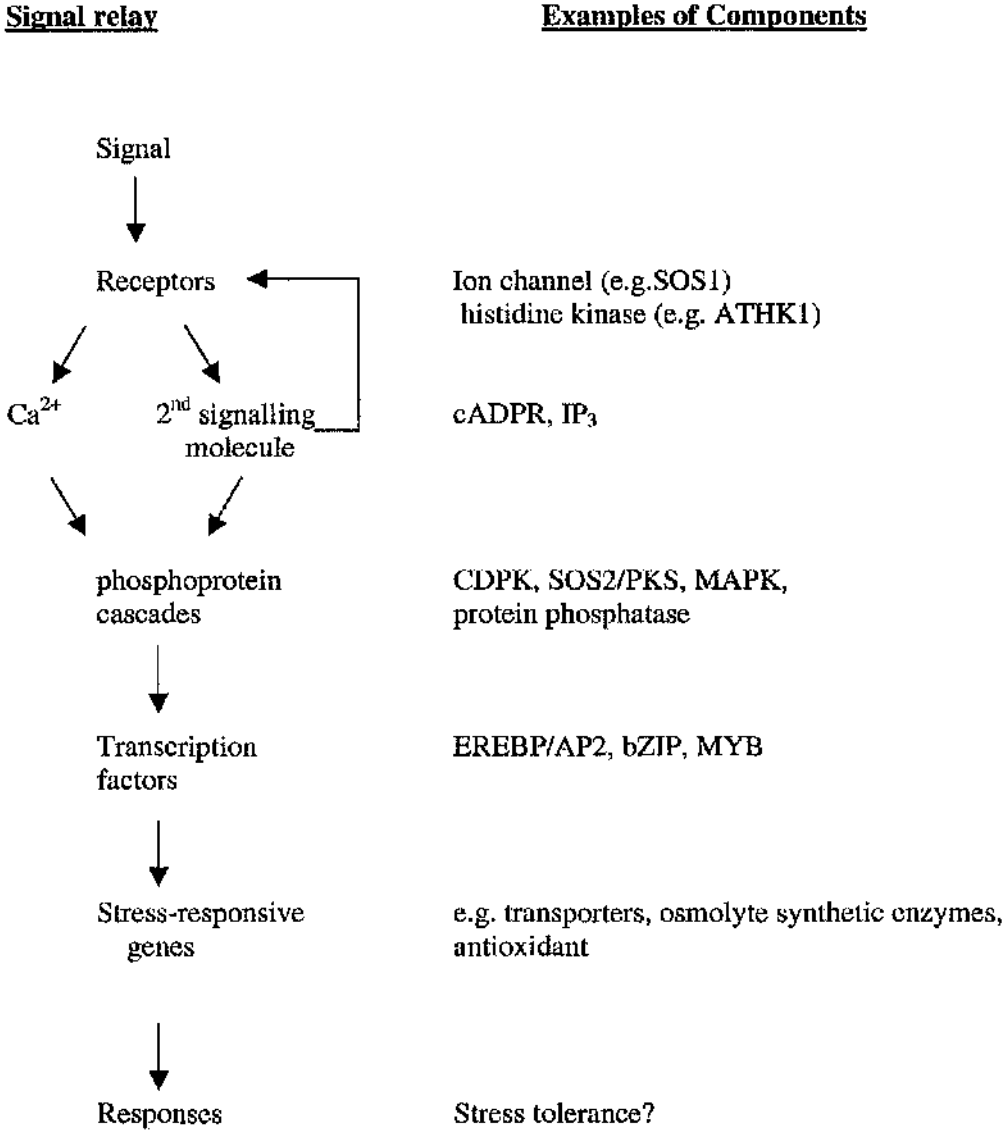


Figure 1.2 A Generic Pathway for the Transduction of Salt Stress Signals in Plants. Once the salt stress has been perceived, a signal transduction pathway is triggered which finally regulates salt tolerance mechanisms. Examples of signalling components in each of the steps are shown. Figure adapted from Xiong *et al.* (2002).

1.5.1 Sensors of Salt Stress.

Plants can sense salt stress through both ionic and osmotic stress signals. Osmotic stress activates the synthesis of the plant stress hormone abscisic acid, which has been shown to play an important role in response to various abiotic stresses including drought, salinity, cold and hypoxia (reviewed by Zhu 2002). In the absence of stress, the ABA mutants *aba1, aba2 and aba3* appear only slightly smaller than wild type plants. These plants are much more susceptible, however, to drought and salt stress (Xiong *et al.* 2001). ABA induces the expression of numerous plant genes, some of which encode signal transduction components such as putative receptors and protein kinases/phosphatases, others encode structural products with roles in plant salt tolerance (Zhu 2002). For example, expression of *AtNHX1,* which encodes a tonoplast Na^+/H^+ antiporter, has been shown to be upregulated by ABA (Shi & Zhu 2002).

Loss of turgor caused by osmotic stress can lead to retraction of the plasma membrane from the cell wall; this could trigger conformational changes in membrane proteins and initiate a signalling cascade. Changes in turgor may be detected by stretch –activated channels or by transmembrane protein kinases. The *Arabidopsis* ATHK1, for example, is a histidine kinase that is thought to function as sensor molecule that transduces extracellular signals to the cytoplasm. Transcript levels of *ATHK1* were induced by salt and low temperature stresses. Increased osmotic pressure is thought to cause a conformational change in ATHK1, inactivating the kinase activity of this protein (Urao *et al.* 1999).

Sodium may be sensed by a membrane receptor. The plasma membrane Na^+/H^+ antiporter is thought to be able to sense Na^+. As mentioned previously the SOS1 protein

has 12 transmembrane spanning domains and an unusually long hydrophilic cytoplasmic tail in the C-terminus that is predicted to reside in the cytoplasm. It has been proposed that the C-terminus can function as a Na^+ sensor (Shi *et al.* 2000).

Calcium is an important signal molecule in transduction pathways that lead to stress gene expression. Salinity stress elicits a transient increase in cytoplasmic Ca^{2+} concentrations (Knight *et al.* 1997). This Ca^{2+} is derived from either influx from the apoplastic space or is released from internal stores such as the vacuole or ER, both of which contain Ca^{2+} concentrations many orders of magnitude greater than those measured in the cytoplasm. Release of Ca^{2+} from these internal stores is controlled by ligand-sensitive Ca^{2+} channels. These ligands are secondary messengers and include inositol 1,4,5-triphophate (IP_3), nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPR). These signalling molecules are synthesised by enzymes in the plasma membrane and are translocated to the vacuole and ER where they function by activating Ca^{2+} channels, releasing Ca^{2+} into the cytoplasm. Transient increases in IP_3 levels have been shown to occur in response to a variety of environmental cues; light, pathogen, gravity and ABA (Carre & Kim 2002; Sanchez & Chua 2001; Stevenson *et al.* 2000). In *Arabidopsis*, IP_3 has been shown to increase in plants under salt stress, the time frame for this increase was correlated with changes in cytosolic Ca^{2+} levels (DeWald *et al.* 2001). Transient increases in cytosolic Ca^{2+} are perceived by various Ca^{2+} binding proteins (Section 1.5.2).

1.5.2 Salt Stress Signal Transduction Pathways.

Once the salt signal has been perceived, a signal transduction pathway is triggered which finally regulates salt tolerance mechanisms. This pathway often occurs by

multiple phosphoprotein cascades that transduce and amplify the information. Many kinases and phosphatases have been implicated in salt tolerance and are thought to function in such pathways, for example SOS2. *SOS2* encodes a serine/threonine kinase with a N-terminal kinase catalytic domain and a unique C-terminal regulatory domain (Liu *et al.* 2000). The catalytic and regulatory domains of SOS2 interact with each other and repress the kinase activity by blocking substrate access to the catalytic site (Guo *et al.* 2001). Investigations using the yeast two-hybrid system demonstrated interactions between SOS2 and SOS3 (Halfter *et al.* 2000). In the presence of Ca^{2+}, SOS3 (see below) interacts with SOS2 though the regulatory domain of SOS2. This interaction activates the SOS2 kinase by making the catalytic site available to substrates. The activated SOS2-SOS3 complex can then elicit salt tolerance effectors, the most well known example being the plasma membrane Na^+/H^+ antiporter SOS1 (Qiu *et al.* 2002).

The SOS2-SOS3 complex is also thought to regulate AtHKT1 activity (Section 1.4.3.2). HKT1 is a K^+ transporter and allows entry of Na^+ into plant cells under saline conditions. In *Arabidopsis*, mutations in AtHKT1 suppressed the salt hypersensitivity of the *sos3* mutant by reducing Na^+ accumulation, suggesting the SOS3-SOS2 complex suppresses AtHKT1 activity (Rus *et al.* 2001).

As mentioned previously, increased cellular Ca^{2+} concentrations have been shown to occur in response to ABA, drought, and salinity. Calcium functions as a second messenger by binding to proteins that can trigger phophoryl relay cascades. There are three major classes of Ca^{2+} binding proteins in plants, the calcium-dependent protein kinases (CDPK), calmodulins, and calcineurin B-like proteins.

The calcium-dependent protein kinases (CDPKs) are involved in stress responses, by binding Ca^{2+} these kinases couple changes in cytosolic Ca^{2+} to phosphory relay

cascades. CDPKs have been identified in many plants but not in animals. CDPKs are serine/theronine protein kinases with a C-terminal calmodulin-like domain with up to 4 EF-hand motifs. Most CDPKs have a myristoylation motif that potentially facilitates membrane association. It has been reported that CDPKs from various plants are induced by a selection of stimuli. For example, McCDPK1 induction by salinity and drought has been demonstrated in *Mesembryanthemum crystallinum* (Patharkar & Cushman 2000). In *Arabidopsis* transcript levels of *AtCDPK1* and *AtCDPK2* are increased by drought and salt stress, but not by cold, heat or ABA (Urao *et al.* 1998). Interestingly, over expression of the CDPK, OsCDPK7, has been shown to confer both cold and salt/drought tolerance in transgenic rice plants (Saijo *et al.* 2000).

CBL1 is a calcineurin B-like protein, which has been described as a positive regulator of salt and drought responses and a negative regulator of cold responses in *Arabidopsis* (Cheong *et al.* 2003). Transgenic *Arabidopsis* plants over expressing *CBL1* display increased tolerance to salt and drought but reduced tolerance to freezing. By contrast, *cbl1* null mutant plants were less tolerant to salt and drought but displayed enhanced tolerance to freezing.

Activation of the Na^+/H^+ antiport activity of SOS1 by salt stress, as mentioned previously, is regulated by the SOS3-SOS2 kinase complex (Qiu *et al.* 2002). SOS3 is a Ca^{2+} sensor protein with 3 calcium binding EF hands and a N-myristoylation motif that is capable of sensing the cytosolic calcium signal elicited by salt stress (Liu & Zhu 1998). In the presence of calcium, SOS3 binds to the autoinhibitory motif in the C-terminal regulatory domain of SOS2, and activates the substrate phosphorylation activity of SOS2 (Halfter *et al.* 2000). The SOS2-SOS3 then functions by activating SOS1 and presumably other targets.

The mitogen-activated protein kinase (MAPK) cascades mediate signal transduction from the cell surface to the nucleus. The basic assembly of MAPK cascades consists of three sequentially activated kinases and is conserved in all eukaryotes. MAPKs are activated by phosphorylation on their threonine and tyrosine residues by dual-specificity kinases, MAPK kinases (MAPKKs). MAPKK kinases (MAPKKKs) are the first component of this phosphorelay system that activates MAPKKs by phosphorylating their serine/threonine residues. The MAPKKKs carry distinct motifs in their sequences that selectively confer their activation in response to different stimuli. Once activated, MAPKs may function in a variety of ways, for example, they can activate transcription factors directly, activate additional signalling components to regulate gene expression or target certain signal proteins for degradation. MAPKs have been implicated in biotic and abiotic signalling pathways (reviewed by Zhu 2002). In alfalfa cells, for example, SIMK is a MAP kinase activated in response to moderate osmotic stress (Munnik *et al.* 1999).

One important step in the control of responses to high salinity appears to be the coordinated transcriptional activation or repression of genes. This requires the activity of specific transcription factors that bind to specific sequences in the promoter regions of target genes (Chen *et al.* 2002). Examples of such promoter regions include the dehydration-responsive elements/C-Repeat (DRE/CRT, ABA-independent) and the ABA responsive elements (ABREs, ABA-dependent). Many ABA responsive proteins contain the ABRE element and several bZIP transcription factors (ABF/AREB) have been identified which bind as dimers to the ABRE elements, for example, AREB1 and AREB2. Many drought or cold responsive genes contain the DRE/CRT element. The transcription factors which bind to this element are called either C-repeat Binding

Proteins (*CBFs*) or Dehydration Responsive element Binding Proteins (*DREBs*). Expression of DREB2A and DREB2B, for example, is induced by dehydration and salt stress (Liu *et al.* 1998). Over expression of CBF/DREB1 has been shown to induce stress-responsive gene expression and increase plant tolerance to cold and drought stress (Liu *et al.* 1998).

1.6 Aims of project.

The objectives of this study were:

1. To investigate mechanisms of ion transport across the plasma membrane of salt tolerant and salt sensitive plant cell cultures.
2. To isolate and characterise *Arabidopsis* activation tagged salt tolerant mutants.

Part of the study was directed towards the comparable characterisation of *Arabidopsis* plant cell cultures (May and Leaver 1993), these included wild type (salt sensitive) and HHS (salt tolerant) lines. Total ion content of the lines subjected to different NaCl concentrations was measured by ICP-OES. Attempts were made to use ion-specific fluorescence probes to report intracellular ion concentrations and location. Attempts were also made to assess the activity of ion channels using electrophysiological techniques, two-electrode voltage clamping (TEVC) and the planar lipid bilayer technique.

Arabidopsis is a glycophytic plant that is sensitive to growth inhibition and damage by salt stress. The habituation of the HHS cell line to high salt conditions, however, suggests that *Arabidopsis* has the genetic potential to be salt tolerant. With this knowledge, a population of *Arabidopsis* activation tagged lines was screened for mutants displaying a salt tolerant phenotype. Once isolated and the salt tolerant

phenotype confirmed, the mutants were characterised using a variety of approaches. TAIL-PCR was used to identify the location of the activation tag within the plant genome. Southern blot analysis was performed on to determine the number of independent vector inserts within each line. Semi quantitative RT-PCR was used to investigate transcript levels of genes located near the activation tag.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Plant material

2.1.1.1 Plant Cell Cultures.

Arabidopsis thaliana (L) Heynh. var. Landsberg erecta photomixotrophic cell suspension culture were obtained from Prof. C. Leaver, University of Oxford (May & Leaver 1993) This culture, which is sensitive to 75mM NaCl, was used to acclimatise cultures for growth on media supplemented with 380mM NaCl.

Atriplex halimus cell culture was generated from root meristem tissue by Dr Nigel Urwin. *A.halimus* is a halophyte and the cell culture can tolerate media supplemented with 400mM NaCl.

2.1.1.2 *Arabidopsis* Activation Tagged Lines.

Arabidopsis activation tagged lines generated by Weigel and co workers (2000) were purchased from the Nottingham *Arabidopsis* Stock Centre (http://nasc.nott.ac.uk/). The background for this collection is Columbia (Col-7). These lines are available as 3 sets. Set 1 (N21995) contains 86 pools of 100 lines; set 2 (N21991) contains 82 pools of 96 lines. Set 3 (N23153) contains 62 pools of 100 lines. Combined these 3 sets provide 22 672 lines divided into 230 pools.

2.1.2 Chemicals.

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich Chemical Co. Ltd., Dorset, UK or Fisher Scientific (UK) Ltd., Loughborough UK

Restriction enzymes were purchased from Promega (UK) Ltd., Southampton UK

Radioisotopes were from Amersham Biosciences, Bucks UK

Primers for PCR were designed using the online Primer3 software (Rozen and Skaletsky, 2000) and purchased from MWG Biotech AG, Ebersberg Germany. The primers were supplied desalted and were resuspended to the appropriate concentration in sterile water before use.

Antibiotics were supplied by Sigma-Aldrich Chemical Co. Ltd., Dorset UK All antibiotics used were dissolved in appropriate solvent and filter sterilised.

2.2 Methods

2.2.1 Growth of *Arabidopsis* and *Atriplex Halimus* Cell Suspension Cultures.

Arabidopsis cell suspension cultures were initiated by May and Leaver (1993). The suspension cultures were grown in MSMO media (Linsmaier & Skoog 1965) supplemented with 3% (w/v) sucrose, 0.5mg L^{-1} α-naphthalene acetic acid and 0.05mg L^{-1} kinetin, pH 5.8.

A.halimus cell cultures were initiated by Dr Nigel Urwin. The suspension cultures were grown in MSMO media (Linsmaier & Skoog 1965) supplemented with 3% (w/v) sucrose, 0.5 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 1 mg l^{-1} 1-naphthalene acetic acid (NAA), pH 5.8.

Arabidopsis and *A.halimus* cultures were grown under continuous light (PPFD 20 µmol m^{-2} s^{-1}) at 20°C, constantly shaken at 150rpm, and sub-cultured every 7 days under sterile conditions by transferring 10ml into a flask containing 90ml medium.

2.2.2 Generation of the *Arabidopsis* HHS (Habituated to High Salt) Cell Line.

Wild type *Arabidopsis* cell culture cannot survive in cell culture media (Section 2.2.1) supplemented with 75mM NaCl. The HHS (Habituated to High Salt) cell line was

adapted from non-treated wild type cell suspension by sub-culturing weekly into progressively higher levels of NaCl. Initially media was supplemented with 70mM NaCl. After several weeks, once the cultures adapted to this NaCl concentration, the NaCl concentration was increased by 10mM. When the cells adapted to this new concentration of NaCl, levels were increased by a further 10mM. This process was repeated and after a period of 2 years the cells were able to tolerate 380mM NaCl.

2.2.3 Measuring Growth Rates of Cell Cultures.

Cell culture was mixed by swirling and a 5ml aliquot removed under aseptic conditions. The aliquot was centrifuged (Sorvall Legend RT) at 500rpm in a graduated tube, total volume and packed cell volume were noted and the volume of cells/ml calculated.

2.2.4 Preparation of Cell Culture Material for ICP-OES Analysis.

Cell culture was mixed by swirling and a 10ml aliquot removed under aseptic conditions. Medium was removed by filtering using a pre-cooled Buchner flask and funnel connected to a vacuum pump. Cells were immediately washed using ice cold distilled water, then stored at -20°C. Washed cells were ground to a fine powder in liquid nitrogen using a pestle and mortar. The powder was transferred to a preweighed 15ml sterile falcon tube, dried overnight at 95°C, then reweighed and the dry weight of the samples calculated. 10ml 5% nitric acid was added to each tube, and left to digest for 2 nights on a shaking incubator. Ion content was measured using a Perkin Elmer Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) model optima 4300 DV (Perkin Elmer, Warrington, UK).

2.2.5 Surface Sterilisation of Seeds.

One Covclor 1000 chlorine tablet (Coventry chemicals Ltd UN 2465) was dissolved in 35ml dH_2O with 10µl Triton X-100. 5ml of this was added to 45ml ethanol, mixed gently by inversion and left at room temperature for 5 min. White precipitate was removed by centrifugation at 2000rpm for 5 min (Sorvall Legend RT). Under sterile conditions, seeds to be sterilised were transferred to an eppendorf tube and soaked in 70% ethanol for 2 min. The ethanol was removed and the seeds were soaked in 1ml bleach for 8 min, mixing occasionally by gentle inversion. The bleach was removed, the seeds washed twice in 70% ethanol then left to air dry. Once all traces of ethanol were removed the seeds were then washed 5 times in sterile dH_2O. The seeds were left in the final wash water for 2 nights at 4°C for stratification before sowing.

2.2.6 Isolation of Salt Tolerant *Arabidopsis* Activation Tagged Mutants.

The Weigel lines were surface sterilised (Section 2.2.5), chilled at 4°C for 2 days, then germinated on agar plates containing 0.75% sucrose, 80mM NaCl and MS media modified to contain 530µM Ca^{2+} and 200µM K^+ (Table 2.1). Seeds were germinated under continuous white light (PPFD 20µmol m^{-2} s^{-1}) at 22°C. Under these conditions approximately 50% of wild type seeds failed to germinate compared with 85% germination under control conditions. The majority of wild type plants that did germinate produced 2 cotyledons, white in colour, before growth arrested. Approximately 1 in 100 wild type plants continued to grow, albeit slowly. Often surviving wild type plants were small and pale yellow/green in colour. Putative salt-tolerant activation tagged mutants were identified as plants with greater shoot growth compared with wild type. After 4 weeks, the surviving plants were transferred to magenta jars containing ½ strength MS and 0.7% agar to recover. After one-week plants

Component	MS (Sigma M5519)	Modified MS
Macro Nutrient Salts (mM)		
Ammonium Nitrate	20.61	1.24
Calcium Chloride anhydrous	2.99	0.53
Magnesium Sulfate anhydrous	1.50	0.09
Potassium Nitrate	18.79	0.19
Potassium Phosphate monobasic	1.25	0.012
Sodium phosphate		0.063
Micro Nutrient Salts (μM)		
Boric Acid	100.00	6.00
Cobalt Choride-6H2O	0.110	0.006
Cupric Sulfate-5H2O	0.10	0.006
Na_2- EDTA	100.00	6.00
Manganese Sulfate-H2O	100.00	6.719
Molybdic Acid sodium salt	1.03	0.0618
Potassium Iodide	5.00	0.05
Ferrous Sulfate-$7H_2O$	100.00	6.00
Zinc Sulfate-$7H_2O$	29.91	1.794
Organics (μM)		
Glycine	26.64	1.596
myo-Inositol	560.00	33.299
Nicotinic Acid (Niacin)	4.06	0.2436
Pyridoxine HCL (vitamin B_6)	2.43	0.1458
Thiamine HCL (vitamin B_1)	0.30	0.0177

Table 2.1: Nutrient Composition of MS Media Used for Screening *Arabidopsis* Activation Tagged Lines.
The MS media used for screening was modified to contain, importantly, 530μM Ca^{2+} and 200μM K^+. This was supplemented with 0.75% sucrose and 80mM NaCl and the pH adjusted to 5.8.

were transferred to soil, grown to maturity and the seeds harvested. The putative mutants selected by primary screening were screened again under identical conditions to confirm salt tolerance. Percentage germination of seeds, size, and vigour of plants were noted. Authentic salt tolerant mutants were categorized according to phenotype, strong or weak, the false positives were discarded.

2.2.7 Isolation of Plant Genomic DNA.

Genomic DNA was extracted from plant tissue using a method adapted from Sambrook *et al.* (1998). Approximately 200mg fresh weight plant material was ground to a fine powder in liquid nitrogen using a pestle and mortar. The powder was transferred to a sterile Eppendorf tube and 600μl of extraction buffer (1.1% CTAB, 110mM tris-HCl pH 8.0, 55mM EDTA pH 8.0, 1.54M NaCl pre-warmed to 65°C) was added immediately followed by 1.56µl 20mg/ml proteinase K/ 50mM tris.HCl pH8.0, 1.5mM calcium acetate. 2% SDS was added and the solution mixed by inversion. The tubes were incubated at 65°C for 1 hour, mixed occasionally by inversion. The tubes were allowed to cool to room temperature and an equal volume of PCI (phenol: chloroform: isoamylalcohol 25:24:1) added. After mixing, the tubes were centrifuged at 13 000 rpm (Eppendorf 5415 D) for 2 min at room temperature. The top phase was transferred to a fresh eppendorf tube and an equal volume of chloroform:IAA (24:1) added. After centrifugation (as described previously) the upper phase was transferred to a fresh tube and the DNA extracted again with an equal volume of chloroform:IAA (24:1). The upper phase was transferred to a fresh Eppendorf and 0.6 volume isopropanol added. The tubes were gently rocked for 1-2 min and then incubated at room temperature for 10 min. To pellet the DNA the mixture was centrifuged for 10 min at 13 000 rpm. The supernantant was discarded and the pellet washed twice with ice-cold 70% ethanol. The tube was centrifuged briefly to consolidate the pellet and all traces of ethanol removed.

The pellet was re-dissolved in 500µl 0.1 x TE and allowed to re-hydrate on ice for 1-2 hours. Contaminating RNA was removed from DNA preparations by addition of 5µl of RNase stock solution (10mg/ml) followed by incubation at 37°C for 1 hour.
Alternatively, genomic DNA was isolated using the Qiagen DNAeasy Plant Kit (Qiagen, West Sussex, UK) following the manufacturer's instructions.

2.2.8 Isolation of Total RNA.

Total RNA isolation was preformed using TRI reagent (Sigma-Aldrich Chemical Co. Ltd., Dorset, UK). Approximately 100mg fresh weight plant material was ground to a fine powder in liquid nitrogen using a pestle and mortar. The powder was transferred to a sterile eppendorf tube and 1ml TRI reagent added. The mixture was vortexed briefly and incubated on ice for 5 min 0.2ml chloroform was added, the sample vigorously shaken for 15 secs before another incubation on ice for 10 min. The mixture was centrifuged (Eppendorf 5417R) at 12 000 x g for 15 min at 4°C. The colourless upper phase containing the RNA was transferred to a fresh tube, 0.5ml of isopropanol added and the tube mixed gently. The sample was allowed to stand on ice for 5 min. The solution was then centrifuged at 10 000 g for 10 min at 4°C and the supernatant discarded. The pellet was washed once with ice cold 75% ethanol. After all traces of ethanol had been removed the pellet was resuspended in 20µl DEPC treated water.

Contaminating DNA was removed from all RNA preparations using Ambion's DNA-*free* Kit (Ambion (Europe) Ltd, Cambridgeshire UK) following the manufacturer's instructions.

2.2.9 Quantification of DNA and RNA.

The quantity and purity of RNA and DNA were determined spectrophotometrically by measuring absorbance at 260 nm and 280 nm (Sambrook *et al.* 1989). An A_{260} nm of 1 is equivalent to a RNA concentration of 40μg/ml and a DNA concentration of 50μg/ml. The purity of RNA and DNA is determined by the ratio of absorbance at 260 nm to absorbance at 280 nm. An $A_{260/280}$ ratio of between 1.8 and 2.0 indicates that the nucleic acid is free from protein contamination. The quality of RNA and DNA was determined by agarose gel electrophoresis and the bands checked for signs of degradation (Section 2.2.10).

2.2.10 Agarose Gel Electrophoresis.

2.2.10.1 Denaturing Agarose Gel Electrophoresis of RNA.

Samples of 1μg RNA were separated by electrophoresis through a 1.3% (w/v) agarose gel containing 10% formaldehyde and 1 x MOPS buffer, pH 7.0 (20mM MOPS, 5mM sodium acetate, 1mM EDTA) as described by Sambrook *et al.* (1989). The RNA samples were mixed with 1% (v/v) formaldehyde, 30% (v/v) formamide, and 1x MOPS pH 8.0. The samples were heated at 65°C for 10 min; snap cooled on ice and loaded on to the gel. The gel was run for 2 hr at 100V in 1 x MOPS pH 7.0.

2.2.10.2 Electrophoresis of DNA and RNA in Non-Denaturing Conditions.

Samples of purified DNA and RNA were checked for integrity and molecular weight distribution using agarose gel electrophoresis (Sambrook *et al.* 1989). 1% (w/v) agarose gels were prepared and run in 1 x TBE (89mM tris-borate, 89mM boric acid, 2mM

EDTA, pH 8.3) containing 0.25μg/ml ethidium bromide. Samples of DNA or RNA were mixed with 6 x loading buffer (0.025% (w/v) bromophenol blue and 40% (v/v) sucrose in water and loaded alongside 1Kb DNA ladder (Promega UK Ltd, Southampton, UK). The gel was electrophoresed at 5V/cm for 1-2 hours depending on the dimensions of the gel.

2.2.11 Isolation of DNA Fragments from Agarose Gel.

The DNA fragment of interest was separated from residual DNA fragments by agarose gel electrophoresis (Section 2.2.10.2). The fragment was excised using a clean, sharp razor blade and transferred to an eppendorf tube. The DNA fragment was purified using a QIAquick Gel Extraction Kit (Qiagen, West Sussex, UK) following the manufacturer's instructions.

2.2.12 TOPO Cloning and Sequencing of PCR Products.

Successful PCR products to be sequenced were cloned into the TOPO vector and then transformed into One Shot TOP10 Chemically Competent *E. coli* cells following the manufacturer's instructions of the TOPO TA Cloning Kit for Sequencing (Invitrogen Ltd, Paisley, UK). Successful transformants were confirmed by colony PCR (Section 2.2.13), plasmid DNA extracted (Section 2.2.14) and sent for sequencing (MWG Biotech AG, Ebersberg Germany).

2.2.13 Colony PCR.

A sterile yellow pipette tip was dabbed onto a bacterial colony then soaked in 5µl dH_2O for 5 min (Sambrook *et al.* 1989). This was then mixed with 1 x ReddyMix (Abgene, Epsom, UK, contains 1.25 units Thermoprime Plus DNA polymerase, 75mM Tris-HCl (pH8.8), 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01% (v/v) Tween 20) 0.2mM each of

dATP, dCTP, dGTP and dTTP plus a precipitant and red dye for electrophoresis) and 100pmol of each primer (forward and reverse). The PCR programme had an initial denaturing step at 94°C for 10 min. This was followed by 25 cycles composed of a denaturing step at 94°C for 30 sec, an annealing step (temp according to primer Tm) for 30 sec and an extension step at 72°C (time according to fragment length). This was followed by a final extension step at 72°C for 5 min. PCR products were analysed by agarose gel electrophoresis (Section 2.2.10.2).

2.2.14 Plasmid DNA Isolation.

A single colony was used to inoculate 5ml of LB broth supplemented with the appropriate antibiotic. The culture was grown overnight at 37°C with constant shaking at 200 rpm. The plasmid DNA was isolated from the overnight culture using the QIAprep Spin Miniprep Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions.

2.2.15 Southern Blot Hybridisation.

2.2.15.1 Restriction Digestion of Genomic DNA for Southern Analysis.

Restriction digestion of gDNA (total reaction volume 35ul) was performed based on a method described by Sambrook *et al.* (1989). 20μg of genomic DNA, appropriate enzyme buffer and water were chilled at 4°C for 2 hours. 10 units of enzyme was added, the mixture gently stirred for 1-2 minutes then incubated at 37°C. After one hour a further 10 units of enzyme was added and the solution mixed. The digests were incubated overnight at 37°C. The digests were loaded on to a large 0.7% agarose gel and run for 8 hours at 50v.

2.2.15.2 Alkaline Blotting of Digested Genomic DNA.

Genomic DNA was digested and separated on an agarose gel (Section 2.2.15.1). The DNA was transferred by alkaline blotting to a nylon membrane (Zeta-Probe GT membrane Bio-Rad Laboratories Ltd.) following the manufacturers protocol. The DNA was depurinated by soaking the gel in 0.25M HCl for 15 min. A wick of Whatman 3MM paper was placed on a horizontal support soaked in 0.4M NaOH and each end dipped in a reservoir of 0.4M NaOH. The gel was placed on top of the wick ensuring that there were no air bubbles between the wick and the gel. A section of zeta probe membrane was cut to a size 1mm larger than the gel, soaked in dH_2O for 5 min then placed carefully on top of the gel. A clean plastic pipette was used to remove any air bubbles and the surface flooded with 0.4M NaOH. Two pieces of Whatman 3MM were cut to the size of the gel, soaked in dH_2O and placed on top of the membrane, again ensuring no air bubbles were trapped. Cling film was placed around the gel sandwiched between the lower wick and the upper 3MM paper to ensure all rising solution passed from the wick through the gel and up into the upper layer of 3MM paper. A stack of absorbent paper towels was then placed on top of the 3MM paper, a glass paper placed on top of the stack with a 300g weight on top. The blot was left overnight to allow the digested DNA to transfer to the membrane. The blot was dismantled and the membrane was marked with a pencil to identify the position of the wells. The gel and the membrane were examined under UV light to assess the transfer of DNA. The membrane was then carefully washed with 2x SSC, left to dry at room temperature before the DNA was crosslinked to the membrane in a GS Gene Linker (Bio-Rad Laboratories Ltd.).

2.2.15.3 Hybridisation.

Pre-hybridisation and hybridisation were carried out in 0.25M sodium phosphate pH 7.2, 7% SDS (according to the Zeta-Probe GT membrane manufacturers protocol). The

denatured, radiolabelled probe was added to the prehybridisation solution. Hybridisation was carried out at 37°C overnight. Membranes were washed four times for 30 min at 65°C, twice with 20mM Na Phosphate 5% SDS, then twice with 20mM Na Phosphate 1% SDS. Southern blots were exposed onto X-ray film (Fuji RX and Konica UK, Feltham, UK) using an intensifying screen at -80°C for 3-5 days.

2.2.15.4 Preparation of Radio Labelled DNA Probes.

PCR was performed on appropriate DNA fragments cloned into TOPO vector (Section 2.2.12). The reaction was performed in a 50µl volume containing 100ng plasmid DNA, 1x Reddymix (Abgene, Epsom, UK, ingredients described in Section 2.2.13) and appropriate primers. The amplification was carried out in a thermocycler under the following conditions: 1 min at 94°C, 20 cycles of 30 seconds 94°C, 30 seconds 55°C, 2 minutes 72°C, then 5 minutes at 72°C. A Qiagen PCR Purification Kit (Qiagen, West Sussex, UK) was used to purify the PCR product. 10ul of the cleaned PCR product was run on a gel to confirm fragment integrity. 25ng of the purified PCR product was used to generate radiolabelled DNA probes using the Rediprime™ II DNA Labelling System (Amersham Biosciences, Bucks UK) according to the manufacturer's protocol. NucTrap Probe Purification Columns (Stratagene, Amsterdam Zuidoost The Netherlands) were used to separate the unincorporated nucleotides from the radiolabelled DNA probe. Once synthesised the probe was denatured by heating to 95-100°C for 5 min followed by snap cooling on ice before adding to the hybridising solution.

2.2.16 Thermal Asymmetric Interlaced (TAIL)-PCR.

TAIL-PCR (based on a method described by Liu *et al.* 1995) was used for isolating the plant genomic sequence flanking the T-DNA activation tag. TAIL-PCR utilises 3 nested specific primers in successive reactions together with a shorter arbitrary primer (AD) of lower melting temperature so that the relative amplification efficiencies of specific and non-specific can be thermally controlled (Fig 2.1). The right border of pSKI015 is unstable and a region (up to 90 bp from the border) can be lost upon insertion into the plant genome, making TAIL-PCR from this border difficult. For this reason TAIL-PCR was always performed using primers which hybridise to the left border side of the vector.

50ng genomic DNA, isolated using a Qiagen DNeasy Plant Kit (Qiagen, West Sussex, UK), was used as template in the first PCR reaction. The 20µl primary reaction mix was comprised of 1 x PCR buffer (50mM Tris-HCl (pH 8.0), 100mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol and 1% Triton X-100), 1.5mM $MgCl_2$, 200µM each of dNTPs, 0.8 units Taq polymerase (Promega UK Ltd, Southampton, UK), 0.2µM specific primer #1 (LB1 ATACGACGGATCGTAATTTGT (Weigel, unpublished) and a degenerate primer (Table 2.2 Liu *et al.* 1995). The PCR reactions were performed on a thermocycler (PTC-200 MJ Research) for details see Table 2.3. The primary TAIL-PCR reaction products were diluted 200 fold and 4µl aliquots directly added to secondary TAIL-PCR reactions (20µl) containing 1 x PCR buffer, 1.5mM $MgCl_2$, 200µM each of dNTPs, 0.6 units Taq polymerase (Promega UK Ltd, Southampton, UK), 0.2µM specific primer #2 (LB2 TAATAACGCTGCGGACATCTA (Weigel, unpublished) and the same degenerate primer used in the primary reaction. After secondary amplification (Table 2.3) the TAIL-PCR reaction products were diluted 100 fold and 5µl aliquots

added directly to tertiary TAIL-PCR reactions (50µl) containing 1 x PCR buffer, 1.5mM $MgCl_2$, 200µM each of dNTPs, 0.8 units Taq polymerase (Promega UK Ltd, Southampton, UK), 0.2µM specific primer #3 (LB3 TTGACCATCATACTCATTGCTG (Weigel, unpublished) and the same degenerate primer used in the primary and secondary reactions. Amplified products from the primary and secondary reactions were analysed by agarose gel electrophoresis. A 70 bp difference in product size consistent with the specific primer positions was used as the criterion for judging product to be insertion specific.

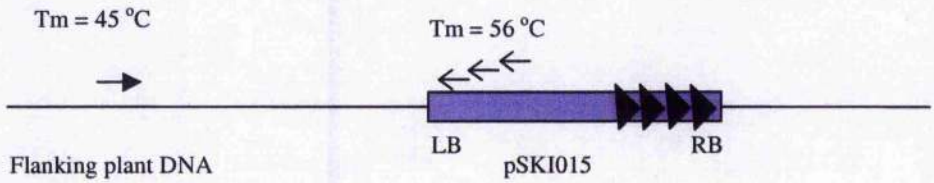


Figure 2.1 TAIL-PCR Amplification of pSKI015 Vector and Adjoining Genomic Plant DNA.

TAIL-PCR was used for isolating the plant genomic sequence flanking the pSKI015 activation-tagging vector. TAIL-PCR is performed in three successive amplification steps at alternating high and low stringency annealing temperatures with a combination of T-DNA-specific primers of high melting temperature (T_m) and degenerate low-T_m primers that can frequently anneal with plant genomic DNA. Genomic DNA is used as the substrate for the first reaction, the product contains not only the segment of interest but also a quantity of spurious fragments as the degenerate primers hybridise throughout the genome. The successive reactions amplify and purify the DNA fragment of interest.

AD (arbitrary degenerate) primers	Sequence	Fold degenerate	Working Concentration (μM)
1.1	TGWGNAGSANCASAGA	128	3
1.2	NTCGASTWTSGWGTT	64	2
2.1	NGTCGASWGANAWGAA	128	3
2.2	AGWGNAGWANCAWAGG	128	3
3	WGTGNAGWANCANAGA	256	4
5	STTGNTASTNCTNTGC	256	4

Table 2.2: Arbitrary degenerate (AD) primers used for TAIL-PCR.
Primer sequence taken from Liu *et al.* (1995) where S = G or C; W = A or T; N = A, C, G, or T.

Reaction	Number of cycles	Thermal settings
Primary	1	93°C, 1 minute 95°C, 1 minute
	5	94°C, 30 seconds 56°C, 1 minute 72°C, 2.5 minutes
	1	94°C, 30 seconds 25°C, 3 minutes Ramp at 0.2°C to 72°C 72°C, 2.5 minutes
	14	94°C, 10 seconds 56°C, 1 minute 72°C, 2.5 minutes 94°C, 10 seconds 56°C, 1 minute 72°C, 2.5 minutes 94°C, 10 seconds 44°C, 1 minute 72°C, 2.5 minutes
	1	72°C 5 minutes
Secondary	12	94°C, 10 seconds 56°C, 1 minute 72°C, 2.5 minutes 94°C, 30 seconds 56°C, 1 minute 72°C, 2.5 minutes 94°C, 30 seconds 44°C, 1 minute 72°C, 2.5 minutes
	1	72°C 5 minutes
Tertiary	20	94°C, 10 seconds 44°C, 1 minute 72°C, 2.5 minutes
	1	72°C 5 minutes

Table 2.3: Thermocycler settings for TAIL-PCR.
The PCR reactions were performed on a thermocycler (PTC-200 MJ Research). Thermocycler settings adapted from Weigel & Glazebrook (2002). Annealing temperatures were reduced from those described in the literature according to the Tm of the specific primers used in this study.

2.2.17 Semi-Quantitative RT-PCR.

Reverse transcription PCR (based on a method described by Fontaine *et al.* 2002) was used to investigate transcript levels of genes located near the pSKI015 vector. Total RNA was extracted from plant tissue using the TRI reagent method (Section 2.2.8). The RNA sample (2.5μg) were mixed with 0.25μM oligo dT for 10 min at 70°C and cooled at 4°C. Reverse transcription was carried out in a reaction mixture (25μL) containing AMV reverse transcriptase buffer (Promega UK Ltd, Southampton, UK), 1mM dNTPs (Promega UK Ltd, Southampton, UK), 1 U μL^{-1} RNase inhibitor (Promega UK Ltd, Southampton, UK) and 0·4 UμL^{-1} AMV reverse transcriptase (Promega UK Ltd, Southampton, UK). The reaction was performed at 48°C for 45 min. The enzyme was then heat-inactivated at 95°C for 5 min and the samples used directly for PCR. PCR reactions were performed using 1μL of each cDNA sample in a reaction mixture (20μl) containing 1 x ReddyMix (Abgene, Epsom, UK) and 0.5 μM of each primer. The primer sequences are indicated in Table 2.4. All PCR products were cloned and sequenced to confirm authenticity (Section 2.2.12). PCR reactions were conducted in a programmable thermocycler (PTC-200 MJ Research) using 23 cycles: 94°C for 5 minutes, 94°C/30 s, 55°C/30 s, 72°C/1 min, and a 5 min final extension step at 72°C. The annealing temperature was adjusted according to the Tm of the primers used. *ACT2* primers were used as a constitutive control. After amplification, the reaction was resolved by electrophoresis on a 1% agarose gel and stained with ethidium bromide. The PCR products were then quantified by scanning densitometry using Quantiscan version 3.0 software (Biosoft Cambridge UK).

Mutant	Gene	Primer Name	Sequence
		ACT2a	CTTACAATTTCCCGCTCTGC
		ACT3s	GTTGGGATGAACCAGAAGGA
JP1	At1g60200	60200 L	GGGCTGCATTAGATGAGAGA
		60200 R	ACAGAAGGCACAGAAGTTCG
	At1g60220	DUB L	GAACTCTGTTAGAGCGGTGAGAA
		DUB R	TGACTATTCCTTGGATCTGCCTA
JP2	At3g59410	GCN2L	AGGGGAGCTACCTCTCAAAT
		GCN2R	ATCTCCTCCATTGGTCAAAA
	At3g59400	59400L	GGAGAACCATCTCGTCAATC
		59400R	TATCATCAGCTGTCGCAAAC
JP3	At5g45900	APG7-Lnew	GCACCTCAGGATTCAATGAC
		APG7-Rnew	ACTCCGATATCACGGTTTCA
JP4	At1g55680	WD-40-L	AGGGGATGATGCTGAGTACA
		WD-40-R	TGTATTCCGCCAGAAATCAT
	At1g55690	Sec14-L	AGAAAGGCAATCCCAATACC
		Sec14-R	GTATTGCAACGGGTTTGTTC
JP5	At5g11040	11040-L	AGGGAGATACTCCTCTCTGCTGT
		11040-R	CAACAAGGATTTGCTAACTCCAC
	At5g11050	MYB64 L	CTTGGATGATCCTTATGACGAAG
		MYB64 R	TCATTGTTGTTGGAAGACAGAAA
JP10	At3g47450	NOS-L	GAAGGCTCTACCGGAAACAT
		NOS-R	TCTCATGCTCTGCTTCATTG

Table 2.4 The Primers used for Reverse Transcription PCR.
RT-PCR was performed based on a method described by Fontaine *et al.* (2002). Primers were designed to the coding sequence for each gene using online Primer3 softare (Rozen and Skaletsky 2000 http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). *ACT2* primers were used as a constitutive control.

2.2.18 Genetic Analysis of Mutants.

Homozygous salt tolerant plants (male parent) were crossed with homozygous *gl1* plants (female parent). *gl1* plants lack trichomes, therefore the F1 progeny of the crosses would be heterozygous for the recessive *gl1* trait and will have trichomes. Unsuccessful crosses can easily be identified by the lack of trichomes on the leaves and stems. Crosses were performed under a binocular microscope by transferring pollen from mature anthers of the salt tolerant mutants to the stigmas of previously emasculated flowers (*gl1*). F2 seeds from successful crosses were plated on agar medium containing 80mM NaCl (Table 2.1).

2.2.19 Using PCR to Genotype *Arabidopsis* Salt Tolerant Mutants.

To identify homozygous and heterozygous plants for each mutant, primers were designed for the plant gDNA flanking the site of insertion of the pSKI015 vector (generally a product size of ~400 bp). If the plant contained a wild type version gene (heterozygous) then a PCR product was obtained. If the plant was homozygous for the pSKI015 insertion (did not contain a wild type version of the gene) then no PCR product would be obtained using these primers as the pSKI015 vector would increase the distance between the primers by ~8kb.

To distinguish between heterozygous and wild type plants, one plant primer (P1 or P2) was used with primers that hybridised only to the pSKI015 vector. A product would be obtained with heterozygous plants but not with wild type plants.

2.2.20 Microarray Analysis.

Plants were germinated on vertical plates containing 0.75% sucrose and MS media modified to contains 530 μM Ca^{2+} and 200μM K^+ (Table 2.1) with either no additional NaCl (control) or supplemented with 80mM NaCl (high salt - long term treatment). RNA was extracted from 2-week-old tissue. Microarray preparation, hybridisation and data analysis was performed in collaboration with Drs. Patrick Armengaud and Anna Amtmann, Univ. of Glasgow (Armengaud *et al.* 2004). The Arizona full genome microarrays used were obtained from D. Galbraith, University of Arizona (http://www.ag.arizona.edu/microarray/).

2.2.21 Extraction and Purification of Plasma Membrane Fractions from Plant Cell Culture.

2.2.21.1 Isolation of Plasma Membrane Fractions from Plant Cell Culture.

Plasma membrane was isolated from *Arabidopsis* cell cultures using a method adapted from Larsson *et al.* (1994). This method can be divided into 2 sections, isolation of microsomal fraction and purification of plasma membrane from the microsomal fraction using two phase partitioning.

All solutions and containers were kept on ice throughout the procedure. At each step of the procedure a sample was taken for marker enzyme analysis. Plasma membrane was purified from 4-day old HHS (240mM NaCl) and wild type *Arabidopsis* cell cultures. Approximately 400ml of each culture was transferred to pre-weighed 50ml centrifuge tubes. The cultures were spun at 300g for 3mins using a bench top centrifuge, and the supernatant discarded. The tubes were re-weighed, and the wet weight of the cells calculated. The cells were then resuspended 5:1 with ice-cold extraction buffer (250mM sucrose, 2mM EDTA, 2mM ATP, 1% (w/v) BSA, 0.6% (w/v) PVP, 0.2mM PMSF,

15mM mercaptoethanol, 4mM DTT, 10% (v/v) glycerol, 70mM tris.HCl (pH8.0) and 250mM potassium iodide. The ATP, PMSF, mercaptoethanol and DTT were added fresh, immediately before use). The tissue was then homogenized using a french press (500 p.s.i.). The homogenate was centrifuged at 10 000g in a Beckman J2-HS centrifuge using a fixed angle rotor (JA-20) for 10 min at 4°C. The pellet, containing the larger organelles such as plastids, mitochondria and chloroplasts, was discarded and the supernatant subjected to a further spin – 50,000g for 30 minutes at 4°C in a Sorvall 65B ultracentrifuge using a fixed angle rotor (T865.1). The resulting pellet, which contained the plasma membrane, was resuspended with 9ml resuspension buffer #1 (330mM sucrose, 3mM KCl and 5mM potassium phosphate pH7.8) and constituted the microsomal fraction.

To purify the plasma membrane fractions from a variety of other cellular membranes present in the microsomal fraction, a series of separations using two aqueous phases of different densities was used. The membrane fragments partition in the two-phases according to their charge surface properties. Plasma membrane fragment concentrate in the upper phase.

The bulk phase system (6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol) was transferred to a separating flask and left overnight at 4°C. Once settled, the lower and upper phases were collected and frozen until required.

The resuspended microsomal material (9g) was then mixed with 27.0g phase mixture (final concentration 6.2% (w/w) polyethylene glycol 3350, 0.33M sucrose, 3mM KCl, 5mM potassium phosphate, pH 7.8). The phase system was thoroughly mixed by inverting 30-40 times and phase separation was facilitated by centrifugation (10 000g for 5 min at 4°C, swing out rotor). Approximately 90% of the upper phase was removed with a Pasteur pipette and re-partitioned twice with fresh lower phase. The final upper

phase was diluted 2 fold with resuspension buffer 1 (0.33M sucrose, 5mM potassium phosphate (pH 7.8) and 3mM KCl), before centrifugation at 4°C for 1 hour at 100 oogKg. The resulting pellet containing the purified plasma membrane fractions was resuspended in 100µL resuspension buffer 2 (250mM sucrose, 3mM KCl, 5mM EDTA, 1mM DTT and 25mM BTP pH 7.5).

2.2.21.2 Determining the Purity of Plasma Membrane Fractions by Marker Enzyme Assays.

2.2.21.2.1 Protein Assay – Lowry method.

The protein content of samples was determined by a modified Lowry procedure (Lowry *et al.* 1951). Triplicate 100µl samples of the protein sample was precipitated overnight by the addition of 100µl 10% (w/v) trichloroactic acid and 100µl 0.02% (w/v) sodium deoxycholate. Samples were centrifuged at 4°C for 15 min at 13 000rpm in a bench top centrifuge. The pellets were resuspended in 1ml 3% (w/v) SDS/NaOH. BSA solutions ranging from 0 – 500µg ml^{-1} was used as standards. To the triplicate samples and the BSA standards 1ml Lowry C solution (100:1:1 3% Na_2CO_3 in 0.24M NaOH: 6% KNa tartrate: 3% $CuSO_4.5H_2O$) was added, vortexed, and incubated at 37°C for 10 min. 1 ml phenol reagent was then added an incubated again at 37°C for 10 min. The absorbance of the unknown protein samples were then compared with the BSA standards of known concentration at a wavelength of 750nm in order to determine the amount of protein present per sample.

2.2.21.2.2 The Vanadate-Sensitive P-type H^+-ATPase Assay.

An enzyme-linked assay was used to measure the rates of the vanadate sensitive P-type ATPase and was based on a method described by Palmgren *et al.* (1990). Here the rate limiting p-type ATPase produces ADP which is required to convert PEP to pyruvate (by

pyruvate kinase) and hence to lactate (by lactate dehydrogenase) which oxidised NADH. The oxidation of NADH to NAD^+ is stoichiometrically linked to the hydrolysis of ATP, and easily followed spectrophometrically at 340nm. The reaction mixture was composed of 250mM sorbitol, 10mM BTP/MOPS (pH7.2), 50mM KCl, 2mM ATP, 1mM EDTA, 1mM DTT, 1mg/ml BSA, 1mM sodium azide. The remaining reagents were added in the following sequence and mixed: 50µl of protein sample, 7.5 units pyruvate kinase and 9 units lactate dehydrogenase (PK/LDH), and 1mM PEP. The solution was left to equilibrate for 4 min before the addition of 2.4µl $MgSO_4$ to start the reaction. To stop the reaction 5mM vanadate was added.

2.2.21.2.3 NADH Cytochrome c Reductase Assay.

This assay is used to detect the presence of tonoplast membrane in protein samples (Hodges & Leonard 1974). 500µl 50mM potassium phosphate buffer (pH7.5); 20µl protein sample (e.g. microsomal fraction), 20µl 50mM potassium cyanide in phosphate buffer and 40µl 0.45mM cytochrome c in phosphate buffer were added to a 1 ml quartz cuvette. The reaction was started by the addition of 20µl 3mM NADH in phosphate buffer. The rate of cytochrome c reduction was monitored at 550nM.

2.2.21.2.4 NADPH Cytochrome c Reductase Assay.

This assay is used to detect the presence of endoplasmic reticulum membrane in protein samples (Hodges & Leonard 1974). 500µl 50mM potassium phosphate buffer (pH7.5); 20µl protein sample (e.g. microsomal fraction), 20µl 50mM potassium cyanide in phosphate buffer and 40µl 0.45mM cytochrome c in phosphate buffer were added to a 1

ml quartz cuvette. The reaction was started by the addition of 20µl 3mM NADPH in phosphate buffer. The rate of cytochrome c reduction was monitored at 550nM.

2.2.21.2.5 Cytochrome *c* Oxidase Assay.

This assay was used to detect the presence of mitochondrial membranes (Hodges & Leonard 1974). 540µl 50mM potassium phosphate buffer (pH7.5), 20µl 0.3%(w/v) digitonin and 20µl protein sample was added to a 1 ml quartz cuvette. The reaction, measured by recording absorbance of the reaction mix at 550mM, was started by the addition of 20µl reduced cytochrome c and stopped by the addition of 20µl 1M sodium azide.

Chapter 3: Characterisation of HHS and WT *Arabidopsis* Cell Lines

3.1 Introduction.

Soil salinity presents an increasing threat to plant agriculture, affecting crop yields in many areas of the world. Salinity induces a wide range of perturbations at the cell and whole plant level. Salt stress results from a number of detrimental processes including a toxic action of Na^+ and Cl^- ions, the impairment of mineral nutrition, a modification on the water status of the plant tissues, and secondary stresses such as an oxidative stress linked to the production of toxic reactive oxygen intermediates.

Despite many decades of intensive research, it is still not known how salt tolerant plant cells maintain ionic balance. There have been advances in our understanding of the regulation of ion transport in a few model glycophyte species (e.g. *Arabidopsis*, rice etc), but the basic cellular mechanisms that maintain favourable ion gradients in halophytes exposed to high salinity are not known. In part, this is due to a lack of research effort focus on halophyte species, but also advances have been confounded by the complexity of the multi-tissued, whole root system. Whilst there is an understandable desire to work with whole roots, the different age of cells, and the different tissues presents the experimenter with results that are often difficult to interpret. For example there may be important transport mechanisms residing on the plasma membrane of endodermal cells that are vital for regulating salt balance in the xylem, and therefore the shoot. The endodermal cells, however, represent ~1% of the mass of roots. Reliable methods for isolating cells from different tissues have not been established. It is therefore difficult to characterise the kinetic properties of transport processes in tissue. Studies are limited, therefore, to interpreting long-term changes (minutes to hours) where several tissues are involved.

Plant cell suspension cultures offer a major advantage over intact tissue for investigating salt tolerance since the complexities superimposed by higher levels of tissue organisation are not present. To survive in high salinity, unlike the intact root system of a plant, each cell in culture must be expressing the required traits for salt tolerance. There is, of course, always the concern that cell suspension cultures will generate results that are artefacts, but at least they will allow studies on single cells that show a range of sensitivities to salinity. This chapter describes experiments performed to investigate the wild type (salt sensitive) and HHS (habituated to high salt) *Arabidopsis* cell cultures. Where possible, comparisons are drawn with cell cultures generated from the halophyte *Atriplex halimus*.

3.2 The Plant Cell Cultures Investigated.

The *Arabidopsis* cell cultures were generated from shoot tissue (May & Leaver 1993). Wild type *Arabidopsis* cell culture cannot survive in MSMO media supplemented with 75mM NaCl. The *Arabidopsis* HHS (Habituated to High Salt) cell line was adapted from wild type cell suspension by growing in MSMO media supplemented with 50mM NaCl; at this salt concentration wild type cells survive and grow slowly. Each week, two aliquots were removed; these were placed in MSMO media supplemented with 50 and 70mM NaCl respectively. Growth was assessed after one week prior to sub-culturing. Each week the culture at 70mM NaCl failed to survive, whereas the culture exposed to 50mM NaCl survived and grew slowly. After 52 weeks, the culture at 70mM NaCl survived and was slowly coaxed (over a further period of 52 weeks) to grow in progressively higher concentrations of NaCl (up to 380 mM NaCl section 2.2.2).

Atriplex halimus cell cultures were generated from root meristem tissue by Dr Nigel Urwin. *A. halimus* is a halophyte and the cell cultures were inherently salt tolerant, surviving in media supplemented with 400mM NaCl.

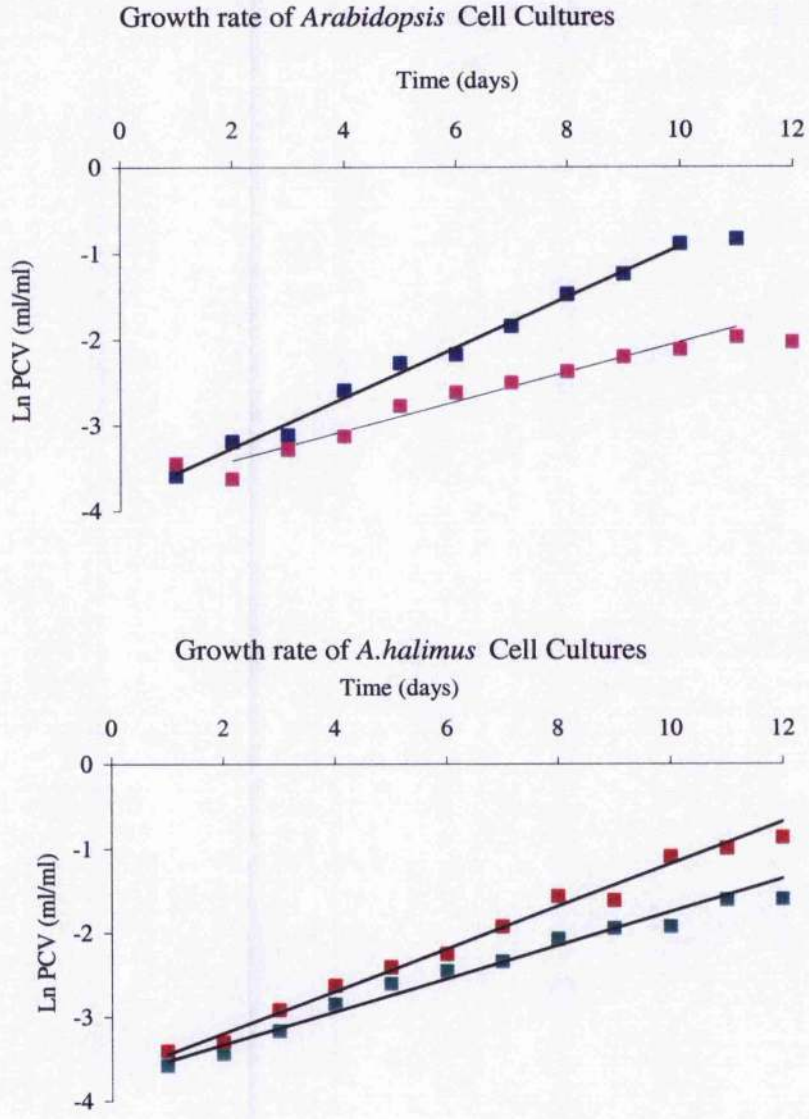
3.3 Growth Rates of *Arabidopsis* and *A.halimus* Cell Cultures.

Under aseptic conditions aliquots were removed daily from *Arabidopsis* wild type (no additional NaCl), *Arabidopsis* HHS (300mM NaCl), *A.halimus* low salt grown (no additional NaCl) and *A.halimus* high salt grown (300mM NaCl). After centrifugation the total volume and packed cell volume were noted and the volume of cells/ml calculated (Section 2.2.3). Wild type *Arabidopsis* cell cultures (0.1mM NaCl) were estimated to have a doubling time of 2.3 days compared with the HHS cell lines grown in 300mM NaCl which had a doubling time of 4 days (Fig 3.1 top graph). *A.halimus* is a facultative halophyte and can grow normally in low or non-saline environments. Cultures without NaCl grow quicker (doubling time 2.8 days) compared with cultures supplemented with 300mM NaCl (doubling time 3.5 days Fig 3.1 bottom graph).

3.4 Investigation of Ion Transport Mechanisms Across the Plasma Membrane of Salt Sensitive and Salt Tolerant Plant Cell Cultures.

Attempts were made to investigate ion transport processes across the plasma membrane of *A.halimus*, *Arabidopsis* HHS (salt tolerant), and *Arabidopsis* wild type (salt sensitive) cell lines (Section 3.2). The experimental approach was to study the biophysical and biochemical properties of these cell lines using a range of techniques (Fig 3.2).

Two-electrode voltage clamping (TEVC) can be used to assess the macroscopic currents from whole cells. Two electrodes are used to impale whole cells; one electrode reports the membrane potential while the other is used to pass current to maintain (i.e. clamp) the voltage at a predetermined value. The major advantage of this technique is that it



Cell Culture	Growth Doubling Time (days)
WT *Arabidopsis* (no NaCl)	**2.3** ± 0.11
HHS *Arabidopsis* (300mM NaCl)	**4.0** ± 0.25
***A.halimus* (no NaCl)**	**2.8** ± 0.15
***A.halimus* (300 mM NaCl)**	**3.5** ± 0.13

Figure 3.1 Growth Rates of Plant Cell Cultures.

Growth of cell cultures was assessed by removing aliquots and determining packed cell volume; each data point represents the average and standard error of single readings from 3 independent cultures. The average and standard errors of the doubling times are given in the above table.

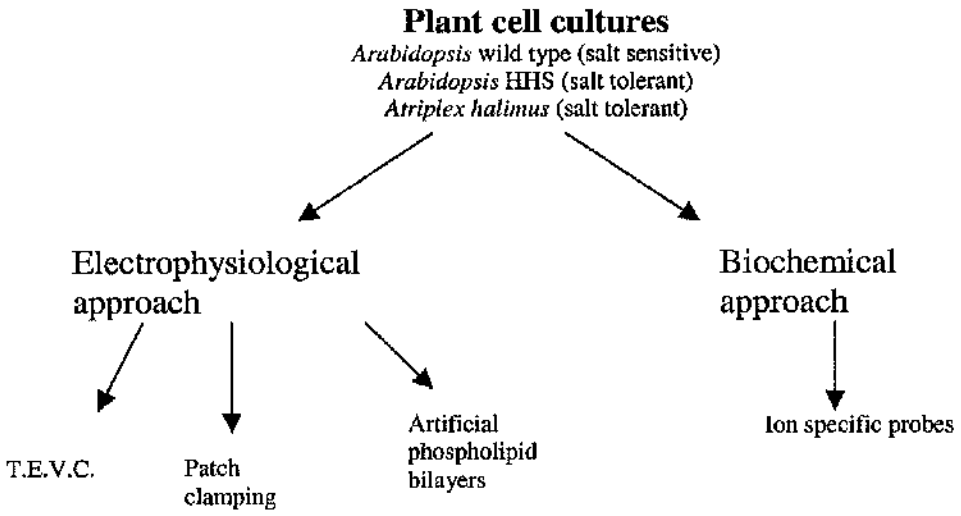


Figure 3.2 Characterisation of Ion transport Mechanisms across the Plasma Membrane of Plant Cell Cultures.

The plant cell cultures used in this study included the glycophytic *Arabidopsis* wild type line, and the halophytic *A.halimus* and *Arabidopsis* HHS lines (Section 3.2).
Plant cell cultures are useful for investigating salt tolerance, as the complexities superimposed by higher levels of tissue organisation are not present. To survive in high salinity, unlike the intact root system of a plant, each cell in culture must be expressing the required traits for salt tolerance.
The experimental approach was to use a combination of biophysical and biochemical techniques to investigate ion transport across the plasma membrane of salt tolerant and salt sensitive cell cultures.
Two electrode voltage clamping (TEVC) can measure macroscopic currents across whole cells. Patch clamping can measure ion channel activity across a small patch of membrane, or across the membrane of a whole protoplast. The planar lipid bilayer technique can be used to investigate single channel activity in purified plasma membrane fractions.
For the biochemical approach whole plant cells can be loaded with ion specific dyes to determine the concentration and cellular location of each ion.

can be used on intact cells inside their cell walls and probably reflects a more physiologically relevant situation compared with patch clamping that utilizes protoplasts. The method is not suitable, however, for cells with plasmodesmatal connections which constitute a 'macro-cell' complex.

Attempts to use the TEVC technique with the *Arabidopsis* cell cultures were largely unsuccessful as impaling the HHS cells proved difficult. The *Arabidopsis* HHS cells were small (~40μm) and similar in shape. Whereas the wild type cells were of varying sizes and shapes but were generally larger than the HHS cells (Fig 3.4). Technical difficulties arose when trying to 'hold' cells in place for impalement. Various techniques were attempted for example, suction pipettes and poly-L-lysine coated slides. Unfortunately the cell walls were remarkably tough and invariably the tip of the electrode would simply bend and snap before impaling the cell. Impalement was attempted with electrode tips of varying shapes and lengths but with no success.

The planar lipid bilayer technique was also used to investigate ion movements across purified plasma membrane fractions. This method requires the preparation of enriched plasma membrane that are subsequently fused with artificial (planar) lipid bilayers. Solutions either side of the lipid bilayer are easily changed, and ion movements across the incorporated plasma membrane monitored. Unfortunately, obtaining fractions of consistently high quality and yields proved difficult (Section 3.4.1) and so it was decided not to continue with this approach.

Ion-specific fluorescence probes e.g. SBFI [Na^+], PBFI [K^+], SPQ [Cl^-], BCECF [H^+] and Calcium green [Ca^{2+}] can be used to determine the concentration and cellular location of each ion. It was hoped these cells could then be used in a series of kinetic experiments to monitor rates of ion flux across the cell membrane (Section 3.4.3). To monitor transport activity across the plasma membrane it was proposed to load

proteoliposomes, prepared by fusing lipid vesicles with enriched plasma membrane vesicle fractions, with the fluorescent probes and determine the kinetics of ion transport under different external ion and pH conditions.

The cellular level of various ions was determined in the *Arabidopsis* HHS (300mM NaCl), control wild type *Arabidopsis* (no NaCl), and salt stressed wild type cells (treated with 50mM NaCl or 100mM NaCl) using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

3.4.1 Purification of Plasma Membrane Fractions from Plant Cell Cultures.

Several of the techniques described in the previous section depend on the successful isolation of high purity and yield plasma membrane fractions from the plant cell cultures. Plasma membrane fragments were prepared from the *Arabidopsis* and *Atriplex halimus* cell cultures by the two-phase partition method (Section 2.2.21.1). Two-phase partitioning systems separate membrane fractions partly according to their surface charge, and partly according to hydrophilic/hydrophobic properties of their outer surface. The plasma membrane fragments specifically distributed to the upper phase of a polyethylene glycol/dextran two-phase system, whereas fragments from the chloroplast, mitochondrial and ER membranes distribute to the lower phase (Albertsson *et al.* 1982). The resulting plasma membrane fragments obtained from these separation procedures are mainly in the form of apoplastic side out ('right side out') vesicles, the orientation can be changed by freeze-thaw cycles (Larsson *et al.* 1994). At each stage of the isolation process, specific enzyme assays were performed as 'markers' for the presence of different intracellular membranes (Section 2.2.21.2). The cytochrome c oxidase assay was used to quantify the presence of mitochondrial membrane: the NADH-cytochrome c reductase assay to detect tonoplast membrane: the NADPH-

cytochrome c reductase assay to detect endoplasmic reticulum: the H^+-ATPase assay to quantify the plasma membrane.

In terms of the purity of the final plasma membrane fraction, marker enzyme assays showed either a very variable batch-to-batch reproducibility or a consistently poor degree of plasma membrane enrichment. Table 3.1 presents typical results of a marker enzyme analysis performed on a microsomal fraction isolated from the *A. halimus* cell lines grown in 0.1 and 300mM NaCl. With both cell cultures the degree of vanadate-sensitive P-type H^+-ATPase enrichment obtained with the plasma membrane-to-microsomal fraction was ~60 fold. With the *Arabidopsis* cell lines, however, a poor degree of plasma membrane enrichment was obtained. With the HHS cells only a 2.9 fold P-type H^+-ATPase enrichment measured with the plasma membrane-to-microsomal fraction (Table 3.2).

In view of these difficulties it was decided not to proceed with this approach. It would be futile to attempt to compare the biophysical properties of transport of cellular fractions whose origin were uncertain and that showed batch-to-batch variation. For this reason, attempts were made to use a voltage clamp impalement method on whole cells in order to characterise differences in ion transport processes between the cell lines.

3.4.2 P-type ATPase Activity of *Arabidopsis* and *A.halimus* Cell Lines.

Although the isolation of plasma membrane from cell cultures by the two-phase partitioning was inconsistent there were batches that proved to generate high yield and purity fractions. The vanadate sensitive P-type H^+-ATPase activity measured from *Arabidopsis* cell lines showed higher levels of activity in HHS cells when compared with wild type cells grown under control conditions (Fig 3.3). This trend was not observed with the *A.halimus* cell lines. The activity measured from high salt grown

0.1 mM NaCl	Specific Activities			Fold Enrichment		
	Microsomal Fraction	Upper Phase	Lower Phase	Upper /Microsomal	Lower /Microsomal	U/L Purity ratio
P-type H^+ ATPase (mol ATP /mg protein /hr)	1.246E-05	7.443E-04	8.8864E-05	59.736	7.132	8.376
NADH cytochrome c reductase (mol CytC red /mg protein /hr)	1.9847E-06	2.7063E-07	3.4631E-06	0.136	1.745	0.078
NADPH cytochrome c reductase (mol CytC red /mg protein /hr)	2.1112E-06	7.9532E-07	7.1271E-06	0.377	3.376	0.112
Cytochrome c oxidase (mol CytC oxd /mg protein /hr)	6.9654E-07	1.6556E-07	4.0661E-07	0.238	0.584	0.407

300 mM NaCl	Specific Activities			Fold Enrichment		
	Microsomal Fraction	Upper Phase	Lower Phase	Upper /Microsomal	Lower /Microsomal	U/L Purity ratio
P-type H^+ ATPase (mol ATP /mg protein /hr)	5.9467E-06	3.585E-04	1.898E-05	60.280	3.192	18.887
NADH cytochrome c reductase (mol CytC red /mg protein /hr)	9.4665E-07	2.9297E-07	5.8508E-07	0.309	0.618	0.501
NADPH cytochrome c reductase (mol CytC red /mg protein /hr)	8.3518E-07	1.4085E-07	1.5004E-07	0.169	0.180	0.939
Cytochrome c oxidase (mol CytC oxd /mg protein /hr)	5.6333E-07	nd	4.4491E-07	-	0.790	-

Table 3.1 Marker Enzyme Assays Performed on Plasma Membrane Fractions Isolated from Low and High Salt Grown *Atriplex* Cells.

Cells were grown to early log phase and harvested. Enriched plasma membrane fractions were prepared using the two-phase polymer method (Section 2.2.21). All fractions obtained during the isolation were assayed for marker enzyme activity as follows: vanadate sensitive p-type H^+-ATPase, plasma membrane: NADH Cyt c reductase, tonoplast: NADPH cyt c reductase, ER: Cyto c oxidase, mitochondria.

The degree of vanadate-sensitive p-type H^+-ATPase enrichment obtained is ~60 fold for both cultures. The data shows that the plasma membrane partitions to the upper phase, but low levels of contaminating membranes from other organelles are detected.

The values represent the average of 5 measurements on each fraction.

0.1 mM NaCl	Specific Activities			Fold Enrichment		
	Microsomal Fraction	Upper Phase	Lower Phase	Upper Phase /Microsomal	Lower Phase /Microsomal	U/L Purity ratio
P-type H^+ ATPase (mol ATP /mg protein /hr)	4.206E-05	1.408E-05	1.856E-06	0.335	0.044	7.585
NADH cytochrome *c* reductase (mol CytC red /mg protein /hr)	4.022E-05	1.338E-06	1.652E-05	0.033	0.411	0.081
NADPH cytochrome *c* reductase (mol CytC red /mg protein /hr)	5.883E-06	2.770E-07	4.384E-06	0.047	0.745	0.063
Cytochrome *c* oxidase (mol CytC oxd /mg protein /hr)	1.904E-06	nd	1.587E-06	-	0.833	-

200 mM NaCl	Specific Activities			Fold Enrichment		
	Microsomal Fraction	Upper Phase	Lower Phase	Upper Phase /Microsomal	Lower Phase /Microsomal	U/L Purity ratio
P-type H^+ ATPase (mol ATP /mg protein /hr)	8.091E-06	2.383E-05	2.364E-05	3.946	2.921	1.008
NADH cytochrome c reductase (mol CytC red /mg protein /hr)	5.379E-06	8.140E-07	3.898E-06	0.151	0.725	0.209
NADPH cytochrome c reductase (mol CytC red /mg protein /hr)	6.904E-07	1.699E-07	1.680E-06	0.246	2.434	0.101
Cytochrome c oxidase (mol CytC oxd /mg protein /hr)	1.761E-06	6.862E-07	2.896E-06	0.390	1.644	0.237

Table 3.2 Marker Enzyme Assays on Plasma Membrane Fractions Isolated from Low and High Salt Grown *Arabidopsis* Cells.

Cells were grown to early log phase and harvested. Enriched plasma membrane fractions were prepared using the two-phase polymer method. All fractions obtained during the isolation were assayed for marker enzyme activity as follows: Vanadate sensitive p-type H^+ ATPase, plasma membrane: NADH Cyt c reductase, tonoplast: NADPH cyt c reductase, ER: Cyto c oxidase, mitochondria.

Poor yields of plasma membrane were generated from the wild type *Arabidopsis* cell line (0.335 fold enrichment), and HHS (2.946 fold enrichment). The fraction obtained from the wild type cells contained, however, less contaminating membranes from other organelles. The values represent the average of 5 measurements on each fraction.

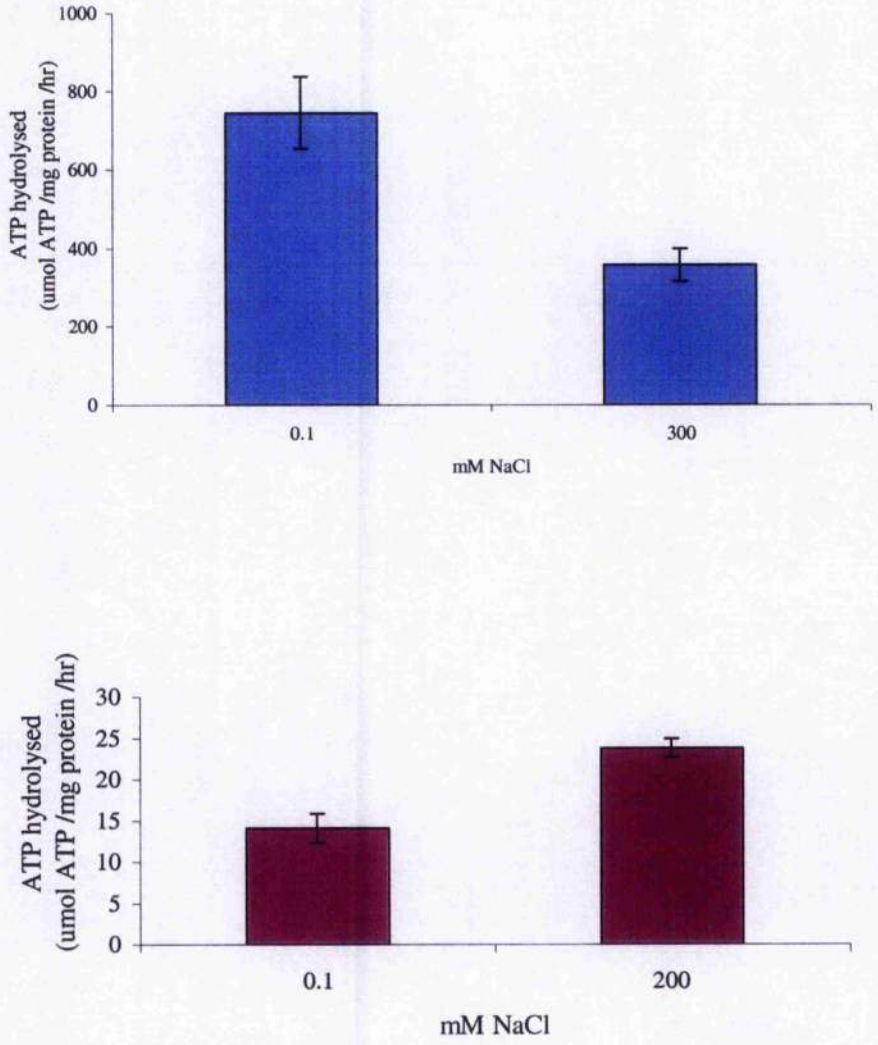


Figure 3.3 p-type H^+-ATPase Activities from 'Enriched' Plasma Membranes of Low & High Salt Grown *Atriplex halimus* (upper) and *Arabidopsis* (lower) Cell Cultures.

The plasma membrane fractions from two independent isolations were used to determine vanadate-sensitive p-type H^+-ATPase activity. The values represent the average of 5 measurements. With *Atriplex*, the vanadate-sensitive H^+ pumping activity is significantly higher ($p < 0.01$) in fractions isolated from cells grown in 0.1mM than those from 300mM NaCl. This is not the case with the *Arabidopsis* cells; here the HHS cells have higher p-type H^+-ATPase levels than wild type cells. Averages and SEs of 3 independent experiments are presented.

A.halimus cells was found to be significantly lower than low salt grown cells. Overall, the P-type H^+-ATPase activity appears to be considerably higher in *A.halimus* cells compared with *Arabidopsis.* The higher contamination found in the *Arabidopsis* plasma membrane fractions may, however, account for the observed lower specific activities. In addition, differences in the purity of fractions isolated from low and high salt grown cultures may account from the observed between-species response to salinity.

3.4.3 Use of Ion-Specific Fluorescence Probes with *Arabidopsis* Cell Cultures.

Cell cultures were incubated with a range of ion-specific fluorescence probes (e.g. SBFI [Na^+], PBFI [K^+], SPQ [Cl^-], BCECF [H^+], and Calcium green [Ca^{2+}] to determine the concentration and cellular location of each ion. It was hoped these cells could then be used in a series of kinetic experiments to monitor rates of ion flux in intact cells. When *Arabidopsis* HHS cells, however, are exposed to the range of probes the dyes appear to move rapidly (<3 min) into small vacuoles/vesicles (between 100-600 nm diameter) in the cytoplasm that subsequently fluoresced brightly, large central vacuoles were also observed but in the short-term these did not fluoresce (Fig 3.4 right). These vesicles are much less prevalent in the wild type *Arabidopsis* cells (Fig 3.4 left). These vesicular bodies are also observed in *A.halimus* and *Beta vulgaris* cell cultures grown in high salt, but not when grown in the absence of NaCl (personal communication Dr PJ Dominy). Thin section E.M. of wild type and HHS *Arabidopsis* cells confirmed the presence of many vesicles in the HHS cell lines that were closely associated with the plasma membrane (Fig 3.4).

To monitor transport activity across the plasma membrane it was proposed to load proteoliposomes, prepared by fusing lipid vesicles with enriched plasma membrane vesicle fractions, with the fluorescent probes and determine the kinetics of ion transport

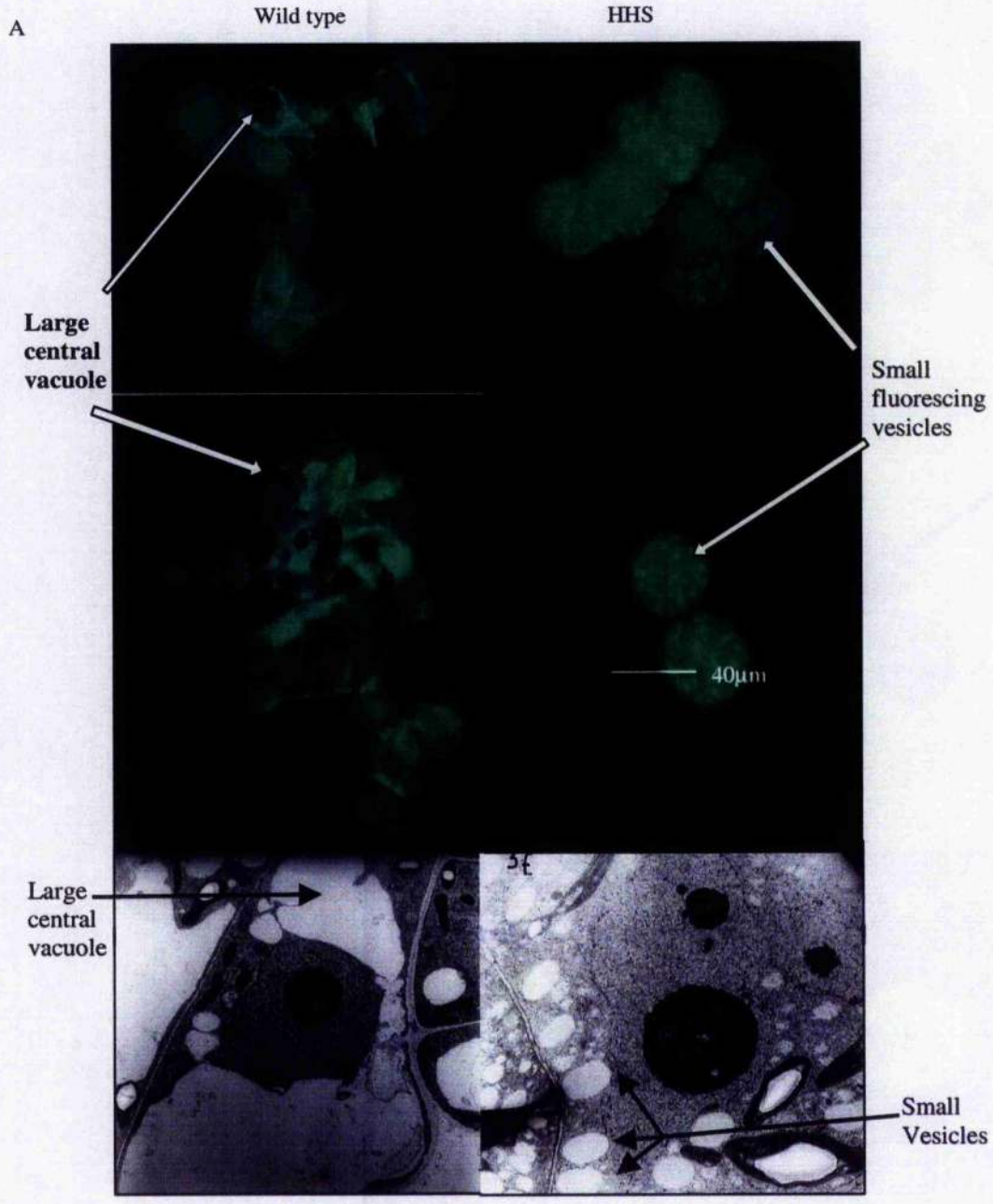


Figure 3.4 The *Arabidopsis* HHS and Wild Type Cells.

Cells from the wild type *Arabidopsis* cultures were a wide variety of shapes and sizes and contained large central vacuoles (left). The HHS cells are habituated to growth in media supplemented with 300mM NaCl; these cells were of similar shape and are smaller than wild type cells. When incubated with fluorescein diacetate the dye moved rapidly (<3 min) into small vesicular bodies found in the cytoplasm. This effect is also observed with other dyes such as SBFI [Na^+], PBFI [K^+], SPQ [Cl^-], BCECF [H^+], etc. Thin section E.M. (x10 000) from wild type (bottom left) and HSS (bottom right) *Arabidopsis* cell lines showed a high degree of vesiculation near the plasma membrane of HHS cells.

under different external ion and pH conditions. This approach would allow the investigation of electro-neutral transport processes that are not detectable by electrophysiological characterization. This approach is dependent, however, on a reliable method for extracting consistently high purity and high yield plasma membrane fractions. This proved difficult to achieve for the plant cell cultures (section 3.4.1) and so this approach was also abandoned.

3.5 Effects of Salinity on Ion Content of *Arabidopsis* Cell Cultures.

Ion content (Na, Fe, K, S, P, Mn, Mg and Ca) of *Arabidopsis* HHS and wild type cells was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Samples were harvested from HHS, control wild type (no NaCl), and salt stressed wild type cell cultures (in MSMO media supplemented with 50 or 100mM NaCl) at daily intervals during growth (from inoculation through log phase of growth, to the stationary phase). Aliquots were taken from the culture flasks, quickly washed with ice-cold distilled water to remove all traces of media. The washed cells were then ground to a fine powder in liquid nitrogen using a pestle and mortar. The dry powder was placed in a preweighed tube and the dry weight calculated. Samples were then digested in 5% analytical reagent grade nitric acid before ion content was measured (Section 2.2.4).

Analysis of the data showed stable growth of the wild type and HHS cell lines, and ion content measurements were very reproducible. However when wild type cells were treated with NaCl, the results were more erratic and large variances were incurred.

3.5.1 Na Content of *Arabidopsis* Cell Cultures.

Over the period of growth measured, wild type control cells maintained a steady Na concentration of ~0.18mg/g dry weight. When exposed to 100mM NaCl, Na levels increased dramatically to a max of ~25mg/g dry weight by day 3 (Fig 3.5). These levels are 6 times greater than those detected in the HHS cells grown in 300mM NaCl. These data suggest that the HHS cell lines have mechanisms for minimising Na content and that these are either not present in the wild type cells or are less effective. These may be mechanisms that reduce Na uptake or increase Na efflux.

3.5.2 K Content of *Arabidopsis* Cell Cultures.

Over the period of growth from lag to log phase, K levels in wild type control cells decreased considerably from ~62mg/g dry weight (day 2) to ~20mg/g dry weight (day 6 Fig 3.6). Since 7-day-old cells are used to generate fresh cultures (Section 2.2.1), the control cells must accumulate ~40mg/g dry weight K during day 1 (early lag phase). This is not observed in the HHS cells, which maintained stable K concentrations of ~6mg/g dry weight over the period of growth, concentrations much lower than measured in wild type control cells. Salt treated wild type cells contained much less K than non-treated wild type cells and did not accumulate K during day 1 of growth.

Sodium toxicity can result by interfering with K^+ nutrition, therefore one of the key elements in salinity tolerance is the capacity to maintain a high cytosolic K^+/Na^+ ratio (reviewed by Maathuis & Amtmann 1999). The K/Na ratio of wild type control cells decreased from 260 on day 2 of growth to 125 on day 6. Upon salt stress, the K/Na ratio dropped considerably (Fig 3.7). At all times the K/Na ratio measured in HHS cells was higher than in salt stressed wild type cells.

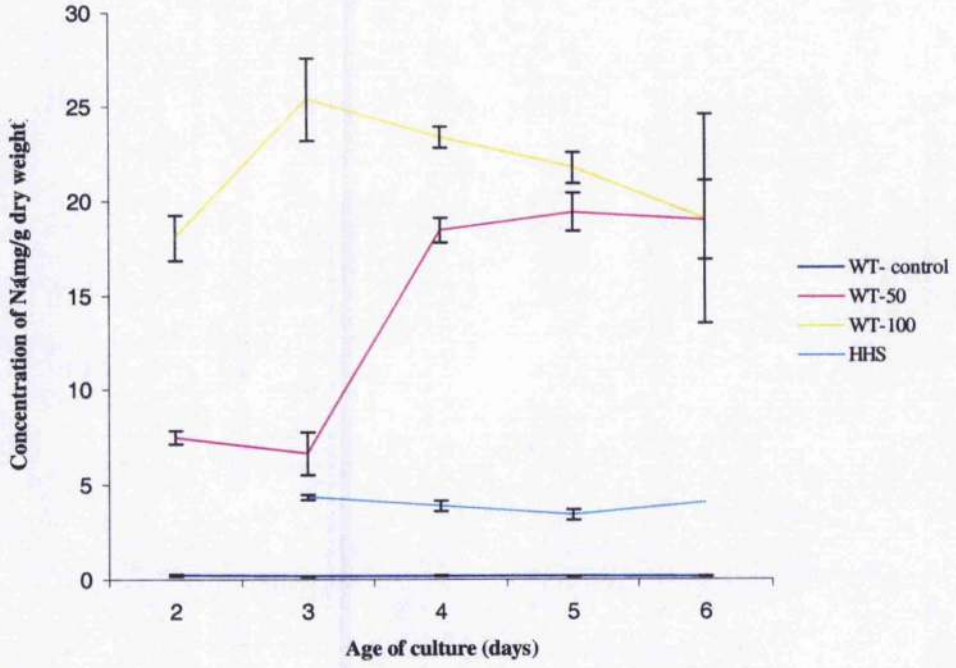


Figure 3.5 Sodium Content of *Arabidopsis* Cell Cultures.

Samples were harvested from HHS (salt tolerant, 300mM NaCl grown), control wild type (no NaCl), and salt stressed wild type cells treated with either 50mM NaCl (WT-50) or 100mM NaCl (WT-100). Sodium content was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Averages and SEs of three independent experiments are presented.

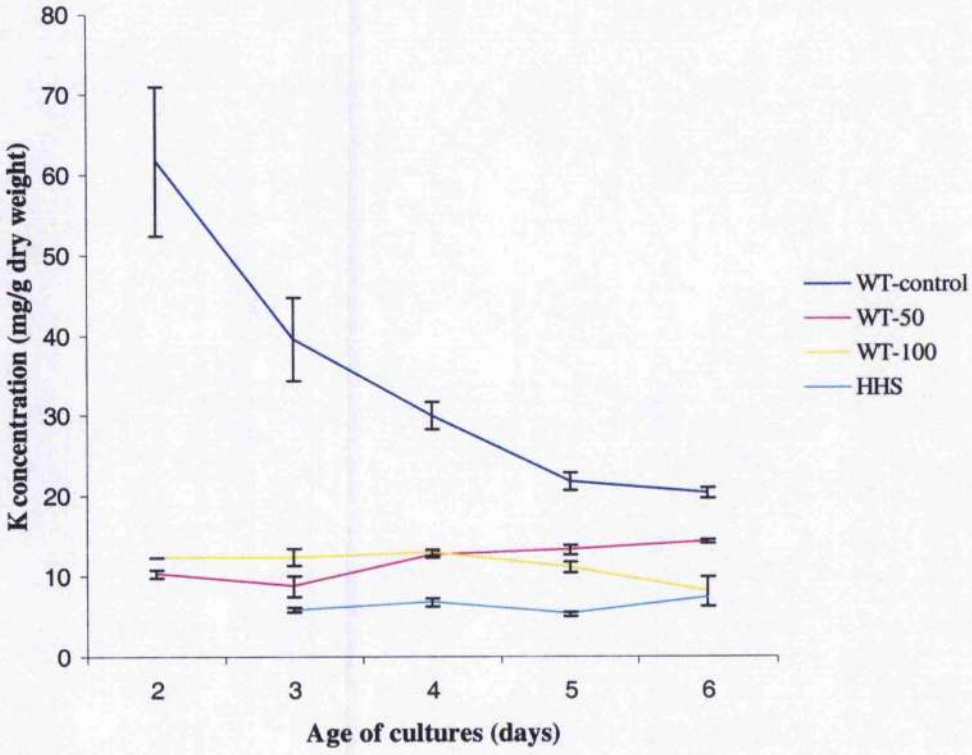


Figure 3.6 Potassium Content of *Arabidopsis* Cell Cultures.

Samples were harvested from HHS (salt tolerant, 300mM NaCl grown), control wild type (no NaCl), and salt stressed wild type cells treated with either 50mM NaCl (WT-50) or 100mM NaCl (WT-100). Potassium content was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Averages and SEs of three independent experiments are presented.

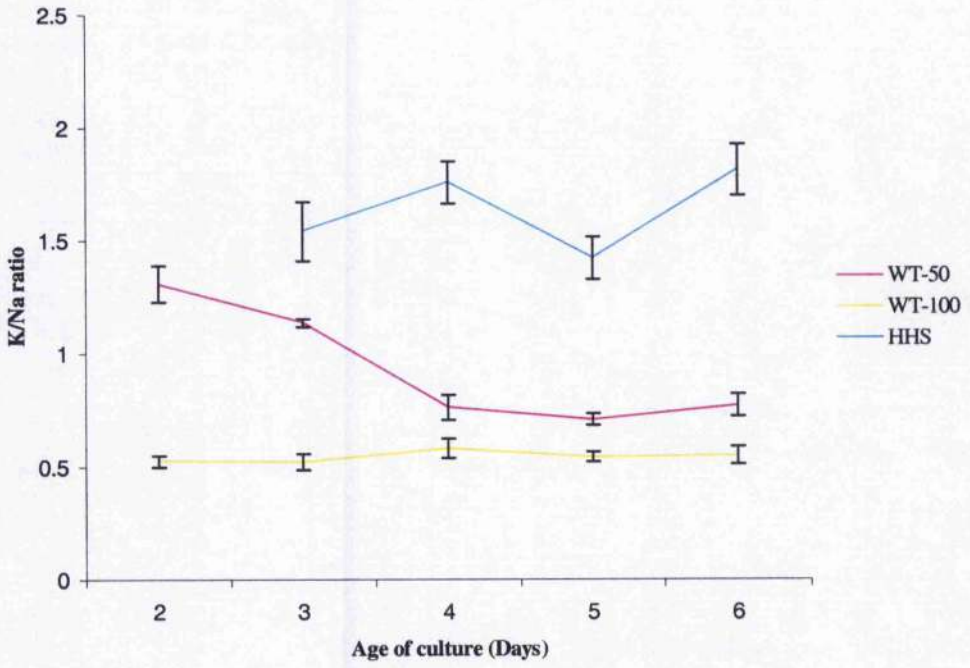


Figure 3.7 K/Na Ratios of *Arabidopsis* Cell Cultures.

Samples were harvested from HHS (salt tolerant, 300mM NaCl grown), control wild type (no NaCl), and salt stressed wild type cells treated with either 50mM NaCl (WT-50) or 100mM NaCl (WT-100). Potassium and Na^+ content was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Averages and SEs of three independent experiments are presented.

3.5.3 Ca Content of *Arabidopsis* Cells Cultures.

Over the period of growth measured, HHS cells maintained a steady Ca concentration of ~0.5mg/g dry weight (Fig 3.8) During the first 4 days of growth the levels of Ca measured in the wild type control cells decreased from ~2.8 to ~1.5mg/g dry weight, from this time point Ca levels increased to ~2.0mg/g dry weight on day 6. The opposite trend was observed with salt stressed wild type cells. These cells contained considerably less Ca on day 2 compared with wild type control, Ca content increased from day 2 to day 4 when Ca levels declined.

3.5.4 Mn Content of *Arabidopsis* Cell Cultures.

At day 2 of growth wild type cells treated with 50 and 100mM NaCl contained ~4 fold and ~2 fold lower levels of Mn respectively, compared with wild type control cells. With the wild type salt treated cultures, Mn levels were found to increase over the period of growth but do not reach levels measured for wild type control cells. The lowest levels of Mn were measured in the HHS cells, wild type control cells contained ~4 fold higher levels of Mn than HHS cells (Fig 3.9).

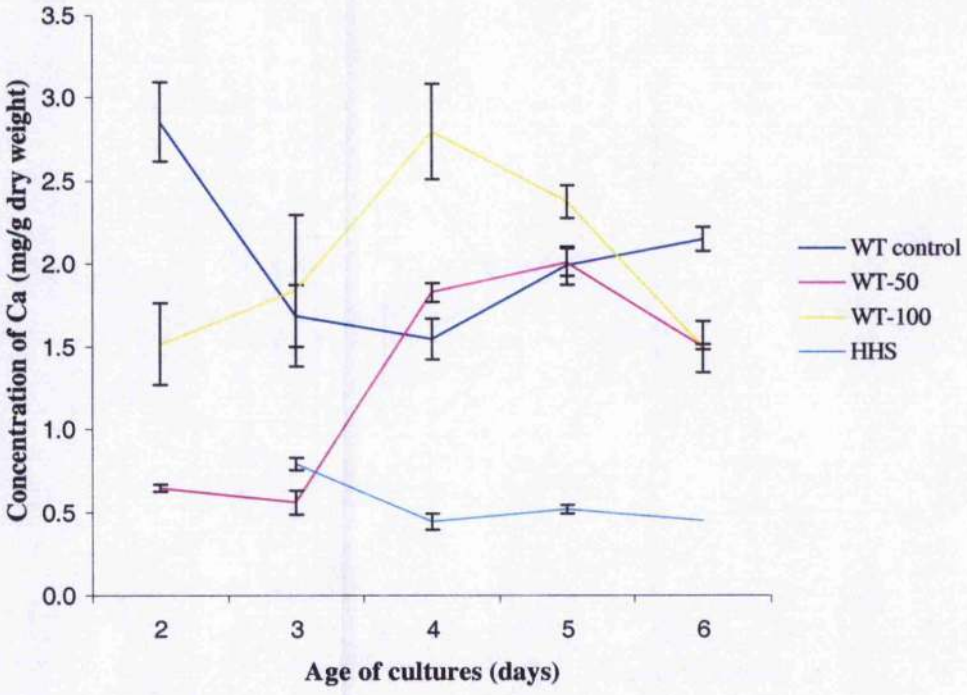


Figure 3.8 Calcium Content of *Arabidopsis* Cell Cultures.

Samples were harvested from HHS (salt tolerant, 300mM NaCl grown), control wild type (no NaCl), and salt stressed wild type cells treated with either 50mM NaCl (WT-50) or 100mM NaCl (WT-100). Calcium content was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Averages and SEs of three independent experiments are presented.

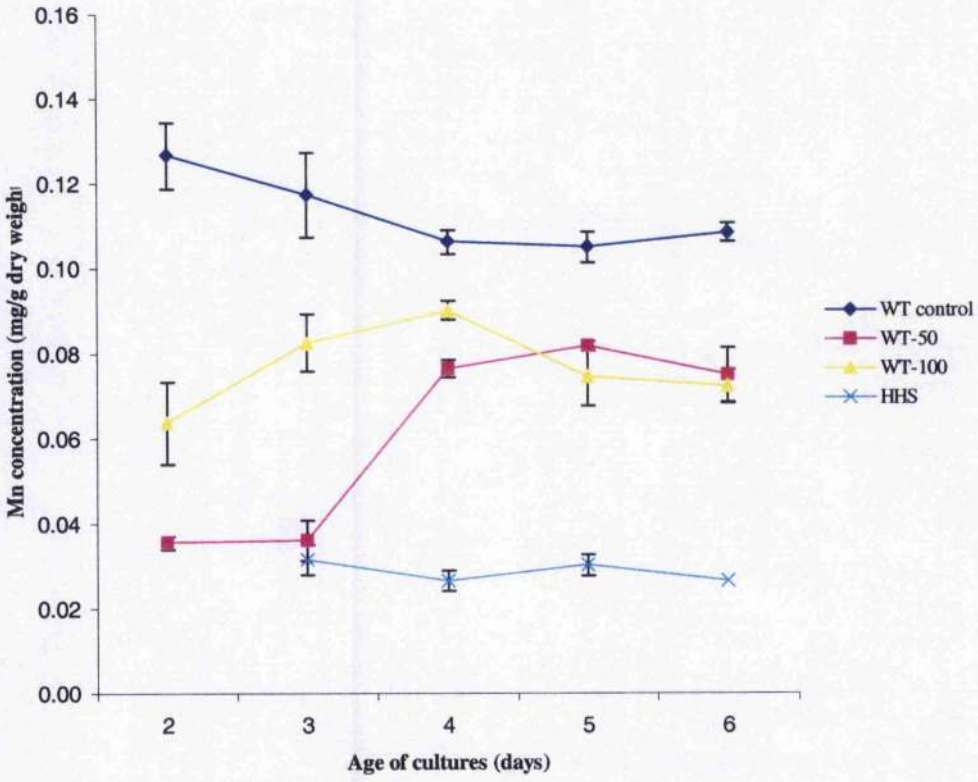


Figure 3.9 Manganese Content of *Arabidopsis* Cell Cultures.

Samples were harvested from HHS (salt tolerant, 300mM NaCl grown), control wild type (no NaCl), and salt stressed wild type cells treated with either 50mM NaCl (WT-50) or 100mM NaCl (WT-100). Manganese content was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Averages and SEs of three independent experiments are presented.

3.5.5 S Content of *Arabidopsis* Cell Cultures.

Wild type control cells contained higher levels of S (~1.25mg/g dry weight) than the HHS cells (~0.75mg/g dry weight Fig 3.10). Day 2 and 3 wild type cells treated with 50mM NaCl contained less S than wild type control, however by day 4 these cells appeared to accumulate S to levels higher then those measured in the wt control cells. At day 2, S levels of wild type treated with 100mM NaCl were comparable with wild type control. While this level remained stable with the wild type control cells, S levels of the WT-100 cells increased.

3.5.6 P Content of *Arabidopsis* Cell Cultures.

Over the period of growth from lag to log phase, P levels detected in wild type control cells decreased considerably from ~25mg/g dry weight (day 2) to ~4mg/g dry weight (day 6 Fig 3.11). Since 7-day-old cells are used to generate fresh cultures (Section 2.2.1), the wild type control cells must accumulate ~20mg/g dry weight P during the first day of growth (early lag phase). This trend is not observed in the wild type cells treated with 50mM NaCl. P levels at day 2 are ~5 fold lower than those measured with the wild type control cells. P content declined to 2mg/g dry weight day 3, these levels were maintained for the remainder of the growth period. At day 6, P levels are comparable with the wild type control cells. Until day 4, the wild type cells treated with 100mM NaCl maintained a steady level of ~15-17 mg/g dry weight P. From day 4 this level decreased so that by day 6 the levels were ~6 mg/g dry weight, comparable with the levels measured in wild type control cells. The HHS cells maintained steady levels of 7-9 mg/g dry weight P over the period of growth measured.

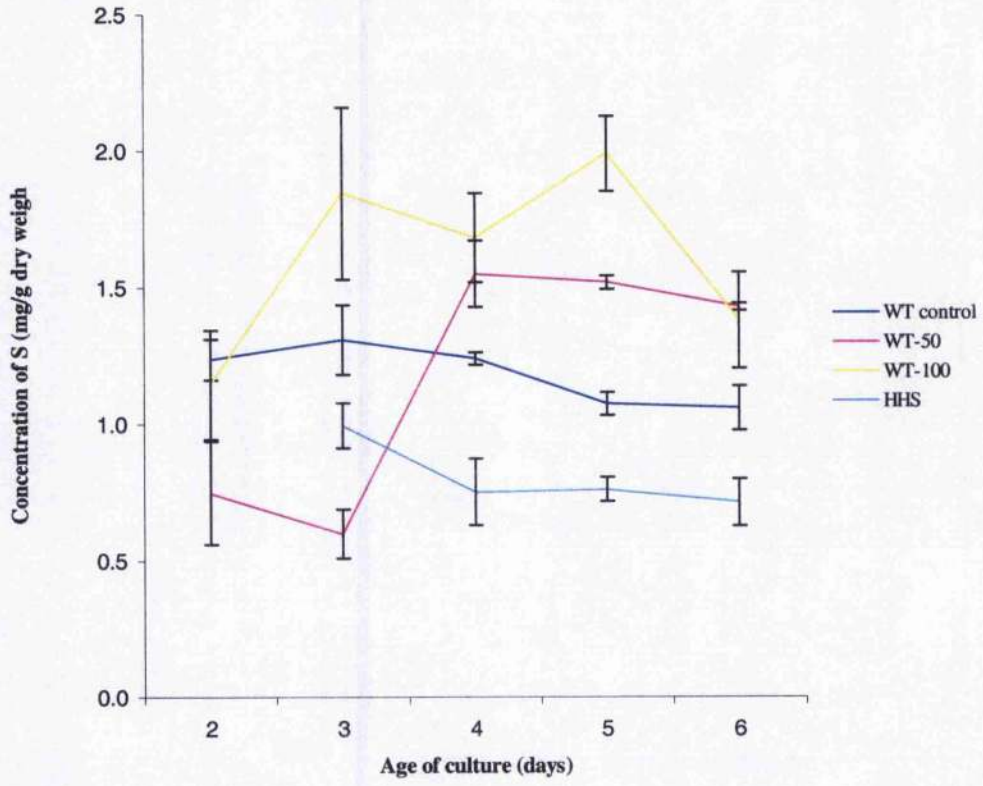


Figure 3.10 Sulphur Content of *Arabidopsis* Cell Cultures.

Samples were harvested from HHS (salt tolerant, 300mM NaCl grown), control wild type (no NaCl), and salt stressed wild type cells treated with either 50mM NaCl (WT-50) or 100mM NaCl (WT-100). S content was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Averages and SEs of three independent experiments are presented.

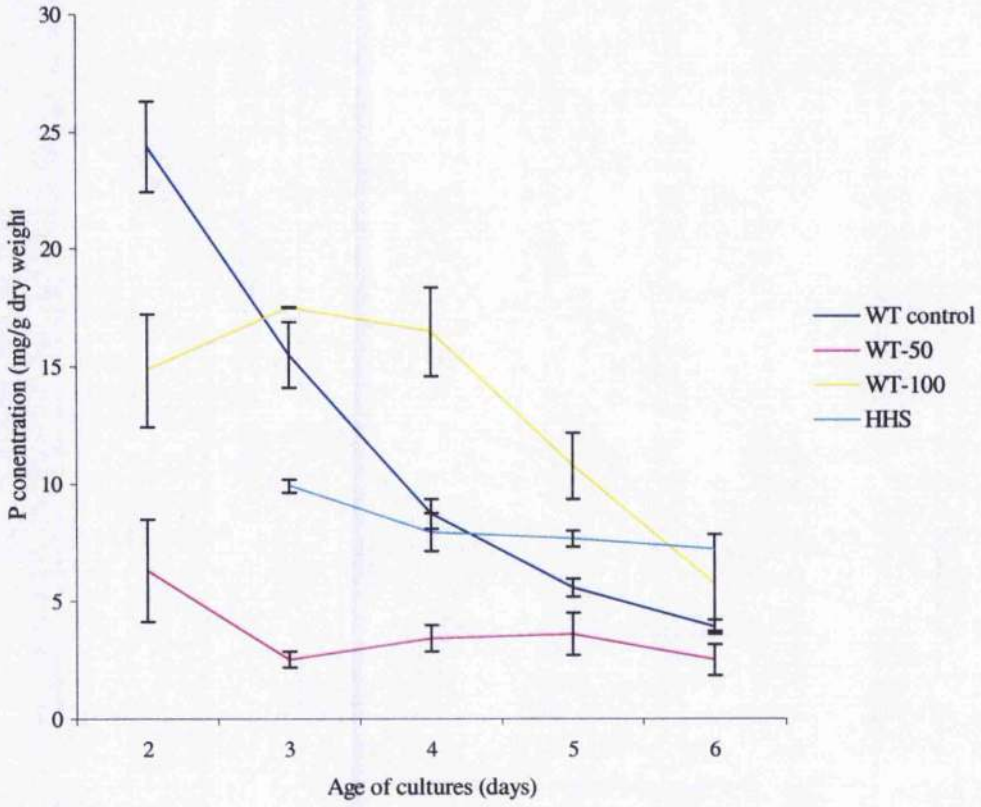


Figure 3.11 P Content of *Arabidopsis* Cell Cultures.

Samples were harvested from HHS (salt tolerant, 300mM NaCl grown), control wild type (no NaCl), and salt stressed wild type cells treated with either 50mM NaCl (WT-50) or 100mM NaCl (WT-100). P content was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Averages and SEs of three independent experiments are presented.

3.5.7 Fe Content of *Arabidopsis* Cell Cultures.

With the HHS cells, the highest levels of Fe (0.06mg/g dry weight) were measured on day 2 of growth (Fig 3.12). Over the growth period these levels decline to ~0.02mg/g dry weight on day 6. Since the cell cultures are initiated with cells from 7-day-old cultures (Section 2.2.1) this new culture must accumulate 0.04mg/g dry weight during the first day of growth. A similar trend was observed with the wild type control cells, on day 2 of growth these cells contained ~0.045mg/g dry weight these levels decreased to ~0.025mg/g dry weight on day 6. On day 2 of growth, wild type cells treated with 50mM NaCl (WT-50) contained the lowest levels of Fe compared with the other cultures. This level increased almost 2 fold by day 4 and from this time onwards Fe levels remained higher with this culture compared with HHS and wild type control cells.

The wild type cells treated with 100mM NaCl (WT-100) contained the highest levels of Fe measured. On day 2 of growth these levels (~0.06 mg/g dry weight) are comparable with the HHS cells but over the growth period these cultures maintained this high Fe content whereas levels declined in the HHS cultures. By day 6 WT-100 cells have 3 fold higher levels of Fe compared with HHS.

3.5.8 Mg Content of *Arabidopsis* Cell Cultures.

The level of Mg measured in the wild type control cells decreased over the period of growth from ~1.5mg/g dry weight on day 2 to ~0.9mg/g dry weight on day 6 (Fig 3.13) When treated with 50mM NaCl wild type cells contain 3 fold less Mg than wild type control cells. On day 2 Mg levels in the WT-50mM NaCl cells increased from ~0.5 to 0.7 by day 4 these levels, comparable with those measured in the HHS cells, were then stably maintained for the remainder of the growth period.

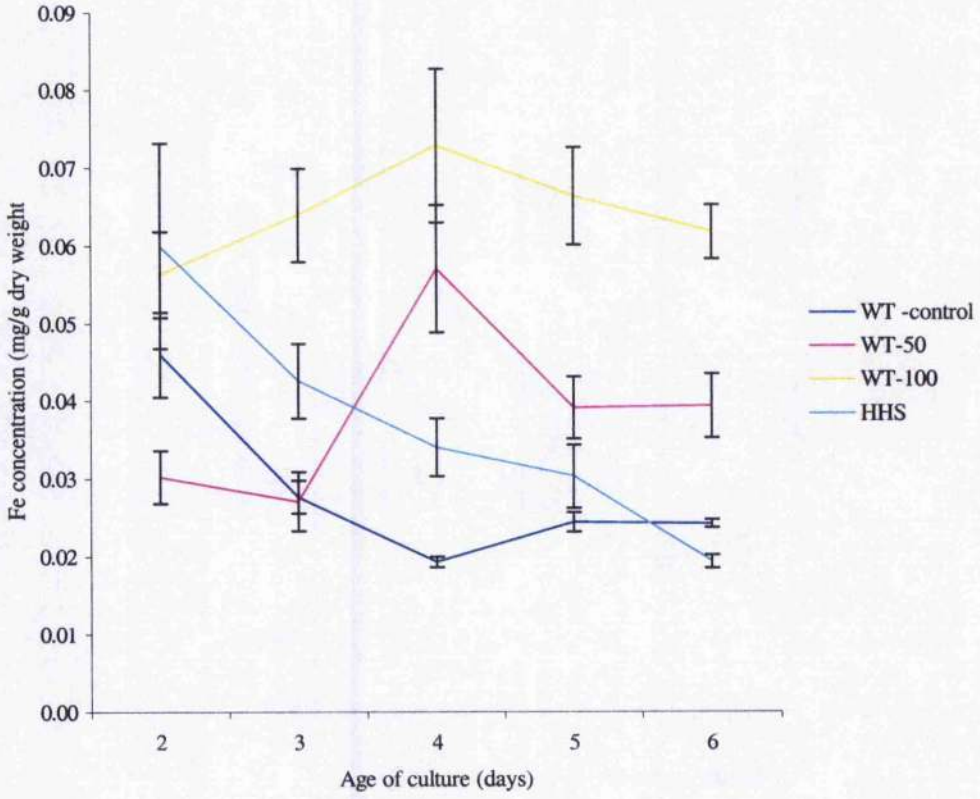


Figure 3.12 Iron content of *Arabidopsis* Cell Cultures.

Samples were harvested from HHS (salt tolerant, 300mM NaCl grown), wild type - control (no NaCl), and salt stressed wild type cells treated with either 50mM NaCl (wt-50) or 100mM NaCl (wt-100). Fe content was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Averages and SEs of three independent experiments are presented.

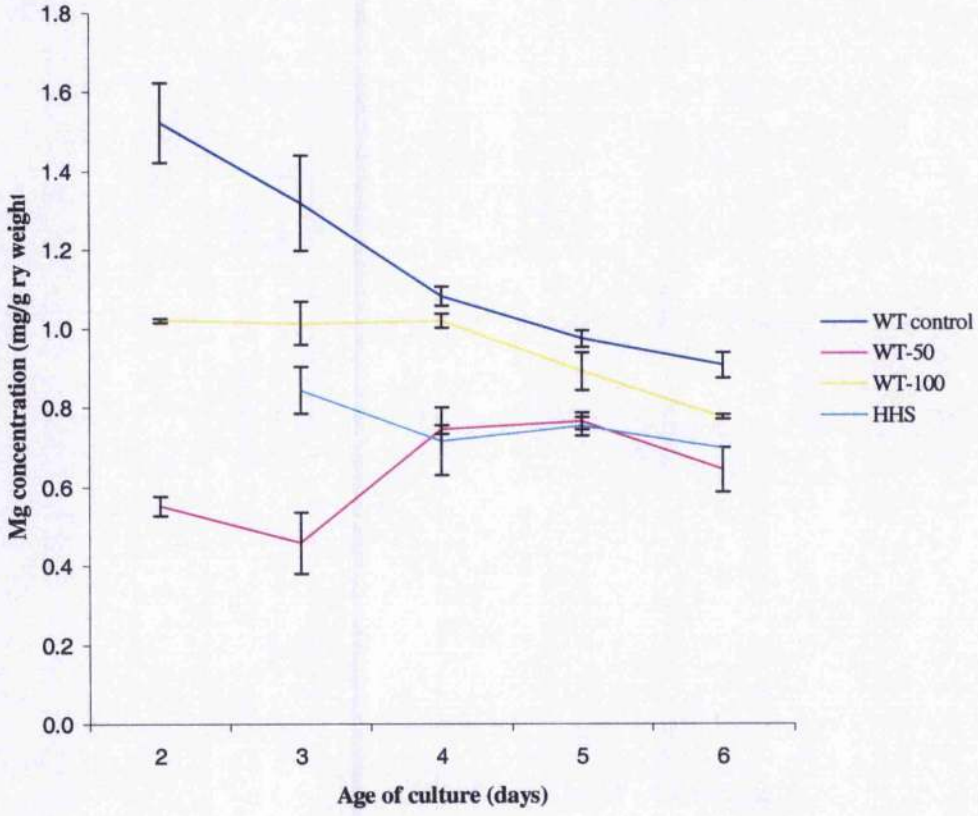


Figure 3.13 Magnesium Content of *Arabidopsis* Cell Cultures.

Samples were harvested from HHS (salt tolerant, 300mM NaCl grown), wild type - control (no NaCl), and salt stressed wild type cells treated with either 50mM NaCl (WT-50) or 100mM NaCl (WT-100). Mg content was measured using a Perkin Elmer Optima 4000 series Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). Averages and SEs of three independent experiments are presented.

3.6 Discussion

Salinity is detrimental to the growth and yield of crop plants. Salt stress results from a number of harmful processes including a toxic action of Na^+ and Cl^- ions on cell metabolism, the impairment of mineral nutrition, a modification in the water status of the plant tissues, and secondary stresses such as an oxidative stress linked to the production of toxic reactive oxygen intermediates. Since salt tolerance is a complex trait involving several interacting properties, there is an increasing interest in studying the physiological behaviour of cells from halophytes in order to identify and better understand salt tolerance mechanisms.

In this chapter, plant cell cultures were used to investigate mechanisms involved in plant salt tolerance. Plant cell cultures offer a major advantage over intact tissue for investigating salt tolerance, as the complexities superimposed by higher levels of tissue organisation are not present. To survive in high salinity, unlike the intact root system of a plant, each cell in the culture must be expressing the required traits for salt tolerance and so important proteins and genes involved in tolerance should be enriched. The plant cell lines investigated in this study included wild type *Arabidopsis* (salt sensitive), HHS *Arabidopsis* (salt tolerant), and *Atriplex halimus* (halophyte).

The wild type *Arabidopsis* cell line is salt sensitive and cannot tolerate 75mM NaCl. The *Arabidopsis* HHS (Habituated to High Salt) cell line was adapted from the wild type cell lines by sub-culturing into progressively higher levels of NaCl, starting with 70mM NaCl and increasing gradually to 380mM NaCl. The development of the HHS cell line suggests that *Arabidopsis* cells have the genetic potential to tolerate high levels of salinity, and perhaps it is the inability of intact plants to coordinate the expression of the appropriate genes in the appropriate tissues that limits the plants ability to grow in the presence of NaCl.

The wild type *Arabidopsis* cell cultures grew quicker than the HHS cells (WT- doubling time 2.3 days compared with 4 days of the HHS lines). The *Arabidopsis* HHS cells appeared to be smaller (~40μm) and similar in shape. The Arabidopsis wild type cells were of varying sizes and shapes. *A halimus* is a facultative halophyte able to tolerate high salt conditions and can also grow normally in low or non-saline environments. *A.halimus* cultures supplemented with 0.1mM NaCl grew quicker (doubling time 2.8 days) than cultures supplemented with 300mM NaCl (doubling time 3.5 days).

When salt treated HHS and *A.halimus* cells were exposed to ion - specific fluorescence probes (*e.g.* SBFI [Na^+], PBFI [K^+], SPQ [Cl^-], BCECF [H^+] and Calcium green [Ca^{2+}]) the dyes moved rapidly into small vacuoles/vesicles that appear in the cytoplasm. These vesicular bodies were less prevalent in the wild type *Arabidopsis* cells. The mechanism(s) that accumulate these dyes in the vesicles is uncertain. Some of the dyes are charged at physiological pH (anionics) and may follow cations that are sequestered into the vesicles. Some of the other dyes, however, are neutral or zwitterionic. In contrast, some (but not all) of the dyes fluoresce brightly when cleaved by esterases, and this might reflect high levels of esterase in the vesicles. A third possibility is that the dyes are treated as xenobiotics and are conjugated in the cytoplasm (to glutathione?) and removed to the vesicles by the operation of an ABC-pump (Theodoulou 2000). Regardless of the mechanisms of dye sequestration, the presence of these vesicles only in salt-resistant cell lines is an interesting and novel observation and may reflect a key strategy for survival in high salinity. Whether they are involved in cellular detoxification or in vesicle trafficking (exocytosis), however, remains to be established.

Attempts were made to investigate single channel activity using the planar lipid bilayer technique. With this technique single channel activity can measured across plasma membrane fractions that are incorporated into an artificial phospholipid bilayer. This technique is dependent on the preparation of enriched plasma membrane fractions. Two-phase partitioning was used to isolated plasma membrane from the cell culture lines. Exhaustive analysis by marker enzyme assays demonstrated that the purity and yield of the plasma membrane fractions was variable with poor batch-to-batch reproducibility, making comparisons between samples difficult. For this reason, this approach was abandoned.

Two-electrode voltage clamping (TEVC) can be used to assess the macroscopic currents from whole cells. Two electrodes, one for passing current and one for measuring voltage, are used to impale whole cells. The membrane potential is held at a desired level, while the current flowing through the membrane is measured. The major advantage with this technique is that it can be used on intact cells inside their cell walls and probably reflects a more physiologically relevant situation than patch clamping that requires wall-less protoplasts, thereby necessitating lengthy preparation procedures. Attempts to use this technique were largely unsuccessful as the single cells from the plant cultures proved difficult to impale.

P-type H^+-ATPase activity was measured using plasma membrane fractions isolated from *Arabidopsis* and *Atriplex* cell cultures. Vanadate sensitive P-type H^+-ATPase activity measured from *Arabidopsis* cell lines showed higher levels in HHS cells compared with wild type cells, but as the purity of these fraction was low it is not possible to attribute this difference to a salt stress response. This trend was not

observed, however, with the *A.halimus* cell lines. The P-type H^+-ATPase activity measured from high salt grown *A.halimus* cells was found to be significantly lower than the fraction from low salt grown cells. Overall, the P-type ATPase activity was found to be considerably higher in *A.halimus* cells compared with *Arabidopsis,* however, due to the low purity of the *Arabidopsis* fraction it is again not possible to attribute this to differences between the species. There are conflicting data in the literature concerning P-type H^+-ATPase activity levels in glycophytes and halophytes. One study demonstrated NaCl induction of P-type H-ATPase mRNA accumulation to be greater in *Atriplex nummularia* than the glycophyte *Nicotiana tabacum* (Niu *et al.* 1993). In another study, the glycophytic *(Lycopersicon esculentum)* and halophytic *(Lycopersicon cheemanni)* plasma membrane fractions were shown to display a similar NaCl-dependent increase in P-type H^+-ATPase activities (Mennen *et al.* 1990). Conflicting results were obtained with *Plantago*, where no difference in P-type H^+-ATPase activities was detected in the salt tolerant and salt sensitive lines observed (Brüggemann & Janiesch 1989).

Sodium chloride can exert its toxic effect on plants by interfering with the uptake of other nutrient ions, especially K^+, leading to intracellular nutrient ion deficiency. The effects of salinity on ion levels of *Arabidopsis* cell cultures were investigated. Ion contents (Na, Fe, K, S, P, Mn, Mg and Ca) were determined for *Arabidopsis* HHS (salt tolerant), wild type (salt sensitive), and salt stressed wild type (media supplemented with 50mM NaCl or 100mM NaCl) cell lines.

With the salt sensitive wild type cells, treatment of NaCl induced an accumulation of Na, Fe and S whilst the levels of Mg, Mn and K were found to decrease. The levels of Ca were found to be initially high in wild type control cells, but these levels then

decreased over the period of growth. The opposite trend was observed with the salt stressed wild type cells. At day 2, these cells contained considerably less Ca than wild type control cells, and over the period of growth Ca levels increased.

As Na^+ toxicity can result by interfering with K^+ nutrition, one of the key elements in salinity tolerance is the capacity to maintain a high cytosolic K^+/Na^+ ratio. The K/Na ratio of wild type control cells decreased from 260 on day 2 of growth to 125 on day 6. Upon salt stress, the K/Na ratio dropped dramatically to ~0.5 (100mM NaCl treatment). At all times the K/Na ratio measured in HHS cell was higher than salt stressed wild type cells, but most interestingly, the HHS cells contained considerably less Na than salt stressed wild type cells, suggesting that the HHS cell lines have mechanisms for minimising Na^+ content that are not present or are less effective in wild type cells; these may be mechanisms that reduce Na^+ uptake or increase Na^+ efflux. Further studies will be required to establish this; one approach might be to use the patch clamp method on protoplasts isolated from these cultures.

Chapter 4: The Partial Characterisation of Nine Salt Tolerant Activation Tagged Mutants.

4.1 Introduction.

Arabidopsis activation tagged lines were used in large-scale gain-of-function screens to identify single sequences that allow seedlings to survive better under NaCl stress. Seeds were germinated on media containing 80mM NaCl and low concentrations of Ca^{2+} and K^+ (530μM and 200μM respectively). Low concentrations of Ca^{2+} and K^+ were chosen as it is well established that high external levels of these cations can ameliorate the toxic effects of Na^+. These conditions seriously impaired wild type growth, therefore any mutants that are more efficient at K^+ uptake, Na^+ efflux, preventing Na^+ uptake, or show an increased sensitivity to the Ca^{2+} response, should flourish and easily be identified in the screen. Fifty mutants were isolated that displayed a strong salt tolerant phenotype; a further 36 had a weak phenotype. This chapter describes partial characterisation of 9 of these lines, named JP1-10. The mutant JP 5 has been more extensively characterised, this work is described in chapter 5.

4.2 Activation Tagging in *Arabidopsis*.

Activation tagging technology was originally developed by Walden and colleagues (Hayashi *et al.* 1992). With this approach a T-DNA vector, containing four copies of an enhancer element from the promoter of the cauliflower mosaic virus (CaMV) 35S promoter, is randomly inserted into the plant's genome through *Agrobacterium* infection. The tetrameric CaMV 35S enhancers can mediate transcriptional activation of nearby genes. The pSKI015 vector (Fig 4.1) was developed to allow large-scale application of enhancer activation tagging to *Arabidopsis* (Kardailsky *et al.* 1999). Using this vector, Weigel *et al.* (2000) found that while the majority of over expressed

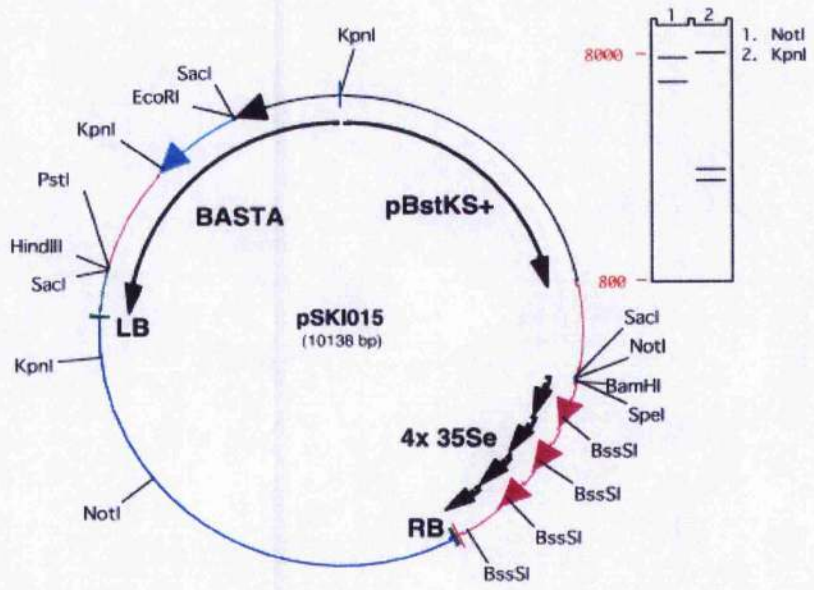


Figure 4.1 The pSKI015 Vector Used to Transform the Weigel *Arabidopsis* Activation Tagged Lines.

Features of the pSKI015 vector (GenBank accession number AF187951) include:

- BAR gene (light blue), this confers resistance to the herbicide glufosinate (Basta) for selection of transgenic plants grown on soil.
- pUC19 sequences with a bacterial origin of replication and an ampicillin resistance gene for plasmid rescue in bacteria.
- 4 copies of the 35S Cauliflower Mosaic Virus (CaMV) promoter enhancers (black arrows).

Using *Agrobacterium* transformation the vector is randomly inserted into the *Arabidopsis* genome, where the 35S CaMV enhancers can activate or suppress the expression of local genes. Figure taken from Weigel *et al.* (2000).

genes were located immediately adjacent to the enhancers, the enhancers could also function in either direction and at a considerable distance (up to 10kb) from the site of insertion. The CaMV 35S enhancers effectively amplify the expression of local genes, and do not induce constitutive or ectopic expression associated with the complete 35S promoter (Weigel *et al.* 2000; Neff *et al.* 1999; van der Graaff *et al.* 2000).

Use of activation tagged lines in gene discovery programmes has several advantages over classical loss-of-function screens. For example, a significant limitation of loss-of-function screens designed to dissect genetic pathways is that they rarely uncover genes that function redundantly, are compensated by alternative metabolic or regulatory circuits, or which are lethal (such as those that play a role in early embryo or gametophyte development). With activation tagged lines, screening for phenotypes is possible in the T1 generation as all mutations are dominant. The tagging vector can, however, insert into the coding region of a gene, which usually results in loss-of-function mutations, in which case the phenotype of these recessive mutants is usually not seen until the T2 generation.

Activation tagging has proved a productive strategy to identify gene function. To date this technology has predominately been applied, with considerable success, to *Arabidopsis* and now the system is being deployed in an increasing number of diverse plant species, including petunia (Zubko *et al.* 2002), Madagascar periwinkle (van der Fits *et al.* 2001; van der Fits & Memelink 2000), poplar (Busov *et al.* 2003), tomato (Mathews *et al.* 2003), tobacco (Ahad A *et al.* 2003) and rice (Jeong *et al.* 2002).

4.3 The Available *Arabidopsis* Activation Tagged Lines.

The Weigel *Arabidopsis* activation tagged lines (Weigel *et al.* 2000) used in this study are available from the Nottingham Arabidopsis Stock Center (http://nasc.life.nott.ac.uk/). This collection is composed of almost 23000 individual lines, distributed as 3 sets N21995, N21991 and N23153. Set N21995 contains 8600 individual lines, divided into 86 pools each with 100 independent lines. Set N21991 contains 7872 lines, 82 pools of 96 lines. Set N23153 contains 6200 lines; a set of 62 pools each with 100 individual lines.

More recently the Scheible and Somerville lines (~63 000 lines) have become available. These lines have been produced using the same vector (pSKI015) as the Weigel lines. Our group has screened this collection for cadmium tolerant mutants with considerable success. Sixteen cadmium tolerant mutants have been identified and eight have been partially characterised. Interestingly, the tagged loci of two of the mutants are annotated as heavy metal-binding proteins, and one is a putative phytochelatin synthase all of which are known to be involved in cadmium tolerance (pers. com. Dr Peter Dominy and Catriona Thomson, University of Glasgow). These results provide confidence that the strategy does work for identifying genes involved in stress tolerance.

4.4 Screening of *Arabidopsis* Activation Tagged Lines for Salt Tolerant Mutants.

The Weigel *Arabidopsis* activation tagged lines were germinated on agar plates containing 0.75% sucrose, 80mM NaCl and MS media modified to contain low concentrations of Ca^{2+} and K^{+} (530μM and 200μM respectively, see Table 2.1). Under these conditions approximately 50% of wild type seeds failed to germinate compared with 85% germination under control conditions (in the absence of NaCl). Of the wild

type seeds that did germinate, growth arrested after the development of the first cotyledons. Approximately 1 in 100 wild type plants continued to grow, albeit very slowly. These plants were small and pale yellow/green in colour (Fig 4.3 a). Putative salt-tolerant activation tagged mutants were identified as plants with greater shoot growth compared with wild type (Fig 4.2). After four weeks, surviving plants were rescued and transplanted in soil, grown to maturity, and the resulting seed used in secondary screens to confirm the salt tolerant phenotype. Percentage germination, size, and vigour of plants were noted. Authentic salt tolerant mutants were categorised according to their phenotype, strong or weak, the false positives were discarded (Fig 4.3). From the 23 000 independent activation tagged lines, 631 putative salt tolerant mutants were isolated from primary screening. Of these putative salt tolerant mutants 50 lines were confirmed to have a strong salt tolerant phenotype, 36 weak, 109 false positive, with the remainder to be investigated (Table 4.1)

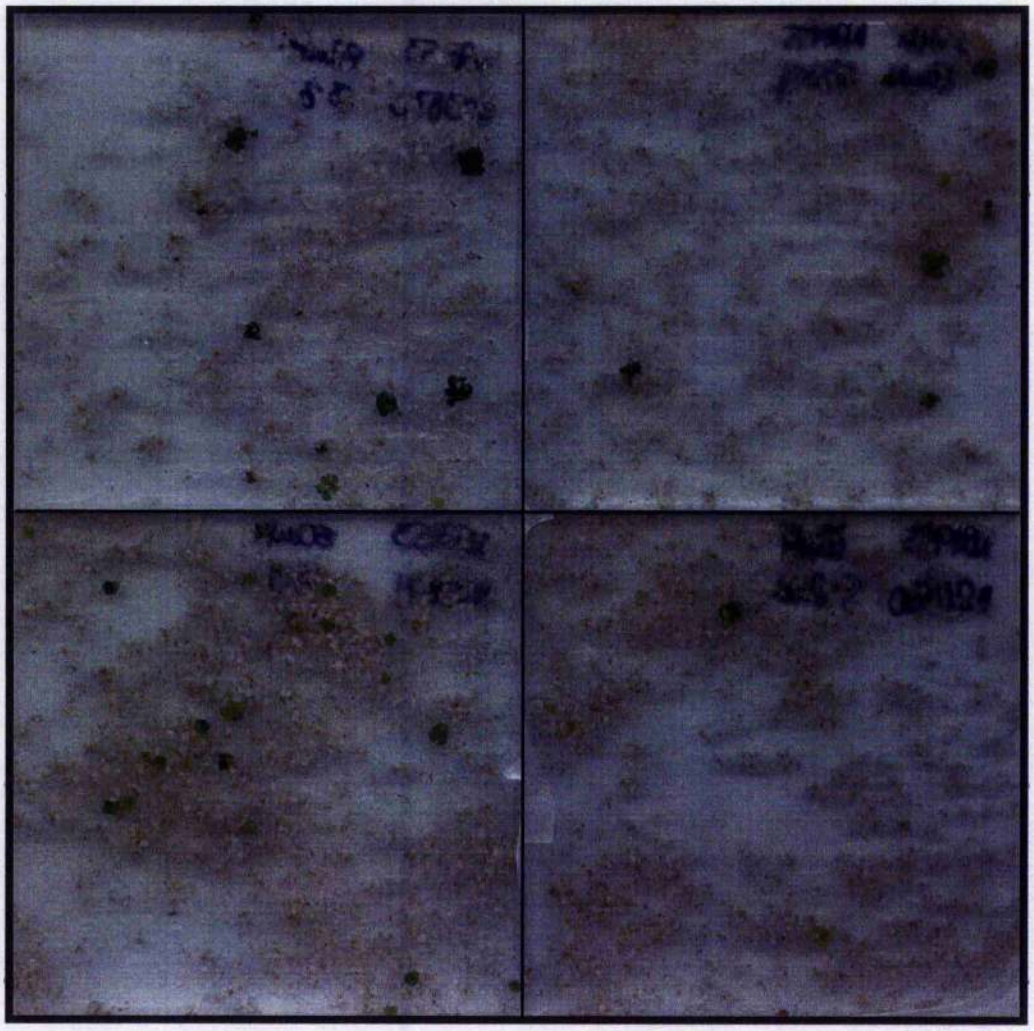


Figure 4.2 Primary Screening of the Weigel Collection of *Arabidopsis* Activation Tagged Lines for Salt Tolerant Mutants.

The Weigel lines were screened on agar plates containing 0.75% sucrose, 80mM NaCl and MS media modified to contain 530μM Ca^{2+} and 200μM K^{+} (Table 2.1). Under these conditions growth of wild type plants is seriously impaired with approximately 50% of seeds failing to germinate. The majority of wild type plants that did germinate produced 2 cotyledons, white in colour, before growth arrested. Approximately 1 in 100 wild type plants continued to grow, albeit slowly. These surviving wild type plants were small and pale yellow/green in colour. Putative salt-tolerant activation tagged mutants were identified as plants with greater shoot growth compared with wild type.

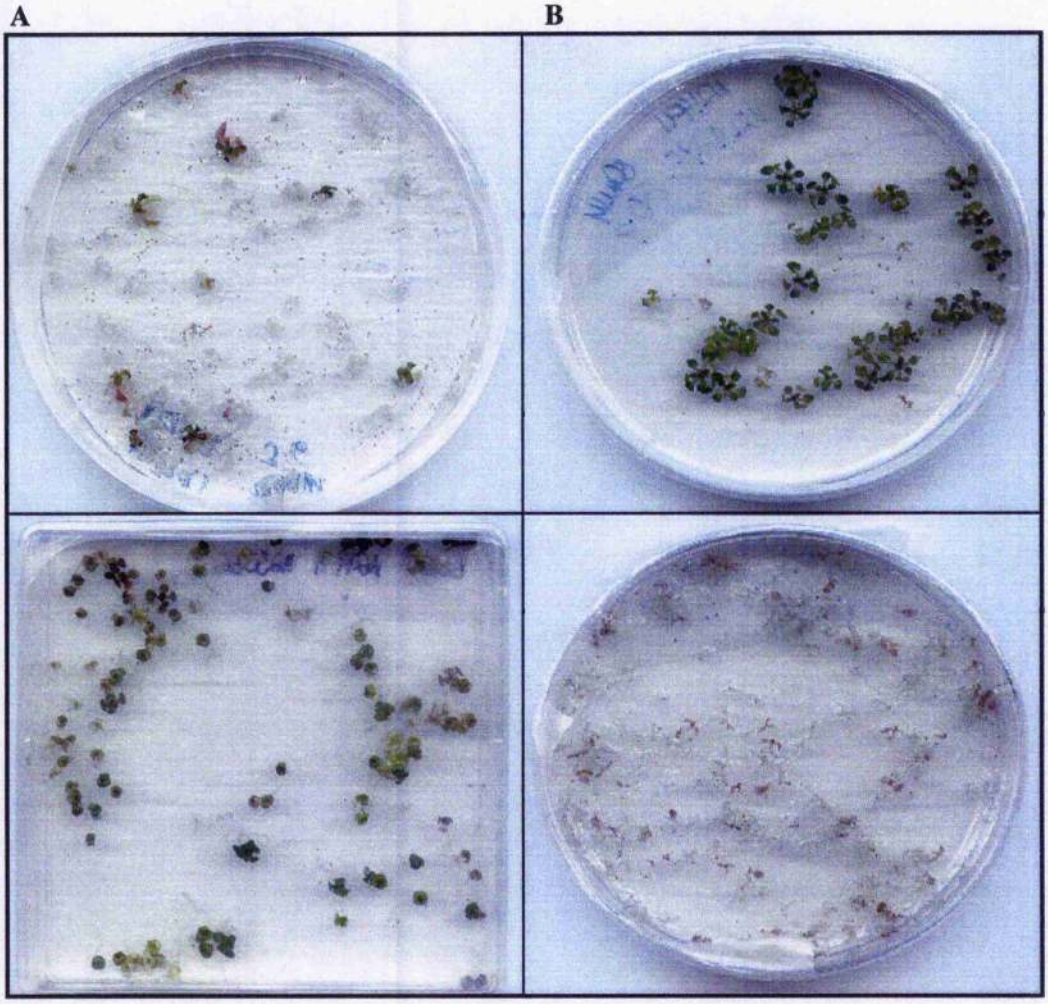


Figure 4.3 Confirmation of Salt Tolerant Phenotype of Mutants Isolated by Primary Screening of the Weigel *Arabidopsis* Activation Tagged Lines.

The Weigel lines were screened on agar plates containing 0.75% sucrose, 80mM NaCl and MS media modified to contain 530μM Ca^{2+} and 200μM K^{+} (Table 2.1), under these conditions wild type growth is seriously impaired **(A).** Six hundred and thirty one putative salt tolerant mutants were selected by primary screening (Fig 4.2). Seed was harvested from these plants, and screened under identical conditions to confirm salt tolerance (secondary screening). Percentage germination of seeds, size, and vigour of plants were noted. Authentic salt tolerant mutants were categorised according to phenotype, strong **(B)** or weak **(C)**, the false positives were discarded **(D)**.

Weigel Set	Primary Screening	Secondary Screening		
	Number of mutants rescued	Strong	Weak	False Positive
N21991 (82 pools)	223 (45 pools)	9 (8 pools)	17 (6 pools)	58 (20 pools)
N21995 (86 pools)	194 (44 pools)	27 (17 pools)	13 (8 pools)	32 (19 pools)
N23153 (62 pools)	214 (29 pools)	14 (8 pools)	6 (4 pools)	19 (6 pools)
	631 (118 pools)	50 (33 pools)	36 (18 pools)	109 (45 pools)

Table 4.1: Number of Mutants Selected from Primary and Secondary Screening of the Weigel *Arabidopsis* Activation Tagged Lines.
The Weigel collection of *Arabidopsis* activation tagged lines were screened for a salt tolerant phenotype on agar plates containing 0.75% sucrose, 80mM NaCl and MS media modified to contain 530μM Ca^{2+} and 200μM K^+ (Table 2.1). In total, 631 putative salt tolerant mutants were selected by primary screening. Seed was harvested from these plants, and screened under identical conditions to confirm salt tolerance (secondary screening). Percentage germination of seeds, size, and vigour of plants were noted. Authentic salt tolerant mutants were categorised according to phenotype, strong or weak, the false positives were discarded (Fig 4.3).

4.5 Characterisation of Activation Tagged Salt Tolerant Mutants.

Once isolated and the salt tolerant phenotype confirmed, the mutants were characterised using a variety of approaches (Fig 4.4).

A high-throughput modified TAIL-PCR protocol was developed to identify the plant genomic sequence flanking the inserted pSKI015 vector (Section 2.2.16). The product generated by tertiary TAIL-PCR was cloned into pTOPO (Invitrogen Ltd, Paisley, UK), amplified in *E.coli,* and then sequenced (Section 2.2.12). Of the 35 mutants analysed, TAIL-PCR products were generated from 19 lines. The sequences were used to interrogate the full *Arabidopsis* genome database using BLAST2 (Altschul *et al.* 1997). The amplified sequences showed 9 of these sequences to have no close homologues; further analysis showed these sequences to be derived from vector sequence. These sequences were generated from concatameric inserts, and TAIL-PCR amplified the junction between the ends of the tandem repeats. TAIL-PCR was only performed using primers designed to the left border, the right border of pSKI015 is generally considered to be unsuitable as a region (up to 90 bp from the border) can be lost upon insertion into the plant genome, making TAIL-PCR from this border difficult (Weigel *et al.* 2000). For this reason TAIL-PCR was always performed using primers which hybridise to the left border of the T-DNA insertion. Perhaps TAIL-PCR using right border primers would be more successful for the mutants in which left border primers amplified only the junction between tandem repeats. False positives are often generated from TAIL-PCR, but these can be detected by the absence of pSKI015 vector sequence from the cloned fragment.

Southern blot analysis was performed on each authentic mutant to determine the number of independent vector inserts within each line. A selection of restriction enzymes was chosen to allow the distinction between a single insertion as one location

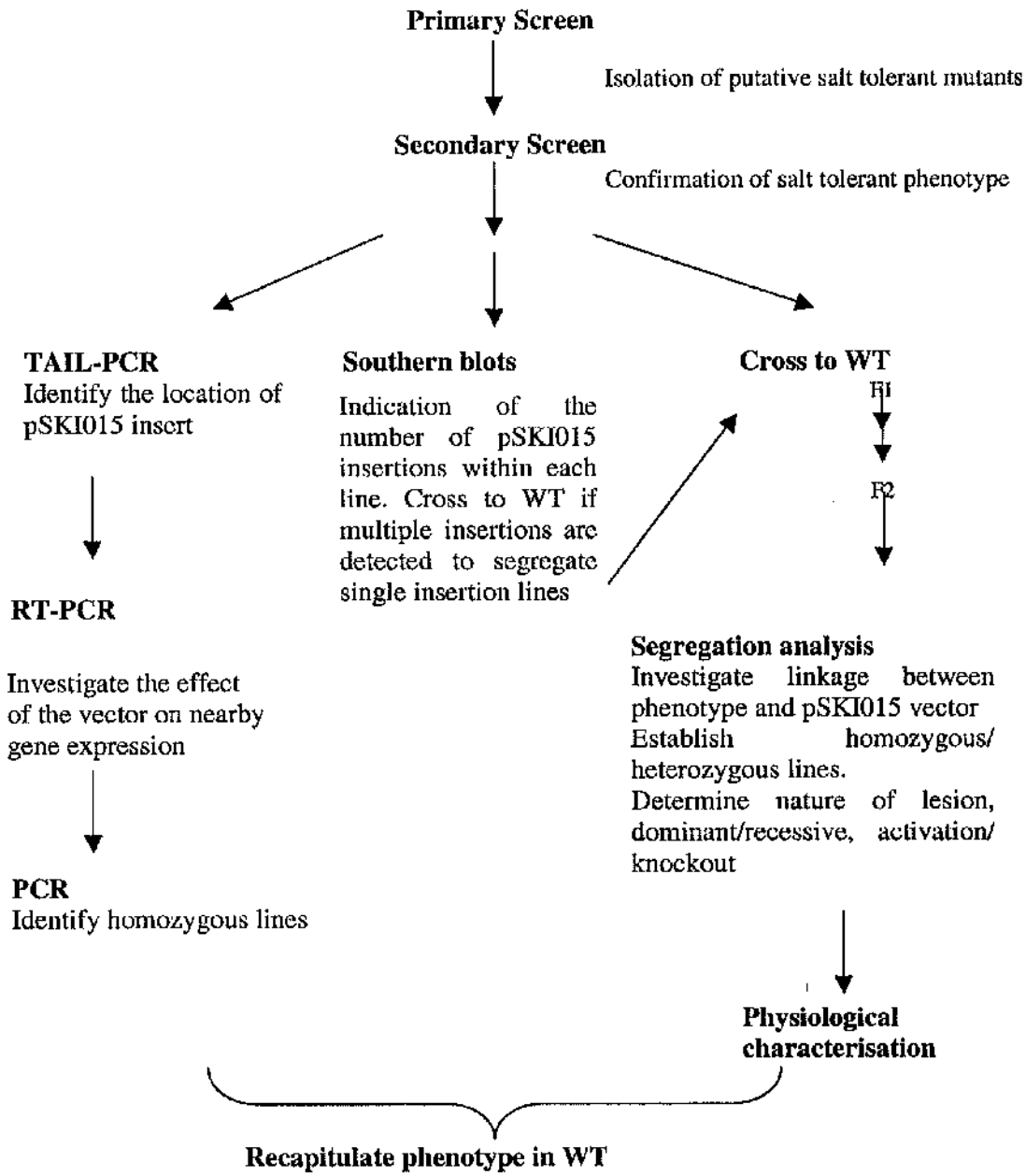


Figure 4.4 The Experimental Approach Used to Characterise the Salt Tolerant *Arabidopsis* Activation Tagged Mutants.

Salt tolerant *Arabidopsis* mutants were isolated from Weigel's collection of activation tagged lines. Genetic crosses with wild type plants determined linkage of the phenotype with the pSKI015 vector. Southern blot analysis indicated the number of pSKI015 insertions. The location of the pSKI015 insert was determined by TAIL-PCR, and the effect of the insert on nearby gene expression was investigated by RT-PCR. Once a candidate gene was identified it was over expressed in wild type to recapitulate the salt tolerant phenotype.

within the genome, multiple independent insertions of the T-DNA vector at different locations, and multiple insertions at a single location. Restriction enzymes *Nde*1 and *Nco*1 are 'hex cutters' cleaving genomic DNA on average every 4096 base pairs; neither enzyme cuts within the pSKI015 vector (~10kbp). Evidence for multiple insertions in the mutant will be provided, therefore, if genomic Southern blots using a fragment of pSKI015 as probe, generates more than one hybridisation band. The restriction enzymes *Pst*1 and *Bam*H1 cut within pSKI015 once, outside the region of probe hybridisation. The presence of multiple bands on genomic Southern blots prepared with these restriction enzymes should distinguish between multiple insertions and the presence of a single insertion within the plant genome. Chalfun-Junior and co workers performed Southern blot analyses on 47 of Weigel's activation tagged lines, chosen at random, and concluded that 26 of the lines contained multiple insertions (Chalfun-Junior *et al.* 2003). Restriction digests were performed, however, using *Hin*dIII an enzyme that cuts the pSKI015 vector once. By using only *Hin*dIII, it is impossible to differentiate between a single insertion of a concatameric sequence and multiple insertions at different loci. No Weigel line investigated in this study showed the presence of multiple insertions of the pSKI015 vector.

Reverse transcription- PCR (Section 2.2.17) was used to investigate transcript levels of genes located near the activation tag. Weigel *et al.* (2000) described 14 activation tagged mutants in which an over expressed gene was always detected immediately adjacent to the multimerized CaMV 35S enhancers on the right border, in none of the examples was the distal gene over expressed as well. Nevertheless, in this study the transcript level of genes each side of the pSKI015 insertion point were investigated.

To investigate linkage between the salt tolerant phenotype and the pSKI015 vector, homozygous salt tolerant mutants (male parent) were crossed with homozygous *gl1*

mutants (female parent). The *gl1* mutation is recessive and the phenotype of the F1 progeny of a successful cross will have trichomes and can be easily identified; unsuccessful crosses will lack trichomes on the leaves and stems. Segregation analysis of the F2 population can determine if the salt tolerant phenotype is caused by a single, recessive nuclear mutation (one quarter of the plants will display the mutant phenotype), or a single, dominant nuclear mutation (either three quarters of the plants will display the mutant phenotype if the mutant was homozygous, or 3/8 will have the mutant phenotype if the original mutant was heterozygous). If other, more complicated, genotypes are responsible for the mutant phenotype, different segregation patterns will be observed.

4.6 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP1.

This mutant was isolated from Weigel set N23153 pool N23846 and has a strong salt tolerant phenotype (Fig 4.5 c) compared with wild type when germinated on modified MS media (Table 2.1) supplemented with 80mM NaCl. BLAST2 interrogation of the full *Arabidopsis* genome database using the fragment cloned by TAIL-PCR, revealed vector DNA adjacent to plant genomic DNA (Fig 4.5 a) indicating a successful TAIL-PCR reaction. The pSKI015 vector inserts between At1g60200 and At1g60220, with right border enhancers adjacent to At1g60200 (Fig 4.5 b). Southern blot analysis using a probe designed to the BAR gene of the pSKI015 vector, showed a multiple insertion at a single location within this mutant (Fig 4.5 d). The PCR (Section 2.2.19) identified the original JP1 mutant as homozygous for the pSKI015 insert. Results from reverse transcription PCR (Section 2.2.17) show gene transcript levels of both At1g60200 and At1g60220 to differ in wild type tissue compared with JP1 (Fig 4.6).

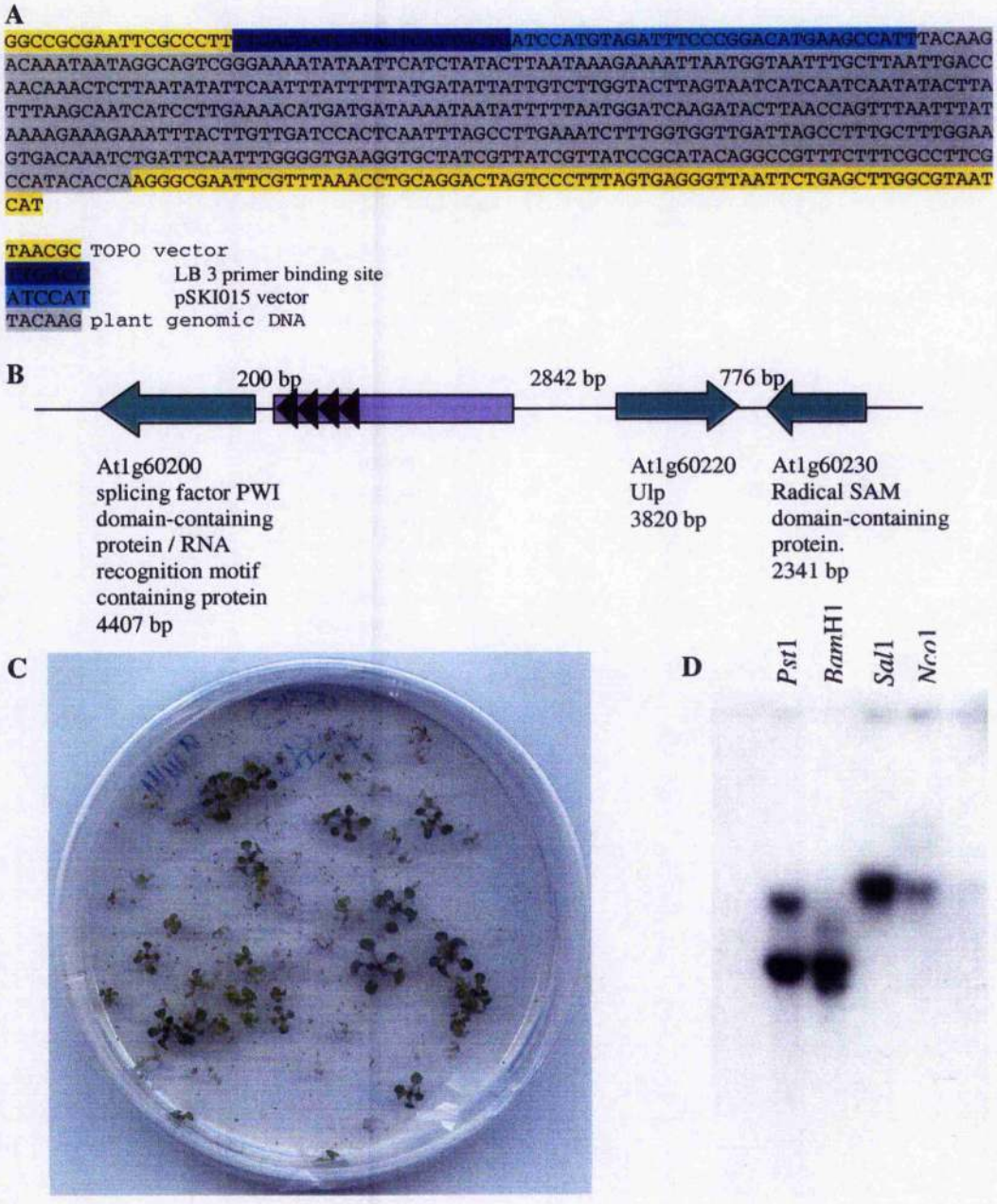


Figure 4.5 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP1.

JP1 demonstrates a strong salt tolerant phenotype **(C);** under these conditions wild type growth is seriously impaired. BLAST interrogation of the full *Arabidopsis* genome database using the sequence of the cloned fragment generated from this mutant by TAIL-PCR, reveals vector DNA adjacent to plant genomic DNA indicating a successful TAIL-PCR reaction. **(A).** The tag inserts with left border adjacent to At1g60200 the right border and enhancer elements are adjacent to At1g60220 **(B).** Gene annotations obtained from The Arabidopsis Information Resource (TAIR http://www.arabidopsis.org/). Southern blot analysis indicates the presence of a single copy, concatamer insertion **(D).**

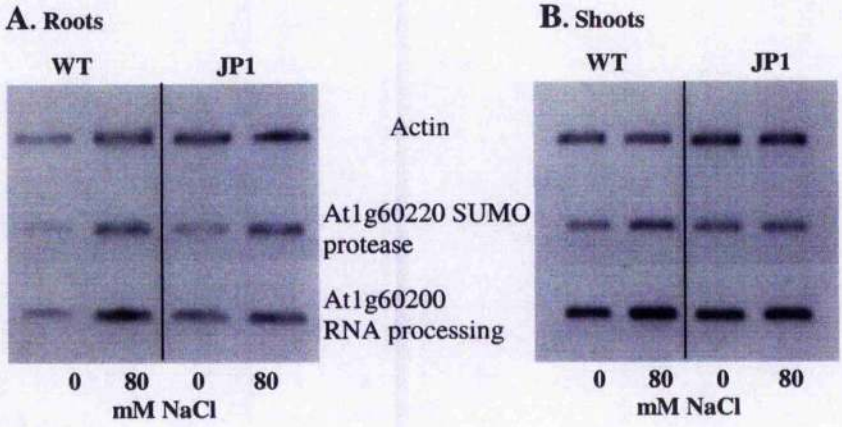


Figure 4.6 Transcript Levels of Genes Located Near pSKI015 Vector are Altered in the *Arabidopsis* Salt Tolerant Mutant JP1.

The RT-PCR (section 2.2.17) was performed using RNA isolated from 2-week-old root (**A**) and shoot (**B**) tissue treated with modified MS (containing 530μM Ca^{2+} and 200μM K^{+}) and supplemented with 0.75% sucrose without (0mM) or with NaCl (80mM). Expression levels were quantified by scanning densitometry using Quantiscan version 3.0 software (Biosoft Cambridge UK). All RT-PCR products were cloned and sequenced to confirm authenticity.

Transcript levels of At1g60220

Roots **(A)** from salt-treated WT plants have a 2-fold higher level of At1g60220 (SUMO protease) transcript levels than control WT roots. With JP1 mutant roots, transcript levels are 1.5-fold higher in salt-treated tissue compared with control.

Salt-treated WT shoots **(B)** have a 1.6-fold higher level of At1g60220 (SUMO protease) transcript levels than control WT plants. This trend is not observed in the JP1 mutant, At1g60220 transcript levels are similar in treated and non-treated shoots.

Transcript levels of At1g60200

Salt-treated WT roots **(A)** have a 1.6-fold higher level of At1g60200 (RNA processing) transcript levels than control WT roots. With JP1 roots, transcript levels of At1g60200 are similar in treated and non-treated tissue. Shoots **(B)** from salt-treated WT plants have a 1.9-fold higher level of At1g60200 (RNA processing) transcript levels than control WT shoots. With JP1 shoots, transcript levels are 1.2-fold higher in salt-treated tissue compared with control.

In both wild type and JP1 roots, At1g60220 transcript levels are higher in salt-treated tissue compared with control. In shoots this trend is observed only with wild type plants, At1g60220 transcripts levels are similar in JP1 shoot tissue regardless of salt treatment.

The transcript levels of At1g60200 are higher in salt treated roots and shoots of both wild type and JP1 compared with those observed when the plants are grown in the absence of NaCl.

4.6.1 At1g60200: a Role for RNA Processing in Plant Salt Tolerance?

The gene At1g60200 comprises 4406 bp from the predicted translation start site to the end of the translation stop codon and codes for a protein comprising 899 amino acids. Features of the protein include both a RRM and a PWI domain. The RNA recognition motif (RRM, also referred to as a RNA-binding region or RNP-1) is often diagnostic of an RNA binding protein. The RNA recognition motif has been found in a variety of RNA binding proteins, including various heterogeneous nuclear ribonucleoproteins (hnRNPs, proteins implicated in regulation of alternative splicing), and in the protein components of small nuclear ribonucleoproteins (snRNPs). The PWI domain (named after an almost invariant Pro-Trp-Ile signature) is found only in eukaryotic proteins. While its function is unknown, the presence of PWI motifs in known splicing factors suggests that it may be important for pre-mRNA processing (Blencowe & Ouzounis 1999). Pre-mRNA splicing, the removal of intervening sequences (introns) from primary mRNA transcripts with the concomitant ligation of protein coding sequences (exons), is a fundamental step in the mechanisms of constitutive and regulated gene expression. While the mode of action is unclear, proteins involved in pre-mRNA processing have been implicated in salt tolerance. Vicente and co-workers expressed an

Arabidopsis cDNA library in salt-sensitive yeast and searched for a sequence that would confer salt tolerance (Forment *et al.* 2002). Seven clones displayed a salt tolerant phenotype and sequencing showed these cDNAs to correspond to just three different genes, a component of the U1 snRNP and two putative 'SR-like' proteins, each involved in pre-mRNA splicing.

4.6.2 At1g60220: a SUMO Protease Involved in Plant Salt Tolerance?

Post-translational modification of proteins plays a critical role in most cellular processes. In ubiquitination, ubiquitin covalently attaches to proteins and targets them for degradation by the large protease complex, the 26S proteasome. Ubiquitin-conjugate formation is accomplished by a multi-enzyme cascade involving several classes of proteins. These include the ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and the ubiquitin-protein ligases (E3) (for a comprehensive review see Hochstrasser 1996). Multi- ubiquitination sometimes requires another factor, a ubiquitin elongation or extension enzyme E4 (Koegl *et al.* 1999). In addition to the ubiquitin system, yeast possesses at least five additional conjugation systems: SUMO (reviewed by Müller *et al.* 2001), autophagy (reviewed by Ohsumi 2001)), RUB1 (Liakopoulos *et al.* 1998), HUB1, and URM1 (Furukawa *et al.* 2000). All have homologs in plants and despite only limited primary sequence homology to ubiquitin at least SUMO, HUB and RUB have the characteristic ubiquitin-fold tertiary structure.

SUMO (small ubiquitin-like modifier) modification is reversible by desumoylation, a process mediated by a family of SUMO proteases (Li & Hochstrasser 1999). The gene At1g60220 is a putative SUMO protease. Unlike ubiquitination, SUMOylation does not appear to target proteins for degradation, but rather alters the target protein function

through changes in cellular localization, biochemical activation, or through protection from ubiquitin-dependent degradation. SUMO conjugation is carried out by an enzymatic pathway that is related to the ubiquitin conjugation pathway. Genes encoding the full-length SUMO tag and the predicted E1, E2, E3 and SUMO-proteases required for SUMO processing, ligation, and conjugate disassembly have been identified in *Arabidopsis* (Kurepa *et al.* 2003). There are 3 major classes of SUMO proteins (ATSUMO1, 2 and 3). During stress, SUMOylation by AtSUMO1 and 2 increase dramatically, but not AtSUMO3 mediated SUMOylation (Kurepa *et al.* 2003). This indicates that target proteins modified by AtSUMO1 and 2 may play critical roles during stress responses. In tomato, T-SUMO has been reported to be involved in stress responses to fungal infection (Hanania *et al.* 1999), Bejarano and co-workers showed NbSCE1 (a SUMO-conjugating enzyme) to play an important role in geminivirus infection of *Nicotiana benthamiana* (Castillo *et al.* 2004). More interestingly, Chua and co-workers suggest that SUMOylation contributes to the regulation of ABA signalling (Lois *et al.* 2003). BLAST2 database searches with the predicted protein sequence of At1g60220 showed high homology at the C-terminal end with known SUMO-proteases from yeast, humans and *Arabidopsis*. All the conserved key active-site amino acids found in other SUMO-proteases are present within the At1g60220 indicating that it is highly likely to be a SUMO-protease. There seems to be less homology at the N-terminus, this region may be involved in specific SUMO-conjugate substrate binding or SUMO protease-specific regulation and the sequence divergence may reflect different substrate specificity or regulation. So far no SUMO-protease has been implicated in stress signalling in plants. It is possible that At1g60220 confers salt tolerance by desumolyation of key proteins.

4.7 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP2

This mutant was isolated from Weigel set N21991 pool N23095 and has a strong salt tolerant phenotype (Fig 4.7 c). BLAST2 interrogation of the full *Arabidopsis* genome database using the fragment cloned by TAIL-PCR, reveals vector DNA adjacent to plant genomic DNA (Fig 4.7 a) indicating a successful TAIL-PCR reaction. The pSKI015 vector inserts between At3g59400 (*GUN4* which participates in plastid-to-nucleus signalling by regulating Mg-protoporphyrin IX, an intermediate in chloroplast biosynthesis Guo *et al.* 2003), and At3g59410 (*AtGCN2*, a protein kinase), with the CaMV 35S promoter enhancers adjacent to At3g59410 (Fig 4.7 b).

Transcript levels of *AtGCN2* are higher in both wild type and JP2 shoots treated with salt compared with control (Fig 4.8 a). These results suggest *AtGCN2* is induced by salt stress, and this level of induction is increased in the JP2 mutant perhaps due to the effect of the pSKI015 vector. Reverse transcription-PCR performed using RNA extracted from *Arabidopsis* cell culture lines (Chapter 3) also suggest *AtGCN2* is salt stress inducible (Fig 4.8 b). With respect to At3g59410 (*GUN4*), salt-treated wild type shoots have 1.4-fold higher level of *GUN4* transcript levels than control plants. With JP2 tissue a similar trend was observed but the fold increase was only 1.1. Levels of *GUN4* were lower in the salt tolerant HHS cell line compared with wild type cells (Fig 4.8 b).

It is possible that the altered levels of *GUN4* or *AtGCN2* cause the salt tolerant phenotype observed with the JP2 mutant. Attempts were made to recapitulate the salt tolerant phenotype in wild type plants, by creating transgenic lines over-expressing *AtGCN2*. This was unsuccessful mainly due to the large size of *AtGCN2*, the gene is reported to comprise 8,577 bp from the predicted translation start site to the end of the translation stop codon and this made cloning difficult.

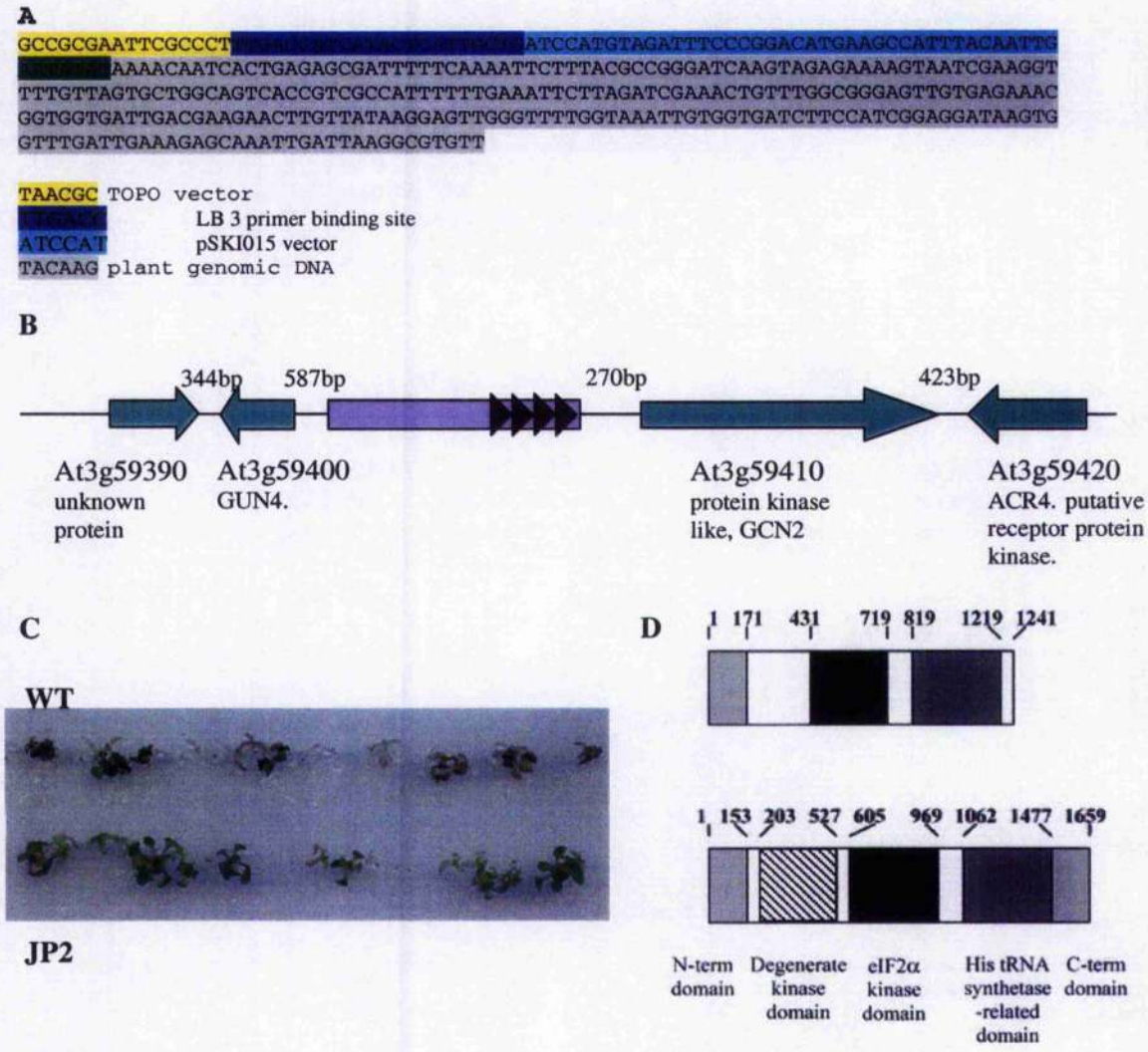


Figure 4.7 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP2.

JP2 demonstrates a strong salt tolerant phenotype (**C** bottom); seeds were germinated on media containing 0.75% sucrose, 80mM NaCl and MS modified to contain 530μM Ca^{2+} and 200μM K^{+} (Table 2.1), under these conditions wild type growth is seriously impaired (**C** top). BLAST interrogation of the full *Arabidopsis* genome database using the sequence of the cloned fragment generated from this mutant by TAIL-PCR, reveals vector DNA adjacent to plant genomic DNA indicating a successful TAIL-PCR reaction (**A**). The tag inserts with left border adjacent to At3g59400 the right border and enhancer elements are adjacent to At3g59410 (**B**) Gene annotations obtained from The Arabidopsis Information Resource (TAIR http://www.arabidopsis.org/). The structures of *Arabidopsis* AtGCN2 (**D** top) and yeast (*S. cerevisiae*) GCN2 (**D** bottom). The numbers shown indicate amino acid sequence positions. AtGCN2 contains both a kinase catalytic domain and a His tRNA synthetase-related domain characteristic of GCN2-type protein kinases.

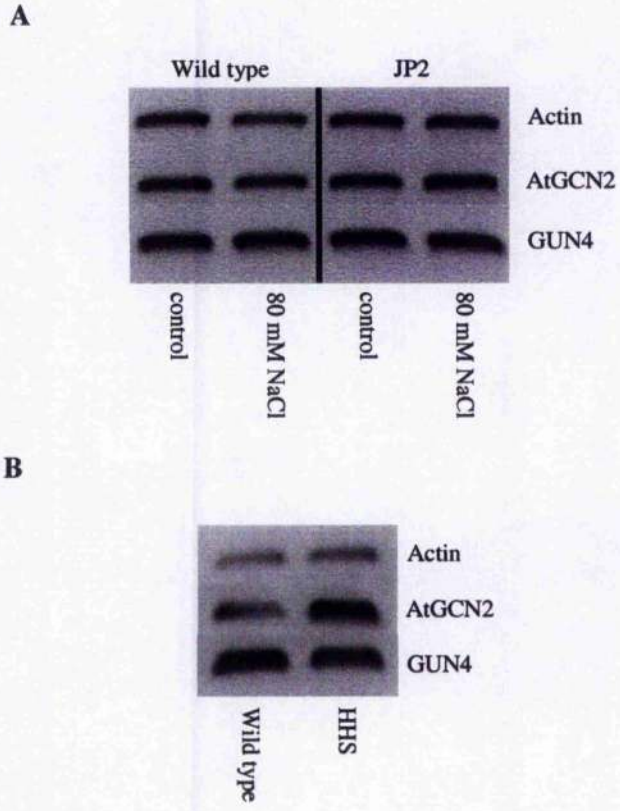


Figure 4.8 Transcript Levels of Genes Located Near pSKI015 Vector are Altered in the *Arabidopsis* Salt Tolerant Mutant JP2.

Transcript levels of At3g59400 (*GUN4*) and At3g59410 (*AtGCN2*) were investigated in wild type and JP2 shoot tissue (**A**), and also in *Arabidopsis* plant cell cultures (**B**). RT-PCR was performed using RNA isolated from 2-week old shoot tissue from plants grown on modified MS media (containing 530μM Ca^{2+} and 200μM K^{+} Table 2.1) without (control) or with NaCl (80mM). *Arabidopsis* wild type cell culture was grown in the absence of NaCl, the HHS cell lines treated with 300 mM NaCl (Section 2.2.1). RNA was isolated from 4 day old cell cultures. Expression levels were quantified by scanning densitometry using Quantiscan version 3.0 software (Biosoft Cambridge UK). All RT-PCR products were cloned and sequenced to confirm authenticity.

Transcript levels of *AtGCN2* are 1.2-fold higher in shoots from salt treated WT plants compared with control WT plants. With JP2 shoots, transcript levels are 1.4-fold higher in salt treated tissue compared with WT (**A**). This salt-induced expression can also be observed in the *Arabidopsis* cell culture lines, where the transcript levels of *AtGCN2* found in the HHS line is 1.4-fold higher than those observed in the wild type cells (**B**).

Salt-treated WT shoots have a 1.4-fold higher levels of *GUN4* transcript levels than control WT roots. With JP2 shoots, transcript levels are only 1.1-fold higher in salt treated tissue compared with control (**A**). Levels of *GUN4* transcripts are lower in the HHS cell line compared with wild type cells (**B**).

At3g59410 (*AtGCN2*) shows 45% similarity to a protein kinase from *Saccharomyces cerevisiae*, *GCN2*. *AtGCN2* contains adjacent eIF2 kinase and His tRNA-synthetase-like domains, characteristic of GCN2-type protein kinases (Fig 4.7 d). Halford and co workers have demonstrated functional conservation between AtGCN2 and yeast GCN2 by complementation of a yeast *gcn2* null mutant with AtGCN2 (Zhang *et al.* 2003). In yeast, GCN2 is involved in controlling the levels of the GCN4 protein (a bZIP transcription factor) by a unique posttranslational mechanism (Fig 4.9). This kinase was originally identified as playing a crucial role in the general amino acid control pathway in yeast, (reviewed by Hinnebusch 1997), where amino acid starvation causes a general reduction in protein synthesis and initiates changes in expression of a large number of genes. More recently, micro array analysis identified 539 yeast genes that are induced by amino acid starvation through the direct action of GCN4 (Natarajan *et al.* 2001). These include genes in every amino acid biosynthetic pathway except cysteine, and genes encoding amino acid precursors. Interestingly, genes involved in amino acid biosynthesis represented only a quarter of the Gcn4 target genes. Numerous other genes encoding protein kinases and transcription factors were identified as targets, suggesting that Gcn4p is a master regulator of gene expression. Studies with yeast have now shown GCN4 to regulate cellular response to a variety of environmental stresses including, amino acid starvation, purine starvation (Marten *et al.* 1999), glucose limitation (Kardailsky *et al.* 1999) and most interestingly, salt stress (Goossens *et al.* 2001; Narasimhan *et al.* 2004).

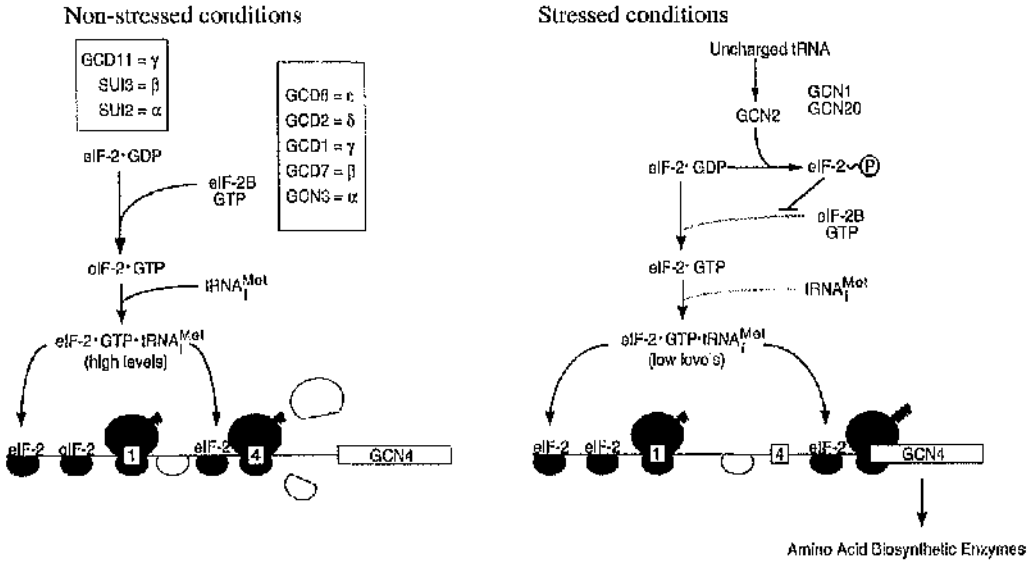


Figure 4.9 A Model for Translational Control of Yeast GCN4 by Phosphorylation of eIF2α by the Protein Kinase GCN2.

GCN4 mRNA is shown with uORFs 1 and 4 and the *GCN4* coding sequences indicated as boxes. The 40S ribosomal subunits are shaded when they are associated with the ternary complex composed of eIF2, GTP, and Met-tRNA$_i^{Met}$ and are thus competent to reinitiate translation; unshaded 40S subunits lack the ternary complex and cannot, therefore, reinitiate. The 80S ribosomes are shown translating uORF1, uORF4, or GCN4, with the synthesized peptides depicted by coils attached to the 60S subunits. Free 40S and 60S subunits are shown dissociating from the mRNA following termination at uORF4 (left panel).

Under non-stressed conditions the eukaryotic initiation factor 2 (eIF2) complexed with GTP delivers Met-tRNA to the 40S ribosome. During this translation initiation process, the GTP is hydrolysed to GDP, and eIF2 is released as an inactive eIF2·GDP complex. Recycling of this complex to the active GTP-bound form requires an exchange factor, eIF2B. Under these conditions, when eIF2·GTP·tRNA$_i^{Met}$ levels are high, GNC4 translation is inhibited. Four short upstream open reading frames (uORFs), present in the leader sequence of the GCN4 mRNA upstream of the coding region, prevent efficient translation initiation at the GNC4 start codon (left panel) GCN4 mRNA is shown with uORFs 1 and 4 and the *GCN4* coding sequences indicated as boxes. Ribosomes that resume scanning following uORF1 translation are forced to reinitiate at uORF4 because they rebind the eIF2.GTP.Met-tRNA$_i^{Met}$ ternary complex before reaching the uORF4 start codon.

In stressed conditions (right panel), such as amino acid starvation (reviewed by (Hinnebusch 1997b), purine starvation (Rolfes & Hinnebusch 1993), glucose limitation (Yang *et al.* 2000), and salt stress (Goossens *et al.* 2001), uncharged tRNA molecules bind to the C-terminal tRNA synthetase domain of GCN2. This results in the activation of the neighbouring kinase domain which phosphorlyates the α subunit of eukaryotic initiation factor-2 (eIF2α) at serine-51. This inhibits the exchange factor eIF-2B and reduces levels of eIF2-GTP. A decreased level of eIF2-GTP enhances the translation expression of GCN4, as ribosomes lacking inititiator tRNA^{Met} cannot recognise the AUG codons at uORFs 2,3 and 4 and continue scanning downstream to *GCN4* instead (Abastado *et al.* 1991; Dever *et al.* 1992). Diagram adapted from Hinnebusch (1997).

4.8 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP3.

This mutant was isolated from Weigel set N21995 pool N20937 and has a weak salt tolerant phenotype (Fig 4.10 c). BLAST2 interrogation of the full *Arabidopsis* genome database using the sequence of the cloned TAIL-PCR fragment as probe, revealed vector DNA adjacent to plant genomic DNA (Fig 4.10 a) indicating a successful TAIL-PCR reaction. The tag inserts within the first intron of At5g45900 (Fig 4.11 b). The PCR (Section 2.2.19) identified the original JP3 mutant as heterozygous for the pSKI015 insert.

The gene At5g45900 contains 10 introns and 11 exons and encodes a 697-amino acid protein with 56 and 62% similarity to yeast and human APG7, respectively. APG7 is involved in the autophagy pathway (Fig 4.11), the process by which intracellular constituents are recycled. Here bulk cytosolic constituents and organelles become sequestered in specialised autophagic vesicles, which then deliver their cargo to the vacuole for degradation. Unlike ubiquitination of proteins, autophagy is believed to be mainly non-selective, targeting proteins for destruction indiscriminately in some situations whole organelles can be targets (Dunn 1994). As a consequence, protein turnover by the vacuole is thought to play less of a role in cellular regulation, but a more prominent role under stress conditions when rapid remobilisation and resorption of nutrients is crucial.

Despite the location of the activation tag within the first intron of At5g45900, an APG7 transcript was detected by RT-PCR from a homozygous JP3 mutant (Fig 4.10 d). RNA used in this experiment was isolated from the shoots of soil-grown wild type and JP3 plants. Levels of expression of the APG7 were in fact higher in the JP3 mutant when

A

CGGCCGCGAATTCGCCCTT[redacted]ATCCATGTAGATTTCCCGGACATGAAGCCATTTACAA
TTG[redacted]TCCGTTTAGGTTAGAGAAGCAAAAAAAAAAGTTGAAAACTTGAAAATCCTCTGTTTTCTGGGCAAGTTGTTATGA
TCCGTTTAGGTTAGAGAAGCAAAAAAAAAGTTGAGAACTTTAAGAGCCTCTGTTTTGTTTAGTAGAGATTATTCAGAT
TTAGAATTTATGTGCTTTTTTGGAACTGAAATTTTCAGGTTTCTATGGACCATGTGGACATCCACAAGTCTCAAATCA
TTTGACTCTTTTATCAGAGTCATTGCCTTTAGATGAACAATCATTGATTGCTAGTACTAGTCATGGAAATAGGAATAA
GTGTCCTGTTCCTGGGATTTTGTATAACACCAATACCGTTGAAAGCTTCAATAAACTTGACAAACAAAGTCTGCTAAA
AGCAGAAGCAAATAAGGTAAATACAAAACTTAGCTCCGTGACTTGGTTTTTACTGGCTCTTGTCTGCAGAAGTTTTAA
CAATTTTGTTGGTGAATTTTAGATTTGGGAGGATATTCAATCAGGAAAAGCTCTTGATGACCCTTCTGTGTTACCAAG
ATTCCTCGTTATCTCCTTTGCAGACCTTAAGAAATGGAGTTTTCGGTATTGGTTTGCGTTCCCTGCGTTTGTACTTGA
TCCTCCTGTAAGTTTGATTGAATTAAAGCCAGCTTCAGAGTATTTTAGTTCTGAAGAGGCTGAATCAGTATCCGCTGC
TTGTTCTAACTCGCAAGGGCGAATTCGTTTAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATCTGAGCTTGGCGT
ATCATGGTCATAGCTGTTTCTGTGTGAATT

TCGCCCTT	TOPO vector
[redacted]	LB 3 binding site
ATCCATGT	pSKI015 vector
TCCGTTTA	plant DNA

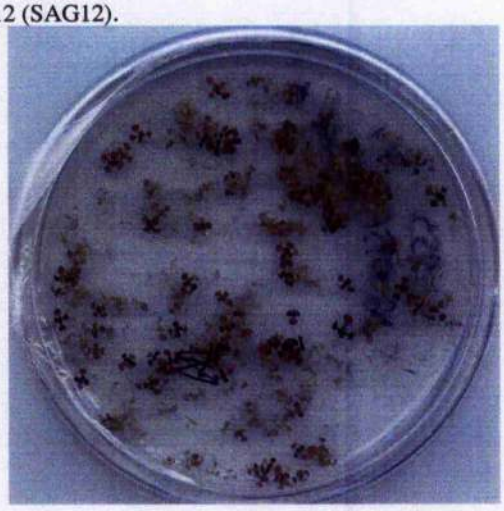
B

At5g45890
Senescence-associated gene 12 (SAG12).

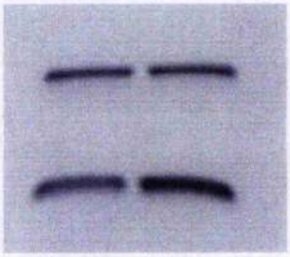
At5g45900
Autophagy 7 (APG7)

At5g45910
GDSL-motif lipase/hydrolase protein.

C



D



WT JP3

Figure 4.10 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP3.

JP3 demonstrates a weak salt tolerant phenotype **(C).** Seeds were germinated on media containing 0.75% sucrose, 80mM NaCl and MS modified to contain 530μM Ca^{2+} and 200μM K^+ (Table 2.1), under these conditions wild type growth is seriously impaired. BLAST interrogation of the full *Arabidopsis* genome database using the sequence of the cloned fragment generated from this mutant by TAIL-PCR, reveals vector DNA adjacent to plant genomic DNA indicating a successful TAIL-PCR reaction **(A).** The tag inserts within the first intron of At5g45900 (APG7) **(B).** Transcript levels of APG7 are higher in the JP3 mutant compared with wild type. The RT-PCR product was cloned and sequenced to confirm authenticity.

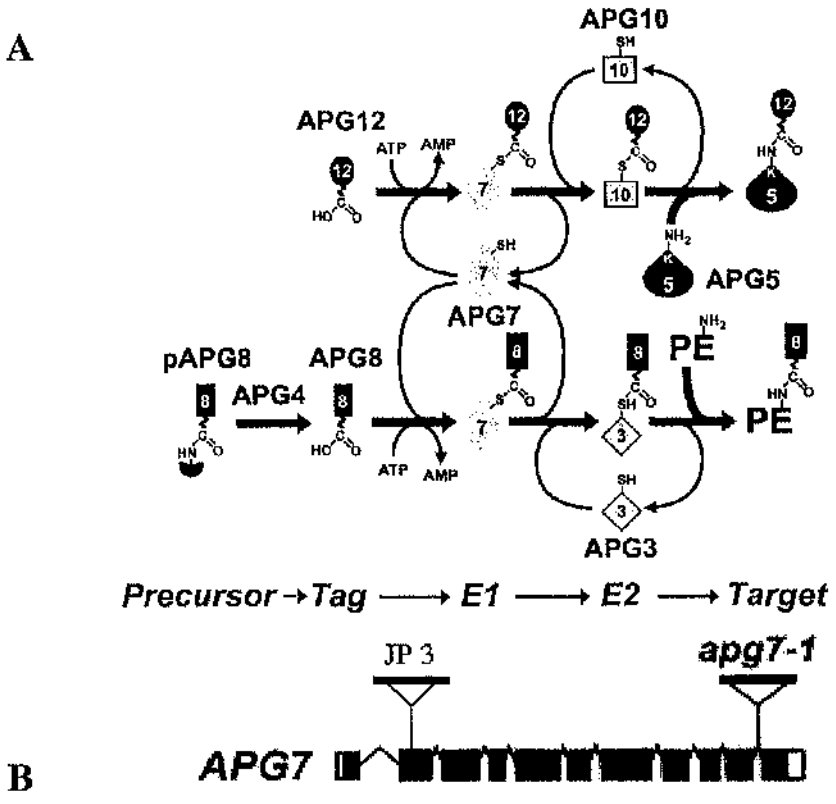


Figure 4.11 The APG conjugation pathways in yeast and the *Arabidopsis APG7*.
The APG conjugation pathways in yeast (**A**). By ATP-dependent reaction cascades involving an E1 and E2s, APG12 and APG8 become covalently attached to their respective targets APG5 and phosphatidylethanolamine (*PE*). Prior to activation, the APG8 precursor is processed by the APG4 protease to remove the extra C-terminal residues and thus expose the penultimate glycine. Both APG12 and −8 are activated by the E1-APG7 and become bound to APG7 by a thiol ester bond. The activated tags are then donated to their respective E2s, APG10 and APG3 by transesterification, and finally attached to their targets by an amide bond. For APG5, a specific lysine (*K*) is involved which forms an isopeptide bond with APG12. For PE, the amide group of ethanolamine is used for attachment to APG8. All important catalytic domains in the yeast APG components are conserved in their *Arabidopsis* counterparts, strongly suggesting that the *Arabidopsis* pathways have similar mechanisms of catalysis. The *AtAPG7* gene (**B**) contains 10 introns (lines) and 11 exons (boxes). The location of the T-DNA insertions in the *apg7-1* and JP3 mutants are shown. Figures taken from Doelling *et al.* (2002).

compared with wild type. Since APG7 controls the first step of the 2 protein conjugation pathways required for autophagy (Fig 4.11; Doelling *et al.* 2002), it is possible that the over expression of APG7 can confer a degree of salt tolerance by increasing the recycling of cellular constituents through the up regulation of the autophagy pathway. Conversely, reducing APG7 protein levels increases sensitivity to nutrient-limiting conditions (Doelling *et al.* 2002). Another mutant, *apg7-1*, deposited in the SALK Knockout collection contains a T-DNA insertion in the final intron of *AtAPG7* and has impaired accumulation of the APG7 protein (Doelling *et al.* 2002). Under limiting nitrogen and carbon levels, *apg7-1* plants demonstrated a poorer growth performance compared with wild type plants. When grown under control conditions, homozygous *apg7-1* seedlings undergo normal growth indicating that the APG7 protein, and by inference the APG8/12 conjugation pathways are not essential for *Arabidopsis* growth and development. It remains to be established whether *apg7-1* has altered sensitivity to high salinity.

4.9 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP4.

This mutant was isolated from Weigel set N23153 pool N23858 and has a strong salt tolerant phenotype (Fig 4.12 c). BLAST2 interrogation of the full *Arabidopsis* genome database using the sequence of the cloned TAIL-PCR fragment as probe, revealed vector DNA adjacent to plant genomic DNA (Fig 4.12 a) indicating a successful TAIL-PCR reaction. The pSKI015 vector inserts between At1g55680 (WD-40 repeat protein family) and At1g55690 (similar to phosphatidylinositol transfer protein) with enhancers pointing towards At1g55680 (Fig 4.12 b). The PCR (Section 2.2.19) identified the original JP4 mutant as heterozygous for the pSKI015 insert. Initial RT-PCR

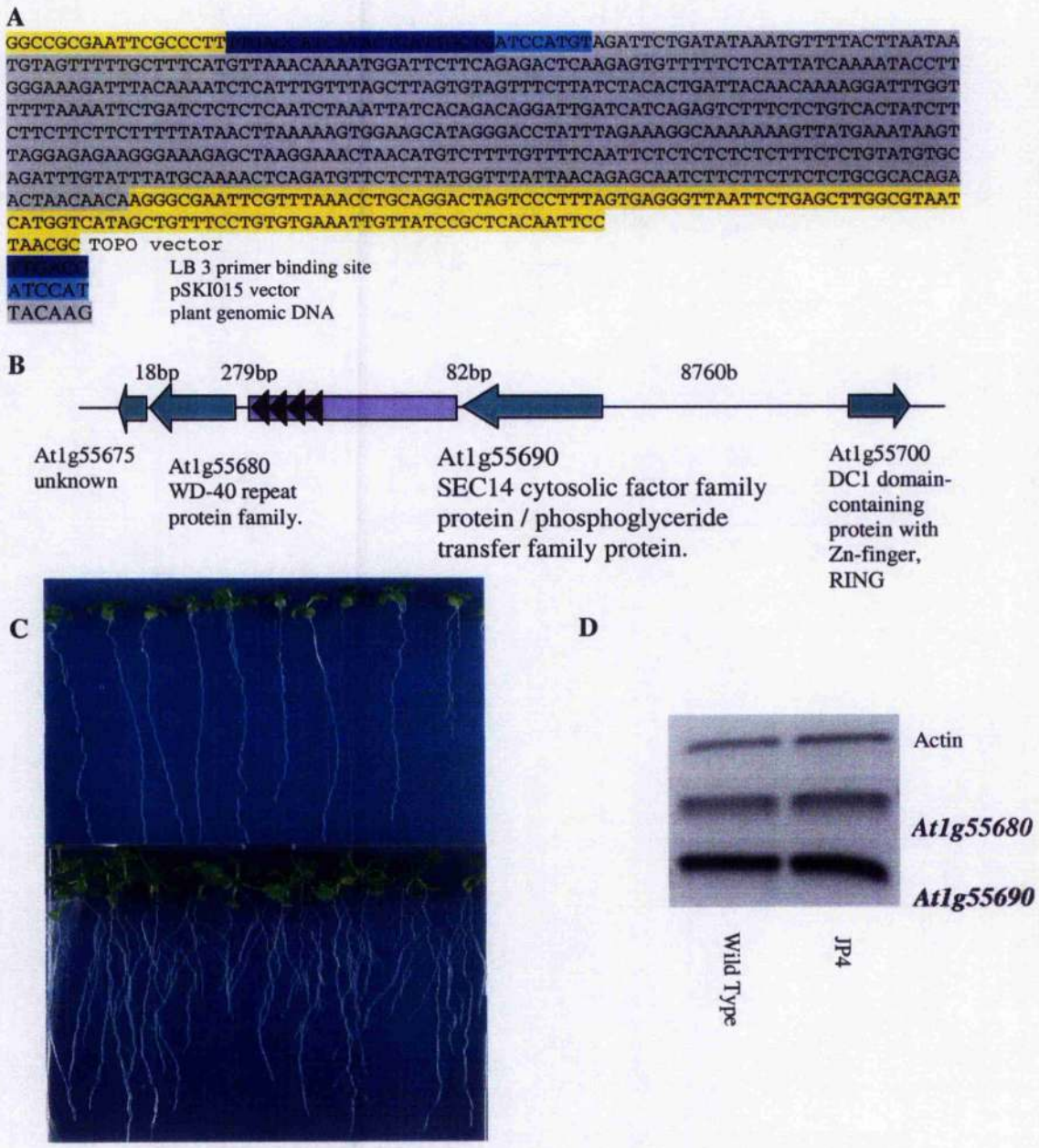


Figure 4.12 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP4.

JP4 demonstrates a strong salt tolerant phenotype (**C** bottom), seeds were germinated on media containing 0.75% sucrose, 80mM NaCl and MS modified to contain 530μM Ca^{2+} and 200μM K^{+} (Table 2.1), under these conditions wild type growth is seriously impaired (**C** top). BLAST searches of the *Arabidopsis* genome database using the sequence of the cloned fragment generated from this mutant by TAIL-PCR, reveals vector DNA adjacent to plant genomic DNA indicating a successful TAIL-PCR reaction (**A**). The tag inserts with left border adjacent to At1g55680 the right border and enhancer elements are adjacent to At1g55680 (**B**). RT-PCR was performed using RNA isolated from shoot tissue of 2 week old soil grown plants. Transcript levels of both genes appear similar in the JP4 mutant and wild type plants.

investigations showed transcript levels of both At1g55680 and At1g55690 to be similar between wild type and JP4 plants. RNA was extracted, however, from plants grown on soil. Future experiments should compare transcript levels of these genes from plants grown under screening conditions (Table 2.1) with/without NaCl.

The WD-40 domain (also called Trp-Asp i.e. WD domain) is composed of a ~40-amino acid stretch typically ending in Trp-Asp (Neer *et al.* 1994). When present in a protein the WD motif is typically found as several (4–10) tandem repeated units and acts as a site for interaction with other proteins. There are 269 *Arabidopsis* proteins containing at least one copy of the WD motif. Of these, 237 contain four or more recognizable copies of the motif (van Nocker & Ludwig 2003). WD-40 repeat containing proteins have been found to play key roles in signal transduction, cytoskeletal dynamics, protein trafficking, nuclear export, and RNA processing, and are especially prevalent in chromatin modification and transcriptional mechanisms (van Nocker & Ludwig 2003).

4.10 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP6.

This mutant was isolated from Weigel set N21995 pool N21360 and has a weak salt tolerant phenotype (Fig 4.13 a). BLAST2 searches of the *Arabidopsis* genome database using the sequence of the cloned fragment generated from this mutant by TAIL-PCR, revealed vector DNA adjacent to plant genomic DNA indicating a successful TAIL-PCR reaction. The pSKI015 vector inserts within At3g11460 (Fig 4.13 c), a gene with no introns within the coding sequence, a common feature of the PPR family (Lurin *et al.* 2004). The PCR (Section 2.2.19) identified the original JP6 mutant as homozygous for the pSKI015 insert.

At3g11460 is 1872 bp long and the protein contains approximately 11 pentatricopeptide repeats (PPR) and one tetratricopeptide repeat (TPR). The PPR family consists of about

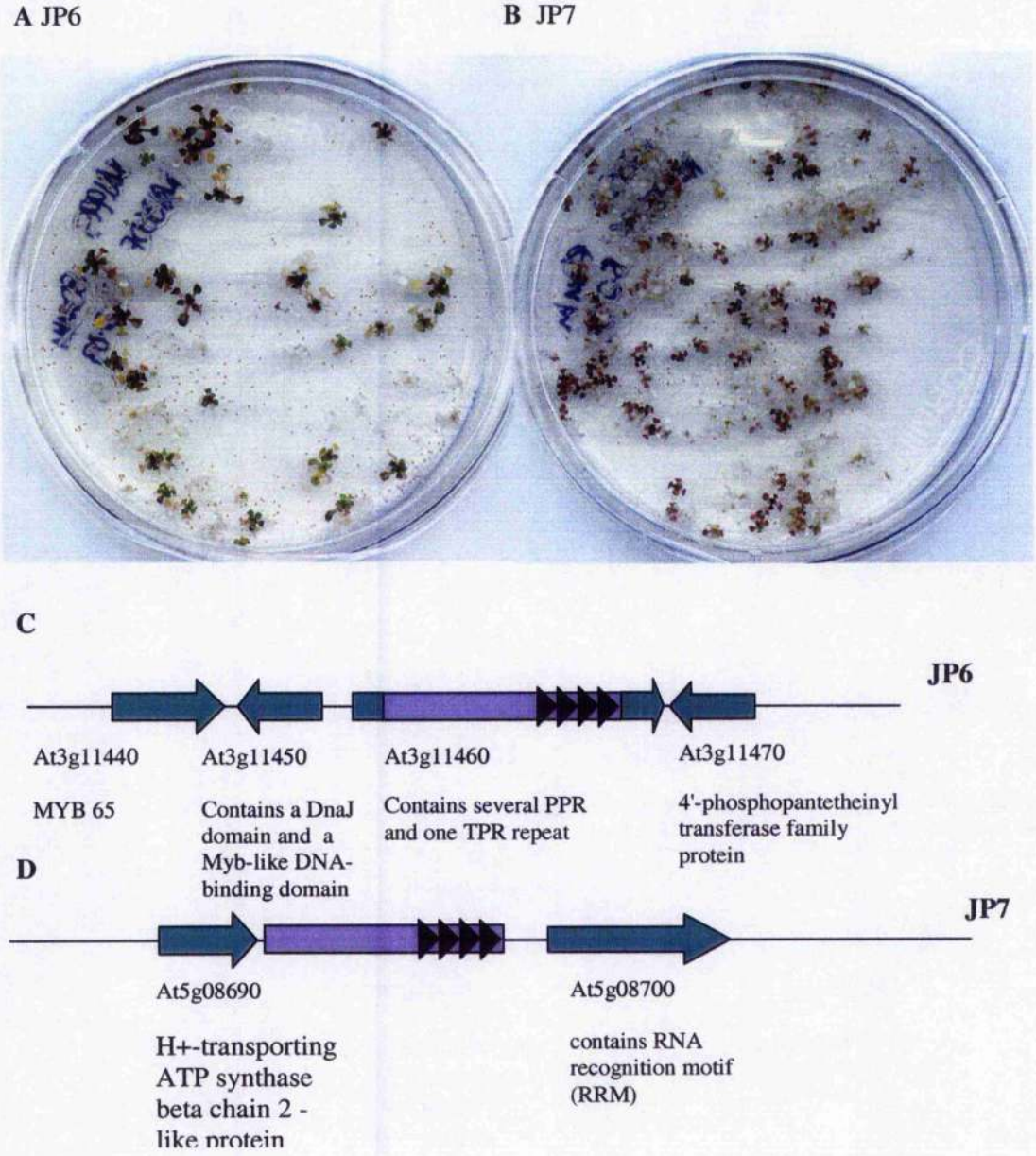


Figure 4.13 Phenotype and location of pSKI015 insertion within the *Arabidopsis* activation tagged mutants JP6 and JP7.

Both JP6 **(A)** and JP7 **(B)** have a weak salt tolerant phenotype when compared with wild type. With the JP6 mutant, the T-DNA was found to be located within At3g11460, a gene containing several PPR repeats and one TPR repeat **(C)**. With the JP7 mutant the pSKI015 vector inserts between At5g08690 and At5g08700, with the enhancers facing At5g08700, a protein containing a RNA-binding region **(D)**.

450 genes in the *Arabidopsis* genome. PPR repeats are about 35 amino acids long, and predicted to be involved in RNA stabilisation or processing in chloroplasts or mitochondria (Small & Peeters 2000). The number of PPR motifs controls its affinity and specificity for RNA and it has been suggested that each of the highly variable PPR proteins is a gene-specific regulator of plant organelle RNA metabolism (Nakamura *et al.* 2004).

The tetratricopeptide repeat (TPR) is a structural motif present in a wide range of proteins. Evidence suggests the TPR mediates protein–protein interactions and the assembly of multiprotein complexes (Lange & Ghassemian 2003). The TPR motif consists of 34 amino acids residues. Proteins containing TPRs are involved in a variety of biological processes, such as cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis, and protein folding.

4.11 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP7.

This mutant was isolated from Weigel set N21991 pool N23073 and has a weak salt tolerant phenotype (Fig 4.13 b). BLAST2 interrogation of the full *Arabidopsis* genome database using the sequence of the cloned TAIL-PCR fragment as probe, revealed vector DNA adjacent to plant genomic DNA indicating a successful TAIL-PCR reaction. The pSKI015 vector inserts between At5g08690 and At5g08700. The PCR (Section 2.2.19) identified the original JP7 mutant as heterozygous for the pSKI015 insert.

At5g08690 is a beta subunit of the ATP synthase enzyme. This ubiquitous, highly conserved enzyme is found in the inner mitochondrial and thylakoid membranes and catalyses the formation of ATP from ADP and Pi using a unique rotary motor mechanism (Yoshida *et al.* 2001).

At5g08700 contains several RRM motifs (discussed in Section 4.6.1). These motifs are commonly found in RNA binding proteins and are thought to be involved in RNA processing.

4.12 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP8.

This mutant was isolated from Weigel set N21991 pool N23086 and has a weak salt tolerant phenotype (Fig 4.14 a). BLAST2 interrogation of the full *Arabidopsis* genome database using the sequence of the cloned fragment generated from this mutant by TAIL-PCR, revealed vector DNA adjacent to plant genomic DNA indicating a successful TAIL-PCR reaction. The pSKI015 vector inserts into the 8th exon of At5g59650 a putative transmembrane serine threonine kinase (Fig 4.14 c). At present no function has been assigned to this putative kinase.

4.13 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP9.

This mutant was isolated from Weigel set N21991 pool N23107 and has a weak salt tolerant phenotype (Fig 4.14b). BLAST2 searches of the *Arabidopsis* genome database using the sequence of the cloned TAIL-PCR fragment as probe, revealed vector DNA adjacent to plant genomic DNA indicating a successful TAIL-PCR reaction. The pSKI015 vector inserts within the 5th exon of At3g14490 (Fig 4.14 d). The PCR identified the original JP9 mutant as homozygous for the T-DNA insertion within the At3g14490 gene. At3g14490 is 2319bp long with 7 exons and encodes a 601 aa protein. At3g14490 is a terpene sythase gene - TPS12 (Lange & Ghassemian 2003). Terpenes are an important class of defense compounds that accumulate in plants after pathogen infection or arthropod injury. There are 40 terpene synthase genes in the *Arabidopsis*

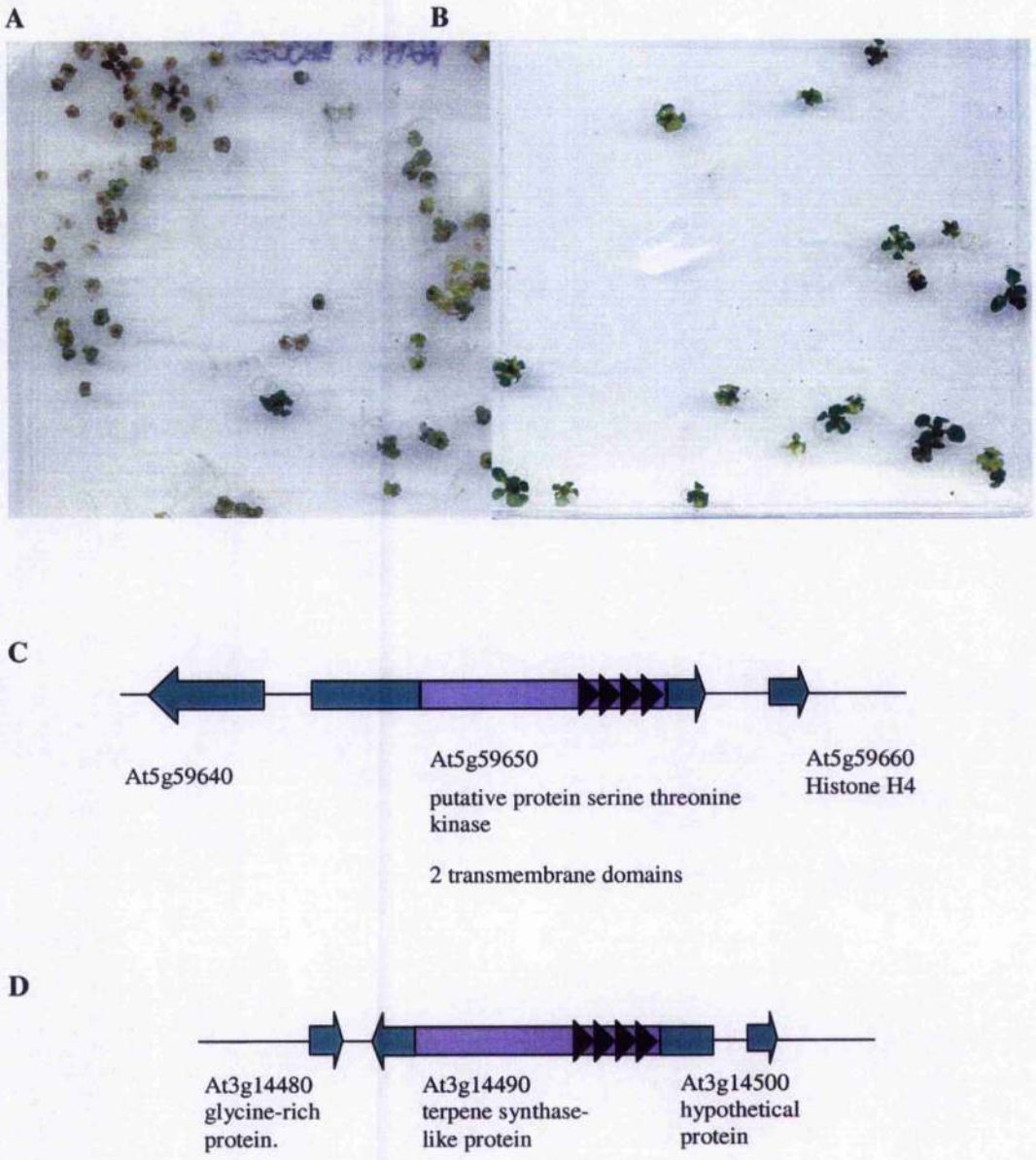


Figure 4.14 Phenotype and location of pSKI015 insertion within the *Arabidopsis* activation tagged mutants JP8 and JP9.

Both JP8 **(A)** and JP9 **(B)** have a weak salt tolerant phenotype when compared with wild type. With the JP8 mutant, the T-DNA was found to be located within At5g59650, a putative protein serine theronine kinase **(C)**. With the JP8 mutant the pSKI015 vector inserts within At3g14490 a terpene synthase-like protein **(D)**.

genome (Aubourg *et al.* 2002). Chen *et al* investigated transcript levels of 32 of these genes and showed 6 (At3g14490 being one) to be expressed only in flowers (Chen *et al.* 2003).

4.14 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP10.

This mutant was isolated from Weigel set N21995 pool N20936 and displayed a weak salt tolerant phenotype (Fig 4.15 a) when compared with wild type. BLAST2 searches of the *Arabidopsis* genome database using the cloned TAIL-PCR fragment as probe, revealed vector DNA adjacent to plant genomic DNA indicating a successful TAIL-PCR reaction. The pSKI015 vector inserts within the last exon of *AtNOS1* (Fig 4.15 b), with the CaMV 35S promoter enhancers adjacent to At3g47440. The PCR (Section 2.2.19) identified the original JP10 mutant as heterozygous for the pSKI015 insertion within the At3g47440 gene.

Reverse transcription-PCR performed using RNA extracted from *Arabidopsis* cell culture lines (Chapter 3) indicated higher transcript levels of *AtNOS1* in the HHS culture (salt tolerant) compared wild type (salt sensitive) cell lines (Fig 4.15 e)

At3g47440 belongs to the MIP family of proteins and is predicted to have water channel activity. Membrane intrinsic proteins (MIPs) are a family of membrane channels that facilitate the bidirectional transport of water and small uncharged solutes such as glycerol. Increasing aquaporin activity could minimise desiccation stress caused by the presence of NaCl. It will not, however, reduce the toxic effects of the Na^+ and Cl^- ions.

AtNOS1 encodes a protein that has been identified as a nitric oxide synthase (Guo *et al.* 2003). Nitric oxide synthase (NOS) catalyses the formation of NO and L-citrulline from L- arginine, NADPH and O_2. The highly reactive molecule NO and its exchangeable redox-activated forms (the nitrosonium cation (NO^+) and the nitroxyl radical (NO^-)) are

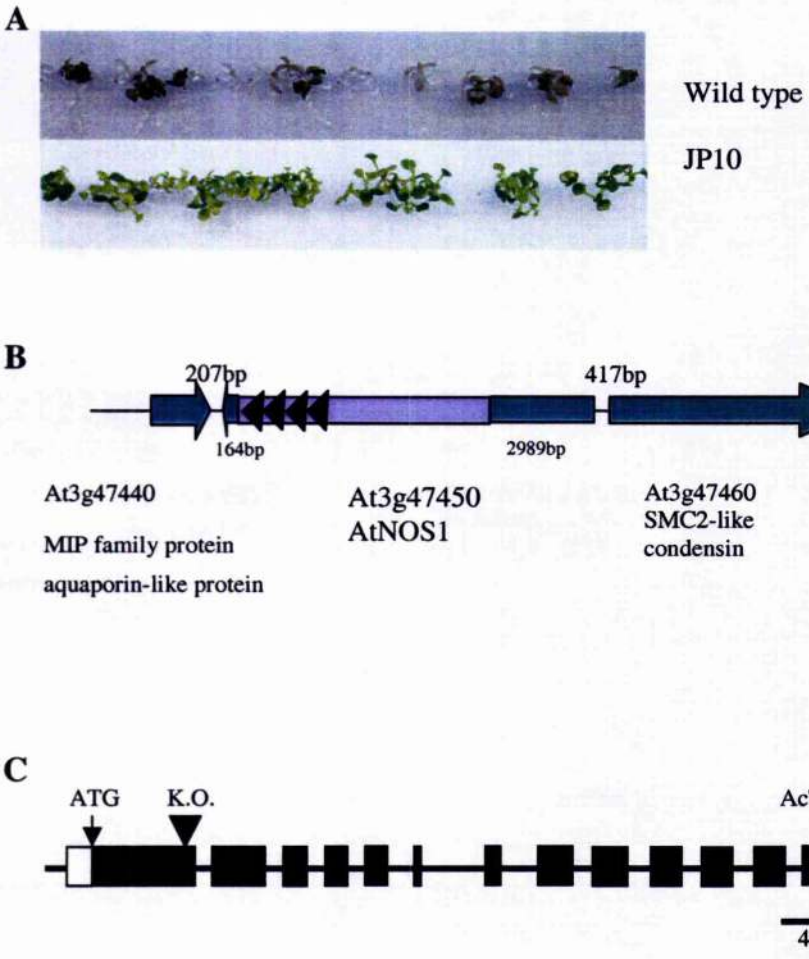

A
Wild type
JP10
B
207bp
417bp
164bp
2989bp
At3g47440
MIP family protein
aquaporin-like protein
At3g47450
AtNOS1
At3g47460
SMC2-like
condensin
C
ATG
K.O.
AcTag
TGA
400 bp
D
Wild type
Homozygous
Heterozygous
E
Actin
AtNOS1
Wild type
HHS

Figure 4.15 Characterisation of the Salt Tolerant *Arabidopsis* Mutant JP10.

When grown on modified MS media (Table 2.1) supplemented with 80mM NaCl, JP10 demonstrated a strong salt tolerant phenotype (A bottom) compared with wild type (A top). BLAST2 interrogation of the full *Arabidopsis* genome database using the sequence of the cloned fragment generated by TAIL-PCR reveals vector DNA adjacent to plant genomic DNA. The tag inserts within the final exon of At3g47450 (*AtNOS1*) with the right border and enhancer elements are adjacent to At3g47440 (B). Schematic diagram of the *AtNOS1* gene showing the insertion sites of the activation tag (AcTag) in the JP 10 line, and the T-DNA insertion site within the knock out line (K.O.) N547882 (C). Black boxes indicate exons. When grown on soil JP10 mutants identified as heterozygous for the pSKI015 insertion at this location, looked similar to wild type plants. Homozygous lines however, were a paler shade of green, and showed reduced shoot and inflorescence growth compared with wild type (D). RT-PCR was performed using RNA isolated from 4-day-old *Arabidopsis* cell cultures. Wild type cell cultures are grown in the absence of NaCl; the HHS cell lines are habituated to growth in 300 mM NaCl (Section 2.2.1). RNA was isolated from 4-day-old cell cultures. *AtNOS1* transcript levels are higher in HHS cell lines compared with wild type (E).

recognized as important intra- and intercellular signalling molecules. NO can interact with both metal- and thiol- containing proteins. Among these target proteins are cellular messengers, ion channels, enzymes, receptors and transcription factors (Lamattina *et al.* 2003). Guo and co-workers (2003) describe a mutant line with a T-DNA insertion within the first exon of *AtNOS1* (Fig 4.15 c). This insertion was shown to reduce NOS activity in the mutant to 25% that of wild type.

By investigating ABA enhancement of NO synthesis in wild type and the mutant line Guo *et al.* (2003) concluded *AtNOS1* is required for ABA-induced NO generation and that AtNOS1-dependent NO generation occurs downstream of ABA in this signalling pathway. Homozygous *AtNOS1* knock out mutant plants were described as pale green with reduced shoot, root and inflorescence growth, and low fertility (Guo *et al.* 2003). The PCR identified the original JP10 mutant to be heterozygous for the pSKI015 insertion at this location. Homozygous mutant lines were identified by the PCR from subsequent generations. When grown on soil these plants showed reduced shoot and inflorescence growth, and fertility was considerably lower, similar to the *AtNOS1* mutant lines described by Guo *et al.* (2003). From this it is tempting to suggest the pSKI015 insertion in the JP10 mutant reduced transcript levels of *AtNOS1*. RT-PCR should be used to investigate this (and also transcript levels of At3g47440 and At3g47460 the genes flanking *AtNOS1*). To test if the JP10 mutants have lower AtNOS1 activity the JP10 homozygous plants could be treated with sodium nitroprusside, an NO donor, which was shown to restore greening, fertility and growth of the AtNOS1 knock out line (Guo *et al.* 2003). Alternatively, insertion of the pSKI015 vector within the last exon of *AtNOS1* could result in C-terminal deletions in the translated product, perhaps where NOS activity is post-translationally controlled. If this is so, truncated NOS may not be subjected to normal cell control mechanisms and it is

this modified version of the AtNOS1 protein that confers the salt tolerant phenotype observed in the JP10 mutant.

4.15 Discussion.

Until recently plant biotechnology has taken a 'hypothesis-led' approach to generate plants that are more tolerant to salt. This approach has been disappointingly unsuccessful, with the notable exception of *AtNHX1*, a vacuolar Na^+/H^+ antiporter which when over–expressed in *Arabidopsis, B. napus* and tomato increased salt tolerance (Apse *et al.* 1999; Zhang & Blumwald 2001;Section 1.4.3.4). Activation tagging presents a 'hypothesis-less' approach to identify gene products that are involved in salt tolerance mechanisms in plants. With activation tagging a T-DNA vector containing 4 copies of the CaMV 35S promoter enhancer, is randomly inserted into the plant genome. In each line the enhancers can mediate transcriptional activation of nearby genes, which may lead to an observable phenotype. Screening large collections of activation-tagged lines is a powerful way of surveying the genome and isolating genes that affect traits of interest, in this case salt tolerance.

Seeds were surfaced sterilised, chilled at 4°C for 2 days, then germinated on agar plates with MS media modified to containing 530μM $Ca^{2+}$and 200μM K^+ and supplemented with 0.75% sucrose and 80mM NaCl (Table 2.1). Stratification of seeds at 4°C for 2 days encouraged synchronised germination of seeds, increasing time for stratification decreased the total germination percentage. Despite stratification, and in the absence of sucrose, plants of varying sizes were observed within wild type populations possibly due to natural variation of seed quality, depending on when/how the seeds were harvested. The addition of sucrose to the screening media was found to minimise this rate of natural variation.

Many studies on salt tolerance use full strength MS media that contain very high levels of K^+ (20mM) and Ca^{2+} (3mM), levels that would not be found in nature. The concentrations of these ions used in this study were more typical of agricultural soil. It is well established that high levels of K^+ and Ca^{2+} ameliorate the toxic effects of Na^+, as the concentration of these ions is increased the 'window' of NaCl a plant can tolerate is widened. At the concentrations of K^+ and Ca^{2+} used in this study, 80mM NaCl was found to the lowest level toxic to Col 7 wild type plants (background of the Weigel lines). Under these conditions approximately 50% of wild type seeds failed to germinate compared with 85% germination under control conditions. The majority of wild type plants that did germinate produced 2 cotyledons, white in colour, before growth arrested. Approximately 1 in 100 wild type plants continued to grow, albeit slowly. Often, surviving plants were pale yellow/green in colour. When screened under these conditions putative salt tolerant mutants were identified as surviving better than wild type. These plants showed greater shoot growth and were greener in colour. The Weigel collection of *Arabidopsis* activation tagged lines, containing 23 000 independent lines, was screened for plants displaying a salt tolerant phenotype. Six hundred and thirty one putative salt tolerant mutants were isolated from primary screening. Seeds harvested from each of these lines were screened again under identical conditions (secondary screening) to confirm the salt tolerant phenotype. Of the 631 putative salt tolerant mutants 50 lines were confirmed to have a strong salt tolerant phenotype, 36 weak, 109 false positive, with the remainder to be investigated (Table 4.1). A large number of the mutants isolated from primary screening were falsely identified as salt tolerant. This is possibly due to small variations in microenvironment e.g. a small pocket of condensation providing an alternative source of water. Every effort was made to create a uniform environment on all Petri dishes.

A strategy was devised to characterise mutants confirmed by secondary screening to display a salt tolerant phenotype. A high-throughput TAIL-PCR protocol was developed to identify the plant genomic sequence flanking the pSKI015 vector. Of the mutants analysed, TAIL-PCR products was generated from 19 lines. Sequence analysis of the PCR products showed 9 of these to contain only vector sequence. These sequences were generated from concatameric inserts, and the junction between the ends of the tandem repeats was amplified. TAIL-PCR was always performed using primers generated to the left border, as the right border is unstable with sequence lost upon integration into the plant genome. To identify the location of the pSKI015 vector within these mutants, and for those mutants where TAIL-PCR generated no product using primers designed to the right border of the insert might be more successful. Alternatively, plasmid rescue could be an option for locating the insert. The pSKI015 vector contains pUC19 sequences with a bacterial origin of replication and an ampicillin resistance gene for plasmid rescue in bacteria. The plant gDNA is digested with restriction enzymes, the fragments ligated, then transformed into *E.coli.* Colonies containing pSKI015 vector, and hopefully the adjacent fragment of plant gDNA, can be selected on media containing ampicillin. The plant gDNA can be sequenced from the left or right borders and BLAST2 interrogation of the full *Arabidopsis* genome database will reveal the location of the pSKI015 vector within the plant genome.

Southern blot analysis was performed on each authentic mutant to determine the number of independent pSKI015 vector inserts within each line. A selection of restriction enzymes was chosen to allow the distinction between multiple independent insertions of the T-DNA vector at different locations, and a single concatamer insertion. In this study Southern blot analysis was successfully performed on 12 independent lines, 7 of these were shown to contain a single concatermic insertion while the

remaining 5 contained one single pSKI015 insertion. No Weigel line investigated in this study showed the presence of multiple insertions of the pSKI015 vector.

To investigate linkage between the salt tolerant phenotype and the pSKI015 vector, and to determine the nature of the lesion (dominant or recessive, activation or knockout), salt tolerant mutants (male parent) were crossed with homozygous *gl1* mutants (female parent). The *gl1* mutation is recessive and the phenotype of the F1 progeny of a successful cross will have trichomes and can be easily identified. Several attempts at these crosses were performed but were largely unsuccessful.

Reverse transcription- PCR was used to investigate transcript levels of genes located near the activation tag. RNA was extracted from plants grown on soil, or on agar media (MS modified to contain 530μM Ca^{2+} and 200μM K^{+}, with 0.75% sucrose) with/without 80mM NaCl. Weigel *et al.* (2000) described 14 activation tagged mutants in which an over expressed gene was always detected immediately adjacent to the multimerized CaMV 35S enhancers on the right border, in none of the examples was the distal gene over expressed as well. Nevertheless, in this study the transcript level of genes each side of the pSKI015 insertion point were investigated. With 2 mutants (JP1 and JP2) genes on both side of the pSKI015 vector showed an altered pattern of expression compared with wild type. With JP4 gene neither side of the insert showed altered expression compared with wild type, although RNA was extracted from soil grown plants that were not salt treated. As the enhancers are thought to function by up regulating the endogenous pattern of gene expression it can be very difficult to detect where and when the up regulation occurs. For example activated genes may be expressed only in root tips, but by extracting RNA from total roots, the increased signal is diluted by message from the bulk root tissue. Alternatively the signal could occur during a particular stage of development, e.g. germination. Therefore investigating gene

transcript levels in 2-week-old tissue is futile. In order to confirm which gene was under the influence of the pSKI015 vector, semi quantitative RT-PCR should be performed with RNA extracted from different plant tissues over a wide range of developmental stages to create a spatial and temporal pattern of expression for each gene.

Alternatively, reporter lines where the gene of interest's promoter drives the expression of luciferase (*LUC*) or *GFP* reporter gene could be used to determine patterns of gene expression. These lines could also be used to characterise which cation (K^+, Ca^{2+} and/or Na^+), or other environment factors (e.g. pH, cold/heat shock), elicit gene activation.

This chapter describes the partial characterisation of 9 salt tolerant activation –tagged *Arabidopsis* mutants summarised in Table 4.2. Although only partial characterised, it is tempting to hypothesis mechanisms of salt tolerance from the information available.

No mutants were identified whose gene product is directly involved in ion transport e.g. a membrane channel or transporter. Regulation of such a channel, however, is a complex process. Upon exposure to salinity, a plant must first 'sense' either the osmotic or ionic stress associated with high NaCl conditions. Once the stress has been detected, signal transduction pathways are initiated that ultimately target proteins which function in salt tolerance (Fig 1.2). With 3 mutants, possible activation of a kinase gene (JP8), NO synthase (JP10) and a WD-40 domain (JP4) containing protein could be involved such a signalling pathways. With mutants JP2, 6 and 7 over expression of RNA processing/stabilising proteins may be involved in post transcriptional regulation of genes involved in salt tolerance, perhaps controlling expression of a membrane channel or a component of a signal transduction pathway.

This chapter presents an exciting collection of salt tolerant mutants but further characterisation is required before definitive conclusions can be drawn.

Possible Role in Salt Tolerance	Mutant
Signalling	
Membrane serine theronine kinase	JP8
NO signalling	JP10
WD-40 domain G-protein	JP4
Cell Reprogramming	
SUMOylation	JP7
Autophagy	JP8
GCN2 kinase	JP9
RNA processing/stabilising	
RNA processing	JP2
RNA binding (PPR)	JP6
RNA binding (RRM)	JP7

Table 4. 2 Summary of Activation Tagged Salt Tolerant Mutants

Chapter 5: Characterisation of The Activation Tagged Salt Tolerant Mutant JP5

5.1 Introduction.

Arabidopsis activation tagged lines were used in large-scale gain-of-function screens to identify single sequences that allow seedlings to survive better under high NaCl, low Ca^{2+} and K^{+} conditions. Activation tagging uses T-DNA containing multimerized cauliflower mosaic virus (CaMV) 35S enhancers (Hayashi *et al.* 1992). The T-DNA inserts randomly within the plant genome and because the enhancers can function in either orientation and at a considerable distance from the coding regions, they can cause transcriptional activation of nearby genes resulting in dominant gain-of-function mutations. Such gene activations may produce novel phenotypes that identify important genes that are either redundant members of a gene family, or are essential for survival; loss-of-function screens would not discover these genes.

Weigel's *Arabidopsis* activation tagged lines were screened for salt tolerant mutants (see Chapter 4). Seven putative salt tolerant mutants were isolated from pool N23153 N23858. In this chapter a series of experiments characterising one of these mutants, JP5, is presented.

5.2 Isolation of Salt Tolerant Activation Tagged *Arabidopsis* Mutants from Pool N23153 N23858.

The isolation of salt tolerant activation tagged mutants is described in detail in section 4.4. Seven putative salt tolerant mutants were isolated by primary screening of pool N23858 Weigel set N23153 (Fig 5.1). Secondary screening confirmed 6 of these to have a strong phenotype; one was a false positive. One mutant, JP5, demonstrated a strong salt tolerant phenotype when compared with wild type (Fig 5.2 a). Under control

conditions (identical media but without NaCl) there was no difference in growth between wild type plants and this mutant (Fig 5.2 c). In the presence of NaCl, however, JP5 plants have shorter roots than wild type (Fig 5.2 b) with an increased number of root hairs compared with wild type (Fig 5.3).

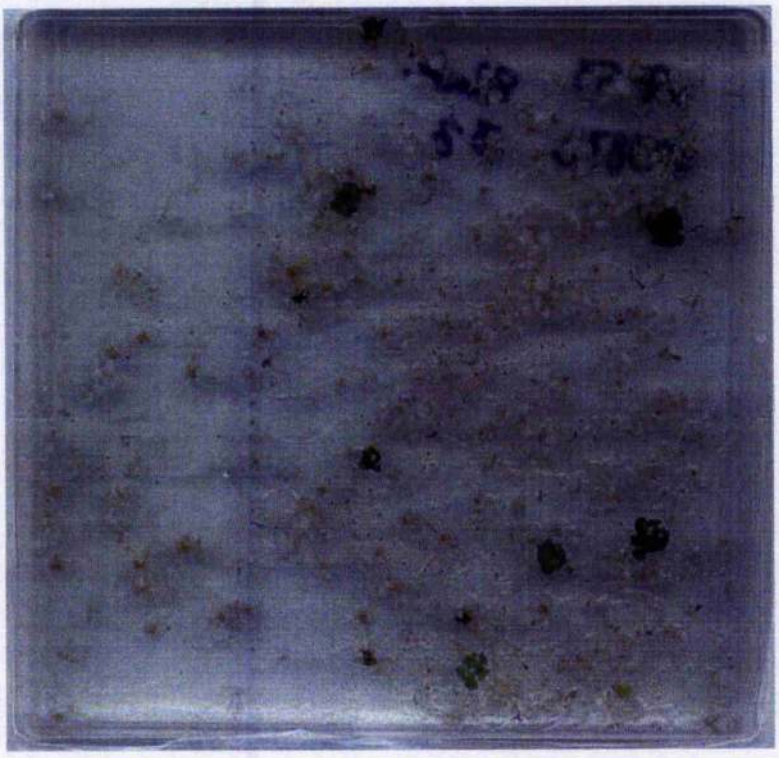


Figure 5.1 Primary screening of *Arabidopsis* Activation Tagged Lines for Salt Tolerant Mutants.

Weigel set N23153 pool N23858 was screened on MS media modified to contain 530μM Ca^{2+} and 200μM K^{+}, supplement with 0.75% sucrose and 80mM NaCl (Table 2.1). Under these conditions growth of wild type plants is severely impaired with approximately 50% of seeds failing to germinate. The majority of wild type plants that did germinate produced two cotyledons, white in colour, before growth arrested. Putative salt tolerant activation tagged mutants were identified as plants with greater shoot growth compared with wild type. Seven putative salt tolerant mutants were isolated from set N23153 pool N23858. Secondary screening under identical conditions confirmed 6 of these to have a strong phenotype one was a false positive.

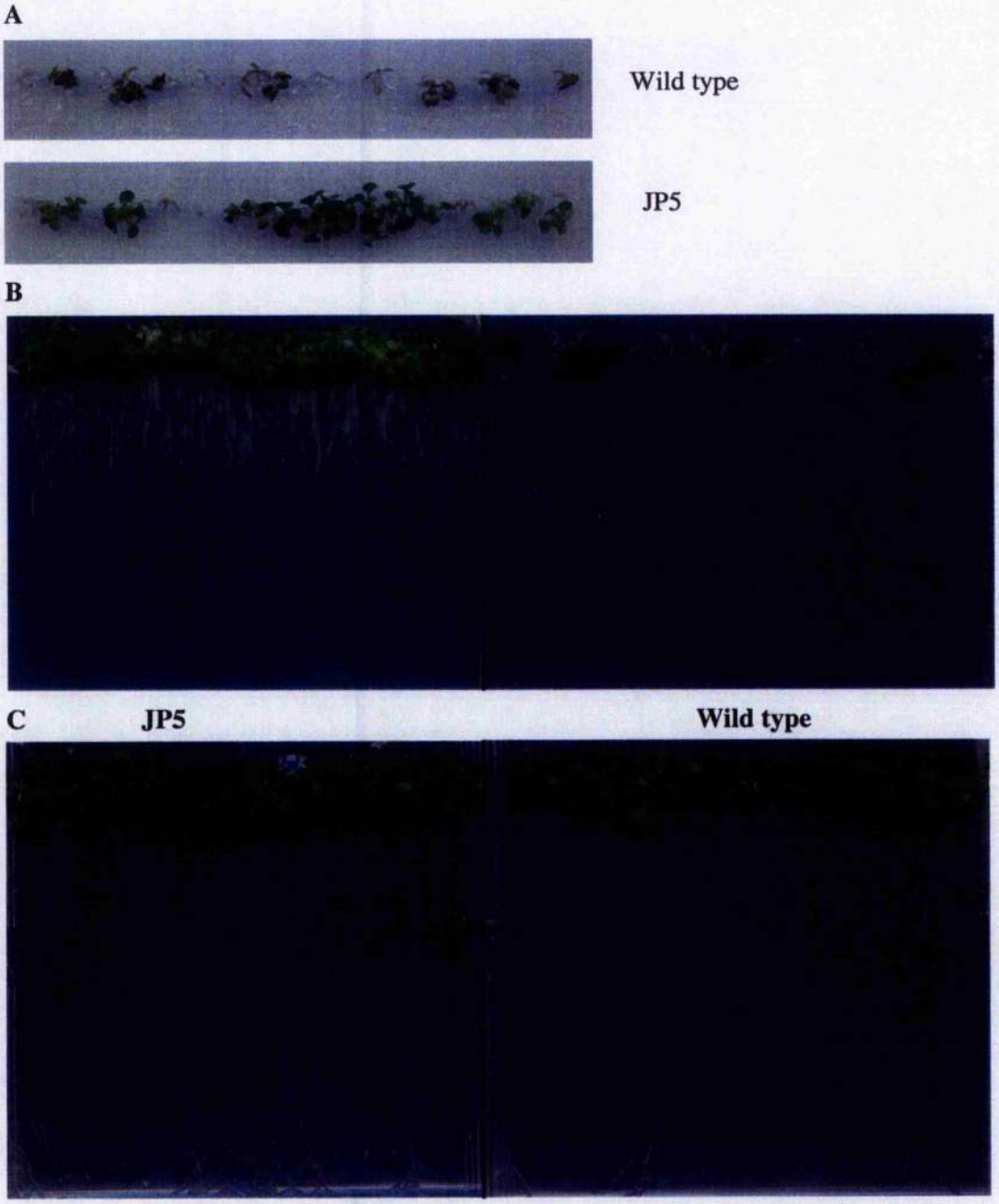
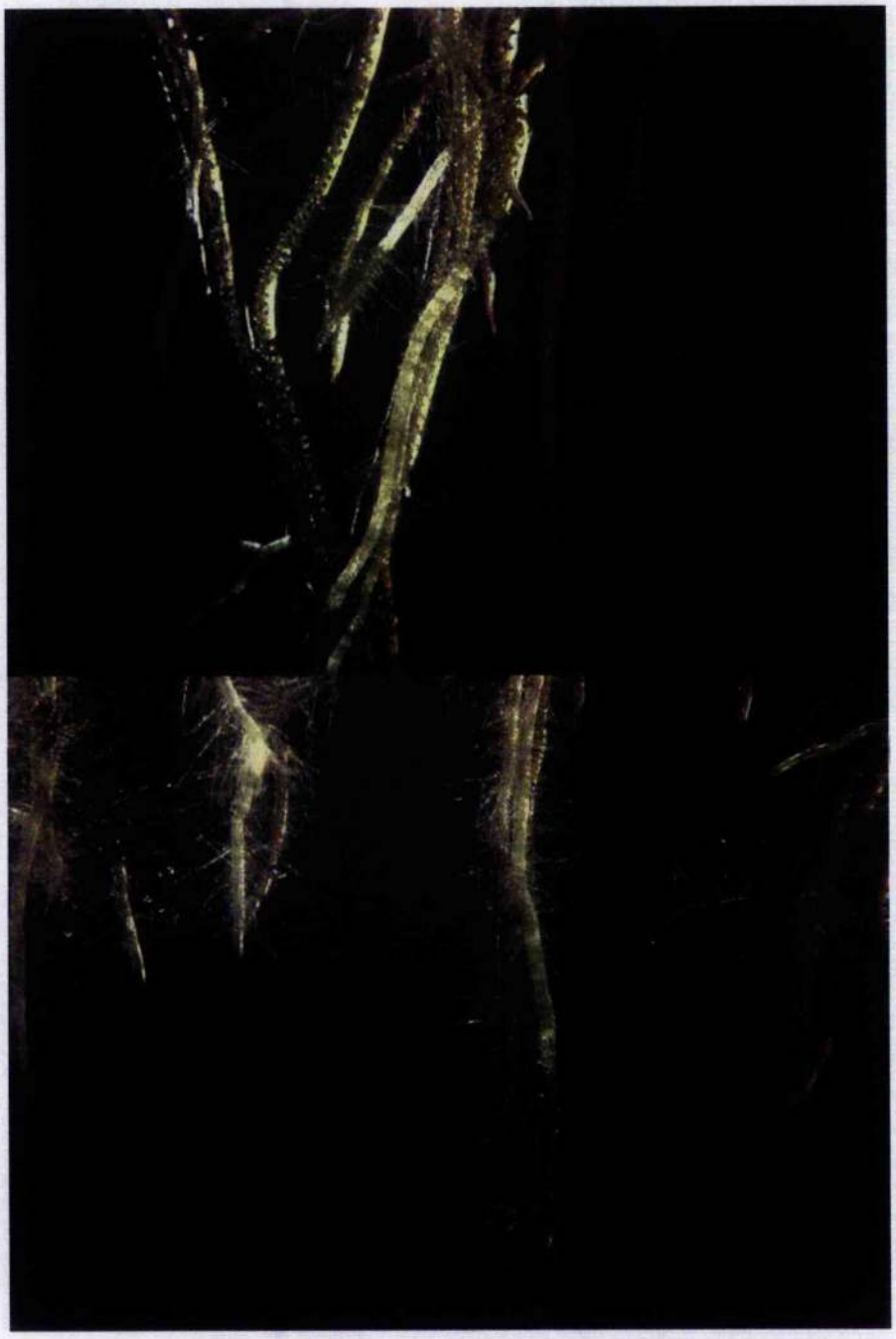


Figure 5.2 Secondary Screening of Salt Tolerant *Arabidopsis* Activation Tagged Mutant JP5.

Secondary screening on media containing 0.75% sucrose, 80mM NaCl and MS modified to contain 530μM Ca^{2+} and 200μM K^{+} (Table 2.1) confirmed JP5 to have a strong salt tolerant phenotype when compared with wild type plants **(A)**. When grown in the presence of 80mM NaCl, JP5 plants have shorter roots **(B)**, with an increased number of roots hairs when compared with wild type (Fig 5.3). In the absence of salt, there is no difference in plant growth between the JP5 mutant and wild type plants **(C)**.

A WT



B JP5

Figure 5.3 Root Morphology of Wild Type and JP5 Plants.

Plants were grown vertically on agar plates containing 0.75% sucrose, 80mM NaCl and MS media modified to contain 530μM Ca^{2+} and 200μM K^{+} (Table 2.1). Under these conditions JP5 roots are shorter than wild type (Fig 5.2). When examined under a low power microscope JP5 roots **(B)** have an increased number of root hairs when compared with wild type **(A)**. When grown in the absence of NaCl there is no difference between wild type and mutant JP5 root growth (Fig 5.2 C).

5.3 Location of Activation Tag within the Genome of JP5.

TAIL-PCR was used to identify plant genomic sequence flanking the inserted T-DNA activation tag (for details on methodology see Section 2.2.16). The product generated by tertiary TAIL PCR (Fig 5.4) was cloned into pTOPO (Invitrogen Ltd, Paisley, UK) and then sequenced (Section 2.2.12). BLAST2 interrogation of the full *Arabidopsis* genome database using the sequence of the cloned TAIL-PCR fragment as probe, revealed vector DNA adjacent to plant genomic DNA (Fig 5.5 a) indicating a successful TAIL-PCR reaction. Comparison of the cloned plant sequence with the *Arabidopsis* genome database indicated that the pSKI015 vector had inserted between genes At5g11040 and At5g11050, with the CaMV 35S promoter enhancers adjacent to At5g11050 (Fig 5.5 b). At5g11040 is an expressed protein of unknown function. At5g11050 is a MYB transcription factor *AtMYB64*. MYB transcription factors contain a common DNA-binding domain that consists of one to three imperfect repeats that are denoted R1, R2 and R3. MYB-like proteins containing one repeat are referred to as MYB1R, those with two as MYBR2R3 and those with three as MYB3R. There are nearly 200 members of the MYB family in *Arabidopsis,* 125 of these are R2R3-type (Stracke *et al.* 2001); AtMYB64 falls into the R2R3 group.

The distance between the insertion point of the pSKI015 vector and *AtMYB64* is 1370 bp (Fig 5.5b). The *AtMYB64* gene is 1743 bp long, contains 3 exons, 2 introns (Fig 5.5c) and codes for a protein consisting of 423 amino acids. One of the introns found within *AtMYB64* occurs at a highly conserved site that intervenes within the coding sequence for one of the MYB repeats, a feature typical of most *Arabidopsis* R2-R3 MYB genes (Penfield *et al.* 2001).

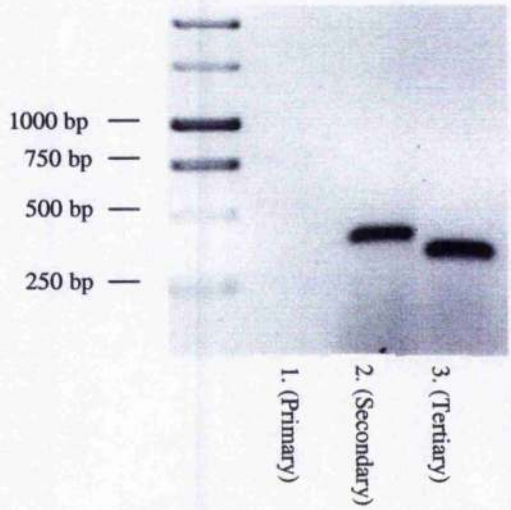


Figure 5.4 TAIL-PCR Amplification of pSKI015 vector and Adjoining Genomic Plant DNA from JP5.

TAIL-PCR (Section 2.2.16) is a 3 step PCR reaction used for isolating the plant genomic sequence flanking the T-DNA activation tag. TAIL-PCR utilizes 3 nested specific primers that bind to the left border of the pSKI015 vector and a series of shorter, arbitrary primers that may hybridise to the plant DNA. The right border of pSKI015 is unstable and a region (up to 90 bp from the border) can be lost upon insertion into the plant genome, making TAIL-PCR from this border difficult. For this reason TAIL-PCR was always performed using primers which hybridise to the left border side of the vector.

Primary TAIL-PCR utilizes the outermost specific primer and 20ng genomic DNA was used as template. The products of primary TAIL PCR are rarely visible by gel electrophoresis (lane 1). The primary TAIL-PCR reaction products were diluted 200 fold and 4µl aliquots directly added to secondary TAIL-PCR reactions as template. After secondary amplification the TAIL-PCR reaction products (lane 2) were diluted 100 fold and 5µl aliquots added directly to tertiary TAIL-PCR reactions (lane 3) as template. A 70 bp reduction in product size was used to confirm that an authentic product had been amplified.

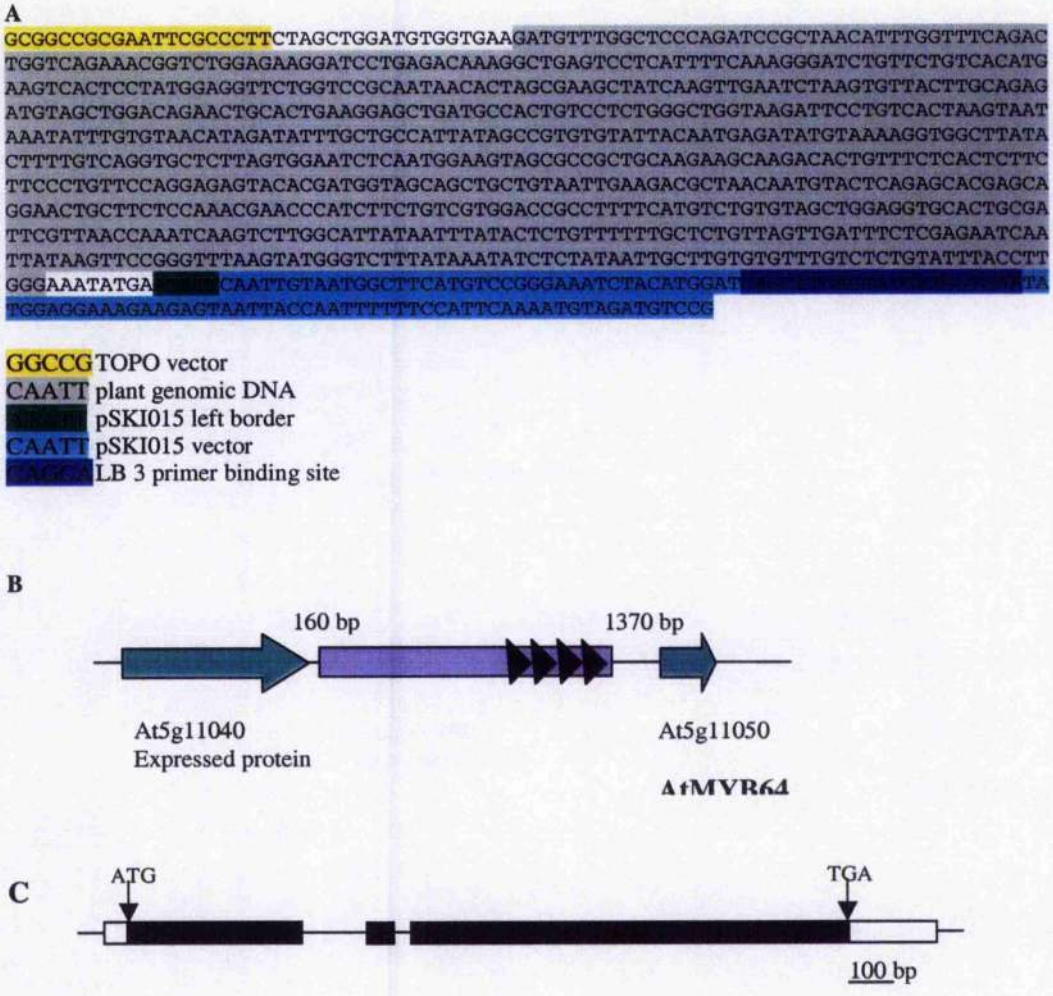


Figure 5.5 Identification of the Activation Tag Insertion Site within JP5.

BLAST analysis of tertiary TAIL-PCR product showed the presence of pSKI015 vector DNA (left border) adjacent to plant DNA (**A**). The tag inserted with left border adjacent to At5g11040, the right border and enhancer elements adjacent to At5g11050 (**B**). At5g11040 is an expressed protein of unknown function. At5g11050 is a putative MYB transcription factor (MYB64). The *AtMYB64* gene is 1743 bp long and contains 3 exons (**C**) AtMYB64 gene structure; open box indicates 5' or 3' UTR; closed box indicates exons.

5.4 The Transcription Factor, *AtMYB64*, is Over-Expressed in the JP5 Mutant.

To examine the effect of the activation tag on the expression levels of nearby genes, semi-quantitative RT-PCR (Section 2.2.17) was performed using primers specific to At5g11040 and At5g11050 (Table 2.4). Transcript levels of both genes were determined in shoots of two-week-old wild type and JP5 plants grown on modified MS (containing 530μM Ca^{2+} and 200μM K^{+}) supplemented with 0.75% sucrose and 80mM NaCl (Table 2.1). The abundance of At1g11040 transcript was estimated to be 1.2 fold higher in wild type when compared with JP5 plants. Interestingly, transcript levels of At1g11050 (*AtMYB64*) were estimated to be 7 fold higher in mutants compared with wild type (Fig 5.6). This pattern of gene activation by the pSKI015 insert is consistent with observations described in the literature (Weigel *et al.* 2000).

5.5 The *Arabidopsis* Salt Tolerant Mutant, JP5, Contains One pSKI015 Insertion.

Genomic Southern blot hybridisation (Section 2.2.15) was performed to determine the number of pSKI015 vector insertions with the mutant genome (Fig 5.7). Genomic DNA isolated from JP5 plants was digested with 4 restriction enzymes, *Pst*1, *Bam*H1, *Nde*1 and *Nco*1. *Nde*1 and *Nco*1 are 'hex cutters' cleaving genomic DNA on average every 4096 base pairs. These restriction enzymes do not cut within the pSKI015 vector, therefore, the number of bands with each digest indicate the number of pSKI015 insertions within the genome. Restriction enzymes *Pst*1 and *Bam*H1 cut once within the pSKI015 vector, outside the region of probe hybridisation; the presence of multiple bands on genomic Southern blots may indicate multiple insertions, or the presence of concatamers within the plant genome. Southern analysis of JP5 genomic DNA digested

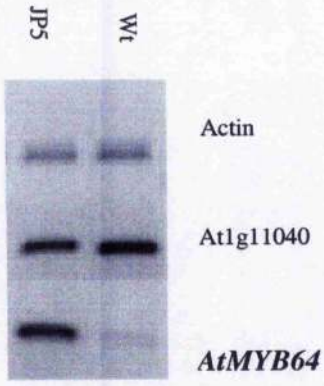


Figure 5.6 Transcript Levels of *AtMYB64* are Higher in the JP5 Mutant than in Wild Type Plants when Grown in the Presence of NaCl.

Semi-quantitative RT-PCR (section 2.2.17) was performed using RNA isolated from 2-week-old shoot tissue treated with modified MS (containing 530μM Ca^{2+} and 200μM K^{+}) supplemented with 0.75% sucrose and 80mM NaCl (Table 2.1).

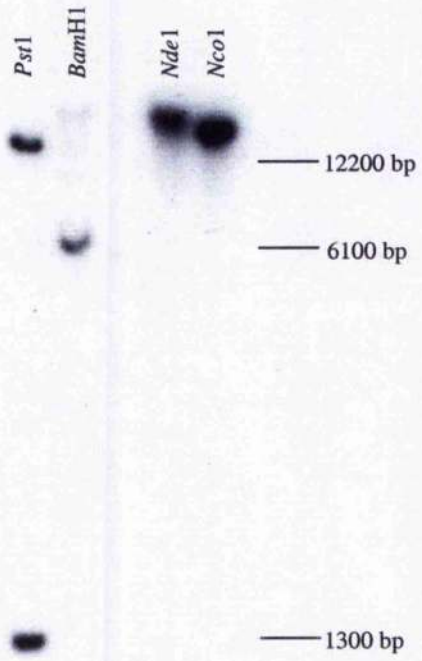


Figure 5.7 Southern Analysis of *Arabidopsis* mutant JP5 indicates the presence of a single concatamer insertion of the pSKI015 vector.

Twenty µg gDNA isolated from JP5 plants was digested with *Pst*1, *Bam*H1, *Nde*1 or *Nco*1. The digested DNA was loaded onto a 0.7% agarose gel and run overnight at 50 volts. Capillary blotting was used to transfer the DNA onto a nylon membrane. The membrane was then probed with a ^{32}P-labelled PCR product amplified over the BAR gene of pSKI015. The membrane was washed at high stringency and autoradiographed for 5 days at $^{-}80^{o}$C (for full details see section 2.2.15).

*Nde*1 and *Nco*1 do not cut within the pSKI015 vector therefore, the number of bands with each digest indicates the number of pSKI015 insertions within the genome. *Pst*1 and *Bam*H1 cut within pSKI015 once, multiple bands indicated multiple insertions, or the presence of concatamers within the plant genome. The banding pattern on this membrane suggests JP5 has a single insertion of a concatamer.

with *Nde*1 and *Nco*1 showed one band; with *Pst*1 and *Bam*H1 two bands are observed. Taken together, these results suggest JP5 has one tag insertion that is a concatamer.

5.6 Over-Expression of *AtMYB64* in *Arabidopsis* Wild Type Plants Confers Salt Tolerance.

To confirm the observed salt tolerant phenotype of JP5 resulted from activation of *AtMYB64*, attempts were made to reproduce the phenotype by generating transgenic *Arabidopsis* lines that over express *AtMYB64*. *AtMYB64* was amplified from JP5 gDNA using *ttgttcgtagatattgatct* and *aacctttcgcaaacgtgcat* as forward and reverse primers, respectively. The resulting PCR fragment was first cloned into pTOPO (Invitrogen Ltd, Paisley, UK) to yield pMYB64-TOPO. The *EcoR*I fragment of pMYB64-TOPO containing the *AtMYB64* gene and the *EcoR*I sites of pTOPO was introduced into the *EcoR*I site of the pMN19 vector, yielding pMYB64-MN19 (Fig 5.8). This vector was designed specifically for recapitulation of the phenotype observed in activation tagged mutants (Weigel *et al.* 2000). The plasmid pMN19 contains multiple cloning sites adjacent to the same tetramerized CaMV 35S enhancers as in the activation-tagging vector pSKI015, to facilitate insertion of the plant gene of interest. *Arabidopsis* wild type plants were transformed with *Agrobacterium tumefaciens* (stain GV3101) harbouring plasmid pMYB64-MN19 (and empty pMN19 as control). T1 plants demonstrating kanamycin resistance were considered successful transformants. To test the salt tolerance of the transgenic plants, T_2 seedlings (10-15 days old) of similar size were transferred to media containing ½ strength MS containing various concentrations of NaCl. The plants transformed with pMYB64-MN19 grew better on ½MS+NaCl than the pMN19 plants (Fig 5.9 a & b). RT-PCR was used to investigate levels of *AtMYB64* expression in the transgenic lines (Fig 5.9 c). RNA was extracted from 8 lines that demonstrated both kanamycin and salt tolerance. *AtMYB64* expression increased in 7 of

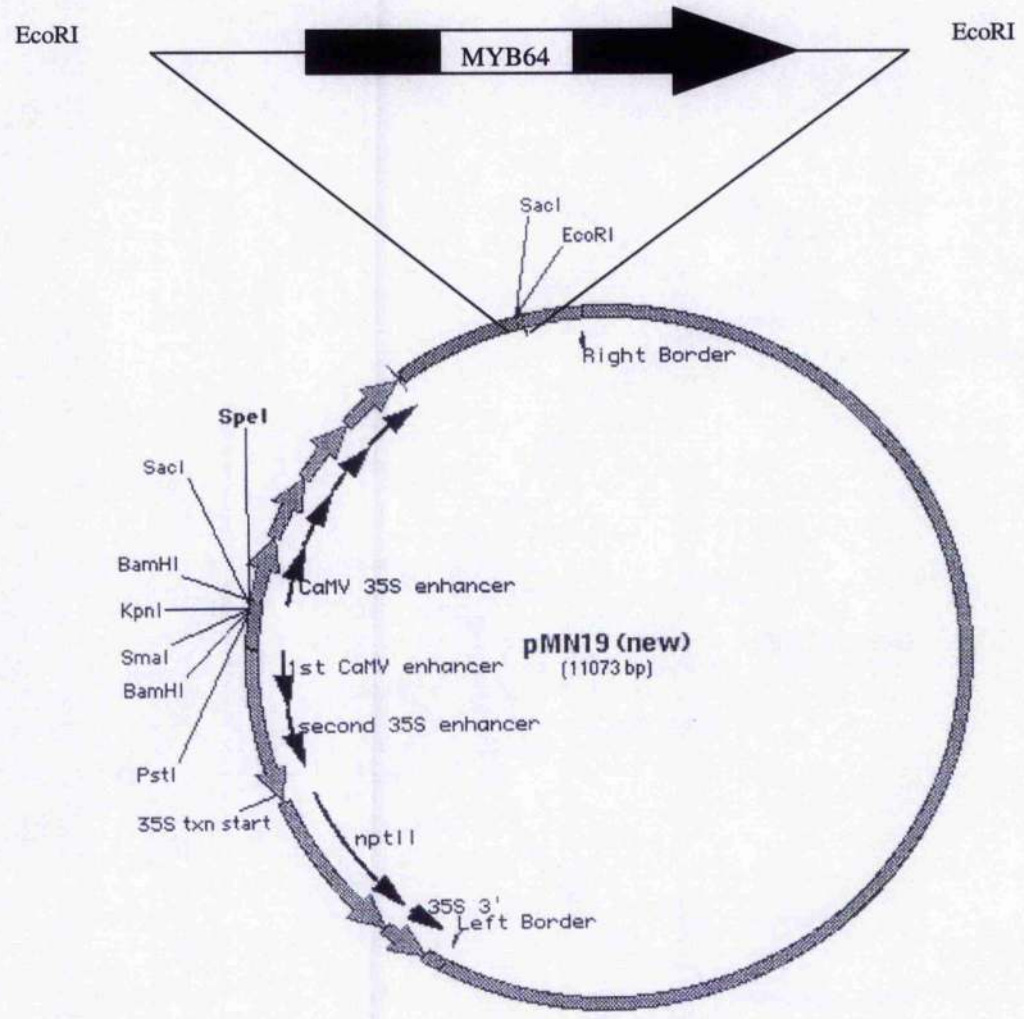


Figure 5.8 The Vector pMYB64-MN19 used for Recapitulation of Salt Tolerant Phenotype in Wild Type Plants.

AtMYB64 was amplified from gDNA and cloned into pTOPO to yield pMYB64-TOPO. The *EcoR*I fragment of pMYB64-TOPO containing the MYB64 gene and the *EcoR*I sites of pTOPO was introduced into the *EcoR*I site of pMN19 to yield pMYB64-pMN19. The plasmid pMN19 contains a kanamycin resistance gene (for selection of successful transformants) and four copies of the CaMV 35S enhancer element, which drives the over expression of the *AtMYB64* gene. *Arabidopsis* wild type plants were transformed with *Agrobacterium tumefaciens* (stain GV3101) harbouring plasmids pMYB64-MN19 and empty pMN19 as control.

Figure 5.9 Wild Type *Arabidopsis* Plants Transformed with pMYB64-MN19 have Increased Levels of *AtMYB64* Expression and are More Salt Tolerant.

Wild type *Arabidopsis* plants were transformed with pMYB64-MN19 and pMN19. Successful transformants (kanamycin resistant) were selected from the T1 generation. T2 transgenic plants were germinated on MSMO/sucrose + 50 μg/ml kanamycin. Seedlings (10-15 days old) with similar growth rates were transferred to media containing ½ strength MSMO and various concentrations of NaCl (5-6 seedlings/jar). After six weeks plants transformed with pMYB64-MN19 survived better on ½ strength MSMO medium containing 160 mM NaCl **(B).** When grown in the absence of NaCl the growth rate of MN19 plants was increased compared to that of pMYB64-pMN19 plants **(A)**. RT-PCR was used to investigate levels of *AtMYB64* expression in the transgenic lines **(C)**. RNA was extracted from 8 lines demonstrating both kanamycin and salt tolerance. *AtMYB64* expression increased in 7 of these 8 lines when compared with wild type.

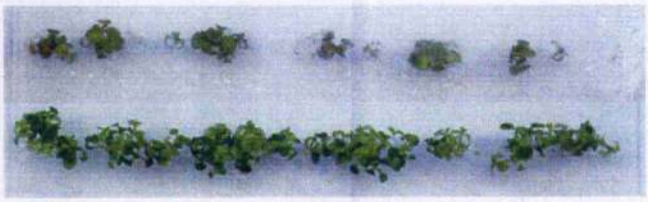
these 8 lines when compared with wild type. This work was performed in collaboration with Dr Ileana Farcasanu.

5.7 The *AtMYB64* Knockout Line is Salt Sensitive Compared with Wild Type

A SALK knockout line (Alonso *et al.* 2003), N595057, was obtained from the Nottingham Arabidopsis Stock Centre (NASC). This line contained a T-DNA insert within the first exon of *AtMYB64*. A homozygous line was identified by PCR (section 2.2.19). When screened on media containing 0.75% sucrose, 75mM NaCl and MS modified to contain 530μM Ca^{2+} and 200μM K^{+} (Table 2.1), N595057 plants were salt sensitive compared with wild type (Fig 5.10 a). When subjected to these conditions 50% of N595057 seeds failed to germinate (compared with 80% under control conditions), the growth rate of germinated plants was greatly reduced with surviving plants paler compared with wild type. These conditions (75mM NaCl) are not lethal to wild type plants, however growth rate is reduced and plants are paler in colour when compared with those grown under control conditions (Fig 5.10 b left panel). Under control conditions there is no difference in the growth or germination rate, between N595057 and wild type plants, suggesting *AtMYB64* is not essential for normal growth of *Arabidopsis* (Fig 5.10 b).

The N595057 knockout line must however, be characterised before definitive conclusions can be drawn. RT-PCR would confirm that the T-DNA insertion does interfere with transcription of *AtMYB64*. The number of T-DNA inserts within the genome should be confirmed by Southern blot hybridisation. Homozygous lines should be crossed with wild type and segregation analysis performed to investigate linkage between the salt sensitive phenotype and the T-DNA vector.

A 75mM NaCl



B Control

Figure 5.10 Putative *AtMYB64* Knockout Line has a Salt Sensitive Phenotype.
A SALK knockout line (Alonso *et al.* 2003), N595057, which contains a T-DNA insert within the first exon of *AtMYB64*, was obtained from NASC. A homozygous line was identified by PCR. When screened on media containing 0.75% sucrose, 75mM NaCl and MS modified to contain 530μM Ca^{2+} and 200μM K^{+} (Table 2.1), N595025 appears to be more salt sensitive than wild type plants (**A**). When subjected to these conditions 50% of N595057 seeds failed to germinate (compared with 80% germination under control conditions). N595057 plants have a reduced growth rate and are paler in appearance compared with wild type. These conditions (75mM NaCl) are not lethal to wild type plants however, growth rate is reduced and plants are paler in colour when compared with those grown under control conditions (**B** left). Under control conditions there is no difference between N595057 and wild type plants, suggesting *AtMYB64* is not essential for normal growth of *Arabidopsis* (**B**).

5.8 Micro Array Assessment of JP5 and Wild Type Transcriptional Responses to NaCl.

The Arizona full genome microarrays (obtained from D. Galbraith, University of Arizona, http://www.ag.arizona.edu/microarray/) were used to assess the transcriptional responses of the JP5 mutant and wild type plants to NaCl. Plants were germinated on vertical agar plates containing 0.75% sucrose and MS media (modified to contain 530μM Ca^{2+} and 200μM K^{+} Table 2.1) with either no additional NaCl (control) or supplemented with 80mM NaCl (high salt – long term treatment). For short-term salt treatment plants were grown under control conditions for 2 weeks before exposure to 80mM NaCl for 4 hours (Fig 5.11 a).

RNA extraction and microarray preparation, hybridisation and data analysis was performed in collaboration with Drs Patrick Armengaud and Anna Amtmann (University of Glasgow; see Section 2.2.20). Genes were sorted to produce a ranked list based on their fold-change (FC; JP5 compared with wild type) i.e. the gene with the highest FC is assigned rank 1 etc.

With all salt treatments no difference in transcript levels of *AtMYB64* were detected between the JP5 mutant and wild type plants. Differences were detected, however, by RT-PCR performed using the same batch of RNA used for microarray analysis (Fig 5.11 b). *AtMYB64* transcript levels were higher in the JP5 mutant compared with wild type plants for all treatments with the highest abundance detected with the long-term salt treatments. No *AtMYB64* transcript was detected in the wild type plants with short-term salt treatment, and only low levels were measured in the JP5 mutant. For future short-term salt treatment investigation, RT-PCR analysis of *AtMYB64* transcript levels over a time period from application of NaCl would establish a temporal pattern of gene

A

Long term salt stress	Short term salt stress	Control
Control media supplemented with 80mM NaCl (2 biological repeats)	Grown for 2 weeks on control media, 80mM NaCl treatment for 4 hours (1 biological repeat)	No NaCl (1 biological repeat)

B

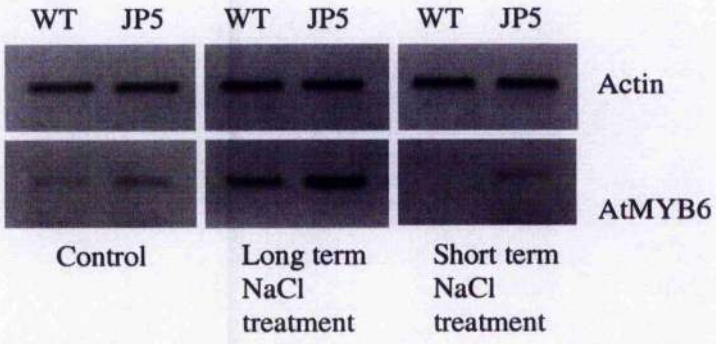


Figure 5.11 Growth Conditions of Plants used for RT-PCR and Micro Array Analysis.

RNA for micro array and RT-PCR analysis was extracted from 2-week-old plants. Plants were germinated on vertical agar plates containing 0.75% sucrose and MS media (modified to contain 530μM Ca^{2+} and 200μM K^{+} Table 2.1) with either no additional NaCl (control) or supplemented with 80mM NaCl (high salt – long term treatment). For short-term salt treatment plants were grown under control conditions for 2 weeks before exposure to 80mM NaCl for 4 hours (**A**).

Micro array analysis detected no change in *AtMYB64* transcript levels. RT-PCR, using the same batch of RNA, detected higher levels of *AtMYB64* transcript levels in JP5 mutants compared with wild type for all treatments (**B**).

expression and from this a more appropriate short-term treatment could be chosen for future micro array analysis.

It is possible that the probes for *AtMYB64* used with these arrays are not specific; there are almost 200 MYB genes in *Arabidopsis* some with high sequence homology to *AtMYB64* (for example *AtMYB119* refer to Fig 5.13). Under all conditions, a statistically significant signal for *AtMYB64* was observed, but there were no differences between any of the samples. This suggests that some other transcript(s) also bind to the '*AtMYB64*-specific' probe on the micro array and swamps the signal that arises from authentic *AtMYB64* cDNA. Unfortunately the specificity of the probes cannot be confirmed, as probe sequence details are not available from the manufacturer. Others using the Arizona chips have encountered similar problems, and it is possible the oligo probes have not been well designed (Dr JJ Milner, University of Glasgow, pers. comm.) Alternatively, the Affymetrix 'GeneChip Arabidopsis ATH1 Genome Array' which contains more than 22,500 probe sets representing approximately 24,000 genes are available. The sequence is available for all probes used in this array (www.affymetrix.com) and BLAST2 interrogation of the full *Arabidopsis* genome database using the sequence provided by the manufacture indicates high specificity of the *AtMYB64* probe.

Not withstanding the problems associated with *AtMYB64*, the Arizona chips did identify a number of transcripts that were differentially abundant. Two biological replicates were used for the long-term salt treatment experiments this data is presented in Tables 5.1 and 5.2. The 4 genes that show the greatest fold change in transcript abundance between the JP5 mutant and wild type are all heat shock proteins (Table 5.1). Dysfunction of enzymes and proteins usually accompanies salt stress; therefore, maintenance of proteins in their functional conformations and preventing aggregation of non-native

AGI	Name	Av. FC	RP UP	FDR (%)
At1g53540	17.6 kDa heat shock protein (AA 1-156)	5.08	12	1.44676E-06
At1g74310	heat shock protein 101 (HSP101)	3.97	36	1.44676E-06
At3g12580	Heat shock protein 70 (HSP70)	4.14	51	1.53718E-06
At5g12030	17.6 kDa heat shock protein (HSP 17.6A)	3.83	66	1.59144E-06
At1g51420	unknown protein (putative sucrose phosphatase)	3.34	172	3.45615E-06
At4g12470	pEARLI 1-like protein	2.78	209	3.59967E-06
At5g48570	peptidylprolyl isomerase	2.43	476	7.17351E-06
At4g12490	pEARLI 1-like protein	2.38	528	7.07305E-06
At1g26680	hypothetical protein	2.31	572	6.89622E-06
At1g61800	glucose-6-phosphate/phosphate-translocator precursor	2.41	611	6.13868E-06
At5g10840	endomembrane protein 70, putative	2.81	728	6.75154E-06
At1g36060	AP2 domain transcription factor RAP2, putative	2.60	980	8.43943E-06
At1g76900	Tub family protein, putative	2.67	1088	8.74486E-06
At3g50970	dehydrin Xero2 (low temp induced protein)	2.20	1104	8.31887E-06
At4g12480	pEARLI 1	2.42	1116	7.91462E-06
At1g56600	water stress-induced protein, putative	2.16	1140	7.63567E-06
At2g05510	putative glycine-rich protein	2.20	1440	9.13743E-06
At5g24110	WRKY family transcription factor (WRKY30)	2.07	1508	9.09047E-06
At3g47420	glycerol-3-phosphate transporter, putative	2.12	1624	9.32356E-06
At3g61830	auxin response factor-like protein	2.22	1785	9.78207E-06
At4g12500	pEARLI 1-like protein	2.02	1848	9.28337E-06
At3g25830	myrcene/ocimene synthase, putative	2.12	2640	1.27315E-05
At1g27730	salt-tolerance zinc finger protein (ZAT10)	2.00	2646	1.22696E-05
At3g16460	putative lectin	2.03	2688	1.20027E-05
At1g59860	17.6 kDa heat shock protein (HSP17.6A-CI)	2.11	2821	1.21467E-05
At3g10020	expressed protein	2.06	2884	1.19898E-05
At5g66690	UTP-glucose glucosyltransferase	2.03	3034	1.2193E-05
At3g09260	glycosyl hydrolase family 1	2.02	3132	1.21808E-05
At5g26280	low similarity to ubiquitin-specific Arabidopsis protease 12	1.95	3151	1.18717E-05
At3g17790	acid phosphatase type 5	2.06	3375	1.23303E-05
At1g07400	17.8 kDa class I heat shock protein (HSP17.8-CI),	2.08	4200	1.48931E-05
At4g22610	putative protein	2.07	4294	1.47914E-05
At1g17710	expressed protein	1.90	4400	1.47355E-05
At1g45210	expressed protein	2.13	4755	1.5494E-05
At5g06320	harpin-induced protein-like	1.85	5109	1.62094E-05
At2g11810	putative monogalactosyldiacylglycerol synthase	1.86	5239	1.61957E-05
At1g01470	late embryogenesis abundant protein, putative	1.85	5400	1.6276E-05
At2g03760	putative steroid sulfotransferase	1.85	5430	1.59673E-05
At1g17330	hypothetical protein	1.89	6440	1.84864E-05
At4g30280	xyloglucan endotransglycosylase, putative	1.95	6650	1.86453E-05
At3g46230	17.4 kDa class I heat shock protein (HSP17.4-CI),	1.83	6674	1.82873E-05
At2g46240	hypothetical protein	1.93	6942	1.85989E-05
At3g25250	kinase	1.83	7138	1.87083E-05
At5g01220	putative protein	1.84	7200	1.84693E-05
At5g52060	putative protein contains BAG domain	1.94	9362	2.35149E-05

Table 5.1 Microarray Analysis of the JP5 Salt Tolerant Mutant.
Plants were grown on agar plants with MS media (modified to contain 530μM Ca^{2+} and 200μM K^{+} Table 2.1) supplemented with 80mM NaCl. Tissue was harvested after 2 weeks growth. Data represents genes up regulated in JP5 mutant compared with wild type plants (average of 2 biological replicates).

AGI	Name	Av. FC	RP DOWN	FDR (%)
At4g25050	acyl carrier - like protein	0.38	15	1.80845E-06
At3g63190	putative chloroplast ribosome recycling factor protein	0.49	147	8.8614E-06
At1g80960	F-box protein-related	0.50	255	7.68591E-06
At3g04400	ribosomal protein L17 like	0.51	297	7.16146E-06
At1g25097	expressed protein	0.52	406	8.15811E-06
At5g14470	putative protein	0.53	621	1.06957E-05
At3g43810	calmodulin 7	0.53	675	1.01725E-05
At5g36790	p-nitrophenylphosphatase-like protein	0.53	704	9.43073E-06
At5g20630	germin-like protein	0.51	704	8.48765E-06
At1g08260	DNA polymerase epsilon catalytic subunit, putative	0.54	1178	1.29112E-05
At4g29350	profilin 2	0.55	1312	1.31816E-05
At1g12900	glyceraldehyde 3-phosphate dehydrogenase like	0.56	1664	1.54321E-05
At1g31190	inositol monophosphatase family protein	0.56	1881	1.61985E-05
At5g12970	anthranilate phosphoribosyltransferase -like protein	0.56	1974	1.58661E-05
At2g22980	putative serine carboxypeptidase 1	0.56	2144	1.61555E-05
At2g29340	putative tropinone reductase	0.57	2220	1.57441E-05
At4g24260	glycosyl hydrolase family 9 (endo-1,4-beta-glucanase)	0.56	2364	1.5834E-05
At5g14910	heavy-metal-associated domain-containing protein	0.57	2775	1.76086E-05
At2g26190	calmodulin-binding family protein	0.58	2952	1.77951E-05
At2g16190	hypothetical protein	0.58	3080	1.76826E-05
At5g58760	transducin family protein / WD-40 repeat family protein	0.58	3120	1.70981E-05
At3g16140	photosystem I subunit VI precursor	0.57	3344	1.75289E-05
At1g74060	putative 60S ribosomal protein L6	0.57	3486	1.75118E-05
At3g55030	phosphatidylglycerophosphate synthase - like protein	0.58	3672	1.77083E-05
At5g02850	hydroxyproline-rich glycoprotein family protein	0.59	4284	1.98651E-05
At5g62490	ABA-responsive protein (HVA22b),	0.59	5060	2.25945E-05
At1g09590	putative 60S ribosomal protein L21	0.58	5180	2.23042E-05
At5g43080	Cyclin A3;1	0.59	6532	2.71558E-05
At1g23060	expressed protein	0.60	6678	2.68374E-05
At5g47190	putative protein	0.59	6864	2.58608E-05
At5g05370	ubiquinone-binding protein (QP-C)-like protein	0.60	7344	2.68308E-05
At2g39100	putative RING zinc finger protein	0.60	7392	2.62119E-05
At5g53200	putative protein	0.60	7420	2.55594E-05
At4g11120	putative translation elongation factor	0.60	8030	2.68923E-05
At4g26360	putative protein	0.61	8275	2.69638E-05
At2g14470	putative helicase	0.60	8378	2.6581E-05
At1g29410	phosphoribosylanthranilate isomerase	0.60	8832	2.73029E-05
At4g37820	putative protein	0.60	9796	2.95259E-05
At5g59300	E2, ubiquitin-conjugating enzyme 7 (UBC7)	0.61	10212	3.00291E-05
At3g25520	ribosomal protein	0.60	10731	3.02894E-05
At5g67620	unknown protein	0.61	10803	3.03326E-05
At2g46560	hypothetical protein	0.61	11070	2.98997E-05
At1g70680	Ca^{2+}-binding EF-hand common family protein	0.61	11160	2.93126E-05
At5g44720	putative protein	0.61	11184	2.88582E-05
At5g37170	O-methyltransferase	0.61	11250	2.96736E-05

Table 5.2 Microarray Analysis of the JP5 Salt Tolerant Mutant.
Plants were grown on agar plants with MS media (modified to contain 530μM Ca^{2+} and 200μM K^{+} Table 2.1) supplemented with 80mM NaCl. Tissue was harvested after 2 weeks growth. Data represents genes down regulated in JP5 mutant compared with wild type plants (average of 2 biological replicates).

proteins is important for cell survival under stress. Heat shock proteins (HSP) have been shown to act as molecular chaperones, which are responsible for synthesis, targeting, maturation, and degradation of proteins in a broad array of normal cellular processes. These HSP can also function by stabilising proteins and membranes under stress conditions. It is well established that plant HSP are expressed in response to a variety of abiotic stresses e.g. temperature, water, salt and oxidative stress (reviewed by Wangxia *et al.* 2003).

Several studies have demonstrated that over expression of a HSP can confer tolerance to abiotic stress. For example, in carrot, over expression of HSP17.7 improves thermotolerance (Malik *et al.* 1999). In *Arabidopsis,* over expression of a chloroplast HSP21 increases resistance to heat stress (Härndahl *et al.* 2005). In tobacco, overexpression of DnaK1, a member of Hsp70 from the halotolerant *Cyanobacterium aphanothece*, improves salt tolerance (Sugino *et al.* 1999). Interestingly, over expression of At5g12030 (*At-HSP17.6A*), the gene to show the fourth highest fold change between JP5 mutants and wild type, has been shown to confer salt and drought tolerance to wild type *Arabidopsis* plants (Sun *et al.* 2001). It is tempting to suggest that over expression of these HSP, perhaps under the control of *AtMYB64*, confers salt tolerance. Before such conclusion can be drawn however, further microarray analysis must be performed and the fold change in transcript levels detected confirmed by RT-PCR or northern blot analysis.

5.9 Discussion

5.9.1 Summary

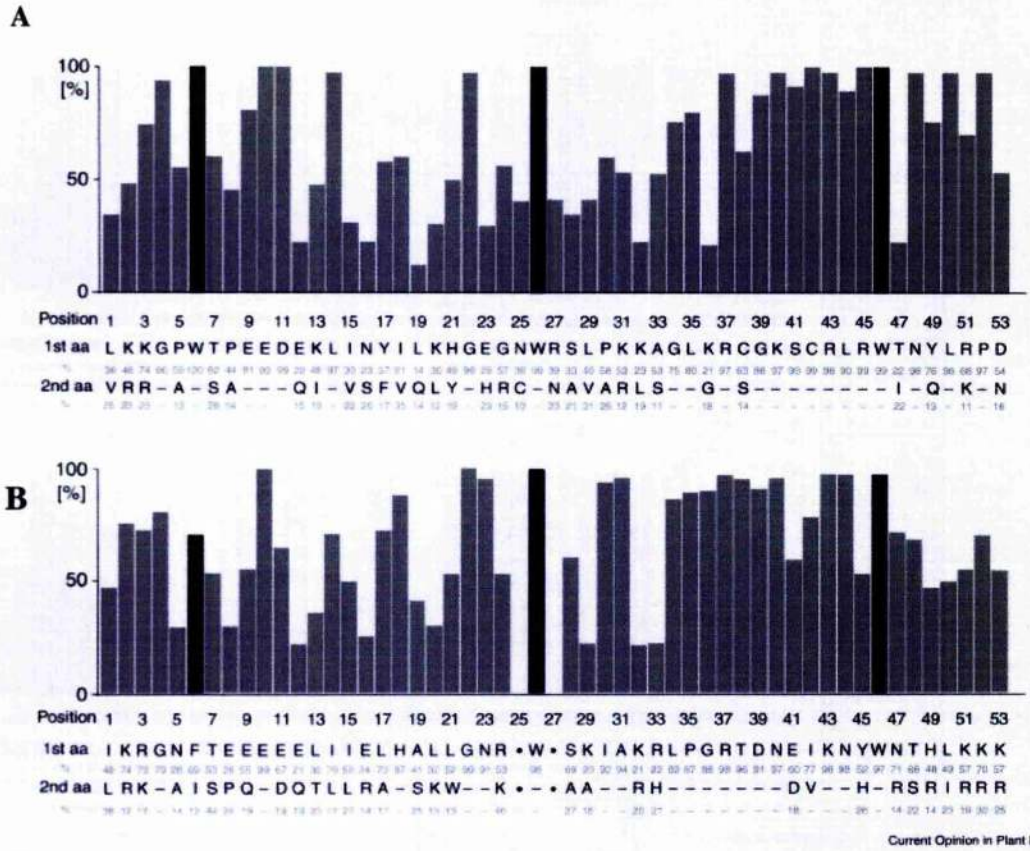
With activation tagging a T-DNA vector, containing four copies of an enhancer element from the promoter of the cauliflower mosaic virus (CaMV) 35S gene, is randomly inserted into the plant's genome through *Agrobacterium* infection. The tetrameric CaMV 35S enhancers can mediate transcriptional activation of nearby genes, which may lead to an observable phenotype. The Weigel collection of *Arabidopsis* activation tagged lines, containing 23000 independent lines, were screened for mutants displaying a salt tolerant phenotype. The mutant JP5 was isolated from pool N23153 N23858. When grown on agar plates containing 0.75% sucrose, 80mM NaCl and MS media modified to contain 530μM Ca^{2+} and 200μM K^{+}; JP5 performed better than wild type. When compared with wild type, this mutant had shorter roots with an increased number of root hairs when grown on media supplemented with NaCl. JP5 contains a single, concatamer insertion located between 2 genes, At5g11040 and At5g11050. The right border and 35S promoter enhancer elements face At5g11050, a MYB transcription factor (*AtMYB64*). When treated with high salt conditions expression of *AtMYB64* was higher in the mutants compared with wild type. Transgenic lines over expressing *AtMYB64* were more salt tolerant than wild type plants and a homozygous line reported to carry a T-DNA insertion in the first intron of At5g11050 (a putative *AtMYB64* knockout) was hypersensitive to salt. Taken together, these results suggest AtMYB64 is a transcription factor that activates salt tolerance mechanisms in *Arabidopsis*.

5.9.2 The MYB Family of Transcription Factors.

Transcription factors are usually defined as proteins that show sequence-specific DNA binding and are capable of activating and/or suppressing gene transcription. These

factors are classified in structural families according to the presence of specific DNA recognition motifs. One of the largest families of transcription factors in *Arabidopsis* is the MYB family. MYB genes encode proteins with DNA-binding domains composed of one, two or three semi-conserved repeats of about fifty-three amino acids each (R1, R2 and R3). This MYB domain is well conserved between MYB proteins of animals, yeast and plants (Rosinski & Atchley 1998). Almost 200 MYB genes have been found in *Arabidopsis,* which can be classified into three subfamilies depending on the number of repeats found in the MYB domain. MYB-like proteins with one repeat are referred to as 'MYB1R' factors, with two as 'R2R3' type factors, and with three repeats 'MYB3R' factors (Rosinski & Atchley 1998). AtMYB64 contains two repeats and so falls into the R2R3-type subfamily, the largest subfamily containing 125 genes in *Arabidopsis* (Stracke *et al.* 2001).

Each repeat within the MYB DNA binding domain encodes three α-helices, with the second and third helices forming a helix-turn-helix (HTH) conformation to intercalate in the major groove of the target DNA with the third α-helix considered to have a recognition role in DNA binding (Ogata *et al.* 1992). Residue changes within this α-helix have been shown to impair DNA-binding activity (Williams & Grotewold 1997). Critical in the formation of the tertiary structure of the MYB motif is a series of three tryptophan residues spaced eighteen or nineteen amino acids apart. These are central to the formation of a hydrophobic core of amino acids that plays a crucial role in sequence-specific DNA binding (Kanei-Ishii *et al.* 1990). The tryptophan residues are generally conserved in all MYB proteins, although in plants the first tryptophan in the R3 repeat is often substituted by another aromatic or hydrophobic amino acid. This is not the case however, with the AtMYB64 protein which contains three tryptophan residues in each repeat (Fig 5.12 b).



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MYB64   MEEQKIQEKSLAHGAAPPLTAVERFLNGQKNEALCFKKQERSIDRPIVKTTRAIEIRNEN 60
R2      ------------------------------------------------------------
R3      ------------------------------------------------------------

MYB64   KENMMFGPRKEKNLAVIGEIVVKGAAKDYTCKDITKKQPYKNIIKGQWTADEDRKLIKLV 120
R2      ----------------------------------------LKKGPWTPEEDEKLINYI
                                                  : ** **.:**.***: :
R3      ------------------------------------------------------------

MYB64   MQHGERKWAVISEKLEG-RAGKQCRERWHNHLRPDIKKDSWSEEEERLLVEAHTRIGNKW 179
R2      LKHGEG-WRSLPKKAGLKRCGKSCRLRWTNYLRPD-----------------------
        ::***   *  : .:*     *.**.** ** *:****
R3      ----------------------------------IKRGNFTEEEEELIIELHALLGNRW
                                           **:..::****.*::* *: :**:*

MYB64   AEIAKLIQGRTENSIKNHWNATKRRQNSKRKHKRSKNADSNSDIDDLSPSAKRPRILEDY 239
R2      ------------------------------------------------------------
R3      SKIAKRLPGRTDNAIKNYWNTHLLKKK--------------------------------
        ::*** : ***:*:***:**:   :::

MYB64   IKNIENNDKNNGENIMTTSGNNVLSTSNYDQFNSEDSTSSLLDDPYDEELVFLKNIFENH 299
R2      ------------------------------------------------------------
R3      ------------------------------------------------------------

MYB64   SLENINLSQGTEITQSSSSGFMIENPKPKPNLYNNTFGTHLGAMVTEPANSSHLASDIYL 359
R2      ------------------------------------------------------------
R3      ------------------------------------------------------------

MYB64   SDLLNGTASSSSSLTFLSSNNNEHAGENELLLPQANSTSERREMDLIEMLSGSTQGSNIW 419
R2      ------------------------------------------------------------
R3      ------------------------------------------------------------

MYB64   FPLF 423
R2      ----
R3      ----
```

Figure 5.12 The MYB DNA Binding Domain of *Arabidopsis* R2R3-MYB Proteins.
Comparison of R2 (**A**) and R3 (**B**) repeats of all 125 R2R3-MYB genes from *Arabidopsis* (taken from Stracke *et al.* 2001). The numbers below the amino acid residues indicate the percentage of times the particular residue appears at the respective positions.
Sequence of the R2 and R3 repeats is highly conserved between *Arabidopsis* MYB proteins. In R2, 25 (of 53) positions are occupied by a single, or two very similar, residues in more than 80% of the proteins. For R3, the equivalent number is 31 (out of 51). AtMYB64 has 57% identity to the consensus sequence generated by Stracke *et al.* 2001 (**C**), with one notable exception. The R3 repeat of AtMYB64 contains 3 tryptophan residues (**C**) commonly found in animal and yeast MYB R3 repeats but is not often present in plant R2R3 MYB proteins (**B**). Black arrows indicate the location of the tryptophan residues.

Stracke and co-workers (2001) compared the amino acid sequence of R2 and R3 repeats from all 125 *Arabidopsis* R2R3 MYB proteins to deduce a consensus sequence and to determine the frequency of the most prevalent amino acids at each position within the repeats (Fig 5.12). With repeat R2, 25 of 53 positions are occupied by a single, or two very similar, residues in more than 80% of the proteins (Fig 5.12 a). For R3, the equivalent number is 31 out of 51 (Fig 5.12 b). AtMYB64 demonstrates a high sequence homology (57%) to the consensus sequence created by Stracke *et al.* (2001), with one notably exception: AtMYB64 contains three tryptophan residues in the R3 repeat, which although common in animal and yeast R3 repeats, are not present in plant R2R3 MYB proteins (Fig 5.12 c). Of the 125 R2R3 MYB proteins investigated, 69% had a phenylalanine replacing the first tryptophan of the R3 repeat (Fig 5.12 b).

The conservation of amino acid sequence within the DNA-binding domain implies a common binding site for MYB proteins but yet MYB transcription factors are involved in a diverse range of cellular processes including:

1. Control of secondary metabolism, particularly in phenylpropanoid metabolism. Over expression of *AtMYB75/PAP1* and AtMYB90/PAP2 results in accumulation of anthocyanins (Borevitz *et al.* 2000) and AtMYB4 represses the synthesis of sinapoyl malate (Jin *et al.* 2000).
2. Control of development and determination of cell fate and identity. *AtMYB0/GL1* is required to initiate the differentiation of trichomes (Oppenheimer *et al.* 1991). AtMYB66/(WER) is involved in root epidermal cell patterning (Lee & Schiefelbein 1999). *LHY* and *CCA1* are MYB-like proteins with a single MYB repeat which regulate circadian rhythms in *Arabidopsis* (Carre & Kim 2002).

3. Mediating signal transduction pathways, such as response to abiotic stress and pathogen attack. *AtMYB2* is induced by drought and salt stress, and also with the onset of treatment with abscisic acid (Urao *et al.* 1996). More recently, AtMYB2 has been shown to bind to and thus regulate expression of a calmodulin isoform (GmCaM4) protein (Yoo *et al.* 2005). Over expression of GmCaM4 was shown to enhance salt tolerance in *Arabidopsis*. AtMYB2 has also been shown to regulate *AtADH1* (alcohol dehydrogenase1) and is thought to be involved in the response to low oxygen (Hoeren *et al.* 1998).

Interestingly, the MYB R2R3 transcription factors described to date are involved predominately in controlling 'plant specific processes'. This may explain why plants have such a large family of MYB proteins compared with animals or yeast (Jin & Martin 1999).

The conservation of amino acid sequence within the DNA-binding domain implies a common binding site for MYB proteins. The MYB domain binds to a specific DNA sequence C/TAACG/TG for most organisms, C/TAACNA/G for *Arabidopsis* (Romero *et al.* 1998). While the MYB domain is highly conserved, the coding sequences downstream of the R2R3 domains are often very divergent (Rosinski & Atchley 1998). This diversity might affect sequence-specific binding and account for some of the differences in DNA-binding specificity between plant MYB proteins (Solano *et al.* 1997).

The 125 R2R3-type MYB genes found in *Arabidopsis* have been categorised into 25 groups on the basis of conserved amino acid sequence motifs present at the C-terminal end of the MYB domain (Fig 5.13, Kranz *et al.* 1998, revised by Stracke *et al.* 2001). These conserved domains might represent activation or suppression domains, and/or domains for the interaction with other proteins. The binding site preference and affinity

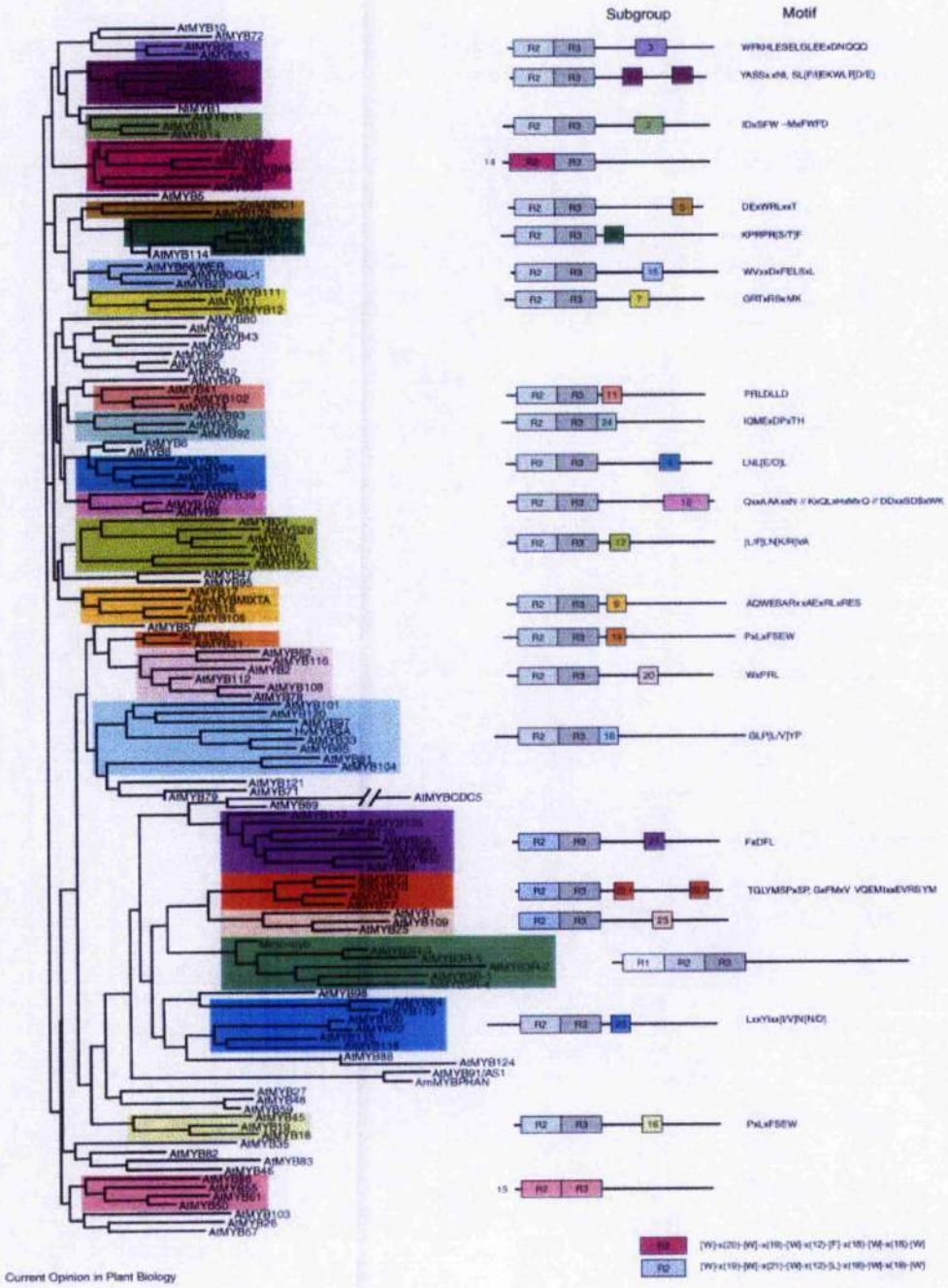

Figure 5.13 Phylogenetic relationship of *Arabidopsis* R2R3-MYB genes.

The *Arabidopsis* R2-R3 MYB genes can be categorised into 25 groups on the basis of conserved amino acid sequence motifs present at the C-terminal end of the MYB domain (figure taken from Stracke *et al.* 2001) *AtMYB119* is the most closely related gene to *AtMYB64* (group highlighted in dark blue).

of MYB proteins is likely to be strongly influenced by other protein factors that interact with them.

AtMYB119 is the protein with greatest similarity to AtMYB64 (Fig 5.13). The MYB DNA binding domain R2 and R3 repeats are highly conserved between AtMYB64 and AtMYB119 (90% identity at the protein level: Fig 5.14 underlined sequence). The sequences downstream and immediately after the R2R3 repeats have a degree of identity of 51% (Fig 5.14). Due to the high degree of similarity between AtMYB119 and AtMYB64 it is possible that these MYB proteins have similar roles, thus AtMYB119 may also be involved in salt tolerance. The most closely related protein to AtMYB75 is AtMYB90 (Fig 5.13 highlighted dark green), both these proteins have demonstrable roles in anthocyanin biosynthesis (Borevitz *et al.* 2000)

5.10 Future Experiments

5.10.1 Characterisation of the Signalling Components Upstream of *AtMYB64.*

Due to the nature of the screen used to identify the salt tolerant activation tagged mutant JP5, it is not known if *AtMYB64* is involved in Na^+, K^+ or Ca^{2+} homeostasis/signalling. Reporter lines, where the *AtMYB64* promoter drives the expression of the luciferase (*LUC*) or *GFP* reporter gene, could be used to determine the spatial and temporal pattern of *AtMYB64* expression, and to characterise which stress factors elicit the activation of *AtMYB64*. Investigation of these reporter lines grown under differing K^+, Ca^{2+} and Na^+ concentrations would establish the amplitude, kinetics and spatial pattern of *AtMYB64* expression in response to these cations. These reporter lines could also be used to investigate what other environmental factors, if any, affect *AtMYB64* expression; treatments could include change in pH, cold/heat shock, ethylene and NO concentration, and pathogen attack for example. The reporter lines could subsequently

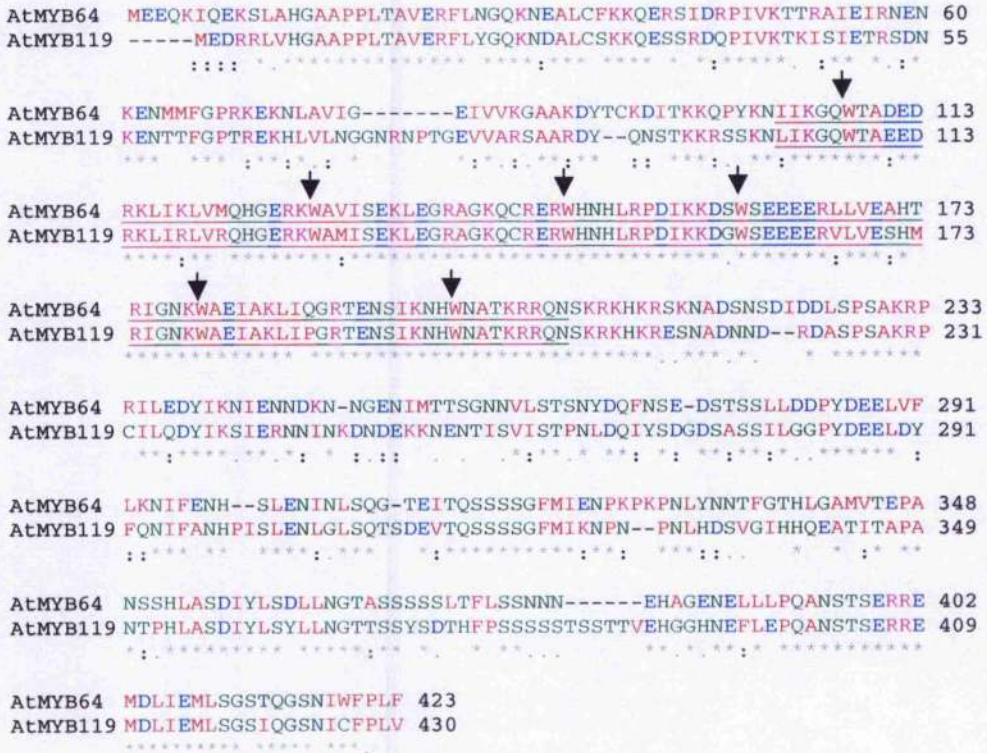

Figure 5.14 Alignment of AtMYB64 and AtMYB119 Protein Sequences.

Black arrows indicate the tryptophan residues of the R2 and R3 repeats.
The MYB DNA binding domain R2 and R3 repeats (underlined sequence above) are highly conserved between AtMYB64 and AtMYB119 (90% identity). The sequences downstream and immediately after the R2R3 repeats have a degree of identity of 51%.

by used as background to undertake a mutagenesis screen to identify components of the upstream signalling pathway. Standard gene knockout techniques could be used (e.g. EMS, neutron bombardment etc.) in loss-of-function screens, but a better approach might be to generate activation tagged lines in this background so that both gain-of-function (signal without salt stress) and loss-of-function (failure to signal in presence of salt) screens can be undertaken.

5.10.2 Methods to identify AtMYB64 targets and downstream responses.

DNA microarrays and ChIP (Chromatin Immuno Precipitation) assays could be used to investigate which genes are directly under the control of the MYB64 transcription factor.

5.10.2.1 Identification of primary MYB64 targets using ChIP

ChIP assays offer the ability to identify the elements that are uniquely recognised by transcription factors. Simply, the ChIP assay combines two steps. First, whole cells are treated with formaldehyde to cross link interacting protein-protein and protein-DNA sequences. This is followed by an immunoprecipitation of protein-DNA complexes with specific antibodies. Once the purified DNA has been recovered universal linkers can be ligated onto the ends of the recovered gDNA fragments. This is used as a template and amplified by the PCR and the product cloned into a suitable vector, sequenced, and then BLAST searches performed in the *Arabidopsis* genome database. The final purification step in the standard ChIP method relies on immunoprecipitation with antibody that is specific to the transcription factor under study. As there are almost 200 MYB transcription factors in *Arabidopsis*, raising a MYB64-specific antibody could be problematic. To avoid generating spurious results, transgenic lines of *Arabidopsis* carrying epitope-tagged AtMYB64 (e.g. HA) could be generated. These plants carrying

an AtMYB64-HA tag chimera could be used with ChIP technology to identify the promoter elements that bind AtMYB64.

5.10.2.2 Identification of AtMYB64 Targets using DNA Micro Arrays

DNA micro array technology could be used to determine genes targeted by MYB64, and related events further downstream. Primary experiments using this technology (Section 5.8) suggested a higher transcript abundance of 7 heat shock proteins in the JP5 mutant compared with wild type plants subjected to long term salt treatment. It is tempting to suggest that over expression of these HSP, perhaps under the control of *AtMYB64*, confers salt tolerance. However, more extensive investigations are required before conclusions can be made. Once a temporal and spatial pattern of *ATMYB64* expression has been established (discussed in section 5.10.1), a more informed choice can be made of the best sampling times and most appropriate stress conditions.

Chapter 6 General Discussion

Soil salinity is an enormous problem affecting many parts of the world. Plant salt stress results from a number of harmful processes including ionic stress, osmotic stress, and secondary stresses such as oxidative stress linked to the production of toxic reactive oxygen intermediates. As most crops are salt sensitive, salinity can have a detrimental effect on crop yield and quality.

Clearly there is interest in identifying sequences in plants that confer salt tolerance. The aim for plant biologists is then to go on to over express (or knockout) these sequences in the major crops in the hope that agricultural yields will be improved. Broadly, two approaches have been used.

The first may be considered to be a 'hypothesis-led' approach. Here, experimenter's use their knowledge to manipulate (activate or suppress) sequences they consider to be important in plants, and these transgenic lines are then tested for improved salt tolerance. Although there have been a few reports where this approach has been successful (e.g. Apse *et al.* 1999), in most reported cases only a moderate level of tolerance has been achieved. There is, however, no doubt that a large number of projects have failed completely, and these are just not reported. It seems the ingenuity of mankind is not yet sufficiently well developed to solve this problem. One of the major barriers for plant biotechnology is, therefore, knowing which genes are important in regulating a plant's response to high salinity. Clearly, the ionic balance of Na^+, Cl^-, K^+, and other nutrient ions are important, but which members of the families of these genes are involved and what governs their posttranslational activities? A great deal of information has been accumulated on how a few model glycophytes regulate ion balance in low salinity, but relatively little is known about how they respond to high salinity. Of greater concern, virtually nothing is known of how halophytes achieve

cellular ionic balance at high salinity, and it is this deficiency that is impairing biotechnological advances in this area. What is required, therefore, is a concerted effort to identify important sequences in halophytes that transport and regulate cellular ion content. Unfortunately, none of the model plants systems are halophytes, and the many advantages offered by these experimental systems, therefore, cannot be bought to bear on the problem. These model systems might, however, provide a more detailed understanding of salt sensitivity in plants.

The second approach may be considered to be 'hypothesis-less', and involves genetic screens of mutated lines for improved salt tolerance. This approach, however, also has its limitations. Conventional genetic screens rely on lines carrying gene knockouts that usually give rise to loss-of-function phenotypes (i.e. they are more salt sensitive that wild type), which makes screening difficult. Gene redundancy is also a significant problem and many important sequences that are members of a gene family may be overlooked by this approach. For example, Zhu and co-workers (Wu *et al.* 1996) have expended an enormous amount of effort on loss-of-function (salt-overly-sensitive) screens on *Arabidopsis*. Whilst this approach has proved to be successful in identifying a number of genes involved in salt tolerance (e.g. the SOS1/SOS2/SOS3) signalling pathway; and this information has been used to look for genetic and biochemical interactors, it remains a distinct possibility that other salt tolerance pathways exist that can not be identified by a conventional gene inactivation (i.e. knockout) approach. Recently, gene activation technology has been developed in plants and this offers a way forward that is not constrained neither by the imagination of the experimenter, nor by the limitations of conventional 'gene knockout screens'.

In Chapter 3 a series of experiments were presented where the experimental advantages of the model plant *Arabidopsis thaliana* were bought to bear on identifying sequences

that might be important in *salt tolerance* as opposed to *salt sensitivity*. An *Arabidopsis* cell culture has been established at Glasgow University that grows well in 380 mM NaCl; WT lines will not survive above 80 mM NaCl. It was hoped that studies on the ion transport processes in these cells would identify which class or classes of transporters are essential for maintaining ionic balance in high salinity (i.e. increased K^+ acquisition, increased Na^+ efflux, etc.). This information could then be used to direct further experiments designed to identify specific sequences that could be manipulated to improve salt tolerance *in planta*, information that is badly needed to direct projects where gene manipulation is used to confer salt tolerance on plants.

In contrast, the activation tagging, genetic approach that has been described in this thesis (Chapters 4 & 5) has proved to be very successful. Approximately 23,000 *Arabidopsis* activation tagged lines (Weigel collection) were screened for improved salt tolerance on low K^+, low Ca^{2+} media. Fifty authentic salt tolerant mutants have been identified, and ten of these selected for further investigation. The site of tag insertion has now been determined in each of these mutant lines, and in most cases the level of transcription of the local genes assessed.

It has been reported that the activation tag is effective up to 10 kbp from its site of insertion (Weigel *et al.* 2000). From this, it can be calculated that approximately 100,000 activation tagged lines will be required to provide 95% genome coverage. As mentioned earlier, only 23,000 lines have been screened to date (the Weigel collection). There is another *Arabidopsis* activation tag collection available, the Scheible & Somerville lines, which contain ~63,000 mutants, and these should also be screened for salt tolerance.

In some of these mutants it is clear how the disrupted gene may confer salt tolerance (e.g. MYB, GCN2, APG7) but other sequences might not have been predicted to be

involved (e.g. NOS, ULP1, RNA modifying proteins; see Chapter 4). Clearly, these mutants have not been fully characterized and more work is required to confirm the role of these sequences in salt tolerance. In particular, it is now imperative to establish the co-segregation of the activation tag and the salt tolerant phenotype in these mutants, and to provide a detailed profile of the expression pattern of the disrupted gene(s).

There is now good evidence that activation tagging offers a new and fruitful way forward for uncovering genes involved in abiotic stress. The identification of mutant JP2, which appears to carry a disruption in a kinase (AtGCN2), gives confidence that the technology works; the closest homolog to this sequence is the yeast ScGCN2 which is known to confer salt tolerance by regulating the translation of ScGCN4, a bZIP transcription factor. It would be interesting to identify the target of AtGCN2 action in *Arabidopsis* as this should provide valuable information on other salt tolerance mechanisms in plants. In addition, to GCN2, the mutant JP5 also provide confidence that this technology works; JP5 has higher transcript levels of a MYB transcription factor, and work detailed in this thesis (Chapter 5) has shown that over expression of this MYB in a WT background also confers salt tolerance; further, *myb* homozygous knockout lines are hypersensitive to salt. More recent experiments have also shown that a double knockout in the ULP1 gene (up regulated in the JP1 mutants) is also hypersensitive to salt. Taken together, these results suggest that activation tagging is an exciting and rewarding development in plant biology for uncovering gene function, particularly in relation to abiotic stress.

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