CHAPTER 1: GENERAL INTRODUCION

1.1 Introduction

Salinity stress is the most significant problem facing plant agriculture in many regions of the world. The increasing number of salt-affected hectares of valuable arable land, combined with the increasing demand for food to feed the ever-rising world population, makes mediation of salinity stress in plants greatly important.

Development of salt tolerance in plants remains elusive, due to the multi-genic nature of the trait and its complexity. Intuitively, cell-types must respond to salt stress individually and co-ordinately, in roots for example, cortical cells need to maximise efflux of sodium, and stelar cells maximise influx to minimise transfer of sodium to the xylem stream and the shoot.

Genes involved in sodium transport in the cell and within the plant have typically been expressed constitutively in transgenic experiments. Due to the cell-specific effects mentioned above, this would be counterproductive to increasing salt tolerance. Constitutive expression of salt tolerance transgenes controlling metabolically expensive processes results in less photosynthate being directed to grain production, even in non-stress environments. This creates a yield penalty. The need for the cell-specific control of salt tolerance genes, and under the induction of salt stress, is important to make significant progress in the development of an understanding of salt tolerance in crops. Considering that plants have similar responses to a variety of stresses, specific control of transgenes may also be useful in the development of crops which are tolerant to other types of stress.

1.2 Rice

1.2.1 Review of importance

1.2.1.1 History

Rice cultivation began in eastern India or western China sometime between 4,000 and 10,000 B.C. (Hoshikawa 1989). The cultivation of rice then moved into the Ganges and Indus River regions in 2,500 – 1,500 B.C., the Near East in 500- 300 B.C., Europe around 100 B.C. and the Americas in the 16th century. Wild rice (*Oryza*

perennis Moench) is believed to be the ancestor of *Oryza sativa* L. and *Oryza glaberrima*, the species grown widely today. Three subspecies of *O. sativa* exist, *indica*, *japonica* and *javanica*, with *indica* being the most cultivated of the three.

1.2.1.2 Use

Rice is an incredibly important crop, accounting for 20% of the caloric intake of people worldwide, with some south-eastern Asian countries deriving up to 75% of their caloric intake from rice (http://www.irri.org/science/ricestat/index.asp). Worldwide, rice production is worth $10 billion US annually, comprising 600 million t of rice grown on 150 million ha (http://www.irri.org/science/ricestat/index.asp). Asian rice production accounts for 135 of the 150 million ha of the rice grown globally.

1.2.1.3 Distribution

Currently, rice is grown on every continent, with its limitations being approximately 50 degrees latitude north and south (Hoshikawa 1989). Rice is grown in four broadly defined ecosystems. Irrigated production zones account for 55% of worldwide rice production, rain-fed lowlands for 31%, uplands for 11% and flood-prone for 4% (http://www.irri.org/science/ricestat/index.asp).

1.2.1.4 Monocot model species

Rice has become a highly useful genetic tool for researchers in a variety of physiological, molecular, genetic and genomic studies. Early studies indicated that rice chromosomes were highly collinear with those in other grass species genomes. As a result, rice became the chosen species to study grass genomics due to its very small genome (389 Mbp) for a cereal crop (International Rice Genome Sequencing Project 2005). However, the unified grass genome model has yet to live up to expectations as there is relatively little sequence data on other grass species and the colinearity of gene order and content that was observed at the recombinational map level is not as evident at the local genome structure level (Bennetzen and Ma 2003).

Whether or not the genome structures of rice and other grasses are similar, rice has been tremendously useful as a model species for monocots with the publication of draft sequences of *indica* and *japonica* varieties (Yu *et al.* 2002 and Goff *et al.* 2002). As well, rice is more similar physiologically to other cereal species than is the dicot model species, *Arabidopsis thaliana*.

1.2.2 Rice morphology

Typically, rice is a semiaquatic annual grass, but it can survive as a perennial in the tropics by producing new tillers after harvest (ratooning). Mature plants vary between 0.4 and 5 m in height with each plant having a main stem and tillers with a terminal flowering head, or panicle. The morphology can be divided into its vegetative phases (germination, seedling and tillering) and reproductive phases (panicle initiation and heading) (Hoshikawa 1989).

1.2.2.1 Germination

The rice grain contains a large starchy endosperm, and is covered by a hull consisting of a palea, lemmas and rachilla (Figure 1.1). Germination begins with dormancy break and water absorption at temperatures between 10 to 40°C. Under aerobic conditions the seminal root is first to emerge through the coleorhiza, but under anaerobic conditions the coleoptile is first to emerge (Hoshikawa 1989).

1.2.2.2 Seedling

The first two leaves to emerge are less than 5 cm in length with the third and following leaves extending to a much greater length (Figure 1.1). With the complete expansion of the third leaf, the fourth one emerges, thus leaf number can be used to describe the growth stage of the seedling. Generally, seedlings grown in a nursery are transplanted to the field at the 6-8 leaf stage (plus one tiller) (Hoshikawa 1989).

1.2.2.3 Tillering

The stem consists of a series of nodes and internodes with about 13 to 16 nodes being common. The upper nodes contain a leaf and a bud which can grow a tiller. Tillering begins when the seedling is self-supporting (about the five-leaf stage), with primary, secondary and potentially tertiary tillers developing (Figure 1.1). Tillers may become independent as they produce their own root structure made up of crown roots and nodal roots. Roots range from 40 cm to more than 1 m in length, depending on the depth of water in the field and the diffusion rate of O_2 through root aerenchyma.

1.2.2.4 Panicle initiation

The life cycle of rice ranges from 3 to 6 months depending on environmental conditions, with 120 days being average. In the tropics, a plant will spend approximately 60 d in vegetative stage, 30 d in reproductive stage and 30 d in ripening

stage. The panicle extends from the stem and has approximately 8-10 nodes at 2-4 cm intervals from which secondary branches develop (Figure 1.1).

1.2.2.5 Heading

Spikelets develop along secondary branches with each spikelet having one fully developed flower with pistil and stamens. Anthesis occurs with the onset of heading and usually is completed within 6 hours (h). The entire panicle will complete anthesis in 7-10 d, a process that is very temperature and humidity sensitive. The ripening stage is divided into sub-stages based on texture and colour of the grains and are referred to as milky, dough, yellow-ripe and maturity.

Figure 1.1: Rice seed, seedling and plant morphology (see http://www.knowledgebank.irri.org/).

1.3 Salinity and Crops

1.3.1 Definition

Soil salinity is a serious issue worldwide. It is estimated by salinity monitoring organisations, such as the United States Salinity Laboratory (USSL) and the UN Food and Agriculture Organization (FAO), that one billion of the 13 billion hectares of land worldwide are salt-affected, including approximately 30% of all irrigated land (Rengasamy 2006). This estimate includes approximately 200 million ha in the Americas, large portions of southern and eastern Europe, 120 million ha in the Middle East, 80 million ha in Africa, 35 million ha in Asia and over 6 million ha in Australia. Soil salinity is an especially acute problem in Australia with projections that 17 million ha will be affected by 2050, much of which is in the western wheatbelt (Rengasamy

2006). One-third of the world's food comes from irrigated land (Munns 2002), thus salinisation of agricultural soils is a critical issue.

Saline soils are defined as loose and sandy soils with significant amounts of water-soluble salts (e.g. sodium, chloride, calcium, magnesium and sulphate) and have an electrical conductivity (EC) greater than 4 dS/m, an exchangeable sodium percentage (ESP) less than 15% and a pH of less than 8.5 (Rengasamy 2002).

Sodic soils are generally dense and clogged, with low soluble salt content, an EC less than 4 dS/m, an ESP of greater than 15% and a pH higher than 8.5. These soils have high concentrations of insoluble sodium carbonate and bicarbonate, which cause a crust over the soil surface (Rengasamy 2002).

Saline-sodic soils are common in arid and semi-arid regions and are an intermediate type of saline soil with an EC greater than 4 dS/m, ESP greater than 15% and pH less than 8.5 (Rengasamy 2002).

1.3.2 Causes

The Biosalinity Awareness Project states, "The principal causes of salinity in our environment are ultimately linked to the redistribution of water and soluble salts, both above and below ground. The problem of salinisation is most acute in the arid and semi-arid regions where both natural processes (evaporation and plant water consumption) and human interventions (land clearing, resource management and irrigation) play their part in the build-up of salts in our soil and water" (see http://www.biosalinity.org).

Primary salinity is a result of evolutionary processes such as evaporation and underground water movements which bring salt to the surface. Sea water influences and climatic changes are also primary causes of salinity (Rengasamy 2006).

Secondary salinity has been caused by human intervention through irrigation, destruction of critical watersheds, and clearing of natural, deep-rooted vegetation for agricultural, industrial and urban development, thereby altering the dynamic equilibrium of water and salt circulation. Some salinity problems are aggravated by application of certain chemical fertilisers and soil amendments (Rengasamy 2006).

1.3.3 Effects on plant health

Salinity stress in plants is a result of ionic and osmotic components, whose effects can be difficult to distinguish in a salt stressed plant. One common feature of salt-stressed plants is an inhibition of root growth, which appears to be related more to the osmotic potential of the external solution than to the Na^+ content of the plant itself (Munns *et al.* 2000; Munns 2002). The same inhibition of leaf elongation by Na^+ content has been observed in several grass species (Cramer 2003). A method potentially useful to separate the effects of the ionic and osmotic components of salt stress is to compare the effects of LiCl to those of NaCl on plant growth (Tester and Davenport 2003). LiCl is toxic at one-tenth the concentration of NaCl and shares many of the transport pathways, thus comparing the effects on plants of solutions equal in molarity would help to separate the components of the salt stress.

Ionic toxicity of Na^+ is largely related to its competition with K^+ for binding sites crucial to metabolic processes which Na^+ cannot fulfil correctly (Bhandal and Malik 1988). Thus, maintaining a low Na^+: K^+ ratio is critical to salt tolerance, and may be more critical to salt tolerance than the absolute Na^+ level itself (Dubcovsky *et al.* 1996; Maathuis and Amtmann 1999).

The osmotic component of salt stress is related to the build-up of Na^+ (and Cl^-) in the apoplastic spaces in leaf tissue from the evaporating water of the xylem stream. With elevated Na^+ levels in the apoplastic space, water is drawn out of leaf cells causing dehydration (Flowers *et al.* 1991). Shoots accumulate more Na^+ than do roots, thus it can appear that they are more sensitive to osmotic and ionic Na^+ stress than roots.

Elevated Na^+ levels also impedes efficient uptake of other nutrients through nutrient transporters, and can result in nutrient deficiency (Silberbush and Ben-Asher 2001, Hu and Schmidhalter 2005).

An obvious result of decrease in plant vigour due to the osmotic and ionic stresses as well as the nutrient deficiencies imposed on plants by salinity stress is a significant penalty in yield (e.g. Quarrie and Mahmood 1993; Katerji *et al.* 2003). The negative effects of shoot Cl^- accumulation on plant health are also significant in species such as grapevine (Storey *et al.* 2003, White and Broadley 2001), but will not be

discussed as the effects of Na^+ are more significant in the majority of crop species since Cl^- transport into cells is inhibited by their negative electrical potential (Munns 2005).

1.3.4 Management based solutions

1.3.4.1 Water and vegetation management

Control of water movement on land is important to reduce the salts present in the soil and to reduce further accumulation of soil salts, especially those resulting from irrigation (Al-Attar 2002; Hillel 2000; Qadir and Oster 2004). Alternatively, trees and other vegetation may be used to change the level of the water table and saline layer of soil, improve soil structure and even to remove salt from the soil (Qadir and Oster 2004).

1.3.4.2 Soil reclamation

Several techniques exist for reclamation of saline soils. Physical manipulation by removing saline topsoil or deep ploughing to bury saline soil can be useful in some instances. Leaching of the salt in the topsoil may, in some cases, flush sufficient amounts of salt out of the topsoil to reclaim it for agricultural use. Improvement of drainage to prevent further salt accumulation also helps remove the salt present in the soil. The addition of calcium and organic amendments has also been useful to combat the effects of salt on plants as well as to improve the structure of the soil (Rengasamy 2006).

1.3.4.3 Use of salt-tolerant or halophytic crops

Halophytes and other salt-tolerant species can help in remediation of saline soils. Salt-tolerant perennials have deep root penetration to improve soil structure and some extreme halophytes can accumulate significant levels of sodium in their above-ground tissues, which could be removed from the land (e.g. Malcolm *et al.* 2003). However, the cost of removing the above-ground biomass would be a drawback to this method on all but high value land (Tester and Davenport 2003).

Nevertheless, in most agricultural systems, mild to moderate soil salinity exists which means salt-tolerant crops may be grown. Wide ranges in salt-tolerance exist among crop species from relatively salt-tolerant species like barley to salt-sensitive species such as rice or wheat. Considering the importance of these crops to many

countries economies and food supplies it is necessary to keep growing these crops, thus improving their salt-tolerance is extremely important.

1.3.5 Physiological adaptations to salinity

1.3.5.1 Cellular processes

1.3.5.1.1 Intracellular compartmentation

Maintenance of low concentrations of Na^+ within the cytoplasm of cells is of utmost importance to the survival of plants in saline environments. The simplest method for plants to achieve this is the sequestration of Na^+ within vacuoles.

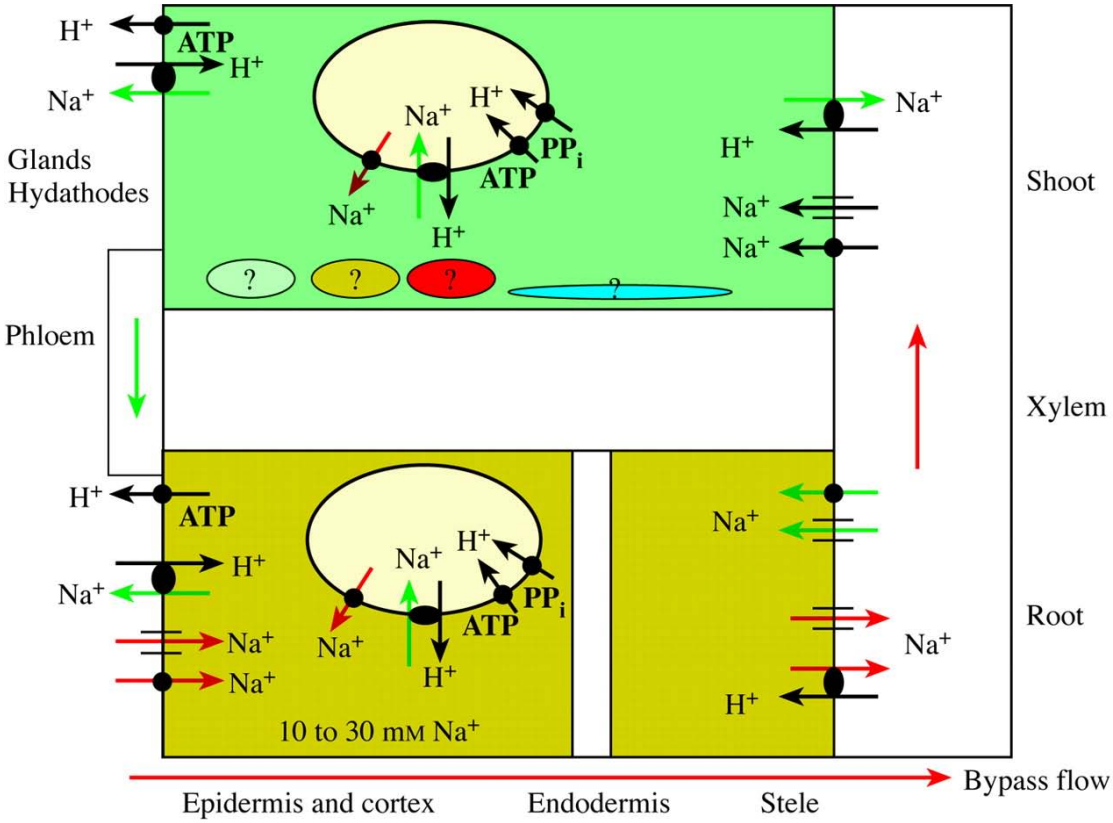


Figure 1.2: Na^+ transport within higher plants (from Tester and Davenport 2003).

1.3.5.1.1.1 Pumping Na^+ into the vacuole

Na^+ that enters the roots and is transported to the leaves must be compartmentalised in the vacuoles in order to avoid build-up of levels of Na^+ which are toxic to cellular proteins in the cytoplasm. Central to this process is the vacuolar Na^+/H^+ antiporter (NHX), which moves Na^+ into the vacuole in exchange for H^+ (Blumwald *et al.* 2000) and may be regulated by the SOS signalling pathway (see

below) (Qiu et al 2004). The original H^+ gradient is created by both vacuolar H^+-ATPase and H^+-pyrophosphatase proteins (Gaxiola *et al.* 2001). Elevated levels of Na^+ increase the activity of the Na^+/H^+ antiporter in the roots of barley (Gabarino and DuPont 1989), tomato (Wilson and Shannon 1995), sunflower (Ballesteros *et al.* 1997), maize (Zorb *et al.* 2005), *Medicago* (Zahran *et al.* 2007), and cotton (Wu *et al.* 2004) but not in salt sensitive rice (Fukuda *et al.* 1998). There are eight *NHX* gene family members in *Arabidopsis* (Yokoi *et al.* 2002) of which only 1, 7 and 8 have functional assignments. *NHX7* is also known as *SOS1* (see below) and *NHX8* has been shown to be a Li^+/H^+ antiporter (An *et al*. 2007)

Constitutive overexpression of the vacuolar transporters appears to increase the salt tolerance of several species. Overexpression of the *Arabidopsis* vacuolar Na^+/H^+ antiporter, AtNHX1, appears to increase salinity tolerance significantly in yeast (Aharon *et al.* 2003), *Arabidopsis* (Apse *et al.* 1999), tomato (Zhang and Blumwald 2001), *Brassica napus* (Zhang *et al.* 2001), and cotton (He *et al.* 2005), while expression of various cereal homologues have been reported to improve the salt tolerance of *Arabidopsis* (Brini *et al.* 2007), rice (Fukuda *et al.* 2004b, Zhao *et al.* 2006), wheat (Xue *et al.* 2004) and barley (Fukuda *et al.* 2004a). Additionally, the overexpression of *NHX1* appears not to alter its possible role in the regulation of cytoplasm and vacuolar pH (Viehveger *et al.* 2002; Fukada-Tanaka *et al.* 2000) and its cation selectivity is regulated by a luminal C-terminus (Yamaguchi *et al.* 2003). The overexpression of *NHX1* in *Arabidopsis* leads to a small increase in shoot Na^+ accumulation (Apse *et al.* 1999), which possibly allowed the cells to maintain a favourable osmotic balance, yet maintain low cytoplasmic Na^+ levels with the Na^+ sequestered to the vacuole. The *nhx1* mutant had much lower Na^+/H^+ and K^+/H^+ exchange capabilities in isolated vacuoles, fewer large epidermal cells and less overall leaf area, indicating NHX1 also plays a developmental role (Apse *et al.* 2003). Overexpression and knockout of the *NHX1* gene in *Arabidopsis* has been shown to significantly and differentially alter the expression of a large number of genes in the plants salt stress response, indicating *Arabidopsis* is able to respond to change in one Na^+ transporter by regulating other genes (Sottosanto *et al.* 2004, 2007)

The overexpression of *AVP1*, the vacuolar H^+-pyrophosphatase, also appears to increase salt tolerance and Na^+ accumulation in *Arabidopsis* (Gaxiola *et al.* 2001). Increased activity of the Na^+/H^+ antiporter must have increased as a result of the *AVP1* overexpression as the vacuolar Na^+ levels of the transformants were higher than those of wild-type plants. This was shown in barley, where expression of the vacuolar H^+-pyrophosphatase, *HVP1*, and the vacuolar Na^+/H^+ antiporter, *NHX1*, was similarly upregulated by salt stress (Fukuda *et al.* 2004a), and are similarly regulated by ABA, auxin and gibberellin (Fukuda and Tanaka 2006). *NHX* and *AVP* genes expressed simultaneously were found to increase salt tolerance beyond the tolerance provided by expression of the genes individually in *Arabidopsis* (Brini *et al* 2007) and rice (Zhao *et al* 2006).

As with all such reports of improved salinity tolerance resulting from constitutive overexpression of a single gene, the reports above must be examined critically. Reports citing purely qualitative evidence of improved salinity tolerance, such as photographs (e.g. Apse *et al*. 1999), require further evidence to quantify the increase in salinity tolerance (e.g. FW or yield measurements). Similarly, reports citing physiologically irrelevant experimental conditions (e.g. Fukuda *et al*. 2004b, where low light levels [photon flux density of 200 μmol m^{-2} s^{-1}] and extremely high Na^+ concentrations [100 mM Na^+ for 7 weeks]) would be more plausible if careful analysis were repeated under more relevant experimental conditions.

Na^+ transport across the tonoplast is bidirectional and dynamic and efflux of Na^+ from vacuoles is thought to occur though non-selective cation channels, which are highly permeable to other cations as well (Demidchick *et al.* 2002). Their activity is presumed to be quite low since they are highly permeable to cations (Na^+, Ca^{2+}), and since it appears there are no major differences in the properties of vacuolar channels of salt-sensitive and –tolerant species (Maathuis and Prins 1990).

1.3.5.1.1.2 Synthesis of osmoprotectants

With the compartmentation of Na^+ within the vacuole, must come the increase of osmoprotectants (compatible solutes) in the cytoplasm to avoid dehydration of the cytoplasm. These solutes do not inhibit biochemical cellular processes, but rather protect them from inorganic ion damage (Shomer-Ilan *et al.* 1991). They are often

soluble, neutral or zwitterionic secondary metabolites such as glycinebetaine (e.g. Sulpice *et al.* 2003) and mannitol or primary metabolites such as proline (Abraham *et al.* 2003; Hien *et al.* 2003), trehalose (Jang *et al.* 2003) and sucrose (Hu *et al.* 2000). Overexpression of genes encoding osmoprotectants generally result in osmotically insignificant levels of the particular metabolite (Chen and Murata 2002), indicating that the role of osmoprotectants in increasing salt tolerance may be to protect protein structures or scavenging of reactive oxygen species (Skopelitis *et al.* 2006). Somewhat problematically, most osmoprotectant transgenic studies have occurred in tobacco, a plant which is more sensitive to the osmotic component of NaCl than the ionic component (Murthy and Tester 1996). A more appropriate species for these studies may be maize, in which a clear relationship between level of osmoprotectant and salt tolerance exists (Saneoka *et al.* 1995).

Related to this is the striking number of salt-tolerance studies using the salt-sensitive *Arabidopsis* as the model plant, but a closely related salt-tolerant plant, *Thellungiella halophila*, may be a more appropriate model for discovering salt-tolerance mechanisms (Volkov *et al.* 2003; Wang *et al.* 2004; Taji *et al.* 2004, Gong *et al.* 2005, Volkov and Amtmann 2006). For instance, one study suggests that *Thellungiella halophila* may be more salt tolerant than *Arabidopsis* due to more efficient production of osmoprotectants (Kant *et al.* 2006).

1.3.5.1.2 Tolerance of high cytoplasmic Na^+

Cytoplasmic reactions have been shown to be quite tolerant of elevated levels of Na^+ (100 mM) in the presence of osmoprotectants in *in vitro* studies (Cheeseman 1988; Shomer-Ilan *et al.* 1991). The cytoplasmic enzymes of some salt-tolerant plants have been shown, *in vitro*, to be tolerant of elevated Na^+ levels, likely due to increased ability to substitute Na^+ for enzyme functions normally requiring K^+ (Flowers and Dalmond 1992). Additionally, the structure of some enzymes in halophytic bacteria provides tolerance to high Na^+ (Dym *et al.* 1995). However, if these enzymes were to be engineered into plants the metabolic efficiency of the plant may drop significantly under non-saline conditions (possibly avoided by stress-inducible induction of osmoprotectants, see Su and Wu 2004; Urano *et al.* 2004). As well, the studies above

must be complemented by *in vivo* studies to prove the validity of using this approach to produce salt tolerant plants.

1.3.5.1.3 Damage response and repair

Salt stress increases the synthesis of osmotins and dehydrins, which have similar properties to chaperones and seem to be responsible for maintenance of protein structure under elevated Na^+ concentrations (Ingram and Bartels 1996; Campbell and Close 1997). Care needs to be taken with these studies as osmotic shock from sudden experimental changes may be responsible for the increase in these protective proteins. Constitutive overexpression of a late embryogenesis abundant (LEA) protein from barley increased salt tolerance in rice (Xu *et al.* 1996), while overexpression of a heat shock protein from a halotolerant bacterium in tobacco increased salt tolerance (Sugino *et al.* 1999). However, the lack of osmotic controls in these experiments makes their results somewhat questionable.

Glycinebetaine (Chen and Murata 2002), putrescine (Galston and Sawhney 1990), spermine (Mansour 2000; Urano *et al.* 2004; Capell *et al.* 2004) and tyramine (Lefevre *et al.* 2001) have all been reported to increase in response to salt stress and to be involved in protective functions such as reducing lipid peroxidation and protecting mitochondrial electron transport reactions (Chen and Murata 2002). The scavenging of reactive oxygen species (ROS) by these compounds is a favoured hypothesis to explain their protective action (Zhu 2001; Xiong *et al.* 2002), but they may also reduce the efflux of K^+ from roots associated with salinity stress and ROS production (Cuin and Shabala 2007). Another, more recent suggestion, is that molecules like polyamines actually block NaCl-induced K^+ efflux by NSCCs, thereby improving the ionic balance of the plant under salinity stress (Shabala *et al.* 2007).

1.3.5.1.4 Genomic-scale observations of alterations in expression of genes

Incredible amounts of data are being developed through the use of microarrays, comparing the expression of genes under salt stress conditions to the expression under normal growth conditions (e.g. Bohnert *et al.* 2001; Kawasaki *et al.* 2001; Seki *et al.* 2001; Chen *et al.* 2002; Ozturk *et al.* 2002, Walia *et al.* 2005, Walia *et al.* 2007). Some of the data indicates that approximately 8% of all genes are transcriptionally altered by

salt stress in *Arabidopsis* and about 70% of the genes affected are distinct from those altered by drought stress (Bohnert 2001). Similar studies in rice (Rabbani *et al.* 2003) and in maize indicated a figure of around 15% of genes being regulated by salt stress (Wang *et al.* 2003). Additionally, the gene expression is dependent on tissue type, developmental stage, and the extent of the stress treatment (Bohnert 2001) as well as being largely ABA-independent (Wei *et al.* 2000). However, the reconciliation of these experiments with the results of previous physiologically based data needs to occur. The new microarray data suggests that the early responses are more important to salt tolerance responses of plants (e.g. Bohnert *et al.* 2001), while older physiologically based data indicated the initial response to salt stress was largely unrelated to the long term tolerance of the plant to salinity stress (Munns 1993; Munns 2002). Additionally, levels of Na^+ used in most microarray experiments to induce salt stress, were likely to be high enough to induce death in *Arabidopsis* (Kilian *et al.* 2007 used 300 mM Na^+) or rice (e.g. Kawasaki *et al.* 2001 used 150 mM Na^+) and future experiments should be undertaken at more relevant levels to increase the validity of the claims (for discussion see Munns 2002). More appropriate stress levels have been used in several more recent experiments done in rice (Walia *et al*. 2005 used 75 mM) and barley (Walia *et al.* 2007 used 100 mM) in collaboration with the U.S Salinity Lab. An *Arabidopsis* study examined the effects of either an 80 mM Na^+ treatment, K^+ starvation or Ca^{2+} starvation on gene expression in roots and found significant overlap in genes being regulated by the three stresses (Maathuis *et al.* 2003). However, the interactions between the three ions in many cellular functions are extensive and complex making interpretation extremely difficult.

1.3.5.1.4.1 Signalling pathways

The onset of salinity stress in plants activates pathways which involve a receptor which perceives the stress, alterations in protein activity, changes in gene transcription via signalling intermediates and phosphoprotein cascades (Leung and Giraudat 1998; Hasegawa *et al*. 2000; Schroeder *et al.* 2001; Xiong *et al.* 2002; Zhu 2002). However, as with the microarray data, the interpretation of the data in many studies is questionable due to the irrelevantly high levels of Na^+ utilised to elicit the stress response (Munns 2002).

1.3.5.1.4.2 Cytosolic calcium activity

One of the first responses to a sudden increase in Na^+ is a significant rise in Ca^{2+} in the cytoplasm of the cell (Knight *et al.* 1997, Kader *et al.* 2007). Sudden increases in Na^+ are not agriculturally relevant, but several components of the Ca^{2+} signal transduction pathway and activity Ca^{2+} transporters are induced by Na^+ stress (Allen and Sanders 1994; Hirayama *et al.* 1995; Wimmers *et al.* 1992). The calcium regulated calcinuerin B-like protein/serine threonine protein kinase (CBL-CIPK) signalling pathway is central to signalling in plants. The *Arabidopsis* genome contains 10 CBLs and 25 CIPKs, while rice has 10 CBLs and 30 CIPKs, indicating the large signalling network potential that exists for this pathway (Kolukisaoglu et al 2004). CBL1, CBL9 and CIPK23 regulate the K^+ transporter, AKT1 which is central to K^+ nutrition (Xu *et al.* 2006). Similarly, SOS2 (CIPK24) and SOS3 (CBL4) regulate SOS1, a Na^+ transporter crucial to the Na^+ response in *Arabidopsis* (see below).

1.3.5.1.4.3 Protein phosphorylation and dephosphorylation

Salinity stress induces protein phosphorylation and dephosphorylation (Xiong *et al.* 2002). A GSK1/shaggy-like protein kinase when overexpressed in *Arabidopsis* caused increased anthocyanin synthesis and the transcription of NaCl stress-responsive genes as if there was a Na^+ stress present (Piao *et al.* 2001). These plants also had increased salt stress tolerance. The plants accumulated Na^+ in the shoot in the same fashion as those which are overexpressing *NHX1* (see above), which may indicate a common pathway is involved. Many other protein kinases and phosphatases have been implicated in the signalling pathway induced by salt stress (Trewavas and Malho 1997; Knight and Knight 2001) including recent reports showing the involvement of the mitogen-activated protein (MAP) kinase signalling pathway (Hua *et al.* 2006, Alzwiy and Morris 2007).

1.3.5.1.4.4 The *sos* mutants

The *sos* (salt overly sensitive) mutants are recessive *Arabidopsis* mutants and display sensitivity to Na^+. The effect of Na^+ on the mutants seems to be ionic rather than osmotic as the mutants are sensitive (unable to maintain root growth) to NaCl and LiCl and not to mannitol. The *sos* mutants are involved in a signalling pathway which

is induced by salt stress and several members of the signalling pathway having been identified (possibly including NHX1, see Qiu *et al.* 2004). The SOS1 protein has a long C-terminal tail which extends into the cytoplasm of the cell and interacts with RCD1, a regulator of oxidative stress responses in *Arabidopsis*. This shows the *sos* pathway functions to relieve oxidative stress and indicates there is some level of cross-talk between salt-stress and oxidative stress tolerance pathways (Katiyar-Agarwal *et al.* 2006). SOS3 is a myristoylated calcium-binding protein which responds to cytosolic Ca^{2+} increases (Liu and Zhu 1997, Ishitani *et al.* 2000; Gong *et al.* 2004) and interacts with SOS2, a serine/threonine protein kinase (Halfter *et al.* 2000; Gong *et al.* 2004). Recently, it was shown that the SOS3-SOS2 interaction occurs in the root, while SOS2 interacts with the SOS3 homolog SOS3-LIKE CALCIUM BINDING PROTEIN8 (SCABP8)/CALCINUERIN B-LIKE10 in the shoot (Quan *et al.* 2007). SOS2 then interacts with SOS1, an Na^+/H^+ antiporter located in the plasma membrane of epidermal and stelar cells in the roots (Shi *et al.* 2002; Qiu *et al.* 2003), increasing SOS1 transcription and the SOS1 activity (Shi *et al.*2000; Qiu *et al.* 2002; Quintero *et al.* 2002). SOS1 may also be a Na^+ sensor and provide feedback to the SOS pathway (Zhu 2002). *sos2* and *sos3* mutants accumulate more Na^+ than wild-type plants and *sos1* mutants accumulate less Na^+ than wild-type plants indicating that SOS2 and SOS3 are likely to control other proteins besides SOS1 (Zhu *et al.* 1998). *sos1*, *sos2* and *sos3* mutations also all affect K^+ nutrition (Zhu *et al.* 1998). *sos4* encodes a pyridoxal kinase and may also be involved in the control of SOS1 (Zhu 2002). The root tips of the *sos5* mutant swell and root growth is arrested under salt stress and *SOS5* has been reported to encode a cell surface adhesion protein and is required for normal cell expansion (Shi *et al.* 2003). The *sos* pathway appears to be conserved in rice as homologs of *SOS1*, *SOS2* and *SOS3* have been identified (Martinez-Atienza *et al.* 2007).

1.3.5.1.4.5 Transcription factors and small RNAs

Response to salinity is a highly coordinated and complicated process involving the induction of transcription of many genes. It is important to identify the transcription factors which coordinate the expression of the target genes (Chen *et al.* 2002; Xiong *et al.* 2002). The promoter regions, which are targets of these

transcription factors, include the dehydration-responsive elements (DREs) (Novillo *et al.* 2004; Dobouzet *et al.* 2003) and ABA-responsive elements (ABREs). However, these elements are more likely to respond transcriptionally to osmotic changes than to Na^+-related stresses. The role of zinc-finger proteins (Mukhopadhyay *et al.* 2004), H-protein promoter binding factors (Nagaoka and Takano 2003), NAC-type transcription factors (Nakashima *et al.* 2007), calcium-binding transcription factors (Kim and Kim 2006) and translation initiation factors (Rausell *et al.* 2003) in regulation of salt tolerance genes have also been examined. While constitutive overexpression of these genes often results in increased tolerance to abiotic stresses it also comes with growth inhibition under unstressed conditions (e.g. Liu *et al.* 1998). However, utilizing a stress-inducible promoter to drive these genes reduced the growth inhibition under unstressed conditions (Kasuga *et al.* 1999).

The importance of small RNAs in the regulation of gene expression is only starting to be realised and the impact this relatively newly discovered class of RNA is having on understanding plant transport regulation is already extremely important (Phillips *et al.* 2007, Sunkar *et al.* 2007). A pair of endogenous natural *cis*-antisense transcripts produce small interfering RNAs when *Arabidopsis* is salt-stressed and these siRNAs are involved in regulating the plant's response to salt-stress (Borsani *et al.* 2005).

1.3.5.1.4.6 Cell-specific signalling responses

Despite the specific responses different cell types have to salt stress very little has been done to study cell types individually. Individual cell type-specific responses are likely to be involved in damage limitation while constitutive responses are likely to be involved in damage repair. Damage limitation is obviously the more desirable response to salt stress. One study by Kiegle *et al.* (2000) examined the changes in cytosolic Ca^{2+} in response to a sudden and large Na^+ stress and found distinctive oscillations in cytosolic Ca^{2+} in endodermal and pericycle cells.

1.3.5.2 Whole plant processes

There are several salinity responses in plants which require the coordination of several cell types each responding to salinity in a different manner. These responses

are largely centred on controlling Na^+ uptake by the roots and its subsequent distribution within the plant tissue.

1.3.5.2.1 Regulation of Na^+ transport to the shoot

Generally, salt tolerance is related to the minimisation of Na^+ reaching the shoot tissue. Two important species where this relationship does not necessarily hold are the relatively salt-tolerant barley and cotton which tolerate higher Na^+ levels within shoot tissue than does a salt-sensitive species, like wheat. Another, perhaps equally important factor, is maintaining a low ratio of Na^+ to K^+ reaching the shoot tissues as Na^+ is largely toxic due to its competition for K^+ binding sites in cells (Gorham *et al.* 1990; Dubcovsky *et al.* 1996; Maathuis and Amtmann 1999; Cuin *et al*. 2003). Increased K^+ efflux from Na^+-stressed root cells creates added difficulty for plants to maintain a low Na^+ to K^+ ratio in the shoot, thus K^+ efflux from roots appears to be a suitable measure of salt tolerance (Chen *et al.* 2005, Chen *et al.* 2007). Despite this, the actual cytosolic Na^+ to K^+ ratio in barley roots does not seem to properly explain salinity-stress induced growth reduction (Kronzucker *et al.* 2006).

1.3.5.2.1.1 Pathways for initial entry to the root

Concentration and voltage favour passive entry for Na^+ from the soil into the root cortical cytoplasm (Cheeseman 1982). However, net accumulation of Na^+ within the cell is due to the difference between passive influx and active efflux. Thus, maximising the ability of the plant to exclude Na^+ from entering root cells is important to salt tolerance (Schubert and Läuchli 1990).

Some discrepancy can be observed in estimates of the rate of Na^+ influx into root cells. Much of this may be explained by the method of measurement utilized in the experiment. When short timecourses are utilized (<3 min) estimates in the range of 0.5-2.0 μmol g^{-1} FW min^{-1} with 50 mM external Na^+ have been found in wheat (Davenport 1998), rice (L. Wang, R. Davenport and M. Tester, unpublished results; D. Plett and M. Tester unpublished results) and *Arabidopsis* (Essah *et al.* 2003). Lower estimates published prior to the studies cited above (Zidan *et al.* 1991; Elphick *et al.* 2001) probably did not take into account the rate of efflux which becomes significant after about 3 min of exposure of the cell to Na^+ (Tester and Davenport 2003).

It is apparent that the rate of Na^+ unidirectional influx in halophytes may be lower than those of glycophytes. The halophytic dicotyledon, *Spergularia marina* was found to have a low rate of influx 0.24 μmol g^{-1} FW min^{-1} at 100 mM external Na^+, significantly lower than those of glycophytes (Cheeseman *et al.* 1985). Similar measurements of halophytic monocotyledons have produced similar results with influx in *Triglochin maritima* being estimated at 0.065 and 0.21 μmol g^{-1} FW min^{-1} at 100 mM external Na^+ (Jefferies 1973) and 0.13 μmol g^{-1} FW min^{-1} at 74 mM Na^+ in *Eleocharis uniglumis* (Shepherd and Bowling 1979).

1.3.5.2.1.1.1 The Ca^{2+}- sensitive pathway

Addition of up to 10 mM Ca^{2+} to the external solution will (generally) reduce the toxic effects of Na^+ through a complex set of effects (Cramer 2002; El-Hamdaoui *et al.* 2003). This effect is, at least partly, due to the inhibition of unidirectional Na^+ influx by Ca^{2+} (also of note is the stimulation of K^+ influx by Ca^{2+}). Interestingly, Ca^{2+} also reduces K^+ efflux stimulated by high Na^+ levels (Cramer *et al.* 1985, Shabala *et al.* 2006).

Liu and Zhu (1998) have interpreted these effects in relation to the SOS signalling pathway. In this model elevated Na^+ stimulates a rise in cytosolic Ca^{2+} which activates SOS3 leading to a change in expression and activity of Na^+ and K^+ transporters. Since *sos3* mutants produce a mutant form of SOS3, they are less sensitive to Ca^{2+} and thus require higher levels of external Ca^{2+} for normal root elongation (Horie *et al.* 2006). However, in wheat, external Ca^{2+} inhibits unidirectional influx of Na^+, which suggests that Ca^{2+} may not necessarily be involved in a signalling pathway (Davenport and Tester 2000).

It is possible that the means of Ca^{2+}-sensitive Na^+ influx is non-selective cation channels (Amtmann and Sanders 1999; Tyermann and Skerrett 1999; White 1999; Davenport and Tester 2000; Demidchik *et al.* 2002, Demidchik and Maathuis 2007). There are many candidates for these channels including the cyclic nucleotide-gated channels (CNGCs) (Leng *et al.* 2002, Gobert *et al.* 2006) and glutamate-activated channels (GLRs) (Cheffings 2001; Lacombe *et al.* 2001, Demidchik *et al.* 2004, Qi *et al.* 2006). Interestingly, the evidence for (Maathuis and Sanders 2001; Essah *et al.* 2003; Demidchik and Tester 2002; Demidchik *et al.* 2004) or against (Leng *et al.*

2002) these channels being the non-selective cation channels seems to depend on whether the experiments are performed in plants or in heterologous systems.

Another important gene to mention in this discussion is *LCT1* from wheat, which, when expressed in yeast, leads to an increase in cation influx and hypersensitivity to Na^+ (Schachtman *et al.* 1997; Clemens *et al.* 1998; Amtmann *et al.* 2001). Addition of external Ca^{2+} reduced Na^+ influx and sensitivity, but the cation profile influxed by LCT1 resembled the profile of endogenous ion transport in yeast, suggesting LCT1 may be stimulating the ion transporters already present (Amtmann *et al.* 2001).

1.3.5.2.1.1.2 Ca^{2+}-insensitive pathway

It is possible that Ca^{2+}-insensitive influx of Na^+ is partially due to a component of the Na^+ influx through non-selective cation channels, as the inhibition of Na^+ by Ca^{2+} is partial (Davenport and Tester 2000). There are several other potential transporters mediating this influx, including those encoded by the *HKT*, *KUP* and *HAK* gene families (Platten *et al.* 2006, Grabov 2007).

TaHKT2;1 is a Ca^{2+}-insensitive transporter from wheat (Schachtman and Schroeder 1994, Tyerman and Skerett 1999), which acts as a high affinity Na^+/K^+ symporter in *Xenopus oocytes* or yeast and in high Na^+ concentrations catalyses low affinity Na^+ uniport (Rubio *et al.* 1995). A screen to identify gene knockouts in *Arabidopsis* complementary to *sos3* revealed several individuals with reduced AtHKT1;1 activity, thus it was suggested the gene is involved in Na^+ influx (Rus *et al.* 2001). It was also suggested that AtHKT1;1 is under control of SOS3 and may simply be suppressed under elevated Na^+ conditions in wild type plants (Zhu 2002). Further studies show the *athkt1;1* mutant has no change in Na^+ influx from wild-type, suggesting another function for AtHKT1;1 (Berthomieu *et al*. 2003, Davenport *et al.* 2007) (see below). However, there are 9 *HKT* genes in rice (see below) and *OsHKT2;1* has been shown to regulate Na^+ uptake into roots under K^+-starvation conditions as it appears Na^+ can partially replace the function of K^+ (Horie *et al.* 2007). Interestingly, similar high-affinity Na^+ uptake was observed in K^+-starved barley roots, but when heterologously expressed in yeast, *HvHKT2;1* was shown to mediate Na^+ (or K^+) uniport, Na^+-K^+

symport, or a mix of both, depending on the construct from which the transporter was expressed (Haro *et al.* 2005).

1.3.5.2.1.1.3 Bypass flow

Apoplastic leakage may be a significant Na^+ entry point for some salt-sensitive plants such as rice, where external Ca^{2+} has little effect on Na^+ uptake and salt tolerance (Yeo and Flowers 1985; Yeo *et al.* 1987). Using an apoplastic dye, it was observed that rice plants with high shoot Na^+ accumulation had high apoplastic water flow (Yadav *et al.* 1996; Yeo *et al.* 1999), indicating leaks in endodermis at root branch points, root apices or simply through permeable endodermis. This pathway also varies between species, with bypass flow in rice being 10 times greater than that of wheat (Garcia *et al.* 1997). This information meshes well with classical observations that the Casparian band is two to three times wider in halophytes than non-halophytes (Poljakoff-Mayber 1975; Peng *et al.* 2004) and salinisation of cotton enhances its formation of the Casparian band and exodermis (Reinhardt and Rost 1995). Apoplastic leakage is reduced by addition of silicon to the growth media, apparently because silicon deposits decrease the gaps in the endodermal and exodermal layer of the rice root (Gong *et al.* 2006).

1.3.5.2.1.1.4 Na^+ efflux out of the root

Maximising efflux of Na^+ from the root may be just as important to improving salt tolerance as minimising influx. Efflux may occur through an Na^+/H^+ antiporter (Blumwald *et al.* 2000), but evidence for this transporter can be difficult to find (Mennen *et al.* 1990). This transporter has been proposed to operate in influx and efflux depending on Na^+ concentration of the growth medium, while stoichiometry of the Na^+/H^+ exchange also depends on the energy costs of the plasma membrane ATPase extrusion of H^+ versus the actual cost of moving an Na^+ ion out of the root cell (Briskin *et al.* 1991; Briskin *et al.* 1995). Addition of Na^+ elevated the transcription of H^+-translocating ATPases in rice and tobacco roots (Niu *et al.* 1996; Zhang *et al.* 1999), but these results have yet to be linked to the increased requirement of the Na^+/H^+ antiporter for H^+-extrusion.

The *Arabidopsis* genome does not encode any Na^+-extruding ATPases as are found in algae (Gimmler 2000) and fungi (ENA1 from *Physcomitrella patens*, see

Benito *et al.* 2002; Benito and Rodriguez-Navarro 2003). A *Ppena1* knockout line had a 40% decrease in growth and significantly lower K^+/Na^+ ratio compared to wild-type moss grown at 100 mM NaCl (Lunde *et al.* 2007), indicating expression of these pumps in crop plants may increase their salt tolerance.

1.3.5.2.1.1.5 Control of net uptake

The rate of unidirectional Na^+ influx is significantly higher than the net rate of influx and this indicates that significant rates of efflux must exist (Jacoby and Hanson 1985; Davenport *et al.* 1997). The fact that unidirectional influx is so high in glycophytes is related to the non-selectiveness of most of the Na^+ transporters (Demidchik *et al.* 2002), which generally function in the uptake of other cations as well (e.g. Ca^{2+}, White and Davenport 2002 and NH_4^+, White 1996). Also, the rate of net uptake of Na^+ in glycophytes appears to be regulated more by the efflux of Na^+ by Na^+-selective Na^+/H^+ antiporters than it is by control of influx (Qui *et al.* 2002).

Net influx is not determined exclusively by the difference between unidirectional influx and efflux, rather internal controls of Na^+ level are also involved (Tester and Davenport 2003). Root Na^+ concentration differs much less significantly than does shoot Na^+ concentration amongst a variety of species and salt tolerance levels (e.g. rice and *Phragmites communis*, Matsushita and Matoh 1991). Root cells apparently detect internal Na^+ concentrations and control Na^+ transporters appropriately as evidenced by data from Munns (2002) which shows a non-linear relationship between root Na^+ concentration and external Na^+ levels. As well, shoot Na^+ concentration is higher than root Na^+ concentration due to the influx from roots and minor efflux levels, while the root may efflux Na^+ to the external media or to shoot tissues. Thus, alterations affecting the net uptake of Na^+ by the root may actually alter the shoot Na^+ levels more significantly. Reid and Smith (2000) showed that inhibiting unidirectional Na^+ influx with Ca^{2+} decreased shoot Na^+ concentration more significantly than root Na^+ concentration. Another pathway controlling Na^+ uptake appears to be external K^+ conditions as K^+ starved rice plants were shown to activate Na^+ uptake mechanisms including *OsHKT2;1* (Horie *et al.* 2007).

1.3.5.2.1.2 Control of xylem loading

An important concept in salt tolerance is that specific cell-types are required to perform different functions to minimise the transfer of Na^+ to the shoot tissues. In order to minimise Na^+ transfer to the shoot, the outer cortical cells of the root need to maximise Na^+ efflux to or minimise influx from the external media, while the inner stelar cells of the root need to maximise Na^+ influx from or minimise efflux to the xylem (Tester and Leigh 2001). For example, the salt-tolerance of a salt-tolerant wheat line and an amphiploid cross between wheat and the salt tolerant wheat grass, *Lophopyrum elongatum*, was related to the minimisation of Na^+ entry to the xylem from the root cortex (Gorham *et al.* 1990; Santa-Maria and Epstein 2001).

The mechanisms that control xylem Na^+ loading and the energetics of Na^+ transfer into the xylem are poorly understood. The loading of Na^+ to the xylem may be an active process, despite the counter-intuitiveness of this idea. Estimates of xylem sap Na^+ concentrations range from 1-10 mM using excised plants (Munns 1985; Shi *et al.* 2002) and spittlebugs (Watson *et al.* 2001), whereas estimates of root cytoplasmic Na^+ concentrations have been made at 10-30 mM using x-ray microanalysis (Koyro and Stelzer 1988) or ion-sensitive electrodes (Carden 1999). An estimate of 100 mV negative inside xylem parenchyma cells relative to the xylem has been made (DeBoer 1999; Wegner *et al.* 1999); the energy difference is mainly a result of the potential difference across the plasma membrane. This situation would create active Na^+ transport into the xylem, detrimental for the plant, but supported by the report that the SOS1 Na^+/H^+ antiporter is preferentially expressed in the xylem symplast boundary in roots and *sos1* mutants accumulate less shoot Na^+ than wild-type (Shi *et al.* 2002).

However, if stelar cytosolic Na^+ levels were closer to 100 mM (Harvey 1985) and xylem Na^+ was closer to 2 mM (Munns 1985), passive leakage of Na^+ to the xylem would be favoured. Alternatively, Na^+ loading could be active at low external Na^+ concentrations and passive at high external Na^+ concentrations (Shi *et al.* 2002).

Xylem loading is controlled by ABA, in the case of K^+ and Cl^- (Roberts 1998, Gilliham 2002). However, control of Na^+ loading by ABA is much less studied. ABA does stimulate H^+ extrusion to the xylem (Clarkson and Hanson 1986), which could stimulate the Na^+/H^+ transport of Na^+ to the xylem.

Other candidates for the control of Na^+ loading to the xylem include the plasma membrane H^+-translocating ATPases, one of which, when knocked out, increases salt sensitivity and shoot Na^+ concentrations (Vitart *et al.* 2001). Inositol stimulates Na^+ transfer to the shoot in *Mesembryanthemum crystallinum*, possibly as a means of lowering osmotic potential in shoots during drought stress (Nelson *et al.* 1999).

1.3.5.2.1.3 Retrieval from the xylem

Removal of Na^+ prior to its arrival in the shoot has been proposed to occur in the mature root (Kramer 1983), mesocotyl (Drew and Läuchli 1987), base of the shoot (Matsushita and Matoh 1992), mature extended shoot (Blom-Zandstra *et al.* 1998) or internodal tissues (Wolf *et al.* 1991). One possibility is that a Na^+-permeable, inwardly rectifying channel in the xylem parenchyma cells moves the Na^+ out of the xylem into the cytosol (Wegner and Raschke 1994). However, the suggestion that the Na^+/H^+ antiporter functions in reverse in this capacity under high Na^+ (Lacan and Durand 1996) is highly unlikely to be thermodynamically possible (Tester and Davenport 2003).

Based on the observation that *sos1* mutants have lower shoot Na^+ concentrations than wild-type at modest salinity and higher shoot Na^+ concentration at high salinity (100 mM), Shi *et al.* (2002) proposed that SOS1 could act as a Na^+-scavenging mechanism at the root xylem-symplast interface. It has been suggested that AtHKT1;1 may have variable activity at different solute levels (Rubio *et al.* 1995). However, the likelihood of *Arabidopsis* plants surviving the experimental conditions of 100 mM Na^+, seriously damage the validity of this idea. The data of Berthomieu *et al.* (2003) suggest that AtHKT1;1 is responsible for Na^+ retrieval from the xylem, which would be the reason the *athtk1;1* mutant has elevated shoot Na^+ levels (see 'recirculation in the phloem').

A recent flurry of information on the function of the *HKT* gene family (Platten *et al.* 2006) has shown that members of the family are indeed responsible for Na^+ retrieval from the xylem and reducing transfer to the shoot tissue. *AtHKT1;1* has been shown to be localised to the stele (Sunarpi *et al.* 2005) and it was shown that *athkt1;1* knockout lines had increased levels of Na^+ in the xylem sap (Sunarpi *et al.* 2005). Flux measurements in *athkt1;1* knockouts showed AtHKT1;1 functions in root accumulation

of Na^+ and retrieval of Na^+ from the xylem, but is not involved in root influx or recirculation in the phloem (Davenport *et al.* 2007). Somewhat counter-intuitively, overexpression of *AtHKT1;1* via it's native promoter resulted in no change in shoot and root Na^+ accumulation, but did result in a decrease in Na^+ tolerance (Rus *et al.* 2004). Large variation in Na^+ accumulation has been observed among *Arabidopsis* ecotypes and some of this variation was explained by a deletion found in the *AtHKT1;1* promoter region in two independent ecotypes, both of which overaccumulate Na^+ in the shoot tissue (Rus *et al.* 2006). Interestingly, this deletion also resulted in increased Na^+ tolerance, indicating there is a complex relationship between Na^+ accumulation and Na^+ tolerance.

Rice has a similar mechanism of xylem retrieval of Na^+. Genetic analysis revealed an important K^+-homeostasis QTL called *SKC1* (Lin *et al.* 2004). The *SKC1* gene was cloned and found to be *OsHKT1;5* (Ren *et al*. 2005). Heterologous expression revealed it was a Na^+ transporter and whole plant analysis indicated it functions in the root xylem parenchyma to retrieve Na^+ from the xylem stream thereby reducing Na^+ accumulation in the shoot (Ren *et al.* 2005).

Flux analysis of a salt-tolerant durum wheat landrace called line 149 revealed two individual traits which provide sodium exclusion to the line are decreased Na^+ transfer to the shoot and increased Na^+ retrieval to the leaf sheath tissue (Davenport *et al.* 2005). Two previously mapped QTLs, *Nax1* and *Nax2*, linked to salt-tolerance in the line, were found to control the two transport traits (James *et al.* 2006). The *Nax2* locus was found to coincide with a sodium transporter related to *OsHKT1;5* in rice, and this gene was shown to be responsible for removal of Na^+ from the xylem in the roots (Byrt *et al.* 2007).

Anoxia in maize roots seems to increase the transfer of Na^+ to shoots at modest salinity, while it has an inhibitory effect at higher Na^+ concentrations (Drew and Dikumwin 1985). Also, transfer cells may be involved in Na^+ removal from the xylem as subjection of maize and bean to modest salinity has been observed to stimulate transfer cell induction (Yeo *et al.* 1977; Kramer *et al.* 1977).

1.3.5.2.2 Recirculation in the phloem

Despite the widely held idea that movement of Na^+ from the shoot through the phloem to the roots is negligible, studies in lupin (Munns *et al.* 1988), *Trifolium alexandrium* (Winter 1982), sweet pepper (Blom-Zandstra *et al.* 1998) and maize (Lohaus *et al.* 2000) indicate significant recirculation is occurring. As well, this process has been linked to salt-tolerance in the salt-tolerant species *Lycopersicon pennellii* (Perez-Alfocea *et al.* 2000) and *Phragmites communis* (Matsushita and Matoh 1991), when compared to their close salt-sensitive relatives.

Also, *sas1* mutants accumulate two to seven times the Na^+ in the shoot as wild type *Arabidopsis* (while maintaining similar root Na^+ levels), which may indicate a role in control of vascular loading and unloading for SAS1 (Nublat *et al.* 2001). Recently, a link has also been drawn between the *sas2* (sodium accumulation in shoots) mutant and inability to recirculate Na^+ in the phloem (Berthomieu *et al.* 2003). The *sas2* locus was found to correspond to the *AtHKT1;1* gene and the *sas* mutants were found to have lower phloem Na^+ concentration, higher shoot Na^+ concentration, lower root Na^+ concentration and increased Na^+ sensitivity. This led to the conclusion that AtHKT1;1 mediates Na^+ loading into the phloem in shoot tissue and unloading in root tissue and that the recirculation of Na^+ from the shoot plays a critical role in Na^+-tolerance (Berthomieu *et al.* 2003) However, the role of AtHKT1;1 has been shown to be in xylem retrieval and not in phloem recirculation (Davenport *et al.* 2007), thus transporters functioning in this regard have yet to be identified.

1.3.5.2.3 Compartmentation within the shoot

Low levels of Na^+ are often observed in young leaves and are sometimes attributed to their low rates of transpiration and short existence (Munns 1993), but also to protection processes (Jeschke 1984; Sibole *et al.* 2003; Wei *et al.* 2003). Movement of Na^+ could occur through phloem and xylem elements towards older 'sacrificial leaves' (Wolf *et al.* 1991) as has been described for other solutes (Pate *et al.* 1979). Using autoradiography, P has been observed to move throughout the plant from a source leaf, while Na^+ did not enter the new leaves and roots (Marschner 1995). Preferential Na^+ accumulation has been observed in leaf epidermal cells, possibly due to non-selective cation channels (Karley *et al.* 2000). Similar observations have been

made in bundle sheath cells which may be a mechanism to avoid Na^+ accumulation in more photosynthetically important cells (Stelzer 1981; Karley *et al.* 2000). Two putative sodium transport genes related to *OsHKT1;4*, were found to be in the *Nax1* (see above) chromosomal region, and these transporters were responsible for retaining Na^+ in the sheath tissue (Huang *et al.* 2006). Given that *HKT* genes have been shown to function in xylem retrieval in several species, it seems likely that homologs of this gene will be identified as being important to Na^+ tolerance in other cereal species, like rice and barley.

Discontinuous distribution of Na^+ within the leaf blades of rice has been observed using radionuclide tracers (Yeo and Flowers 1982). Sodium accumulated in the older leaves before the younger ones and to a decreased concentration in salinity tolerant varieties. Time of exposure and difference in growth rates between the lines could not explain the difference in Na+ distribution between the leaves. This has important ramifications for experimental sampling of leaf blade tissue in rice meaning valuable comparisons between cultivars may only be made when precisely the same leaf blade is harvested from each plant.

1.3.5.2.4 Salt glands

Salt glands are found in many halophytes. They function by moving salt into apoplastic space where it accumulates and is pushed out of the leaf by bulk flow of water due to the negative osmotic potential created by the Na^+ accumulation. This process is generally limited to salt marshes where water is not a limiting factor, but grasses have also been observed to have bicellular glands which secrete salt (Amarasinghe and Watson 1989; McWhorter *et al.* 1995). A wild relative of rice, *Porteresia coarctata* can grow on 25% sea water and has salt-secreting microhairs (Flowers *et al.* 1990). Recently it was shown that an epidermal bladder cell-less mutant of the common ice plant was more salt-sensitive than the wild-type since the EBCs play an important role as water reservoirs and in Na^+ sequestration (Agarie *et al.* 2007). Some halophytes get around the problem of using bulk flow to push out accumulated Na^+ by utilizing 'salt hairs' which accumulate salt and water then die, thereby reducing transpirational losses. Alternatively, hydathodes, which release water

by guttation during low transpiration periods, may be able to be adapted into salt glands through transformation of specific cell-types with Na^+ transporting genes.

1.3.5.2.5 Control of transpiration- stomatal closure

Robinson *et al.* (1997) attributed the salt-tolerance of *Aster tripolium* to its ability to close stomata in response to leaf apoplastic Na^+, whereas salt-sensitivity was attributed to an inhibition of stomatal closure by Na^+ in *A. amellus*. However, it seems that both glycophytes and halophytes tend to have reduced stomatal conductance in elevated Na^+ conditions (Ball 1988; James *et al.* 2002). Given the low productivity of crops in saline conditions is largely due to the low rates of transpiration imposed by osmotic stress, improving stomatal control is unlikely to improve crop salt-tolerance.

1.4 Cell-specific genetic technology

1.4.1 Gene traps

Gene identification has relied on mutagenesis, which interrupts gene function and results in a phenotype. This method depends on the creation of an obvious phenotype from the mutation, which is problematic for two main reasons. First, many genes are functionally redundant, thus despite knocking out one gene another may be fully or mostly redundant and no obvious phenotype will result. Second, genes which are expressed temporally, spatially or conditionally are easily missed by mutation screens which do not, by necessity, examine all aspects of a mutant plant (Springer 2000).

To address these issues, gene trapping was developed and has led to the functional characterisation of many genes, which have been previously difficult to identify and properly characterise. Twenty-five years ago a system was developed in bacterial genetics that utilised random insertions of the *lacZ* reporter gene in the genome to identify genes (Casadaban and Cohen 1979). LacZ protein was produced when it was inserted next to a gene. This 'tag' could then be utilised to identify the gene's sequence and the cellular localisation of the protein it encodes. Three main types of gene traps have been developed: gene traps, promoter traps, and enhancer traps.

1.4.1.1 Gene trapping

Gene traps contain a promoterless reporter gene (e.g. Green Fluorescent Protein [GFP] or β-glucoronidase [GUS]) along with one or more splice acceptor sites preceding the reporter gene. This sequence is randomly inserted in the genome (using T-DNA or transposable elements) and when the insertion is within an intron a transcriptional fusion results and the reporter gene is expressed (Springer 2000).

This system was used successfully in *Arabidopsis* where a variety of genes were identified through the random insertion of a neomycin phosphotransferase II (*nptII*) reporter gene (Babiychuck *et al.* 1997). Twenty of the lines were analyzed and twelve were identified as insertion mutants, which led to the authors suggesting an 80% success rate for tagging of *Arabidopsis* genes. Chin *et al.* (1999) utilized the *Ac/Ds* transposon system to deploy a gene trap in rice, which resulted in 80% of the plants containing *Ds* elements that transposed away from the T-DNA and 30% transposing in the next generation. In an analysis of *Arabidopsis* genome function, 12-41% gene trapping efficiency was achieved using the firefly luciferase gene as the reporter gene (Yamamoto *et al.* 2003). Stress-responsive genes have been identified by gene trapping in rice, where after a treatment of 5°C, 53 lines displayed an up-regulation of GUS activity and 9 lines showed a down-regulation of GUS activity (Lee *et al.* 2004). Notably, of the 62 genes up- or down-regulated by cold treatment, 16 were also responsive to ABA treatment. Gene trapping was used to identify secreted and membrane spanning proteins by comparing GUS activity before and after treating plants expressing gene traps with tunicamycin. This treatment allowed the lines expressing trapped proteins that had been routed through the secretory pathway to express *GUS* (Groover *et al.* 2003).

1.4.1.2 Promoter trapping

Promoter traps also contain a promoterless reporter gene, but promoter traps must be inserted in an exon to form a transcriptional fusion which will result in reporter gene expression (Springer 2000).

Promoter trapping was used successfully in *Arabidopsis* to identify genes involved in embryogenesis. A screen using T-DNA inserts containing the *GUS* reporter gene identified 74 of 430 lines with GUS activity in the siliques (Topping *et*

al. 1994). Genes expressed specifically in the root tip, cotyledon, shoot and root apices and in the root cap were identified in a screen for genes involved in polar organisation of embryos and seedlings (Topping and Lindsey 1997). Genes specific to the flower, fruit and seedling of tomato were identified in a screen using transposon dissemination of the *GUS* reporter gene (Meissner *et al.* 2000). Promoter traps were also used successfully in the identification of the acyl-CoA oxidase gene (*AtACX3*) (Eastmond *et al.* 2000), the *EXORDIUM* gene in embryos, apical meristems and young leaves (Farrar et al 2003) and the nucleic acid helicase gene (*HVT1*) in the tapetum and vascular tissue (Wei *et al.* 1997).

Stress responsive genes were identified in rice using a luciferase reporter gene. Genes responding to sugar, salt and ABA stimuli were identified in 753 of 20, 261 transformed lines (Alvarado *et al.* 2004). Promoter trapping was used in *Lotus japonicus* to identify novel root and nodule associated genes by screening for changes in GUS activity following inoculation with the symbiont *Mesorhizobium loti* (Buzas *et al.* 2005).

1.4.1.3 Enhancer trapping

In enhancer traps, the reporter gene is fused to a minimal promoter (e.g. minimal 35S promoter) containing a TATA box and transcription start site, but transcription does not occur unless the T-DNA lands close to an enhancer element which activates transcription of the reporter gene (Springer 2000). Enhancer traps have a higher rate of reporter gene expression since insertion within a gene is not required for expression, but this creates more difficulty when the trapped gene is to be identified (Springer 2000). Enhancer traps have been used for many years in *Drosophila* to identify genes which have been difficult to characterise by classical mutation analysis (Brand and Perrimon 1993; Phelps and Brand 1998; Duffy 2002).

Michael and McLung (2003) used the enhancer trap system to identify genes involved in circadian clock regulation in *Arabidopsis*. In 36% of the lines expression of luciferase was circadian-responsive indicating the tagged gene was involved in the function of the circadian clock. An interesting study utilising the enhancer-trap system identified three genes within the genome of the maize pathogen, *Ustilago maydis*, which were plant-induced (Aichinger *et al.* 2003).

The addition of minimal promoter elements upstream of *GUS* within a T-DNA vector doubled GUS activity in a comparative study of the promoter and enhancer trap systems in rice (Jeong *et al.* 2002). Using the *Ac/Ds* transposition system the *Ac/Ds* and reporter gene may be transformed as one cassette into plants (Greco *et al.* 2003) or *Ds* element and reporter gene may be transformed into one line and the *Ac* transposase element may be transformed into a separate line. This allows controlled dispersal of the enhancer trap into the genome following crossing of the two types of transgenic plants, especially in plants with low transformation efficiencies. This technique was used by Ito *et al.* (2004) who found 6% of their F_2 lines had transpositions of the enhancer element. Tissue-specific GUS activity was identified and a relationship was identified between frequency of transposition and panicle maturity.

1.4.2 GAL4-GFP enhancer trap

An important adaptation of the enhancer trap utilises the yeast transcription activator *GAL4* as a type of reporter gene. The GAL4 system was first used in *Drosophila* and continues to be an important tool in the functional characterisation of *Drosophila* genes (Brand and Perrimon 1993; Phelps and Brand 1998; Duffy 2002). A modified *GAL4* gene (fused to VP16) has been fused to a minimal CaMV 35S promoter and a modified *GFP* gene which is driven by the GAL4 upstream activating sequence (UAS) (see http://www.plantsci.cam.ac.uk/Haseloff/Home.html, Engineer *et al.* 2005). When this construct is positioned under control of an endogenous enhancer or promoter element, *GAL4* is activated and in turn activates the UAS which drives expression of the *GFP* reporter gene.

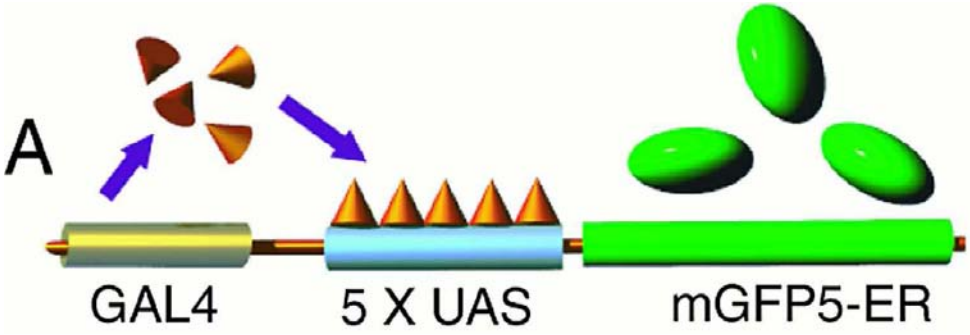


Figure 1.3: The GAL4-GFP enhancer trap (from Tester and Leigh 2001).

1.4.2.1 Construction

Several modifications needed to be made to the system to transfer it from *Drosophila* to *Arabidopsis*. The yeast *GAL4* gene was fused to the activation domain of the herpes simplex virus (VP16) to allow for expression in plants (Haseloff and Hodge 1997) as expression was somewhat unreliable previously (Galweiler *et al.* 2000). The jellyfish green fluorescent protein (GFP) also required some modification to function optimally in *Arabidopsis*. These alterations included the removal of a cryptic intron, modification of *GFP* to allow GFP to localize in the endoplasmic reticulum (ER) instead of the nucleus, improving the thermotolerance of the GFP protein and altering its spectral properties to allow for better imaging (Haseloff 1999; Haseloff *et al.* 1997).

1.4.2.2 Use in *Arabidopsis*

One of the most important uses of this enhancer trap system is the ability to express a gene of interest ectopically (Springer 2000). A library of *Arabidopsis* enhancer trap lines has been generated, which contains lines with various cell-type specific GFP fluorescence patterns or other interesting patterns (see http://www.plantsci.cam.ac.uk/Haseloff/Home.html, also Poethig lines, University of Pennsylvania) indicating the T-DNA may have landed next to a gene expressed in specific cell-types. A gene of interest can then be expressed in a specific cell-type by fusing the gene to the UAS elements and retransforming the line or by crossing the line containing the enhancer trap with one containing the UAS/gene of interest fusion (Springer 2000).

The system has been used to trans-activate genes of interest in *Arabidopsis* roots. Whole cell calcium currents in epidermal cells in the root apex, mature epidermal cells, cortical and epidermal cells from the elongation zone and mature pericycle cells were monitored using *Arabidopsis* lines expressing *GFP* in specific cell-types (Kiegle *et al.* 2000a). The system was used to target the calcium reporting protein, aequorin, to specific cell types to make *in vivo* measurements of changes in cytosolic free calcium concentrations in specific cell-types in response to drought, salt and cold stress (Kiegle *et al.* 2000b). In another study, root xylem pole pericyle cell-specific and young lateral root primordial-specific lines were chosen to express

diphtheria toxin in order to ablate those specific cells to study the effect on root branching development (Laplaze *et al.* 2005).

1.4.2.3 Use in rice

The GAL4 enhancer trap system has recently been introduced into rice. Using *uidA* (*GUS*) as a reporter gene, 31,443 GAL4/VP16-UAS transgenic lines were generated (Wu *et al.* 2003). The authors reported that 94% of the transformants contained the T-DNA insert, 42% of which were single T-DNA insertions. The flanking sequences in 200 lines with GUS activity were examined and almost all identified genes annotated in the rice sequence databases.

The system has also been recently introduced into rice with GFP as the reporter protein. Over 10,000 transformants were generated, and 1,982 T_0 adult lines, 2,684 seed lines and 2,667 T_1 seedling lines were screened for GFP fluorescence with an overall expression rate of approximately 30% (Johnson *et al.* 2005). Cell-type specific expression patterns were observed in stamens, carpels, and lodicules in flowers, root caps, epidermal cells and protoxylem of roots. Transactivation of the *uidA* reporter gene in lines expressing *GAL4* in a cell-type specific manner, resulted in similar *GFP* and *uidA* expression patterns (Johnson *et al.* 2005), raising the possibility of expressing transgenes in cell-type specific manner in rice. Alternatively, specific genes could be silenced in specific cell-types using RNA interference (RNAi) techniques (Miki and Shimamoto 2004) or in combination with a system to allow control of induction of gene expression (Chen *et al.* 2003), providing transgenic plants for important physiological studies of genes of interest.

Utilisation of the GFP reporter system has several advantages over the GUS reporter system. The GUS system requires tissue destruction to perform the histochemical GUS stain, whereas the GFP system may be screened under a microscope and thus plants may be further propagated and screened at a later date. The staining protocol for identification of GUS activity can lead to staining artefacts, skewing actual expression patterns. The GFP system also allows the isolation of single cells and the transactivation of genes in single cells which is not possible with the GUS system (Johnson *et al.* 2005).

1.4.3 Chemically-inducible gene expression

1.4.3.1 Introduction

The development of genetic tools to control gene expression allows transgene expression to be controlled temporally and, in some cases, spatially and quantitatively. Transgenic studies have often been hampered by the negative effects constitutive expression of some transgenes have on plants. Controlling the induction of genes through external means can overcome this problem and allow study of transgenes in plants (Wang *et al.* 2003). Endogenous chemically inducible promoters exist for control of plant gene expression including the benzothiadiazole-inducible *PR-1a* system (Gorlach *et al.* 1996) and the safener-inducible *In2-2* system (De Veylder *et al.* 1997). However, due to the endogenous nature of these systems their application tends to have pleiotropic effects in plants and can affect plant growth (Padidam 2003). Thus, use of non-endogenous chemical inducers to control gene expression is preferable.

1.4.3.2 Bacterial repressor-operator systems

1.4.3.2.1 Tetracycline regulation

Two gene expression regulation systems isolated from *Esherichia coli* are regulated by tetracycline. The tetracycline-inducible system uses a constitutively expressed Tet repressor (*TetR*) element which produces TetR that binds to a target promoter causing a conformational change which inhibits expression of the gene of interest (Gatz *et al.* 1992). The addition of tetracycline inhibits the binding of TetR to the target promoter and transcription of the gene of interest occurs. The system can increase expression of a *uidA* reporter gene 500-fold with the addition of tetracycline (Gatz *et al.* 1992). However, toxicity of the system to plants due to the high level of the intracellular repressor is a drawback to the system (Corlett *et al.* 1996). The system was used successfully to shut down transcription of a transgene to study the decay rates of mRNA and protein levels (Weinmann *et al.* 1994).

The TetR repressor can be turned into an activator element using the VP16 activation domain from Herpes simplex virus (Gossen and Bjard 1992), thus requiring tetracycline application to inhibit the expression of the target gene. This system allowed more stringent control of the transgene and was used successfully in *Arabidopsis* (Love *et al.* 2000).

1.4.3.2.2 Pristinamycin regulation

Utilising the same principle as the tetracycline-regulated system, pristinamycin-inducible and –repressible systems (derived from *Streptomyces coelicolor*) were developed for control of gene expression (Frey *et al.* 2001). The system has yet to be proven to work at the whole plant level, but is promising as its promoter and operator module are separate. Thus tissue specific promoters may be replaced for the constitutive promoter allowing spatial and temporal control of gene expression (Frey *et al.* 2001).

1.4.3.3 Transcription activation systems from fungi

1.4.3.3.1 Copper induction

This system utilises the yeast copper detoxification-metallothionein gene by placing the *ace1* (activating copper-MT expression) transcription factor gene under control of a constitutive promoter (Mett *et al.* 1993). Application of $CuSO_4$, through foliar spray or root drenching, changes the conformation of ACE1 allowing it to bind to the ACE1-binding site thereby driving gene expression. The system successfully achieved a 50-fold increase in GUS activity and has been used successfully in *Arabidopsis* (RNA silencing), tobacco, and *Lotus corniculatus* (Mett *et al.* 1996; Potter et al 2001), despite the phytotoxicity of $CuSO_4$ at high concentration.

1.4.3.3.2 Ethanol induction

The ethanol-inducible system is derived from *Apergillus nidulans* and is comprised of two cassettes: *p35S:alcR* and *palcA:CAT* (Caddick *et al.* 1998). *p35S:alcR* consists of a constitutive promoter driving the *alcR* gene (coding the repressor AlcR) and *palcA:CAT* contains a minimal 35S promoter with the upstream activator region of the *alcA* promoter and a reporter gene (e.g. *CAT*) (Salter *et al.* 1998). The application of ethanol through vapours (Sweetman *et al.* 2002) or root drenching allows AlcR to bind to the *alcA* activator region and induce reporter gene expression (Caddick *et al.* 1998). Induction appeared to be rapid and reversible in *Arabidopsis* (Roslan *et al.* 2001), tomato (Garoosi *et al.* 2005) and *Populus* (Filichkin *et al.* 2006) and has been successfully combined with tissue-specific promoters to have spatial and temporal control over transgenes (Deveaux *et al.* 2003; Maizel and Weigel 2004). The ethanol induction system has also been used to drive double-stranded RNA

silencing of gene expression, which allows some of the pleiotropic effects of gene silencing driven by constitutive promoters to be avoided (Chen *et al.* 2003). However, drawbacks may be the volatility of ethanol, the induction of endogenously produced ethanol by anoxia and induction of other genes with ethanol application (Vreugdenhil *et al.* 2006). These drawbacks were especially evident in cell suspension culture conditions, so the receptor domain of the rat glucocorticoid receptor was fused to *AlcR* to make the switch responsive to dexamethasone when used in tissue culture (Roberts *et al.* 2005). AlcR also responds to other less volatile alcohols, ketones and acetaldehyde (Junker *et al.* 2003, Schaarschmidt *et al.* 2004), which may produce more controlled results than ethanol. Of note, this system has been hailed as the "first step toward a new green revolution" (Tomsett *et al.* 2004).

The ethanol switch was combined with the GAL4-UAS to use in combination with GAL4-GFP enhancer trap lines. This allows expression of transgenes to be controlled both spatially, by transactivation in enhancer trap lines, and temporally, by the ethanol switch (Jia *et al.* 2007, Sakvarelidze *et al.* 2007).

1.4.3.4 Steroid receptor-based activation of transcription

1.4.3.4.1 Glucocorticoid induction

The glucocorticoid receptor (GR) in animal cells resides in the cytoplasm and combines with heat-shock proteins (Dittmar *et al.* 1997). When a glucocorticoid (e.g. dexamethasone) binds to GR it is released from the complex and moves to the nucleus to regulate gene expression. The system was first applied in tobacco cells and upon dexamethasone application plants showed a 150-fold increase in gene expression (Schena *et al.* 1991). In plants, the hormone binding domain of GR was fused with the DNA-binding domain of yeast *GAL4* and the activation domain of VP16 and put under control of a constitutive promoter (Aoyama and Chua 1997). This complex (GVG) is constitutively expressed and bound in the cytoplasm (as in animal cells), until it is released by the glucocorticoid. GVG travels to the nucleus and induces expression of the gene of interest, which is cloned downstream of a minimal 35S promoter and six copies of the GAL4 upstream activating sequence (GAL4 UAS). The system was modified for use in rice by reducing the UAS copies to four and replacing the 35S with *Gos2*, a rice promoter (Ouwerkerk *et al.* 2001).

The LhG4/pOp system utilises a two component switch for control of gene expression via dexamethasone application. The artificial transcription factor LhG4 was fused to the GR domain (see above), thus with the application of dexamethasone LhG4 binds to the target promoter (pOp) and transcription of the gene-of-interest is induced. The switch has been successfully in *Arabidopsis* (Craft *et al.* 2005) and tobacco (Samalova *et al.* 2005). It also has been utilised as an enhancer trap (Rutherford *et al.* 2005) and can be used for tissue specific transactivation of genes (Baroux *et al.* 2005).

The system has been used successfully in regulated overexpression of genes (Aoyama and Chua 1997), the expression of toxic heterologous genes (Kawai-Yamada *et al.* 2001), activation of endogenous genes (Ren *et al.* 2002), developing antibiotic marker-free systems (Zuo *et al.* 2001) and the construction of a high level mRNA amplification system (Mori *et al.* 2001). However, dexamethasone has been shown to cause some growth defects (Amirsadeghi *et al.* 2007) and induce defence-related genes (Kang *et al.* 1999).

1.4.3.4.2 Estrogen induction

The estrogen gene induction system consists of a transcription factor (XVE) made up of the binding domain of a bacterial repressor (LexA), the transactivating domain of VP16 and the regulatory region of the human estrogen receptor (Zuo *et al.* 2000). β-estradiol activates the constitutively produced XVE, which binds to a promoter consisting of a minimal 35S promoter and eight copies of the *Lex* operator. The system has high efficiency and specificity making it an attractive tool for control of gene expression (Zuo *et al.* 2000).

1.4.3.4.3 Ecdysone agonist induction

Ecdysone agonists, including RH5992 (tebufenozide) and methoxyfenozide, have been used to induce gene expression with their respective systems. Tebufenozide utilises the ecdysone receptor of *Heliothis virescens* (EcR) as a part of a transactivator which activates the gene of interest (Martinez *et al.* 1999). The methoxyfenozide inducible system uses the EcR and GAL4 binding domain to induce expression of the promoter comprised of five copies of the GAL4 response element (Padidam and Cao 2001). The attractiveness of this system is that both of these chemicals are currently utilised as agrochemicals in field applications making it a possibility for field testing.

The system has been used successfully in transcriptional interference studies (Padidam and Cao 2001).

1.4.3.5 Dual control system

Bohner *et al.* (1999) combined the tetracycline repressible system with the GVG (glucocorticoid) system to create a gene expression system which is activated by dexamethasone and repressed by tetracycline. By combining the TetR, GR and VP16 domains the protein fusion (TGV) was designed which controlled the expression of a reporter gene fused to modified *tet* operators. The system has been used to drive reporter gene expression (15 – 100% of the 35S promoter driven expression), but has similar drawbacks to the GVG (dexamethasone) system (Bohner and Gatz 2001). It has been used to study local expression of the isopentenyl transferase gene by inducing constitutive expression of the gene with dexamethasone and then turning the gene off locally with tetracycline (Faiss *et al.* 1997).

1.5 Project Aims

It is known that the native expression of most transporters in plants is specific to certain tissue-types and is often upregulated by stress. It is intuitive then that expressing a transporter constitutively would not allow maximum benefit to the plant to be achieved.

Hypothetically, expression of Na^+ transporters in root cortical cells to maximise efflux to or minimise influx from the soil, and in root stelar cells to maximise influx from or minimise efflux to the xylem stream will minimise transfer of sodium to the xylem stream and the shoot. Also, expression of transporters specifically during periods of stress would minimise expense of a plant's energy during periods of low stress and thereby maximise plant productivity. Thus, the aim of this project is to express Na^+ tranporters in specific root cell-types of rice, and induced by stress, to improve our understanding of Na^+ transport and Na^+ tranporters and to develop Na^+ tolerant rice. The following paragraphs describe more specific aims of this project.

A library of rice GAL4-GFP enhancer trap lines will be screened with a mild (i.e. agriculturally relevant) Na^+ stress in order to identify regulatory elements which are

up- or down-regulated by Na^+ stress in specific cell-types. These regulatory elements could be used to express Na^+ transporters.

Vector constructs will be developed combining the GAL4 UAS with the ethanol inducible gene expression system. These constructs will be transformed into rice to enable cell-type specific, ethanol-inducible expression of Na^+ transporters.

Two Na^+ tranporters, AtHKT1;1 and PpENA1 will be expressed cell-type specifically in rice and the effects on Na^+ transport in the transgenic plants will be examined.

A method will be developed to study unidirectional influx into the roots of rice using the radioactive tracer $^{22}Na^+$. This method will be used to characterise the transgenic plants that will be developed.

CHAPTER 2: UNIDIRECTIONAL $^{22}Na^+$ INFLUX IN RICE

2.1 Introduction

The influx of Na^+ into root epidermal cells is passive, likely through voltage-independent non-selective cation channels, as it is energetically favourable due to concentration gradient and cellular voltage (Tester and Davenport 2003). Na^+ also enters plant roots apoplastically, entering through gaps in the Casparian band of the exodermis or at root branch points and apices. Rice appears to be subject to elevated apoplastic uptake of Na^+ with estimates that it is 10 times higher than in wheat (Garcia *et al.* 1997). It has been shown that the exodermal barrier is 30 times more permeable to apoplastic water flow than is the endodermal barrier (Ranathunge *et al.* 2003), thus influx of Na^+ into rice roots may be relatively unrestricted prior to reaching the endodermis. However, most of the Na^+ entering rice roots must be effluxed back to the soil to prevent toxic accumulation of Na^+ within the shoot.

Na^+ influx rate into roots is perhaps best measured using the radioactive tracer $^{22}Na^+$. This technique allows Na^+ entering the root to be quantified over a given time period, thus the rate of influx may be determined. Crucial differences in Na^+ influx were measured between *Arabidopsis* and its closely related Na^+-tolerant relative *Thellungiella halophila*, indicating that regulation of Na^+ influx can play an important role in reducing Na^+ transfer to the shoot (Wang *et al.* 2006). The characteristics of Na^+ influx can be explored using mutants and channel blockers in combination with $^{22}Na^+$ tracer analysis to describe the contributions of the various Na^+ uptake pathways to total uptake (Davenport and Tester 2000, Essah *et al.* 2003). For example, it was demonstrated that AtHKT1;1 is not involved in Na^+ influx in *Arabidopsis*, as *athkt1;1* knockouts showed no change in Na^+ influx rate from wild-type plants (Essah *et al.* 2003, Davenport *et al.* 2007). The technique was used to exclude two other HKT family members as possible Na^+ influx transporters when it was shown *Nax1* and *Nax2* from durum wheat (later shown to be homologs of *OsHKT1;4* and *OsHKT1;5*) function in retrieval of Na^+ from the xylem in the sheath and root (Davenport *et al.* 2005, James *et al.* 2006). Influx experiments have also been used to describe the effect of Ca^{2+} in

reducing Na^+ influx rate was quantified in maize (Zidan *et al.* 1991), bread wheat (Davenport *et al.* 1997) and in *Arabidopsis* (Essah *et al.* 2003).

The rate of unidirectional Na^+ influx is best measured within the first 2 to 5 min of exposure to $^{22}Na^+$, since this is the estimated time required for a cell to equilibrate with an external solution containing 50 mM Na^+ (Tester and Davenport 2003). At approximately three min a decrease in apparent Na^+ influx rate occurs due to a significant portion of the cellular Na^+ being actively effluxed to the external solution. Underestimations of Na^+ influx rate have been made in the past due to lengthy influx times (30 min: Zidan *et al.* 1991) or extremely long rinsing times which allows the cellular $^{22}Na^+$ to be effluxed to the rinse solution (2 x 10 min: Elphick *et al.* 2001).

Despite the fact that a large proportion of Na^+ uptake to the shoot in rice appears to be the result of apoplastic flow, the question remains whether variation exists between rice cultivars in terms of Na^+ influx. One recent study examined the $^{22}Na^+$ influx rate into *japonica* rice (Horie *et al.* 2007). It was shown that under K^+-starved and low Na^+ conditions, rice catalysed the passive influx of Na^+ (via OsHKT2;1) in an attempt to compensate for a K^+ deficiency. The experiments were conducted at 0 mM K^+ and 0.1 mM Na^+, thus were likely only applicable to microsites within most agricultural fields.

In an attempt to characterise Na^+ influx into rice roots, the protocol used to analyse unidirectional Na^+ influx in cereals was adapted for rice. Using this protocol, a group of *indica* rice lines was examined at an agriculturally relevant Na^+ concentration to determine if there were measurable differences in Na^+ influx rate in rice germplasm. Additionally, transgenic rice lines with altered root Na^+ transport properties were analysed to determine if the genetic modifications had altered Na^+ influx (see Chapter 6).

2.2 Materials and Methods

2.2.1 Plant material

The two cultivars studied in most experiments were Na^+-sensitive Nipponbare (*Oryza sativa* ssp *japonica*) and Na^+-tolerant Pokkali (*Oryza sativa* ssp *indica*). In Experiment 8 (Table 2.2), nine additional *indica* cultivars ranging in Na^+ accumulation

and tolerance were examined: Kalurundai, Nona Bokra, FL478, Kullurundai Vellai, IR29, IR28, IR36, NSICRC 106 and SAL208 (obtained from IRRI, The Philippines).

2.2.2 Growth conditions

All experiments used the following protocol for seed preparation and seedling growth. Prior to imbibition, seeds were sterilised with 70% ethanol for one minute, and excess ethanol was decanted. Sodium hypochlorite (30% of the purchased product – e.g. White King – which generates about 1% available chlorine) plus a few drops of Tween20 was added to the seeds for 30 minutes, then the seeds were washed thoroughly (at least five times) with RO water. Sterilisation was done in multi-well plates (using modified syringes to move solutions) or in tubes, depending on the number of individual lines being used.

Surface sterilised seeds were placed in deep cell culture dishes (Petri dishes) on filter paper with 5 mL RO water. The dishes were wrapped with Parafilm to reduce evaporation and contamination and placed in a growth chamber with 28°C days and 24°C nights (16 h full light and high humidity). Experiments 1 and 2 (Table 2.2) were conducted using seedlings grown on large Petri dishes for 10 d.

After five days of growth on dishes, seedlings were transplanted to 10 L hydroponics boxes fitted with aeration pumps and tops designed to hold 1.5 mL Eppendorf tubes (maximum 50 plants per box – even if top is designed to hold 100 tubes). Eppendorf tubes were prepared by cutting off approximately 7 mm of the bottom of the tube off (to leave a hole big enough to fit the rice seed through) and removing the cap. Seedlings were placed in the tube by gently pushing the shoot and seed through the hole in the bottom of the Eppendorf tube and lodging the seed in the bottom of the tube. The seedling was able to rest on the seed until it was big enough to support itself without falling into the tank. The solution was aerated to eliminate zones of nutrient depletion surrounding the roots. The seedlings were grown for two days in the tank before aeration was started.

To minimise nutrient depletion and microbial growth, the nutrient solution was replaced with fresh nutrient solution after the first week of growth, and then replaced every three to four days, depending on the size and number of plants per tank. Care

was taken to not splash growth solution on the plants as this would have affected ion concentration measurements of shoot tissue.

The seedlings were allowed approximately 7 d to grow on nutrient solution before stress application (i.e. stress was added with the first solution change). Experiments 3 through 8 (Table 2.2) were conducted using seedlings that had been grown in hydroponics for 14 d.

Five stock solutions (Table 2.1) were added to the required volume of RO water and the solution pH was not adjusted as it was approximately 5.5. The pH was not adjusted during plant growth since the solution was changed frequently.

Table 2.1: ACPFG growth solution for hydroponic rice culture.

Solution	Salts	Formula Weight	Final concentration (mM)
	Macronutrients		
1	NH_4NO_3	80	5
	KNO_3	101.1	5
2	$Ca(NO_3)_2 \cdot 4H_2O$	236.1	2
3	$MgSO_4 \cdot 7H_2O$	246.5	2
	KH_2PO_4	136.1	0.1
4	NOTE:No 4		
5	NaFe(III)EDTA	367.1	0.05
	Micronutrients		(μM)
6	H_3BO_3	61.8	50
	$MnCl_2 \cdot 4H_2O$	197.9	5
	$ZnSO_4 \cdot 7H_2O$	287.5	5
	$CuSO_4 \cdot 5H_2O$	249.7	0.5
	Na_2MoO_3	242	0.1

No solution 4 (silicon) was added to this growth solution as it had been observed to cause severe Fe^{3+} or Ca^{2+} deficiencies when added to new hydroponics tanks (constructed with silicon glue).

2.2.3 Pre-treatment

Experimental conditions are summarised in Table 2.2. Experiments 1 and 2 were conducted using roots excised 1 h prior to influx treatments. Excised roots were treated in unlabelled influx solution consisting of concentrations of NaCl, 0.5 mM KCl and 0.1 mM $CaCl_2$ activity (Table 2.2 - adjusted to compensate for the drop in Ca^{2+} activity from the addition of various Na^+ concentrations using the program Visual MINTEQ ver 2.40b – KTH, Department of Land and Water Resources Engineering, Stockholm, Sweden). Solutions were shaken gently (35 rpm) to keep solutions aerated and reduce boundary layer effects.

Experiments 3 to 8 (Table 2.2) were conducted using intact plants that had been treated in the hydroponic tanks for 2 or 5 d with the influx solution concentration of NaCl and the compensatory amount of $CaCl_2$. Hydroponic tanks were moved from the glasshouse to the flux bay 1 h prior to experimentation and remained on aeration throughout the experimental period prior to influx treatment.

2.2.4 Influx treatments

All experiments were conducted under a fluorescent light bank at 22°C to 26°C. Following pre-treatment excised roots were blotted dry with paper towels and placed in 50 mL of influx solution (various mM NaCl, 0.5 mM KCl and 0.1 mM $CaCl_2$ activity) labelled with 0.05 µCi/ml of $^{22}Na^+$ (Amersham/GE Healthcare, Sydney, Australia) on rotating (35 rpm) shakers (Ratek, Boronia, Australia).

The roots of intact plants were blotted dry after removal from hydroponic tank. Roots were placed in the influx solution by fixing the Eppendorf tube holding the plant to the side of the influx solution vessel using Power Tack adhesive (Acco, Australia) on shakers (35 rpm).

2.2.5 Rinse solution, time and temperature

Following influx treatment, excised roots were removed from the influx solution and extra influx solution was allowed to drip off roots. Roots were rinsed quickly (for a few sec) in a large volume (approx 1 l) of room temperature rinse solution (various mM NaCl, 0.5 mM KCl and 10 mM $CaCl_2$) to displace apoplastically bound $^{22}Na^+$ then rested in tea strainers in two successive rinses in 500 ml of ice cold

(4°C) rinse solution (2 min + 2 min) on shakers (35 rpm). Rinse solutions were changed every 4 h.

Intact plants were removed from the influx solution and extra influx solution was allowed to drip off roots. Roots were rinsed quickly in room temperature rinse solution then all roots were excised below the Eppendorf holder into tea strainers for two successive rinses in ice cold rinse solution (various rinse times) on shakers (35 rpm). An exception to this was in Experiment 3 (Table 2.2) which used RT (23°C) rinses. Shoot radioactivity was examined in Experiments 4 and 5 (Table 2.2).

2.2.6 Weighing, scintillation counter

After rinsing, roots were blotted dry with paper towels and immediately weighed in 6 ml scintillation vials (PerkinElmer, Melbourne, Australia). Four ml of scintillation fluid (Ecolume, MP Biomedicals, Sydney, Australia) was added to each vial and each vial was shaken and measured using a liquid scintillation counter (Beckman Coulter LS6500, Gladesville, Australia). Shoot samples (leaf sheaths and blades) were placed in 10 ml scintillation vials for weighing and 8 ml of scintillation fluid was added prior to counting. Immediately prior to and immediately following each experiment three 20 μL samples of the influx solution were placed into scintillation vials, scintillation fluid was added and shaken, to enable calculation of specific activity for each influx solution. (This allowed conversion of the counts of radioactivity in the roots to an amount of chemical Na^+, and thus a conversion to a flux.) Blank vials were also measured to allow subtraction of background radiation from the measurements of the samples.

Table 2.2: Summary of conditions in each experiment used to develop a unidirectional influx experimental protocol for rice. Highlighted in red are the important changes made to the protocol in each experiment.

Experiment #	Cultivars	Influx Concentration Na^+ (mM)	Influx Concentration Ca^{2+} (mM)	Time Points (min)	Pretreatment	Excised Roots/ Intact Plants	Rinse Time and Temperature	Rinse concentration Na^+ (mM)	Number of Reps	Growth Conditions	Leaf Samples	Figure #
1	Nipponbare, Pokkali	10, 50	0.16, 0.24	1, 2, 5, 10	1 hr, influx solution	Excised	2 min + 2 min ice cold	50	5	Petri Dishes	No	1
2	Nipponbare	10	0.16	0.25, 0.5, 0.75, 1, 2, 3, 5, 10	1 hr, influx solution	Excised	2 min + 2 min ice cold	50	6	Petri Dishes	No	2
3	Nipponbare	10	0.16	2, 10	2 d, ACPFG + 10 mM NaCl + 0.14 mM CaCl2	Intact	2 min + 2 min ice cold	10	8	Hydroponics	10 min	3
4	Nipponbare	10	0.16	2	2 d, ACPFG + 10 mM NaCl + 0.14 mM CaCl2	Intact	2 min + 2 min OR 0.5 min + 0.5 min, RT OR ice cold	10	6	Hydroponics	No	4
5	Nipponbare	10	0.16	0.5, 1, 2, 5, 10, 15, 20	5 d, ACPFG + 10 mM NaCl + 0.14 mM CaCl2	Intact	2 min + 2 min ice cold	10	6	Hydroponics	0.5, 1, 2, 5, 10, 15, 20 min	5
6	Nipponbare	30	0.21	0.5, 1, 2, 5, 10, 15, 20	5 d, ACPFG + 30 mM NaCl + 0.19 mM CaCl2	Intact	2 min + 2 min ice cold	30	6	Hydroponics	No	5
7	Nipponbare	3	0.13	0.5, 1, 2, 5, 10, 15, 20	5 d, ACPFG + 3 mM NaCl + 0.13 mM CaCl2	Intact	2 min + 2 min ice cold	3	6	Hydroponics	No	5
8	Indica lines, Nipponbare	30	0.21	2	5 d, ACPFG + 30 mM NaCl + 0.19 mM CaCl2	Intact	2 min + 2 min ice cold	30	3 to 6	Hydroponics	No	6

2.3 Results and Discussion

2.3.1 Experiment 1

A common cereal $^{22}Na^{+}$ uptake protocol (e.g. Davenport and Tester 2000) was the starting point to developing a protocol that would work in rice. A time course was run using roots from the cultivars Nipponbare and Pokkali (grown in Petri dishes) at 10 and 50 mM Na^{+}. The roots were excised 1 h prior to influx experiments and pre-treated in the 10 or 50 mM influx solution without ^{22}Na. Roots were rinsed for 2 min and 3 min in ice cold rinse solution (to reduce active efflux of Na^{+}) containing 50 mM Na^{+} and 10 mM $CaCl_2$ (to displace apoplastically bound Na^{+}).

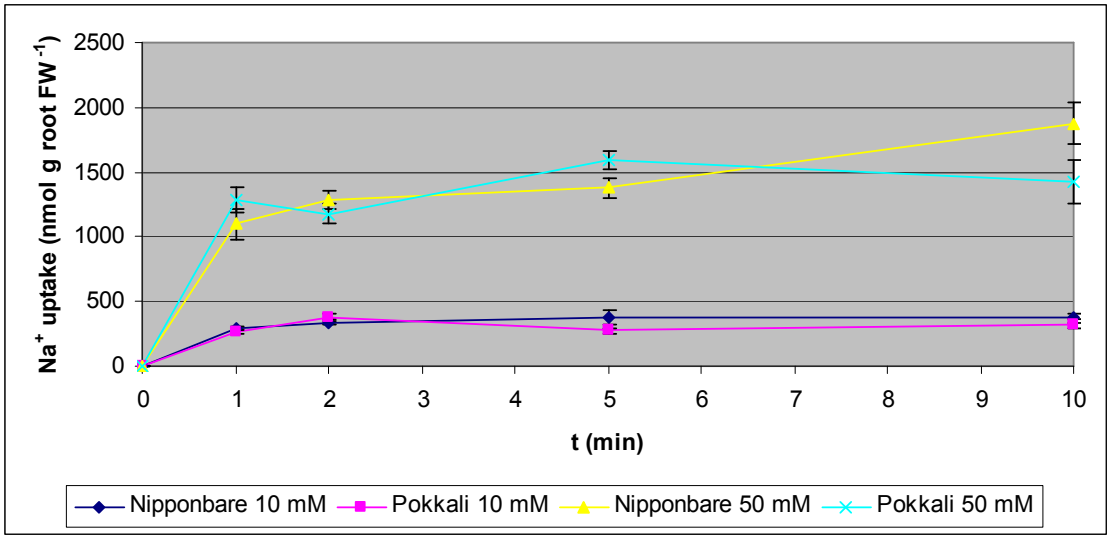


Figure 2.1: Root Na^{+} uptake (measured as $^{22}Na^{+}$ uptake) over 10 min in Nipponbare and Pokkali in 10 and 50 mM influx Na^{+} concentrations (at 0.1 mM Ca^{2+} activity). Data points represent mean $^{+}$/- standard error of means, n = 5.

The Na^{+} uptake measurements quickly reached apparent maxima around 1 min for both the 10 and 50 mM influx solutions (small increase for the 50 mM treatment between 1 and 10 min) and for both cultivars (Figure 2.1). The Na^{+} uptake in the 50 mM treatment did not plot smooth curves, but rather the points rose and fell from 1 to 10 min indicating there was some degree of inaccuracy in the measurements. Also, the rapid increase in uptake to 1 min needed to be resolved by increasing the time points prior to 1 min to further resolve the influx rate at the short time points.

2.3.2 Experiment 2

In an attempt to increase the confidence in measurements, Pokkali was dropped from Experiment 2 to enable an increase in the number of replicates within a single cultivar. Only the 10 mM influx solution was used to further increase the focus on one influx concentration. Na^+ influx prior to 1 min was further resolved by adding 0.25, 0.5 and 0.75 min time points to Experiment 2.

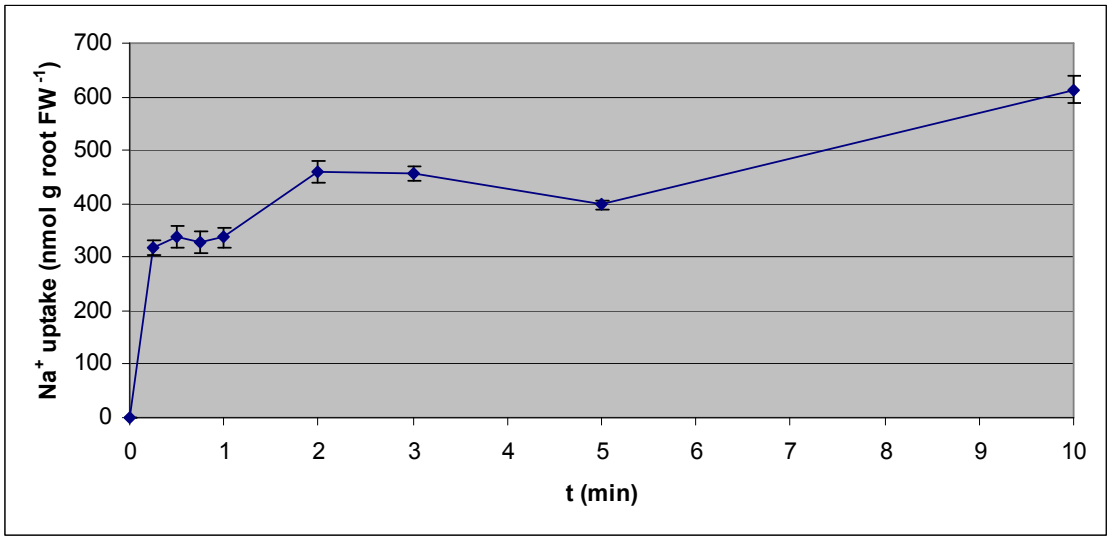


Figure 2.2: Root Na^+ uptake (measured as $^{22}Na^+$ uptake) over 10 min in Nipponbare in 10 mM influx Na^+ concentration (at 0.1 mM Ca^{2+} activity). Data points represent mean $^+$/- standard error of mean, n = 6.

Na^+ uptake rose very quickly to the 0.25 min time point then levelled off before rising at a much slower rate from 1 to 10 min (Figure 2.2). This quick rise to the first time point with reduced rate increase following the first time point closely resembled Experiment 1 as did the rise and fall of the measured uptake following the first time point (and the actual numbers themselves). The initial rapid rise in root Na^+ indicated that the Na^+ uptake was probably not being accurately measured since it seemed highly unlikely that the roots could be loaded to the point of equal influx and efflux of ^{22}Na within 15 sec. The most likely cause of this was apoplastic contamination, due to insufficient rinsing of extracellular $^{22}Na^+$ after the influx period. Potentially, excising the roots prior to influx was damaging them sufficiently to allow highly uneven $^{22}Na^+$ uptake through cut sites. Also, the roots were rinsed in 50 mM Na^+ solution (from Experiment 1) and

this may have affected the ability of the rinse solution to displace the $^{22}Na^{+}$ loaded into apoplastic space in the roots.

2.3.3 Experiment 3

Intact plants grown in hydroponic tanks were used in Experiment 3 to determine if the problems with data from Experiment 2 were the result of damaged roots. Plants were grown in ACPFG nutrient solution (see above) and were pre-treated for 2 d prior to the experiment in ACPFG solution supplemented with 10 mM NaCl and 0.19 mM $CaCl_2$ to help decrease any osmotic shock on the plants upon transfer from the hydroponic tank to the influx solution container. The plants were grown for 14 d at the time of the experiment, as opposed to the 10 d plants from dishes used in the previous two experiments. Also, a 10 mM NaCl rinse solution was used to prevent any osmotic adjustment effects occurring during rinse periods. As well, only two time points were used to allow more replicates to be examined per time point to reduce error.

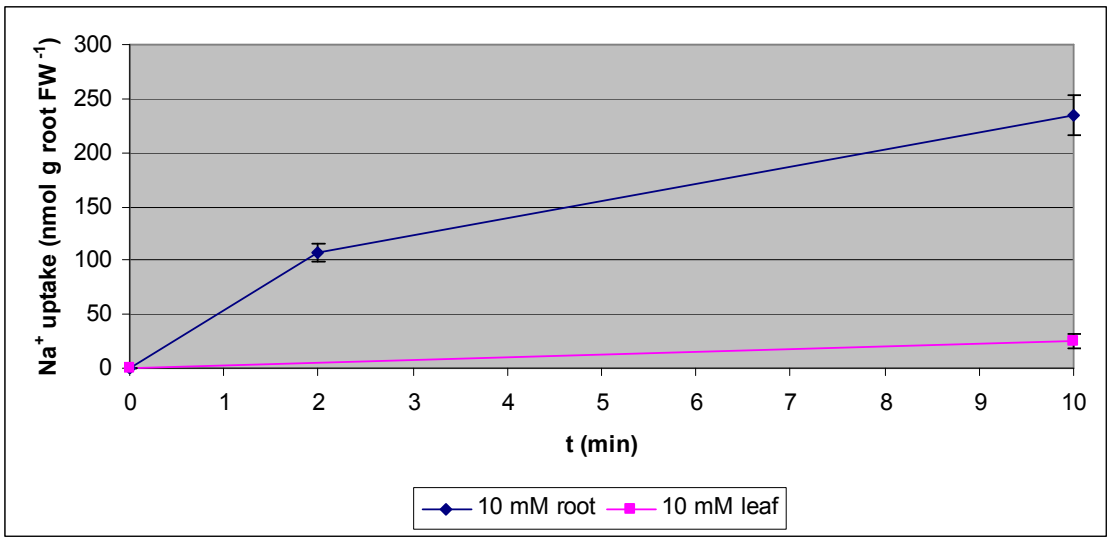


Figure 2.3: Root and shoot Na^{+} uptake (measured as $^{22}Na^{+}$ uptake) over 10 min in Nipponbare in 10 mM influx Na^{+} concentration (at 0.1 mM Ca^{2+} activity). Data points represent mean $^{+}$/- standard error of means, n = 8.

Using intact plants for influx measurements decreased the Na^{+} uptake measurement at 2 min from approximately 400 nmol/g FW (Figures 2.1 and 2.2) to 100 nmol/g FW (Figure 2.3). This indicated that excised roots were perhaps too damaged to accurately measure Na^{+} uptake because there was significant $^{22}Na^{+}$ entering the cuts in

the root and creating the appearance that equivalent amounts of Na^+ were being actively taken up by the roots regardless of time point. This is likely the reason the measured Na^+ uptake decreased from previous experiments. Also, the use of a pre-treatment and rinse solutions at the same Na^+ concentration as the influx solution likely reduced variability due to osmotic effects. $^{22}Na^+$ could be measured at 10 min giving an indication of how quickly Na^+ is transferred to the shoot. This closely resembles the rate found in durum wheat (Davenport *et al.* 2005) and *Arabidopsis* (Davenport *et al.* 2007), in which measurement of shoot $^{22}Na^+$ also occurred at 10 min.

The Na^+ uptake increased 2.5-fold from 2 min to 10 min, which was similar to the Experiment 2. This also indicates that the rapid influx to 2 min in excised roots is an artefact of the excision.

Questions remained about the employed rinsing protocol of 2 min + 2 min rinses in ice cold rinse solution. However, rice has a large apoplastic spaces compared to other cereals and the effect of rinse time on apparent Na^+ uptake measurements needed to be determined. Also, it was suggested that since rice is a tropical species, the ice cold rinses which are appropriate for temperate cereals may damage the membranes more significantly in rice leading to increased efflux of $^{22}Na^+$ and thus an underestimation of the Na^+ uptake.

2.3.4 Experiment 4

To determine the effect of the rinse length and temperature on the Na^+ uptake measurements intact plants were placed in the influx solution for 2 min and were rinsed for either 1 min (30 s + 30 s) or 5 min (2 min + 2 min) at ice cold (4°C) or room (25°C) temperatures. If the rice roots were being damaged by the ice cold rinse treatment the measured Na^+ uptake should be lower for the ice cold rinses than for the RT rinses.

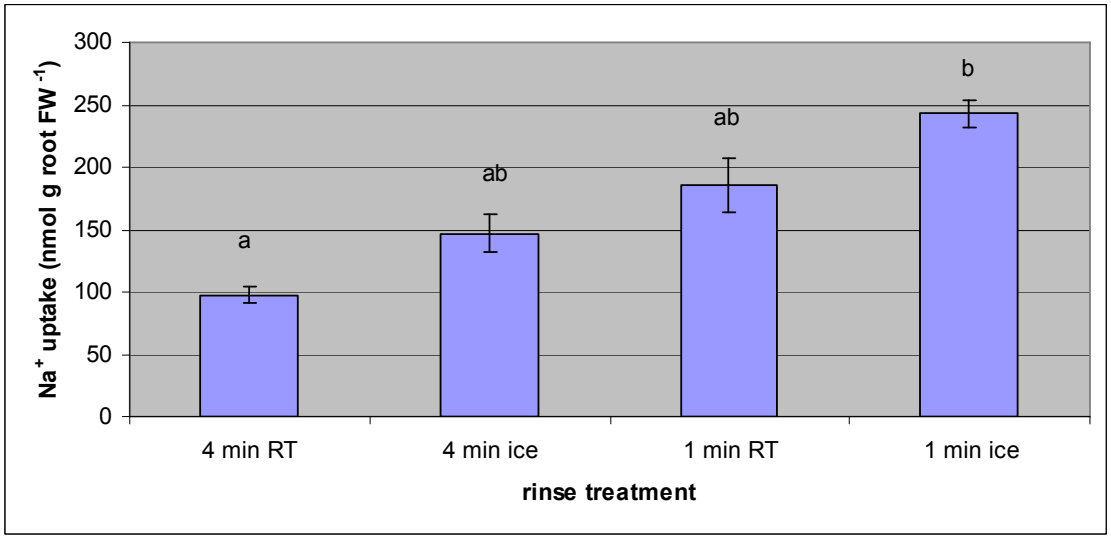


Figure 2.4: Na^+ uptake (measured as $^{22}Na^+$ uptake) in 2 min in Nipponbare in 10 mM influx Na^+ concentration (at 0.1 mM Ca^{2+} activity). Rinse treatments were 2 min + 2 min at room temperature (4 min RT), 2 min + 2 min ice cold (4 min ice), 0.5 min + 0.5 min at room temperature (1 min RT), and 0.5 min + 0.5 min ice cold (1 min ice). Bars represent mean $^+$/- standard error of means, n = 6. Bars with the same letter(s) above are not significantly different at $P<0.05$ according to the Kruskal-Wallis and Dunn's Multiple Comparisons tests.

As would be expected the 4 min rinse times resulted in lower apparent Na^+ uptake measurements since there is more time for active efflux of $^{22}Na^+$ from the roots to the rinse solution (Figure 2.4). The ice cold rinsed roots had higher Na^+ uptake than the RT rinses at both 1 and 4 min indicating that the cold was not damaging the rice roots significantly, but it was slowing down efflux as intended.

Since it appeared that the use of pre-treated, intact plants grown in hydroponics and cold rinses was providing reliable measurements of Na^+ influx, the next step was to run time courses at different Na^+ concentrations to determine a suitable concentration to run further experiments at and to determine a suitable time point to measure unidirectional Na^+ influx.

2.3.5 Experiment 5 – 10 mM, Experiment 6 – 30 mM, Experiment 7 – 3 mM

Three successive experiments were run using pre-treatments (5 d), influx solutions and rinse solutions at Na^+ concentrations of 3, 10 and 30 mM (with compensatory $CaCl_2$ to maintain Ca^{2+} activity in the pre-treatment and influx solutions, and 10 mM $CaCl_2$ in the rinse solutions).

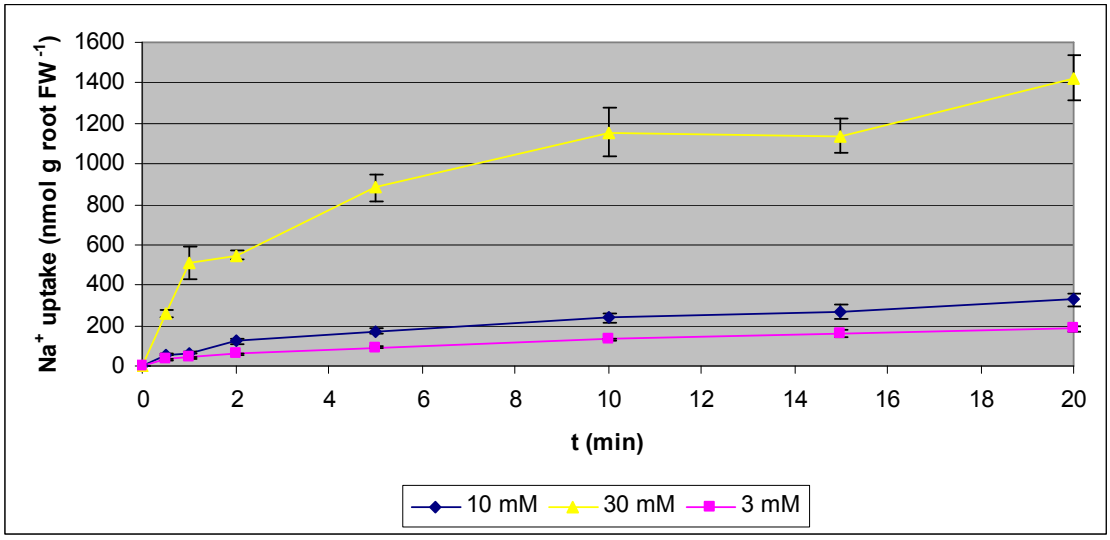


Figure 2.5: Na^+ uptake (measured as $^{22}Na^+$ uptake) over 20 min in Nipponbare in 3, 10 and 30 mM influx Na^+ concentrations (at 0.1 mM Ca^{2+} activity). Data points represent mean $^+$/- standard error of means, n = 6.

The curves from all three concentrations appeared relatively smooth and thus it appeared the methodology was solid (Figure 2.5). It was very difficult to determine at which time point Na^+ uptake decreased most significantly (signalling a significant efflux component to the net flux) at both 3 and 10 mM influx concentrations. Also, the influx measurements at 30 mM were likely the most agriculturally relevant for studies of rice in saline soils (the lower concentration not generally having a very significant effect on growth), thus it was decided to carry out further experiments using an influx solution concentration of 30 mM Na^+. It is evident that at 30 mM influx concentration, the influx rate drops most significantly between the 1 and 2 min time points, indicating that efflux has begun to significantly impact the Na^+ uptake measurement. It is unlikely that this drop in influx rate is due to a downregulation of unidirectional influx since this would imply either a sufficiently high cellular $^{22}Na^+$ concentration has been reached to eliminate the $^{22}Na^+$ gradient or there has been a downregulation of Na^+ transport at the molecular level. Neither of these options seems probable given the brief influx time. To minimise the impact of efflux on Na^+ uptake measurements it was decided that influx measurements would be best made at 2 min. Also, measurements made at 1 min would likely have larger errors than at 2 min (since roots have less chance to take up ^{22}Na before rinsing and so errors due to apoplastic contamination will be proportionately greater; and

timing errors will be more significant). The same observations have been made for *Arabidopsis* (Essah *et al.* 2003) and wheat (Davenport 2000), despite the large differences in morphology between the plants.

2.3.6 Experiment 8

Eleven rice lines were examined using the protocol to determine if there was measurable variation in Na^+ influx in rice germplasm or if significant apoplastic flow eliminates the variation. Ten of the lines were from the subspecies *indica* (all obtained from IRRI, The Philippines), while Nipponbare was included as the lone *japonica*. The eleven rice lines differed greatly in both Na^+ accumulation and Na^+ tolerance (J Sundstrom and O Cotsaftis, unpublished data). Nona Bokra and Pokkali are Indian landraces that are commonly used in breeding programs as a source of Na^+ tolerance genes since they both exclude Na^+ and are extremely Na^+ tolerant (Ren et al. 2005, Walia et al. 2005). IR28, IR29 and IR36 are cultivars bred by IRRI for yield, but are all Na^+-sensitive, although IR28 has been shown to have efficient Na^+ exclusion (Walia et al. 2005, Bohra and Doerffling 1993). FL478 is a Na^+-tolerant breeding line resulting from the cross of Pokkali and IR29 and is an efficient Na^+-excluder (even better than Pokkali) and is high-yielding (Walia et al. 2005). SAL208 (also known as IR63731) is a Na^+-tolerant breeding line derived from a cross between Nona Bokra and IR28 and is an efficient Na^+-excluder and appears to have some degree of tissue tolerance to Na^+ (Zeng et al. 2004). NSICRC 106 (also known as IR61920) is a moderately Na^+-tolerant breeding line (Zeng et al. 2004). Kalurundai and Kallurundai Vellai survived the 26 December, 2004 tsunami in Sri Lanka and the ensuing sea water flooding, thus are potential new donor lines for Na^+ tolerance breeding in rice. Thus, a wide range of Na^+ tolerances (and likely tolerance mechanisms) was represented among the lines.

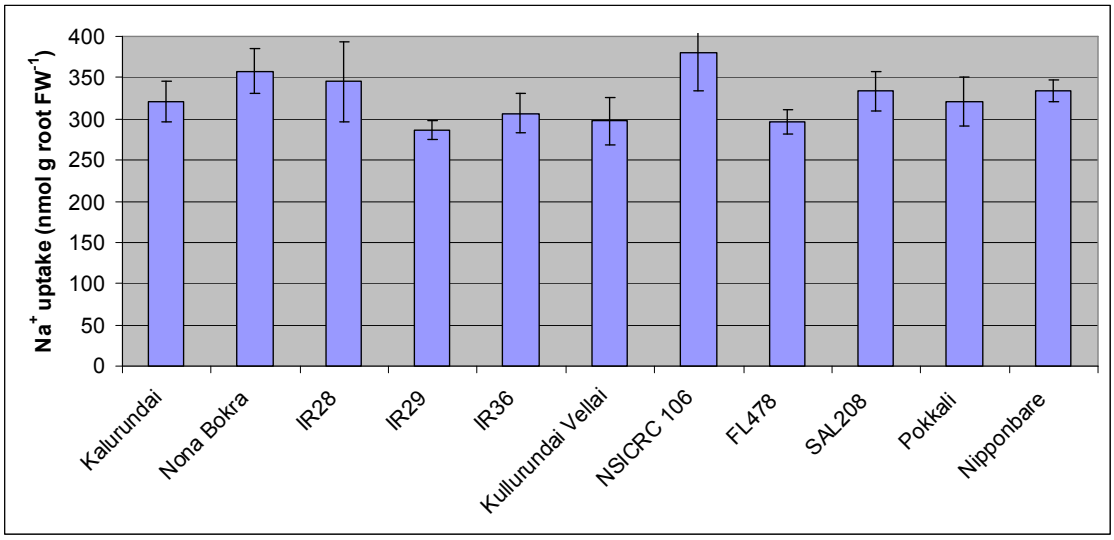


Figure 2.6: Na^+ uptake (measured as $^{22}Na^+$ uptake) in 2 min in 11 rice cultivars in 30 mM influx Na^+ concentration (at 0.1 mM Ca^{2+} activity). Data points represent mean $^+$/- standard error of means, n = 3-6. No significant differences exist between means at $P<0.05$ according to the Kruskal-Wallis and Dunn's Multiple Comparisons tests.

There is a wide range in unidirectional Na^+ influx between the rice varieties (Figure 2.6). There is more than 30 % difference between the line with the lowest influx (IR29) and the highest (NSICRC 106). Interestingly, despite the large difference in influx between the lines there was no obvious relationship between low influx and Na^+ accumulation or tolerance. In fact the lowest influx was found in IR29, a Na^+-sensitive cultivar, and the highest influx was in NSICRC 106, a moderately Na^+ tolerant breeding line and Nona Bokra, a Na^+ tolerant landrace. Even the Na^+ sensitive japonica, Nipponbare, had lower influx than Na^+ tolerant Nona Bokra.

Table 2.3: Summary of Na^+ uptake, rank, FW, average leaf number, and reported Na^+ tolerance in 11 rice cultivars. Green highlights indicate relatively small-sized cultivars, tan highlights indicate relatively large-sized cultivars.

Line	Na^+ Uptake (nmol/gFW)	s.e.	Na^+ Uptake Rank	Root FW (g)	Shoot FW (g)	Total FW (g)	Leaf Number	Na^+ Tolerance
IR29	286.5	10.8	1	0.094	0.158	0.252	3.2	sensitive
FL478	296.3	14.9	2	0.198	0.411	0.608	4.8	tolerant
Kullurundai Vellai	297.8	28.8	3	0.254	0.371	0.625	3.8	tolerant?
IR36	306.9	24.0	4	0.085	0.162	0.247	3.8	sensitive
Pokkali	321.0	29.5	5	0.152	0.363	0.515	4.0	tolerant
Kalurundai	321.5	24.5	6	0.200	0.300	0.500	4.0	tolerant?
SAL208	334.0	23.7	7	0.119	0.211	0.330	4.0	tolerant
Nipponbare	334.3	13.2	8	0.126	0.168	0.294	3.8	sensitive
IR28	345.0	48.1	9	0.104	0.158	0.262	3.7	sensitive
Nona Bokra	358.0	27.5	10	0.193	0.377	0.570	4.5	tolerant
NSICRC 106	380.5	46.3	11	0.124	0.193	0.317	3.8	moderate

There was also no correlation between plant size and Na^+ influx since neither FW nor leaf number explained the variation in Na^+ uptake (Table 2.3). Thus, despite the large differences in Na^+ influx measured among rice varieties, it is not an important determinant of Na^+ tolerance in rice. This indicates that other Na^+ transport processes, such as transport of Na^+ across the endodermis, Na^+ loading to the xylem in the root, Na^+ retrieval from the xylem in the root or Na^+ sequestration in the sheath tissue, are likely more crucial to determining the Na^+ accumulation and/or tolerance in rice. However, the differences between the lines indicates there is some genetic variation among the lines which could be explored further for breeding purposes. Of note, Pokkali, IR29, and FL478 all have relatively low Na^+ uptake while Nona Bokra, IR28 and SAL208 all have relatively high Na^+ uptake, indicating that it is likely Na^+ influx is a heritable trait.

2.4 General Discussion

A protocol has been developed to measure unidirectional Na^+ influx in rice. Interestingly, the protocol is very similar to one recently developed for use in durum wheat (Davenport *et al.* 2005), a temperate cereal with very different morphology to rice. The protocol here was used successfully to show a 30 % difference in unidirectional Na^+ influx exists between rice varieties and that it is poorly correlated to the Na^+ accumulation and tolerance. This method will be used in Chapter 6 to determine if rice lines expressing *AtHKT1;1* in specific cell-types of the root have been altered in Na^+ influx.

Table 2.4: Summary of 11 experiments measuring Na^+ influx using $^{22}Na^+$. The influx concentration, time point at which Na^+ uptake was measured and the influx rate at the given time point are listed. The influx rate was divided by the influx concentration and is reported as a single number in the final column.

Reference	Species	Cultivar/Ecotype	Influx Concentration (mM)	Time Point (min)	Na^+ Uptake (nmol/gFW)	Influx Rate (nmol/gFW/min)	Influx Rate / Influx Concentration (nmol/gFW/min / mM influx solution)
Davenport 2007	Arabidopsis	gl-1	50.0	2	1000	500	10
Essah 2003	Arabidopsis	C-24	200.0	2	5000	2500	13
Wang 2006	Arabidopsis	Col-0	100.0	2	2250	1125	11
Wang 2006	Thellungiella	Shandong	100.0	2	1100	550	6
Davenport 2000	Bread Wheat	Hunter	100.0	5	7665	1533	15
Davenport 2005	Durum	Tamaroi/Line 149	25.0	2	200	100	4
James 2006	Durum	Nax1/Nax2	25.0	2	300	150	6
Horie 2007	Rice	WT Nipponbare	0.1	2	2	1	10
Plett 2007	Rice	WT Nipponbare	30.0	2	400	200	7
Elphick 2001	Arabidopsis	Col-0	50.0	5	5320	89	2
Zidan 1991	Maize	Halamish	100.0	30	4600	153	2

The protocol gives strikingly similar results to those obtained in previous examinations of unidirectional Na^+ influx using $^{22}Na^+$ (Table 2.4). An estimation of unidirectional Na^+ uptake at (2 min or as close to 2 min as possible) was made from previous reports. Uptake was converted to an influx rate and this rate was divided by the Na^+ concentration in the influx solution. This provided a number that allowed comparison of influx across experiments regardless of influx solution concentration. The higher numbers were from studies which used a very high influx Na^+ concentration (Essah *et al.* 2003) or very short rinse times and low rinse Na^+ concentration (Davenport and Tester 2000). The lower numbers resulted from studies which used extremely long rinse times (Elphick *et al.* 2001) or reported influx at a long time point (Zidan *et al.* 1991), which in both cases likely resulted in an underestimation of unidirectional Na^+ influx rates.

A recent report indicates that traditional unidirectional measurements of radiotracer influx have greatly underestimated influx by ignoring radiotracer efflux into rinse solutions (Szczerba *et al.* 2006). The method developed here and similar methods used in most of the studies mentioned in Table 2.4 avoid such underestimation by using short influx times (e.g. 2 min), thereby not allowing a significant radiotracer efflux component to reduce influx measurements, and through use of ice cold rinse solution and short rinse times, which are sufficient to wash apoplastically bound radiotracer from root samples, but prevent a large efflux of radiotracer.

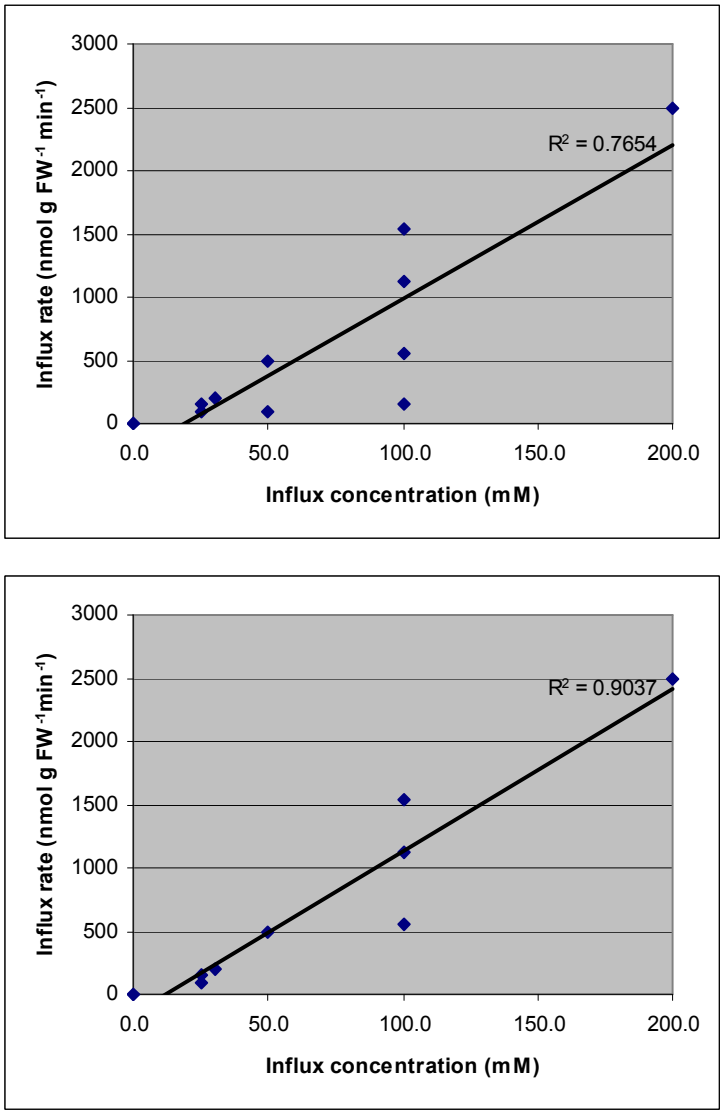


Figure 2.7: Influx rate is plotted against influx concentration for the studies presented in Table 2.4. All studies are included in the top figure, while the Elphick 2001 and Zidan 1991 data points are removed from the bottom figure. The R^2 value is significantly higher when the two studies are removed from the calculation.

Interestingly, if the low numbers are removed from the analysis (for aforementioned reasons), the remaining numbers are all within a factor of 3 and influx rate and influx concentration are highly correlated (Figure 2.7), regardless of the species being studied or the concentration of the Na^+ in the influx solution. Even the Horie *et al.* 2007 report, which used an extremely low Na^+ influx concentration (0.1 mM), had a number within the range found for all studies and similar to the number from this study

for rice. This indicates that the protocol developed for rice in this study is valid and useful for further characterisation of unidirectional Na^+ influx in rice lines.

CHAPTER 3: Na^+-REGULATED ENHANCERS

3.1 Introduction

Expression of transgenes using Na^+ stress inducible regulatory elements is advantageous in improving the Na^+ stress tolerance of crop plants. For example, ABA-inducible expression of trehalose biosythesis genes resulted in more Na^+ tolerant plants (due to elevated trehalose production) without the decrease in growth observed in a line expressing the genes constitutively (Garg *et al.* 2002). However, surprisingly few Na^+ inducible promoters or enhancers have been isolated.

Three types of gene traps are commonly used to isolate regulatory elements. Gene trap populations of *Arabidopsis* and rice are produced by inserting a promoterless reporter gene randomly into the genome (Sundaresan *et al.* 1995, Jeon *et al.* 2000). Gene::reporter gene fusions are identified visually when the reporter gene has been inserted in an intron (gene trap) or an exon of a gene (promoter trap). A third gene trap, the enhancer trap, relies on genomic enhancers activating a randomly inserted minimal promoter/reporter gene to produce reporter gene expression. These populations may then be screened to identify lines expressing the reporter gene indicating the successful trapping of a genetic element. Characteristics of the trapped element may be identified by the pattern of reporter gene expression which can give some indication of function or tissue location. The element may also be cloned and used to drive expression of a transgene in a similar manner to the trapped gene/enhancer element.

Gene trap populations have been used to isolate genetic elements involved in numerous plant processes. An *Arabidopsis* promoter trap library was generated by inserting a promoterless firefly *luc* (*luciferase*) to report expression of trapped promoters (Alvarado *et al.* 2004). This population was screened by applying Na^+, glucose or ABA treatments to the germinated seed and observing changes in luciferase levels. Many stress-responsive promoters were successfully cloned from the lines showing expression changes. In another study, populations of *Arabidopsis* gene trap and enhancer trap lines were subjected to oxygen-deprivation and multiple differentially expressed genes were identified by changes in GUS activity (Baxter-

Burrell *et al.* 2003). This strategy has also been used in a *GUS* gene trap library in rice to successfully identify cold responsive genes (Lee *et al.* 2004).

In this study a population of rice GAL4-GFP enhancer trap lines (Johnson *et al.* 2005) was screened with a mild (agriculturally relevant) Na^+ stress in order to identify elements which respond to Na^+ stress. These genes may be involved in Na^+ tolerance and their identification could provide cell-type specific, Na^+ stress-inducible promoters. The stress regulated enhancers may also be regulated by other stresses and thus useful in improving tolerance to other stresses as well.

3.2 Materials and Methods

3.2.1 Plant materials

Development of the GAL4-GFP enhancer trap library is described in Johnson *et al.* 2005.

3.2.2 Seed preparation

Ten seeds each of 824 T_1 lines were sterilised in 24-3 ml well plates. The seed was treated with 70 % ethanol for 2 min, then for 30 min in a solution of 30% bleach (1 % available chlorine) and a few drops of Tween20 detergent. The seed was rinsed 3 times in dH_2O with the third rinse lasting 10 min and placed in labelled Petri dishes with a single filter paper. The seed was allowed to dry then washed with 10 ml of dH_2O to remove any traces of bleach. Five ml of dH_2O was added to each dish and the dishes were sealed with Parafilm. Imbibing seed was placed in a growth chamber at 28°C under fluorescent tubes and Grow-lux lighting.

3.2.3 T_1 seed screen and microscopy information

Seed was screened in the Petri dishes 16 h after imbibition. The seed was examined using a Leica MZ FLIII stereomicroscope fitted with an ultraviolet (UV) light powered by a Leica HBO 100 powersource. The seed was observed through a GFP Plus fluorescence filter set [*GFP*2, 480 nm excitation filter (bandwidth of 40 nm) and 510 nm barrier filter] filter and images were obtained using a Leica DC300F camera and transferred onto a computer to be captured in Adobe Photoshop. All images were assembled into FileMaker Pro 5.5 database for ease of storage. The seed dishes were returned to the growth chamber.

3.2.4 T_1 seedling screen

Seven d following the seed screen the developing roots, endosperm and shoots of seedlings were examined for GFP fluorescence, photographed and images entered into the database. GFP negative lines were separated from GFP positive lines for ease of sorting later in the screening process.

3.2.5 Na^+ screen

Immediately following the seedling screen the 5 ml of dH_2O water was removed from germination dishes and replaced with 5 ml of 40 mM NaCl and 0.5 mM $CaCl_2$. The dishes were resealed with Parafilm and placed back into the growth chamber for 3 d. The lines which had been GFP positive in the previous seedling screen were compared to the pictures taken during the previous screen. If a change was noted in the intensity or location of the GFP fluorescence or in the number of seedlings displaying GFP fluorescence, the seedlings were photographed and the changes noted and entered into the database. The lines which had been GFP negative in the previous seedling screen were examined and if they displayed GFP fluorescence after the Na^+ treatment they were photographed and the changes were noted and entered into the database. The lines which showed no GFP fluorescence in any screen were discarded. The lines displaying GFP fluorescence during any screen were sorted, the individual seedlings showing GFP fluorescence were saved, while null genotypes not showing GFP fluorescence (due to segregation) were discarded.

3.2.6 Re-screen of T_1

To confirm the GFP fluorescence patterns observed in the first screen 10 additional T_1 seeds of the 18 lines selected as showing Na^+ regulated expression patterns were re-screened in exactly the same manner as above. Following the Na^+ screen the lines were planted in the glasshouse to obtain T_2 seed. Plants were grown in a glasshouse at SARDI (Adelaide) with average temperatures of 30°C days and 20°C nights, full sun, and an average humidity of 60%.

3.2.7 T_2 screen

Six lines were chosen to re-examine in the T_2 generation based on the confirmation of GFP fluorescence changes observed in the T_1 generation. Twenty T_2 seeds from each T_1 plant were examined in two dishes of 10 seeds each (as above).

Following the seedling screen 7 d after germination, one of each pair of dishes was treated with the Na^+ solution and one was treated with RO water. GFP fluorescence was observed 3 d following Na^+ treatment.

3.3 Results and Discussion

3.3.1 Initial T_1 screen

824 T_1 rice lines were screened, according to the above protocol, at three stages: seed (16 h following initial imbibition), seedling (one week following germination) and second seedling (one week and 3 d following germination – after addition of salt stress). GFP fluorescence (all screens, any tissue) was observed in 275 lines (33.4%), including 105 lines (12.7%) showing GFP fluorescence in the seed screen, 185 lines (22.5%) with shoot fluorescence (8 shoot specific lines), and 245 (29.7%) lines with root fluorescence (63 root specific lines) (Table 3.1).

Upon addition of the Na^+ stress, 79 lines (9.6%) were noted as having a change in GFP fluorescence from the initial seedling screen. This included 62 lines (7.5%) which had observable up-regulation of GFP fluorescence and 17 lines (2.1%) which had any observable down-regulation of GFP fluorescence. After careful observation of the photographs, 18 lines (2.2%) were chosen for re-testing due to more pronounced GFP fluorescence changes.

Table 3.1: Frequency of GFP fluorescence in various tissues of rice GAL4-GFP enhancer trap lines.

Tissue	Number	% of Total Lines	% of Fluorescing Lines
TOTAL SCREENED	824		
Any Fluorescence	275	33.4	100.0
Seed	105	12.7	38.2
Endosperm	43	5.2	15.6
Endosperm Specific	4	0.5	1.5
Seedling	252	30.6	91.6
Shoot	185	22.5	67.3
Guard Cell or Trichome	23	2.8	8.4
Shoot Specific	8	1.0	2.9
Root	245	29.7	89.1
Root Specific	63	7.6	22.9
Na^+ REGULATION			
Na^+ regulated	79	9.6	28.7
Upregulated	62	7.5	22.5
Downregulated	17	2.1	6.2
T_1 lines re-screened	18	2.2	6.5
Upregulated	12	1.5	4.4
Downregulated	6	0.7	2.2
Confirmed in T_1	9	1.1	3.2
Upregulated	5	0.6	1.8
Downregulated	4	0.5	1.5
Confirmed in T_2	2	0.2	0.7
Upregulated	2	0.2	0.7
Downregulated	0	0.0	0.0

3.3.2 Re-screen of T_1

The 10 T_1 seeds from the 18 lines (Figures 3.1 to 3.18) chosen in the previous screen were moved to Adelaide and screened in the same manner as described previously (Table 3.1). It would have been preferable to Na^+ stress half the seed and leave half on RO water, but 5 seed in each treatment may not have been sufficient to provide an accurate measure of the GFP fluorescence in the line since the seed was still segregating for the GAL4-GFP enhancer trap. The seedlings were sown into soil in order to provide enough T_2 seed to properly analyse the Na^+ stress response of each line with a proper control.

Several of the lines originally characterised with elevated GFP fluorescence in response to Na^+ stress displayed much brighter GFP fluorescence prior to Na^+ stress than in the original screen. Whether this was a result of slightly different conditions in the re-screen or whether the lines had been misclassified in the original screen is difficult to determine. Potentially, very minor differences in the growth conditions, such as the pH of the water, could have affected the response of the trapped enhancer element. Some of the lines originally characterised with reduced GFP fluorescence in response to Na^+ stress had little or no GFP fluorescence prior to Na^+ stress. This is likely also related to minor differences in growth conditions or simple misclassification in the original screen.

The strongest three putative 'upregulators' and strongest three putative 'downregulators' were selected to re-examine in the T_2 generation based on the apparent regulation by Na^+ stress.

3.3.3 T_2 screen

The seedlings from responding lines were planted and harvested and T_2 seed was examined from each T_1 plant to ensure any effects of gene silencing would be taken into account. The seed were once again grown in Petri dishes as previously, but 2 dishes of 10 seeds were germinated for each T_1 line. After 7 d one of the dishes for each plant was given a Na^+ treatment and the RO water was replaced in the other dish in order to determine if the regulation in GFP fluorescence was actually a result of Na^+ stress or if it was a developmental response.

The putatively 'upregulated' lines AVN D05, AVP D11, AVS G07 appeared to have significant upregulation of GFP fluorescence in response to Na^+ stress. AVN D05 (Figure 3.9) showed weak GFP fluorescence in the root cap prior to Na^+ stress, and strong GFP fluorescence following Na^+ stress in the rest of the root and in the shoot collar. However, this significant upregulation was equal between Na^+ treated and untreated plants, thus it appears this is a developmentally regulated enhancer element rather than one which is regulated by Na^+ stress. The enhancer trap insertion in this line was located in Os_10g36210, a putative valyl-tRNA synthetase (CIRAD, Montpellier). AVP D11 (Figure 3.11) had weak to medium GFP fluorescence prior to Na^+ stress and following Na^+ stress this fluorescence became medium to bright. The fluorescence appeared to be brighter in the Na^+ treated dish than in the control dish, thus it appears as though this enhancer element is regulated by Na^+. However, the regulation is relatively minor with fluorescence moving from weak to medium or medium to bright with the application of Na^+, thus this line would not be able to significantly upregulate expression of a transactivated gene with the addition of Na^+ stress. It is possible that this line would respond with a larger upregulation of GFP fluorescence if the Na^+ stress was increased, but the stress required to upregulate the GFP fluorescence may be agronomically damaging for rice. The enhancer trap insertion in this line was located in Os_04g42220, a putative GPI-anchored protein (CIRAD, Montpellier). AVS G07 (Figure 3.12) had medium GFP fluorescence in the root and shoot prior to Na^+ stress. Fluorescence following Na^+ stress increased to bright fluorescence following Na^+ stress and appeared to be brighter in the Na^+ treated seedlings. However, again the upregulation is likely not sufficient to significantly change expression of a transactivated transporter. The enhancer trap insertion in this line was located in Os_11g23960, a putative retrrotransponson protein from the Ty3-gypsy subclass (CIRAD, Montpellier).

The putatively 'downregulated' lines AVK C01, AVL C07, AWC E12 appeared to downregulate GFP fluorescence in response to Na^+ stress and thus were re-examined in the T_2 generation. AVK C01 (Figure 3.6) did not show any GFP fluorescence prior to Na^+ stress as it had in previous T_1 screen. Most likely the GAL4-GFP enhancer trap construct was silenced in the T_2 generation. The enhancer trap

insertion site could not be determined for this line (CIRAD, Montpellier). AVL C07 (Figure 3.8) showed weak root cap fluorescence prior to Na^+ stress and following Na^+ stress the root cap fluorescence disappeared. However, the actual downregulation of GFP fluorescence was minor and there was little difference between the Na^+ stressed seedlings and the unstressed seedlings, so this line is unlikely to be useful for further evaluation. The enhancer trap insertion site could not be determined for this line (CIRAD, Montpellier). AWC E12 (Figure 3.15) showed strong GFP fluorescence in most cell-types of the root and shoot prior to Na^+ treatment. After Na^+ treatment the fluorescence in the root remained strong while fluorescence in the shoot disappeared. However, both the Na^+ and control seedlings showed the same response, thus it is likely a developmental enhancer element that has been trapped in this line. Nonetheless, the element may be interesting for constitutive transactivation of a gene constitutively for the first 7 d, followed by root-specific transactivation thereafter. The enhancer trap insertion site could not be determined for this line (CIRAD, Montpellier).

Line: AUQ G09	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
No fluorescence	
Comments: Medium root fluorescence following Na^+ treatment. Fluorescence started midway down the crown root with no fluorescence observed in the youngest crown roots. Lateral roots branching off the fluorescing crown root region also showed GFP fluorescence.	
T_1 Phenotype Confirmed?: NO	
Comments: No fluorescence observed after Na^+ treatment.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.1: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AUQ G09.

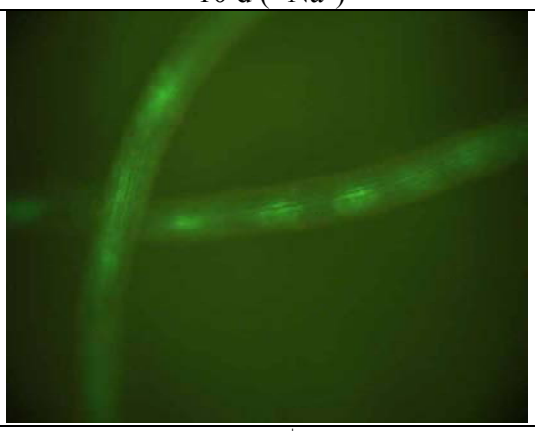
<table>
<tr><td>Line: AUX C04</td><td>Putative Na^+ Regulation: Upregulated</td></tr>
<tr><td colspan="2">T_1 Phenotype</td></tr>
<tr><td>7 d ($-Na^+$)</td><td>10 d ($+Na^+$)</td></tr>
<tr><td>No fluorescence</td><td>  </td></tr>
<tr><td colspan="2">Comments: Medium fluorescence in cortex of crown roots following Na^+ treatment (no lateral roots present at the time to photograph). No fluorescence in crown root tips.</td></tr>
<tr><td colspan="2">T_1 Phenotype Confirmed?: NO</td></tr>
<tr><td colspan="2">Comments: Fluorescence observed in root and shoot prior to Na^+ treatment.</td></tr>
<tr><td colspan="2"></td></tr>
<tr><td colspan="2">T_2 Phenotype: NOT EXAMINED</td></tr>
<tr><td>$-Na^+$</td><td>$+Na^+$</td></tr>
<tr><td></td><td></td></tr>
<tr><td colspan="2">Comments:</td></tr>
</table>

Figure 3.2: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AUX C04.

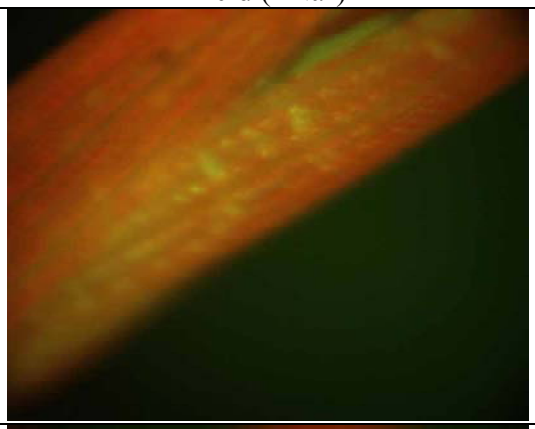
Line: AUZ E10	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Root GFP fluorescence during initial seedling screen, but following Na^+ treatment shoot fluorescence appeared in leaf tips, vascular elements, and guard cells.	
T_1 Phenotype Confirmed?: NO	
Comments: Root and shoot fluorescence observed prior to Na^+ treatment.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.3: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AUZ E10.

Line: AVG C08	**Putative Na^+ Regulation: Downregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Shoot fluorescence decreased after Na^+ treatment response to weak or no fluorescence. Root fluorescence decreased significantly with the top, right picture representing a rare bit of remaining fluorescence.	
T_1 Phenotype Confirmed?: NO	
Comments: No fluorescence observed prior to Na^+ treatment	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.4: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVG C08.

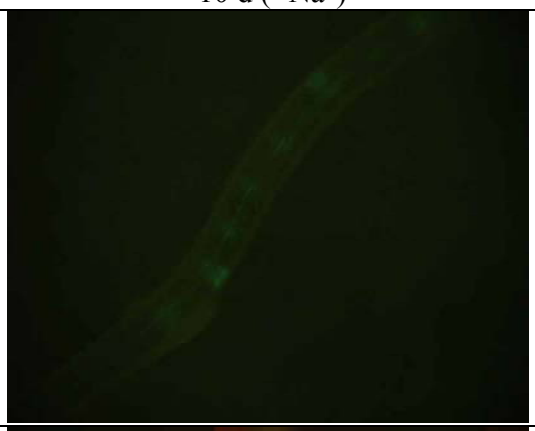
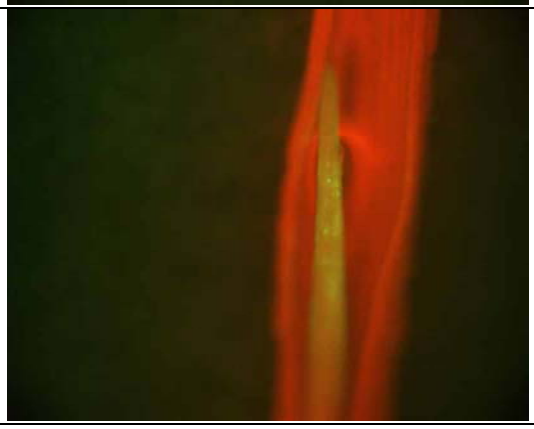
Line: AVH E10	**Putative Na^+ Regulation: Downregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: The root tip fluorescence observed at the initial seedling screen disappears following Na^+ treatment, but the leaf fluorescence has possibly increased following Na^+ treatment.	
T_1 Phenotype Confirmed?: YES	
Comments: Minor downregulation observed in the root, but medium shoot fluorescence was observed prior to Na^+ treatment and was not subsequently downregulated by Na^+.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.5: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVH E10.

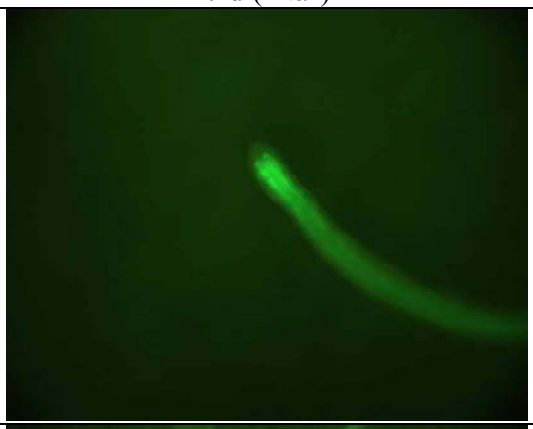
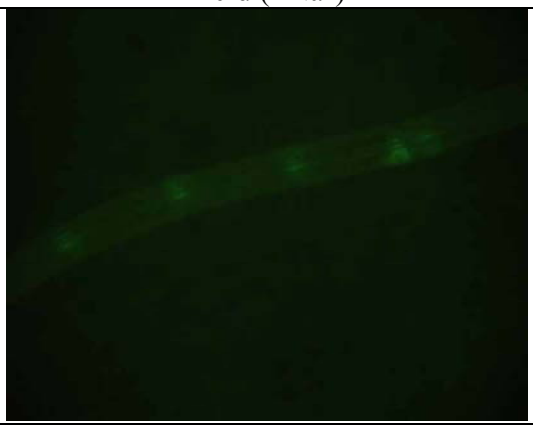
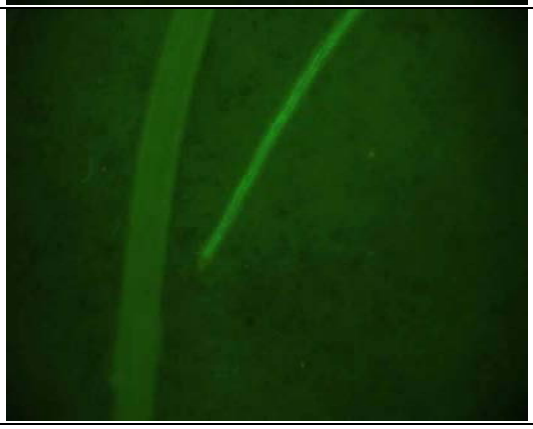
Line: AVK C01	**Putative Na^+ Regulation: Downregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Three of the plants originally had GFP fluorescence in their roots. Following the Na^+ treatment, the two larger plants had no root GFP fluorescence whatsoever. The youngest of the replicates had some faint expression in what appeared to be emerging lateral roots, but nothing like the fluorescence seen at the first screen.	
T_1 Phenotype Confirmed?: YES	
Comments: Downregulation of fluorescence was less pronounced than originally observed.	
T_2 Phenotype: EXAMINED	
$-Na^+$	$+Na^+$
No fluorescence prior to Na^+ treatment.	No fluorescence prior to Na^+ treatment.
Comments: Appears this line has been silenced in the T_2 generation.	

Figure 3.6: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVK C01.

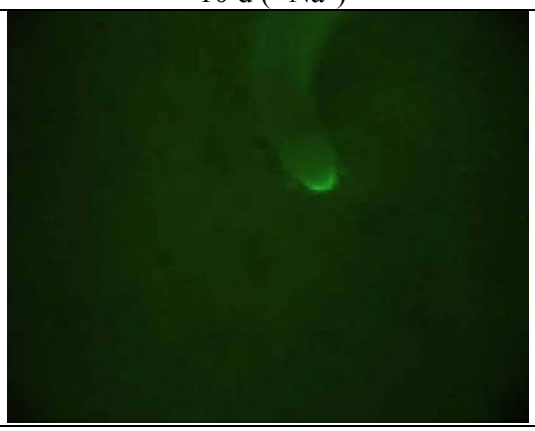
Line: AVK D10	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
No fluorescence	
Comments: No fluorescence was observed at the initial seedling screen. However, following Na^+ treatment, it appeared there was medium root cap specific fluorescence in the roots.	
T_1 Phenotype Confirmed?: NO	
Comments: No fluorescence observed following Na^+ treatment.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.7: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVK D10.

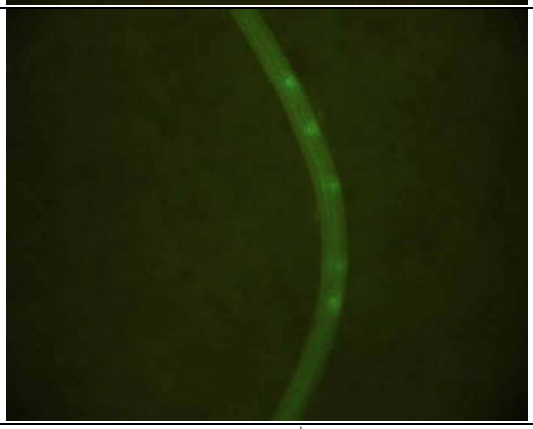
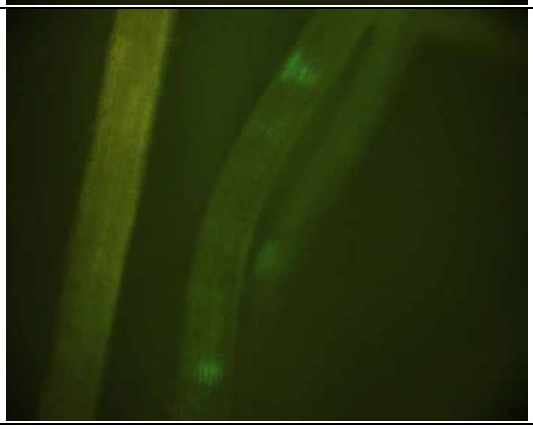
Line: AVL C07	**Putative Na^+ Regulation: Downregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Following Na^+ treatment a large decrease in fluorescence was observed. Root tip fluorescence was completely gone except for one root tip which had very weak fluorescence. There was also one root section with very weak fluorescence in the emergence zone of lateral roots, but crown root fluorescence completely disappeared.	
T_1 Phenotype Confirmed?: YES	
Comments: Fluorescence prior to Na^+ treatment is weak, but downregulated by Na^+.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.8: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVL C07.

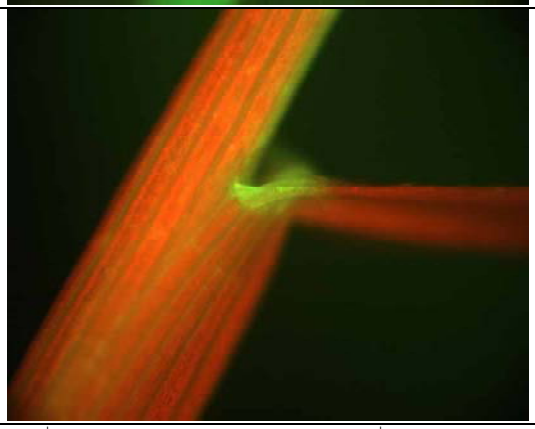
Line: AVN D05	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Root tip-specific fluorescence before Na^+ treatment. Following Na^+ treatment, entire root had fluorescence as well as some shoot collar fluorescence.	
T_1 Phenotype Confirmed?: YES	
Comments: Upregulation of root cap fluorescence to whole root fluorescence with some shoot collar fluorescence following Na^+ treatment.	
T_2 Phenotype: EXAMINED	
$-Na^+$	$+Na^+$
Root cap fluorescence upregulated to whole root and shoot collar fluorescence.	Root cap fluorescence upregulated to whole root and shoot collar fluorescence.
Comments: Appears the upregulation is developmentally regulated rather than Na^+ regulated.	

Figure 3.9: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVN D05.

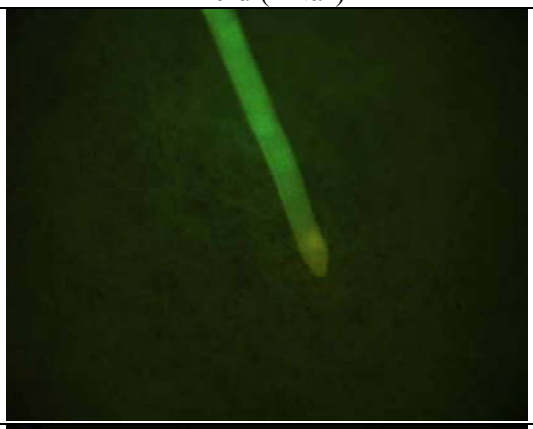
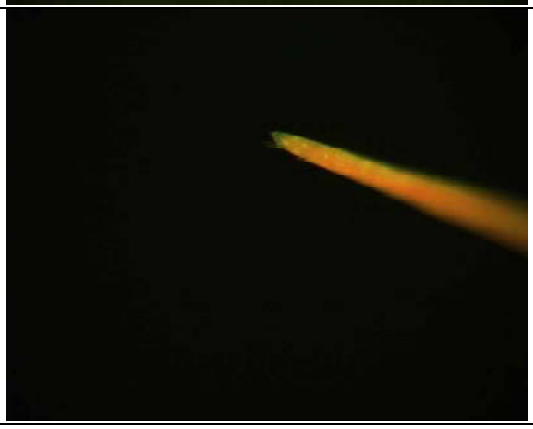
Line: AVP C11	**Putative Na^+ Regulation: Downregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Following Na^+ treatment, medium GFP fluorescence noted in the root and leaf tips prior to Na^+ treatment disappeared. Other root and shoot fluorescence remained, possibly not as bright as before Na^+ treatment.	
T_1 Phenotype Confirmed?: NO	
Comments: No fluorescence observed prior to Na^+ treatment.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.10: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVP C11.

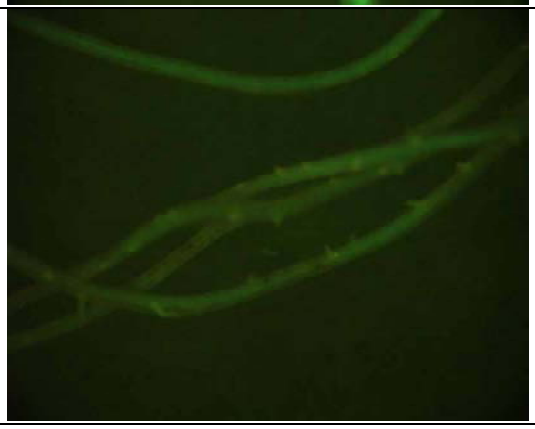
Line: AVP D11	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
No fluorescence	
No fluorescence	
Comments: Following Na^+ treatment medium fluorescence was observed in the entire crown and lateral root (except possibly tips). May be cortical fluorescence.	
T_1 Phenotype Confirmed?: YES	
Comments: No fluorescence observed prior to Na^+ treatment, and medium fluorescence observed in primarily lateral roots following Na^+ treatment.	
T_2 Phenotype: EXAMINED	
$-Na^+$	$+Na^+$
Upregulation of root fluorescence from weak to weak-medium.	Upregulation of root fluorescence from weak to bright.
Comments: Appears this line has trapped an enhancer element that is weakly upregulated by Na^+.	

Figure 3.11: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVP D11.

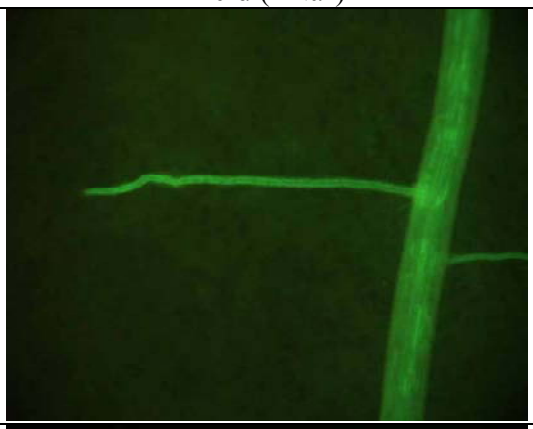
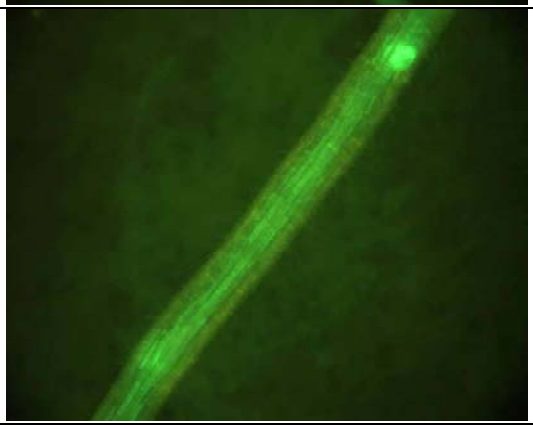
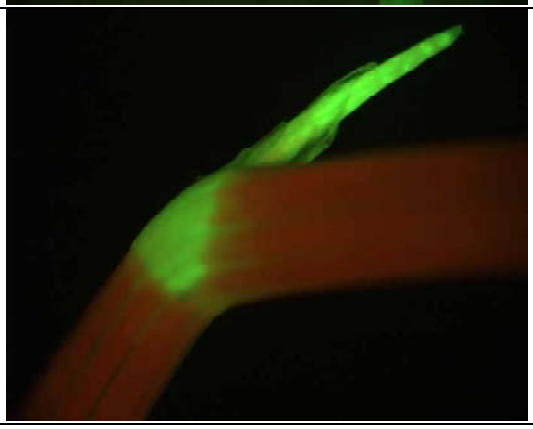
Line: AVS G07	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: At the initial seedling screen medium cortical root fluorescence was observed with no shoot fluorescence. Following Na^+ treatment root fluorescence became bright cortical fluorescence and bright shoot fluorescence was observed in the collar of the shoot as well as vascular fluorescence. Most striking was the emerging leaf which had bright epidermal fluorescence following Na^+ treatment.	
T_1 Phenotype Confirmed?: YES	
Comments: Root fluorescence was observed to be upregulated by Na^+ treatment from medium to bright, but medium shoot fluorescence was observed prior to Na^+ treatment which was upregulated to bright by Na^+ treatment.	
T_2 Phenotype: EXAMINED	
$-Na^+$	$+Na^+$
Medium root and shoot fluorescence prior to Na^+ treatment was upregulated to medium-bright fluorescence.	Medium root and shoot fluorescence prior to Na^+ treatment was upregulated to bright fluorescence.
Comments: Appears to be upregulation of fluorescence by Na^+, but only marginal compared to the $–Na^+$ treatment.	

Figure 3.12: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVS G07.

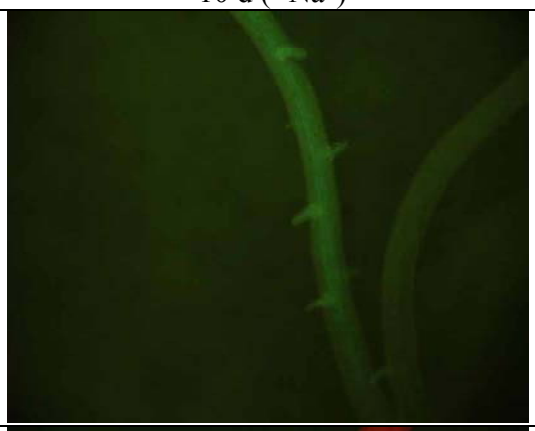
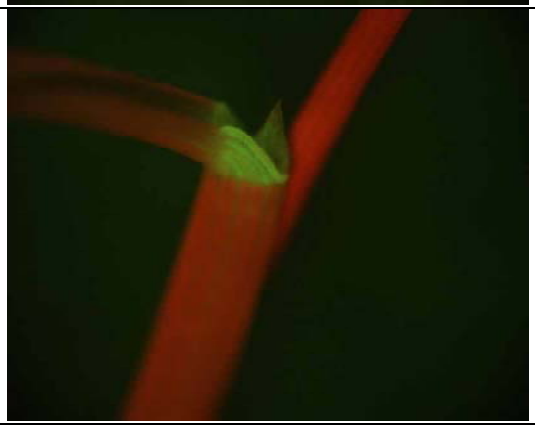
Line: AVT D09	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
No fluorescence	
No fluorescence	
Comments: Following Na^+ treatment faint-medium root cortical fluorescence appeared and medium shoot collar fluorescence appeared as well.	
T_1 Phenotype Confirmed?: NO	
Comments: Root fluorescence following Na^+ treatment was weak, and weak shoot collar fluorescence was upregulated to medium fluorescence by Na^+ treatment.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.13: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVT D09.

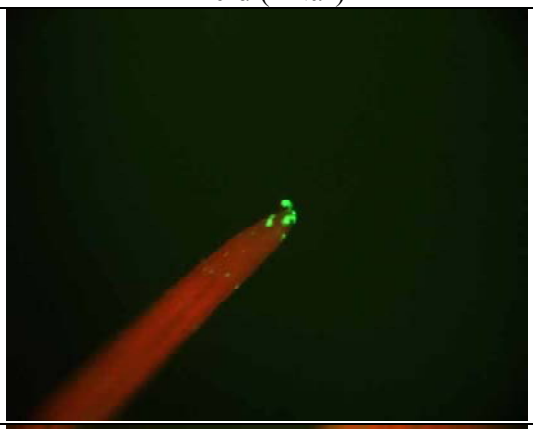
Line: AVY H12	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Following Na^+ treatment it appeared that there was increased trichome fluorescence and the hydathodes showed fluorescence as well. The root fluorescence remained stable in root tips following Na^+ treatment.	
T_1 Phenotype Confirmed?: NO	
Comments: Root tip fluorescence appeared to increase from weak to medium following Na^+ treatment, but no shoot fluorescence was observed prior to or following Na^+ treatment.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.14: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AVY H12.

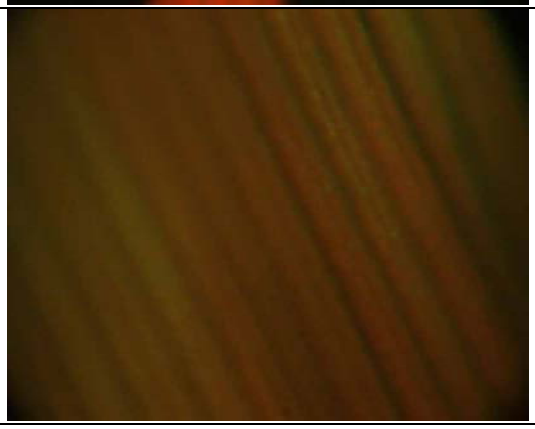
Line: AWC E12	**Putative Na^+ Regulation: Downregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Following Na^+ treatment all observed shoot fluorescence (vascular, guard cells, trichomes, leaf tips) disappeared. Root fluorescence remained medium-bright following Na^+ treatment.	
T_1 Phenotype Confirmed?: YES	
Comments: Medium-bright root fluorescence remained following Na^+ treatment, but medium-bright shoot fluorescence disappeared following Na^+ treatment.	
T_2 Phenotype: EXAMINED	
$-Na^+$	$+Na^+$
No change in root fluorescence, but large downregulation of shoot fluorescence.	No change in root fluorescence, but large downregulation shoot fluorescence.
Comments: Appears the downregulation is developmental as it is the same between $–Na^+$ and $+Na^+$ treatments.	

Figure 3.15: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AWC E12.

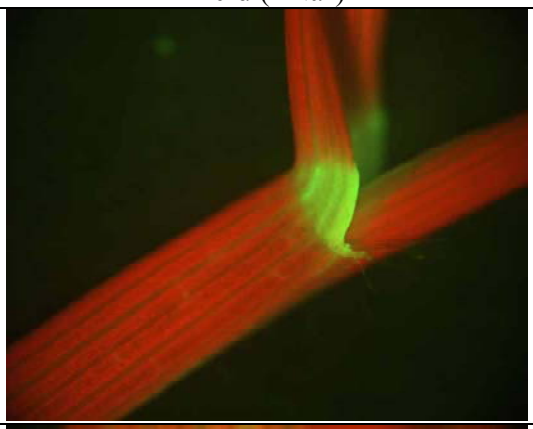
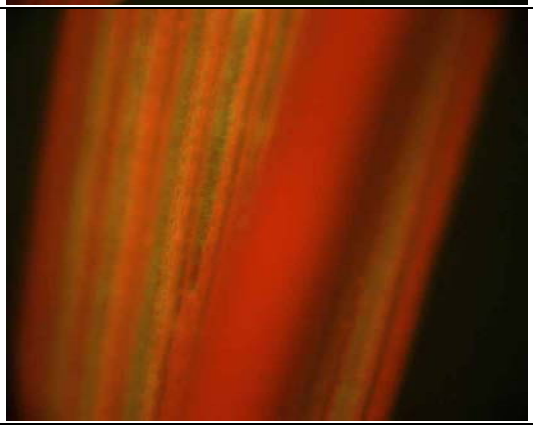
Line: AWD D07	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Following Na^+ treatment, root fluorescence remained the same, but shoot collar, leaf tip and vascular fluorescence was observed.	
T_1 Phenotype Confirmed?: YES	
Comments: Root fluorescence was unchanged following Na^+ treatment, but weak-medium shoot collar and vascular fluorescence was observed following Na^+ treatment.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.16: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AWD D07.

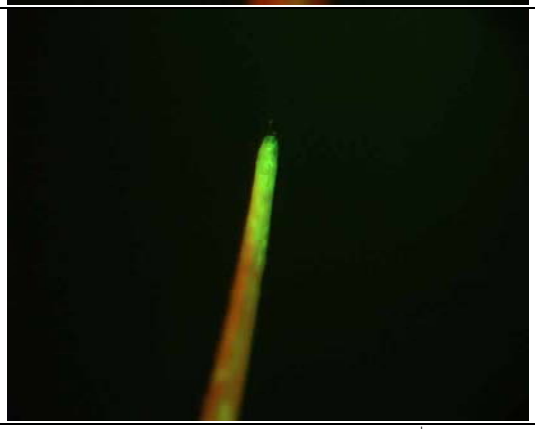
Line: AWD G08	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Shoot collar, vascular and tip fluorescence was observed following Na^+ treatment in addition to the root fluorescence prior to Na^+ treatment.	
T_1 Phenotype Confirmed?: YES	
Comments: Medium root fluorescence remained following Na^+ treatment, but medium fluorescence of shoot collar, vasculature, and leaf tips observed following Na^+ treatment.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.17: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AWD G08.

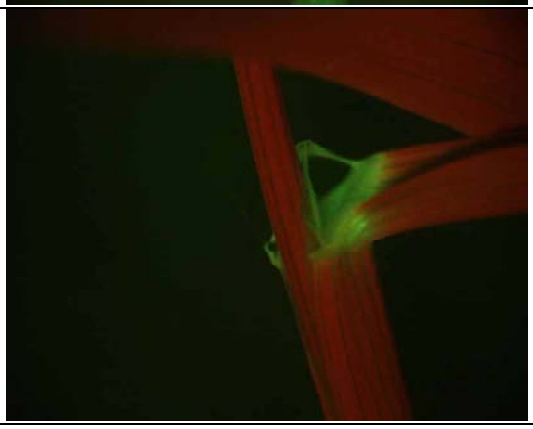
Line: AWF E04	**Putative Na^+ Regulation: Upregulated**
T_1 Phenotype	
7 d ($-Na^+$)	10 d ($+Na^+$)
	
	
Comments: Following Na^+ treatment, root fluorescence went from weak to medium-bright and medium shoot collar and vascular fluorescence appeared.	
T_1 Phenotype Confirmed?: NO	
Comments: Root fluorescence increased slightly from weak-medium to medium, while shoot fluorescence also increased from weak-medium to medium.	
T_2 Phenotype: NOT EXAMINED	
$-Na^+$	$+Na^+$
Comments:	

Figure 3.18: Summary of GFP fluorescence in response to 30 mM Na^+ treatment in line AWF E04.

3.4 General Discussion

The percentage of plants examined in this study that displayed GFP fluorescence (before and after Na^+ treatment) was very similar to that found in a previous screen of the population (Johnson *et al.* 2005) and is very similar to an *Arabidopsis GUS* enhancer trap library that was also screened for stress-regulated enhancer elements (Baxter-Burrell *et al.* 2003) (Table 3.2). As expected, the percentage of the population that expresses the reporter gene in enhancer trap populations is higher than in gene and promoter trap populations because expression of the reporter gene is not dependent on insertion of the trap within a gene (Lee *et al.* 2004, Alvarado *et al.* 2004). Baxter-Burrell *et al.* (2003) report a much higher frequency of trapping success in their gene trap population than Lee *et al.* (2004) or Alvarado *et al.* (2004). The gene trap used in the population screened by Baxter-Burrell *et al.* (2003) was modified from the traditional gene trap as it contains three splice acceptor sites and an intron upstream of the *GUS* reporter gene (Sundaresan *et al.* 1995). This means the reporter gene will be expressed when the trap is inserted in both introns and exons, thus increasing the trapping frequency.

Table 3.2: Comparison of data from this study with other studies which have screened gene trap populations for stress-regulated genetic elements including Alvarado *et al.* (2004), Lee *et al.* (2004) and Baxter-Burrell *et al.* (2003).

	This Study	Alvarado	Lee	Baxter-Burrell	
Trap Type	Enhancer	Promoter	Gene	Gene	Enhancer
Stress Applied	Na^+	Na^+	Cold	Low O_2	Low O_2
Reporter Gene	*GFP*	*luc*	*GUS*	*GUS*	*GUS*
Total Population	824	20,261	15,586	800	600
Overall Expression[(a)]	275 (33.4)	753 (3.7)	309 (2.0)[(d)]	224 (28.0)	210 (35.0)
Stressed Population	824	215[(b)]	15,586	800	600
Regulated by Stress[(e)]	79 (28.7)	18 (8.4)[(c)]	62 (20.1)	129 (57.6)	89 (42.4)
Upregulated by Stress[(e)]	62 (22.5)	11 (5.1)[(c)]	53 (17.2)	50 (22.3)	15 (7.1)
Downregulated by Stress[(e)]	17 (6.2)	7 (3.3)[(c)]	9 (2.9)	79 (35.3)	74 (35.2)

[(a)] Percentage of total population of lines expressing reporter gene before and after stress.
[(b)] 215 lines expressing luciferase were randomly chosen to subject to stress treatments.
[(c)] Numbers are for Na^+ regulated promoters only. ABA, glucose, cold and touch regulated promoters are not included in these figures.
[(d)] Calculated based on Jeon *et al.* 2000 which reported 2.0 % of this population showed GUS activity.
[(e)] Percentage of the population which expressed reporter gene before or after stress, rather than on the entire population.

Even though the successful trapping frequency is higher for enhancer trap populations, the proportion of the trapped elements that are regulated by stress should be similar among all three traps, since the proportion of enhancer, genes and promoters involved in a given stress response is likely similar. A wide variation exists between the observed proportions of stress-regulated trapped elements, thus it would seem there is a factor other than trap type which creates this variation. The response to different stresses may involve different proportions of the genome, but between the two studies of the Na^+ stress response there was a large difference in Na^+ regulated elements reported (29% vs. 8% for the Johnson and Alvarado populations, respectively). Alvarado *et al.* (2004) found much a much lower percentage of Na^+ regulated elements perhaps because they used a much higher throughput method than this study and may

have missed subtle changes in reporter gene expression as a result. Also, Alvarado *et al.* (2004) did not look at root expression, thus may have missed a significant portion of the changes in expression.

Baxter-Burrell *et al.* (2003) found an exceptionally large proportion of the trapped genes and enhancers in their populations were regulated by stress (57.6 and 47.4 %, respectively). It seems doubtful that approximately half of the genetic elements in *Arabidopsis* would be regulated by O_2 deprivation, but if the stress was too severe this may be an indication of a more general death response by the plant. Also, Baxter-Burrell *et al.* (2004) found that a higher frequency of trapped enhancers and genes were downregulated by O_2 deprivation than upregulated. This is the reverse to that found in the other studies of stress regulated genetic elements, which all found more upregulated than downregulated genes in response to stress. It seems that O_2 deprivation response is unique among stress responses in plants and may result from decreasing metabolism in response to low O_2 availability.

There appears to be promise for screening enhancer trap libraries for Na^+ responsive elements, but the protocol used in this study may need improvement to increase the successful isolation of Na^+ stress regulated enhancers.

It is quite likely that around 1 ml of water remained in the Petri dishes when the water was removed and the Na^+ solution was added. If this was the case, the actual Na^+ concentration that the plants were challenged with was only 33.3 mM (1 ml water + 5 ml 40 mM NaCl), which is a relatively mild Na^+ stress, even for Nipponbare rice. A larger Na^+ stress would increase the expression response of Na^+ regulated enhancers, thus increasing the difference in GFP fluorescence observed before and after Na^+ stress. It is likely that this increase would also increase the chance of isolating enhancers that are regulated by osmotic stress rather than Na^+ ion toxicity. However, the relationship between the two is closely linked, thus an enhancer regulated by one stress is likely to be regulated by the other and would still be a valuable genetic element regardless. This would increase the chances of identifying a responsive enhancer and the exact nature of the regulation could be determined later.

The screen was hindered by the growth requirements of rice. Two large *Arabidopsis* GAL4-GFP enhancer trap libraries are available (J Haseloff, University of

Cambridge and S Poethig, University of Pennsylvania), which could be screened more quickly since space requirements and seed limitations are less of an issue with *Arabidopsis*.

This screen examined over 800 lines, but only approximately 30 % of the lines displayed GFP fluorescence at any point during the screen, so the actual number of useful lines that was screened was around 250. If 5% of those lines have trapped Na^+ stress-regulated enhancers, only 12 or 13 lines would have been expected to be regulated by Na^+. The screen identified 18 lines as being putatively regulated by Na^+; but only two lines appeared to truly be regulated by Na^+. This is 0.8% of the population which showed GFP fluorescence and about 0.25% of the entire population (again, possibly related to the mild Na^+ stress that was applied). A larger population of 4,000 lines would yield 10 potentially useful lines, and could possibly be increased with a larger Na^+ stress. Again, this would be more reasonably accomplished in *Arabidopsis*.

Changes in GFP fluorescence in the present study had to occur within 3 d. Synthesis of GFP would occur immediately following the upregulation of the enhancer element, with transcription upregulated in a matter of minutes and translation within a matter of hours. Thus, upregulation of most enhancer elements would likely be observed accurately. However, GFP degradation may require longer than 3 d or insufficient quantity of GFP may degrade by 3 d, thus obscuring the true downregulation of GFP fluorescence in response to Na^+ stress. However, in the line AWC E12, complete downregulation of GFP fluorescence was observed in the shoot, which would imply that GFP has had sufficient time to completely degrade. The regulation of the trapped enhancer in that line was found to be a developmental signal, thus the fluorescence could have been decreasing even before the Na^+ treatment was applied, which would have given GFP more than 3 d to degrade. BOR1::*GFP* fusion protein was found to be completely degraded in 24 h after downregulation of the fusion protein by boron supply in *Arabidopsis* (Takano *et al.* 2003). Whether GFP alone is degraded at this rate is still a question, though it does imply that 3 d is sufficient for GFP degradation to occur.