Transcription Factors Important in the Regulation of Salinity Tolerance

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List of Abbreviations

%	Percent
[α-32P]dCTP	Radioactive deoxycytosine triphosphate
A. thaliana	*Arabidopsis thaliana*
AA	Amino acid
ABA	Abscisic acid
ABF	ABA responsive element-binding factor
ABREL	ABA response element-like
AGRF	Australian genome research facility
amiRNA	Artificial micro RNA
ANOVA	Analysis of variance
AP2	APETALA2
Arabidopsis	*Arabidopsis thaliana*
AREB	ABA responsive element binding protein
AtCBL	*Arabidopsis thaliana* calcineurin B-like Ca^{2+} binding protein
ATHK1	A. thaliana histidine kinase 1
bHLH	Basic helix-loop-helix
BLAST	Basic Local Alignment Search Tool
BLASTN	Basic Local Alignment Search Tool Nucleotide
BLASTP	Basic Local Alignment Search Tool Protein
bp	Base pair
BSA	Bovine serum albumin
bZIP	Basic-domain leucine-zipper
CaCl2	Calcium chloride
CaM	Calmodulin
CBF	C-repeat binding factor
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
CE1	Controlled environment 1
CIPK	CBL-interacting protein kinase 1
CM	Conserved DNA motifs
CPA1	Cation proton antiporter 1
CRT	C-repeat
cv.	Cultivar
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate mix
DRE	Dehydration responsive element
DREB	Dehydration responsive element binding protein
EC_e	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
EE	Evening element
EMSA	Electrophoretic mobility shift assay

ERE	Ethylene responsive element
EREBP	Ethylene responsive element binding protein
ERF	Ethylene responsive factor
EST	Expressed sequence tag
GA	Gibberellic acid
GAP	Glyceraldehyde 3-phosphate
GFP	Green fluorescent protein
GTE	Glucose TE
GUS	Beta-glucuronidase
HKT	High affinity K^+ transporter
HOG1	High osmolarity glycerol response 1
HPLC	High performance liquid chromatography
HTH	Helix-turn-helix
IG	Indole-glucosinate
JRE	Autonomous jasmonate-response element
K+	Potassium ion
LB	Lysogeny broth
LEA	Late embryogenesis abundant
MAFFT	Multiple sequence alignment program
MAPK	Mitogen-activated protein kinase
$MgCl^2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
mRNA	Messenger ribonucleic acid
MTIDK	A matrix derived from myosins, paramyosins, tropomyosins, intermediate filaments type I - V, desmosomal proteins and kinesins
Myb	Myeloblastosis
Na+	Sodium ion
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
O. sativa	Oryza sativa
PCR	Polymerase chain reaction
PP2C	2C protein phosphatase
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
QTL	Quantitative trait loci
R40	RNaseA diluted to 40µg/mL
RCD1	Radical induced cell death
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SIP	SOS3-interacting
Sln1	Synthetic lethal of N-end rule 1
SNRK2	Snf1-related protein kinase 2
SOS	Salt overly sensitive
SSC	Saline-sodium citrate
ssp.	Sub species
TAE	Tris-acetate-EDTA

T-DNA	Transfer DNA
TE	Tris-EDTA
TPA	The Plant Accelerator
uidA	β-glucuronidase
UV	Ultra violet
X-gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt
ZT	Zeitgeber time

Abstract

Salt tolerant plants are able to survive in saline soils by the virtue of an array of channels and pumps that minimise sodium entry into roots and loading into the xylem, as well as the sequestration of sodium in the vacuole of the cells of both root and shoot. Regulation of genes involved in conferring salt tolerance is thought to occur *via* a network of transcription factors. In this project, the aim is to identify transcription factors that are important in regulating genes involved in salinity tolerance.

Affymetrix Rice 57K GeneChip data from a previous project were used to analyse gene expression with and without salt stress in the shoots and roots of the salt sensitive *Oryza sativa* cultivar IR29 and the salt tolerant cultivars FL478, IR63731 and Pokkali. Transcription factors showing differential expression between the salt sensitive and salt tolerant cultivars were identified and confirmed by qRT-PCR. Six transcription factors with confirmed expression patterns were selected and transgenic rice plants were generated either constitutively or salt inducibly over-expressing each of the transcription factor coding sequences. Plants were also made expressing artificial microRNAs designed to reduce levels of transcripts of each transcription factor.

The altered expression of five transcription factors, *OsOrphan19*, *OsEREB67*, *OsbHLH17*, *OsLUX* and *OsMYB54* affected plant salinity tolerance, as evidenced by changes in Na^+ and K^+ accumulation and plant fresh weight. These five transcription factors show significant homology to other previously known stress responsive genes thus suggesting their involvement in plant stress responses. Further experiments such as chromatin immunoprecipitation sequencing and RNA-sequencing of transgenic plants need to be performed to identify the target promoters and downstream genes, respectively, to determine the precise role of these transcription factors in plant responses to salt stress.

Declaration

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Chapter 1. Literature review

1.1. Importance of cereals

Cereal production is of vital importance to the Australian economy. In the 2007-2008 growing season, over 13 million hectares were sown to wheat alone and almost 27 million hectares sown to all crops for grain production (Pink, 2010). For the year ending 30 June 2008, the gross value of wheat production was $5.3 billion, however losses occur each year as a result of abiotic stresses such as salinity (Commonwealth of Australia, 2001; Pink, 2010). One estimate puts the cost at $150 million each year (GRDC, 2010).

1.2. The problem of salinity

Soil salinity is a major problem reducing global crop productivity. With the rapidly expanding world population expected to increase by a third from six billion in 1999 to nine billion by 2042, global food supply will need to be increased dramatically (United States Census Bureau, 2007). To increase food production, new arable land must be found and the productivity of existing cultivated land improved. However, this is difficult considering rising soil salinity levels and soil degradation due to cultivation. Saline and sodic soils cover 397 million hectares (ha) and 434 million ha of the earth's surface, respectively (FAO, 2000). Currently, 45 million ha (19.5%) of the 230 million ha of irrigated land are salt affected and 32 million ha (2.1%) of the 1 500 million ha of dryland agriculture are salt affected (FAO, 2000). Of the salt affected area, 57.9 million ha is human-induced (FAO, 2000). In Australia alone, salinity is estimated to cost farmers, local government and government agencies $251 million annually (Australia, 2001).

Soil salinity can arise from natural causes (primary salinisation) and by human intervention (secondary salinisation). Natural causes include rainfall, windblown salt from the ocean, tsunamis and rock weathering. Human causes vary depending on the use of irrigation. Irrigation-induced salinity is the result of poor water management practices, such as over-application, inadequate drainage and seepage from nearby water canals (Umali, 1993). Non-irrigation induced salinity is caused by two factors. The first being dryland salinity, which is the result of rising water tables, due to the clearing of deep rooting flora that keep the water table down. The second is transient salinity, which results from low rainfall and high evaporation, combined with sodic soils with

poor water transmission properties, which cause salts to accumulate in the root zone (Rengasamy, 2002).

Soil is classified as saline if the electrical conductivity (EC_e) of saturated soil is greater than 4 dS m^{-1} (Staff, 1954). Saline soils accumulate ions of sodium, potassium, magnesium, calcium, chloride, sulphate, carbonate and bicarbonate (FAO, 2000). Sodium is the main ion that accumulates to toxic levels within the plant, however carbonates may be present at toxic levels within the soil (Rengasamy, 2002). Sodic soils have poor water transmission properties due to low aggregate stability and therefore porosity, resulting from the leaching of salts from the soil when the principle cation is sodium (Rengasamy & Olsson, 1991).

1.3. The effect of salt on plants

There are two primary effects of salt upon plants. The first is an osmotic effect (Munns & Termaat, 1986). The osmotic effect is immediate, reducing the water potential of the soil and consequently the amount of water available to the plant. The osmotic effect reduces both shoot and root growth within seconds (Munns & Termaat, 1986; Passioura & Munns, 2000). One pathway for osmotic stress signalling occurs via a mitogen-activated protein kinase (MAPK) cascade, involving transient activation of salt stress-inducible MAPK, for which transcripts accumulate to a maximum between 8 and 16 minutes following the imposition of osmotic stress (Munnik *et al.*, 1999). The genes activated downstream of the cascade have not been identified. The reduction in shoot growth is most likely due to hormonal signals such as cytokinins, abscisic acid (ABA), gibberellic acid (GA) or ethylene, produced in the root (Munns & Termaat, 1986). Once the plant has adjusted to the change in osmotic potential, growth resumes at a lower rate (Munns, 2002).

The second is an ion toxicity effect. Toxicity due to the accumulation of sodium and/or chloride ions takes time to affect plant growth and function. Sodium has been shown to be the primary toxic ion in *Triticum aestivum* (bread wheat) (Husain *et al.*, 2004; Kingsbury & Epstein, 1986; Kinraide, 1999), however another study showed that sodium and chloride act synergistically in their toxic affects upon bread wheat (Martin

& Koebner, 1995). Sodium has been shown to be the primary toxic ion in rice (Chi Lin & Huei Kao, 2001; Hong *et al.*, 2009; Tsai *et al.*, 2004), have less importance in *Arabidopsis thaliana* (Møller & Tester, 2007) and have minimal importance in soybean (*Glycine max*) (Luo *et al.*, 2005; Pantalone *et al.*, 1997), citrus (Storey & Walker, 1998) and grapevine (Walker *et al.*, 2004), where chloride is the primary toxic ion. This thesis primarily concerns rice, so sodium tolerance will be the focus.

Sodium accumulates in the older leaves, inducing early senescence. If new leaves are produced at a faster rate than the old leaves die, the plant will survive under saline conditions. Ions will be sequestered in the vacuole of the cells of the shoot and root, and if the salt stress continues, ions accumulate in the cell wall, effectively dehydrating the cell and causing cell death (Munns & Passioura, 1984). As a monovalent cation, sodium ions are similar to potassium ions and will replace potassium as a cofactor in enzymes. Sodium does not perform the same function as potassium, rendering the enzymes inactive (Greenway & Munns, 1980). Since the enzymes of glycophytes and halophytes are equally sensitive to a low potassium/sodium ion ratio in the cell, it is the ability to exclude or sequester sodium ions that confers salinity tolerance (Greenway & Munns, 1980).

1.3.1. Sodium entry into plant cells

Some of the main routes of Na^+ entry into the plant include voltage-independent non-selective cation channels and potassium transporters (Blumwald *et al.*, 2000; Demidchik & Tester, 2002; Schachtman & Liu, 1999). An example of the latter is the barley high affinity K^+ transporter *HvHAK1*, that mediates low affinity Na^+ influx under salt stress in the millimolar range (Santa-Maria *et al.*, 1997). In rice under salt stress, a significant proportion of Na^+ reaches the xylem through the apoplast, possibly due to degradation of the symplast (Yeo *et al.*, 1987). Influx may also be through Na^+ specific transporters, such as *AtHKT1;1* in *A. thaliana*, which mediates Na^+ specific uptake in *Xenopus laevis* oocytes and causes Na^+ hypersensitivity in *S. cerevisiae* (Uozumi *et al.*, 2000).

1.4. How plants deal with salinity

Plants have many and varied mechanisms for dealing with soil salinity. The tolerance of a given crop species is dependent on its ability to exclude sodium from the root (James *et al.*, 2006a), retrieve sodium from the xylem (Davenport *et al.*, 2007), return sodium from the shoot to the root through the phloem (Berthomieu *et al.*, 2003), sequester sodium ions in the vacuole of shoot and root cells and accumulate compatible solutes such as proline, glycinebetaine and sugars in response to increases in the sodium concentration of the soil (Aharon *et al.*, 2003; Bohnert *et al.*, 1995; Shi & Zhu, 2002; Yokoi *et al.*, 2002). Plants also increase the synthesis of late embryogenesis abundant (LEA) proteins including dehydrins to protect cellular structures such as proteins and membranes (Ingram & Bartels, 1996; Verslues *et al.*, 2006). Salt tolerant plants, such as barley, may also engage in cellular and subcellular partitioning of toxic ions, away from important cellular processes, such as photosynthesis (James *et al.*, 2006b). Plants will perform one or more of these activities depending on their specific tolerance mechanism, which can be broadly categorised into osmotic tolerance, Na^+ exclusion and tissue tolerance (Munns & Tester, 2008). Osmotic tolerance involves the accumulation of compatible solutes to maintain water uptake despite the reduced soil water potential. Na^+ exclusion, as the name suggests, involves actively excluding sodium from the shoot by preventing sodium from entering the root and pumping sodium out of the root. Tissue tolerance involves sequestering Na^+ ions in the vacuole away from important cellular processes and also the accumulation of compatible solutes to balance the osmotic potential across the tonoplast.

The Na^+ content of the shoot has traditionally served as a measure of the ability of a plant to exclude Na^+, which correlates with its salinity tolerance. The greater the ability to exclude Na^+, the more salt tolerant the plant is. This is true when comparing bread wheat and durum wheat, with bread wheat having a greater ability to exclude Na^+ and a higher salinity tolerance (Colmer *et al.*, 2006). Low sodium accumulation in bread wheat is controlled by the *Kna1* locus (Dvořak *et al.*, 1994) and by the genes *Nax1* and *Nax2* in durum wheat (James *et al.*, 2006a). The relationship between low shoot tissue sodium concentration and salt tolerance cannot be applied to the more salt tolerant barley however, which maintains higher Na^+ concentrations in the cell (Gorham *et al.*, 1990). Barley achieves this through a higher tissue tolerance and the sequestration of sodium ions (Gorham *et al.*, 1990). In tobacco, Na^+ and Cl^- ions can accumulate in the

vacuole to concentrations of 780 mM and 624 mM, respectively, whilst cytoplasmic concentrations are maintained below 100mM (Binzel *et al.*, 1988).

The efficacy of using Na^+ exclusion as a measure of salinity tolerance has been questioned in a recent study. In two experiments involving 38 and 21 genotypes of bread wheat, increased biomass and yield did not correlate with Na^+ exclusion (Genc *et al.*, 2007). Salinity tolerance also did not correlate with a high K^+:Na^+ ratio (Genc *et al.*, 2007). This is likely a species specific phenomenon.

The K^+:Na^+ ratio has been shown to be a better indicator of tolerance (Asch *et al.*, 2000). The K^+:Na^+ ratio is highly correlated with yield, where a higher K^+:Na^+ ratio is associated with greater yield under salt stress (Asch *et al.*, 2000).

For plants growing in saline environments, there is a threshold in salt concentration below which yield will remain at 100% and above which there will be a linear relationship between the decrease in yield and the increase in salinity (Maas & Hoffman, 1977). Both the threshold and the rate of the decrease in yield will differ for different plant species. There is significant variation in the salinity tolerance of different cereal species. The relative salt sensitivities of cereals, from the tolerant to moderately sensitive are; *Hordeum vulgare* (Barley), *Triticum aestivum* L. ssp. *aestivum* (Bread Wheat), *Triticum turgidum* L. ssp. *durum* (Durum Wheat) and *Oryza sativa* (Rice) (Francois *et al.*, 1986; Maas & Hoffman, 1977). The threshold NaCl concentration in hydroponic solution at which Barley yield is affected is approximately 60mM, equating to a shoot Na^+ concentration of around 400mmol.kg^{-1} DW (Tavakkoli *et al.*, 2011). When grown in soil supplemented with Na^+ salts to 120mmol.kg-1 DW, plant growth was reduced by 11-25% depending on the cultivar (Tavakkoli *et al.*, 2011). The NaCl threshold at which rice yield is affected is 1.3dS.m^{-1}, which is approximately 15mM (Motamed *et al.*, 2008).

Salt tolerant cereals maintain low cytoplasmic Na^+ concentrations, accumulate Na^+ in the vacuole and may also engage in subcellular partitioning of ions (James *et al.*, 2006b). The salt tolerant barley cv. Franklin accumulates Na^+ and K^+ in mesophyll cells whilst maintaining lower concentrations in epidermal cells, relative to the salt sensitive durum wheat cv. Wallaroi (James *et al.*, 2006b). The maintenance of a higher K^+/Na^+ ratio and ion accumulation in the vacuole in barley also means that leaf Na^+ must accumulate to a higher concentration before inhibition of photosynthesis is comparable to salt sensitive wheat (James *et al.*, 2006b).

Plants indicate the sensing of a stress through the use of signalling molecules, such as abscisic acid (ABA) and calcium ions.

1.5. Signalling molecules in salt stress response

1.5.1. ABA

ABA accumulates in response to osmotic stresses such as drought, salt and freezing which activate downstream signalling cascades, allowing the plant to respond to the stress (Bray, 1993; Chen & Gusta, 1983).

In response to abiotic stress in *A. thaliana,* the PYR/PYL/RCAR ABA receptors bind ABA and inhibit the activity of group A type 2C protein phosphatases (group A PP2C), such as ABI1, ABI2, HAB1, HAB2 and PP2CA (Ma *et al.*, 2009b; Park *et al.*, 2009; Santiago *et al.*, 2009; Vlad *et al.*, 2009).When not bound to ABA, group A PP2C's regulate the activity of SnRK2s through dephosphorylation of serine and threonine residues in the activation loop (Umezawa *et al.*, 2009; Vlad *et al.*, 2009). ABA bound PP2C releases Snf1-related protein kinase 2 (SNRK2) serine/threonine kinases which phosphorylate transcription factors such as ABA responsive element binding proteins (AREB), ABA responsive element-binding factors (ABF) and basic-domain leucine-zipper (bZIP) transcription factors (Chae *et al.*, 2007; Fujita *et al.*, 2009; Furihata *et al.*, 2006; Kobayashi *et al.*, 2005).

AREB1/ABF2, *AREB2/ABF4* and *ABF3* are induced by dehydration, NaCl and exogenous ABA (Fujita *et al.*, 2005). Overexpression of each individually enhances plant drought tolerance (Fujita *et al.*, 2005; Kang *et al.*, 2002; Kim *et al.*, 2004). AREB1, AREB2 and ABF3 require ABA for full activation. In response ABA and water stress, the bZIP transcription factors AREB1, AREB2 and ABF3 form homo- and hetero-dimers and interact with SRK2D/SnRK2.2 (Yoshida *et al.*, 2009). The ABFs bind AREB in target gene promoters (Choi *et al.*, 2000). The *areb1 areb2 abf3* triple mutant has reduced drought tolerance and was more resistant to ABA, than any single or double mutants, suggesting overlapping functions. The *areb1 areb2 abf3* triple mutant treated with 50μM ABA, dehydration stress and 250mM NaCl stresses displayed reduced expression of genes responsive to ABA, dehydration, osmotic, and/or

salt stresses, suggesting that the plants are impaired in water stress- and ABA-dependent gene expression (Yoshida *et al.*, 2009).

The upstream signalling events responsible for the accumulation of ABA are not known definitively, however, two osmosensing protein kinases, histidine kinase 1 (ATHK1) and cytokinin response 1 (Cre1), have been identified in *A. thaliana* that are functionally similar to a yeast osmosensor synthetic lethal of N-end rule 1 (Sln1) (Reiser *et al.*, 2003; Urao *et al.*, 1999). *ATHK1* was overexpressed in the yeast mutant *sln1-ts*, which has constitutive activation of the high osmolarity glycerol response 1 (HOG1) mitogen-activated kinase (MAPK) cascade at 37ºC. *ATHK1* suppressed the temperature sensitive phenotype by suppressing HOG1 hyperactivation (Urao *et al.*, 1999). Overexpression of *ATHK1* in the *sln1Δ sho1Δ* yeast double mutant, lacking two osmosensors, restored tolerance to high osmolarity. This was achieved through activation of the HOG1 MAPK cascade (Urao *et al.*, 1999). Similarly, expression of *Cre1* in *sln1-ts* mutant yeast complemented the temperature sensitive phenotype by suppressing HOG1 hyperactivation (Reiser *et al.*, 2003). High osmolarity treatment of *sln1-ts* mutant yeast complemented with either *CRE1* or *SLN1* induced activation of HOG1, indicating that *CRE1* and *SLN1* are negatively regulated by osmotic stress (Reiser *et al.*, 2003). These osmosensing proteins may regulate ABA accumulation, but the link has not yet been identified.

As well as regulating gene expression in response to osmotic stresses, ABA also regulates seed development, inducing the expression of *LEA* genes (Chandler & Robertson, 1994), and also regulates stomatal aperture by activating or inhibiting ion transporters in guard cells (Bray, 1997) in a Ca^{2+} –dependent and –independent manner (Hetherington & Brownlee, 2004; Levchenko *et al.*, 2005; Marten *et al.*, 2007). The phosphatase-kinase pair ABA-insensitive 1 and open stomata 1 ABI1-OST1/SRK2E/SnRK2–6 regulates the guard cell anion channel SLAC1. In *Xenopus* oocytes, the coexpression of *OST1* and *SLAC1* resulted in anion currents similar to guard cells. Expression of *ABI1* then prevented SLAC1-related anion currents, by a direct interaction with SLAC1 and a physical interaction and with OSTI leading to inhibition of the kinase independently of phosphatase activity (Geiger *et al.*, 2009; Lee *et al.*, 2009).

1.5.2. Calcium

Free cytosolic calcium ($[Ca^{2+}]_c$) is a messenger responsive to many different stimuli. The calcium signal is represented by its concentration, spatial and temporal localisation and oscillation frequency (Dolmetsch *et al.*, 1998; Holdaway-Clarke *et al.*, 1997; Li *et al.*, 1998; Li *et al.*, 2004; Ng & McAinsh, 2003), which can be visualised using fluorescent dyes and the luminescent protein aequorin (Read *et al.*, 1993).

Calcium has been implicated in stomatal closure (McAinsh, 1990), responses to drought and salinity (Knight *et al.*, 1997), cold (Knight *et al.*, 1996) and heat shock (Gong *et al.*, 1998) amongst other responses outlined in reviews (Ng & McAinsh, 2003; Sanders *et al.*, 1999).

Calcium in the cytoplasm remains in the micromolar range, which is conducive to signalling as a 10 to 20 fold increase can occur with the release of a relatively small amount calcium from intracellular or extracellular locations. The calcium signal is relayed to the target proteins or processes, through the action of calcium sensors known as EF-hand proteins (Ikura, 1996). One family of calcium sensors is the calmodulin (CaM) family, which contain four EF-hand Ca^{2+} binding domains (Zielinski, 1998). CaM do not have enzymatic activity on their own but rather bind proteins in the cytoplasm or nucleus in a Ca^{2+} dependent manner, either activating or inhibiting their activity (Zielinski, 1998). The interaction between CaM and the specific target protein determines the concentration at which four Ca^{2+} ions are bound, how rapidly the target protein is activated upon a Ca^{2+} signal and how rapidly the target protein is down-regulated upon removal of the Ca^{2+} signal (Zielinski, 1998).

Another family of Ca^{2+} binding proteins is the *A. thaliana* calcineurin B-like Ca^{2+} binding protein (AtCBL) family, which includes members such as *AtCBL4/SOS3* which is induced by salt and *AtCBL1* which is induced by drought, cold and wounding (Kudla *et al.*, 1999; Liu & Zhu, 1998). The CBL4/SOS3 protein interacts with other members of the SOS pathway, such as the protein kinase CIPK24/SOS2 (Halfter *et al.*, 2000), while CBL1 protein interacts with Mn^{2+}-dependent CBL-interacting protein kinase 1 (CIPK1) (Shi *et al.*, 1999). The SOS pathway is discussed in Section 1.5.4, below.

Plants have evolved many different transport systems for regulating ion homeostasis. Only some of these will be discussed here. They include, HKT, NHX and the SOS pathways.

1.5.3. High affinity K^+ transporter (HKT)

High affinity K^+ or Na^+ uptake is mediated by HKTs. HKTs have been isolated from wheat (Schachtman & Schroeder, 1994), *A. thaliana* (Uozumi *et al.*, 2000), rice (Horie *et al.*, 2001) and the ice plant *Mesembryanthemum crystallinum* (Su *et al.*, 2003). HTKs will be discussed using the nomenclature proposed by Platten *et al.* (2006).

These K^+ transporters are believed to have evolved from bacterial K^+ channel proteins via gene duplication events (Durell *et al.*, 1999). HKTs contain four M1PM2 motifs, consisting of four transmembrane portions, M1 and M2, each joined by the hairpin segment, P-loop, to form $M1PM2_A$, $M1PM2_B$, $M1PM2_C$ and $M1PM2_D$ (Durell *et al.*, 1999; Maser *et al.*, 2002; Rodriguez-Navarro, 2000). The highly conserved P-loops on the outer surface of the transporter each contain a glycine-tyrosine-glycine motif, the terminal "filter" glycine of which is critical for K^+ specificity (Maser *et al.*, 2002). HKTs may be dependent upon H^+ for K^+ transport as occurs in yeast (Bihler *et al.*, 1999; Lichtenberg-Fraté *et al.*, 1996), or dependent on Na^+ for K^+ transport (Rubio *et al.*, 1995; Tholema *et al.*, 1999).

The first characterised HKT, *TaHKT2;1*, was isolated from a *Triticum aestivum* cDNA library, made from root material grown in K^+ free medium (Schachtman & Schroeder, 1994). Expression of *TaHKT2;1* in K^+ uptake deficient *Δtrk1 trk2* double mutant yeast (strain CY162), allowed growth in medium with 30 μM K^+. Voltage clamp studies in *Xenopus* oocytes determined that TaHKT2;1 was a H^+/K^+ symporter, transporting ~1 H^+ for every K^+ ion. *In situ* hybridisation determined that *TaHKT2;1* was expressed in root cortical cells and cells surrounding leaf vascular tissue (Schachtman & Schroeder, 1994).

A *TaHKT2;1* homolog, *AtHKT1;1*, was isolated from *A. thaliana* (Uozumi *et al.*, 2000). The measurement of Na^+ and K^+ currents in *Xenopus laevis* oocytes expressing *AtHKT1;1*, determined that AtHKT1;1 functioned as a Na^+ specific uptake transporter but was unable to mediate K^+ uptake (Berthomieu *et al.*, 2003; Uozumi *et al.*, 2000). *AtHKT1;1* expression caused Na^+ hypersensitivity in *S. cerevisiae* and was also unable to rescue K^+ uptake deficient *S. cerevisiae* (Uozumi *et al.*, 2000). Immunostaining using an anti-AtHKT1;1 antibody and an *AtHKT1;1* promoter *uidA* gene fusion determined that AtHKT1;1 was localised to the plasma membrane of xylem parenchyma cells in leaves but not xylem vessel tubes or phloem cells (Sunarpi *et al.*, 2005). In another study, an *AtHKT1;1* promoter *uidA* gene fusion showed GUS activity in the phloem tissues of roots, leaves and flower peduncles and *in situ* hybridisation also found *AtHKT1;1* expression in root stelar tissue (Berthomieu *et al.*, 2003). In the same study, Northern blots determined that *AtHKT1;1* was expressed predominantly in the roots in both salt free and salt containing growth medium (Berthomieu *et al.*, 2003).

The *A. thaliana athkt1-3* (Ws and Col-0), *athkt1-1* and the fast neutron *athkt1* disruption allele FN1148 mutants, all had significantly increased Na^+ and lower K^+ concentrations in their xylem sap at 75 mM NaCl, relative to wild type Col-0 (Sunarpi *et al.*, 2005). Similarly, *A. thaliana sas2-1* mutants, which have a mutation in the *AtHKT1;1* gene, accumulate 35-40% more Na^+ in the aerial portions of the plant, excluding the stem, compared to wild type plants (Berthomieu *et al.*, 2003). Consistent with low xylem sap K^+, shoot K^+ was lower in all *athkt1* mutants, compared to wild type plants. Also, the root K^+ concentration was higher in all *athkt1* mutants, relative to wild type plants. All mutants had reduced phloem Na^+ compared to wild type plants. High Na^+ and low K^+ in the xylem suggests that Na^+ and K^+ transport are coupled and that *AtHKT1;1* functions in Na^+ unloading from xylem vessels into xylem parenchyma cells (Sunarpi *et al.*, 2005). High Na^+ in the aerial portions of the mutants but not in the roots, low Na^+ in the phloem and expression of *AtHKT1;1* in phloem tissues also suggests that AtHKT1;1 functions in Na^+ loading into the phloem in the shoots and unloading in the roots (Berthomieu *et al.*, 2003).

In rice, the two HKTs *OsHKT2:1* and *OsHKT2:2* were first isolated from Nipponbare and Pokkali, respectively (Horie *et al.*, 2001). Heterologous expression of *OsHKT2:1* in K^+ uptake deficient *Δtrk1 trk2* double mutant yeast (strain CY162), did not complement

the mutant phenotype, however *OsHKT2:2* allowed growth at 0.1 mM KCl. Heterologous expression of *OsHKT2:1* and *OsHKT2:2* in Na^+ sensitive *Δena1-4* mutant yeast (strain G19), caused growth inhibition at 50 mM and 100 mM NaCl, and complete inhibition at 150 mM. Inhibition by *OsHKT2:1* was greater than *OsHKT2:2*. Voltage clamp studies expressing *OsHKT2:1* and *OsHKT2:2* in *Xenopus* oocytes found large inward currents for Na^+. Only *OsHKT2:2* elicited small K^+ currents in the absence of Na^+ and large currents in presence of Na^+. *OsHKT2:1* did not mediate K^+ uptake at all. It was determined that *OsHKT2:1* was a Na^+ transporter and *OsHKT2:2* was a K^+/Na^+ symporter (Horie *et al.*, 2001).

In rice there are seven HKT Na^+ transporters and two HKT pseudogenes (Garciadeblas *et al.*, 2003). BLAST searches using the amino acid (AA) sequence of wheat TaHKT2;1, *A. thaliana* AtHKT1;1 and rice OsHKT2:1 and OsHKT2:2, identified eight rice HKTs (*OsHKT2;1*, *OsHKT2;3*, *OsHKT2;4*, *OsHKT1;1-5*) in cv. Nipponbare (Garciadeblas *et al.*, 2003). *OsHKT1;4* and *OsHKT1;5* could not be cloned as cDNA synthesis stopped at the second exon. *OsHKT1;2* was a non-functional gene with three internal stop codons. The five remaining HKTs were expressed in the K^+ uptake deficient *Δtrk1 trk2* double mutant yeast (strain CY162), and except for *OsHKT1;1*, caused a growth penalty over the negative control, at 1 mM K^+. *OsHKT2;1* and *OsHKT1;1* were found to encode Na^+ transporters, while *OsHKT2;3*, *OsHKT1;3* and *OsHKT2;4* were unable to mediate Na^+ transport, even in medium with 0.1 μM K^+. OsHKT2;1 had a K_m of 20 μM for Na^+, a V_{max} of 15 nmol mg^{-1} min^{-1} and a K_i for K^+ of 70 μM. OsHKT2;1 did not mediate K^+ uptake, even at 100 μM K^+. OsHKT1;1 had a K_m of 11 mM for Na^+, a V_{max} of 33 nmol mg^{-1} min^{-1} and a K_i for K^+ of 4 mM (Garciadeblas *et al.*, 2003). This indicates that *OsHKT2;1* and *OsHKT1;1* encode high and low affinity Na^+ transporters, respectively.

A phylogenetic tree using an alignment of the P_A loops and $M2_D$ helix determined that most HKTs were very divergent, except for the group of OsHKT2;1, Po-OsHKT2;2, TaHKT2;1, OsHKT2;3 and OsHKT2;4 (Garciadeblas *et al.*, 2003). Of the group, only OsHKT2;1 did not have a conserved glycine in the P-loop, instead having a serine. A new nomenclature proposed for the naming of HKTs clarifies the differences between different HKTs. Members of subfamily 1 have a serine in the first pore loop of the

protein, while members of subfamily 2 have a glycine. The serine makes the transporter Na^+ specific, while the glycine makes the transporter K^+ specific (Platten *et al.*, 2006).

1.5.4. SOS

The salt overly sensitive (SOS) pathway was first identified in *A. thaliana* and has more recently been found in rice (Martinez-Atienza *et al.*, 2007; Wu *et al.*, 1996). The SOS pathway contains three main proteins, SOS1, a plasma membrane Na^+/H^+ antiporter, CIPK24/SOS2, a protein kinase and CBL4/SOS3, a calcium sensor. In response to an external stimulus, such as Na^+, transient increases in cytoplasmic Ca^{2+} occur. *CBL4/SOS3* encodes a calcium sensor that has high sequence identity with the yeast calcineurin B subunit and animal neuronal calcium sensors (Cyert & Thorner, 1992; Schaad *et al.*, 1996). It is predicted to have three EF-hand Ca^{2+} binding sites and also requires N-myristoylation for activity (Ishitani *et al.*, 2000; Liu & Zhu, 1998). Myristoylation facilitates reversible membrane attachment (Johnson *et al.*, 1994). Myristoylated and Ca^{2+} bound CBL4/SOS3 becomes active and will bind CIPK24/SOS2, recruiting it to the plasma membrane. The *sos3-1* allele has a three AA deletion in the second EF-hand, which reduces the ability of CBL4/SOS3 to activate the kinase activity of CIPK24/SOS2 and also reduces the capacity of CBL4/SOS3 to bind calcium, indicating that calcium is necessary for the activation of CIPK24/SOS2 by CBL4/SOS3 (Ishitani *et al.*, 2000).

CIPK24/SOS2 contains an N-terminal catalytic domain with serine/threonine kinase activity and a C-terminal regulatory domain, both of which were essential for activity (Liu *et al.*, 2000). In a yeast two-hybrid screen, the C-terminal regulatory domain of CIPK24/SOS2 was found to interact with CBL4/SOS3 (Guo *et al.*, 2001; Halfter *et al.*, 2000). The regulatory domain alone interacted more strongly than full length CIPK24/SOS2, indicating the N-terminal kinase interferes with binding. Serial deletions of the regulatory domain determined that a 21 AA region between Met^{309} and Arg^{330} was necessary and sufficient for binding. This was designated the FISL motif. In an *in vitro* assay, FISL was found to bind specifically to CBL4/SOS3 (Guo *et al.*, 2001). Binding was not dependent on calcium (Guo *et al.*, 2001; Halfter *et al.*, 2000). In a yeast two-hybrid assay, the CIPK24/SOS2 C-terminal regulatory domain and the N-terminal kinase were found to strongly interact (Guo *et al.*, 2001). This suggests that

autoinhibition keeps CIPK24/SOS2 in an inactive state, the regulatory domain blocking substrate binding to the kinase active site.

A superactive CIPK24/SOS2 kinase was created by deleting the FISL motif and mutating the active loop sequence, changing Thr^{168}-to-Asp. The superactive CIPK24/SOS2 was unaffected by the presence or absence of CBL4/SOS3 (Guo *et al.*, 2001). The *sos2-5* mutation has a substitution of a glycine residue with glutamate in the catalytic domain, which abolishes kinase activity and prevents autophosphorylation of CIPK24/SOS2, inhibiting CIPK24/SOS2 function (Liu *et al.*, 2000). Binding of CBL4/SOS3 to CIPK24/SOS2 is required for activation of the CIPK24/SOS2 catalytic domain, which phosphorylates serine and threonine residues and Ca^{2+} is required for CBL4/SOS3 activation of the CIPK24/SOS2 kinase, indicating that CIPK24/SOS2 acts downstream of CBL4/SOS3 (Halfter *et al.*, 2000). *sos2sos3* double mutants displayed comparable NaCl sensitivity to *sos2* mutants. The lack of an additive effect of the two mutations indicates that they function as a part of the same pathway (Halfter *et al.*, 2000).

The CBL4/SOS3-CIPK24/SOS2 complex then phosphorylates the C-terminal auto-inhibitory domain of the plasma membrane Na^+/H^+ antiporter SOS1, which becomes activated and pumps Na^+ out of the cell (Quintero *et al.*, 2011). The *SOS1* gene contains 22 introns and 23 exons, encoding a peptide of 1,146 AAs with 12 predicted transmembrane domains (Shi *et al.*, 2000). The transmembrane regions share high sequence identity with Na^+/H^+ antiporters from Chinese hamster and *Pseudomonas aeruginosa* (Counillon & Pouyssegur, 1993; Utsugi *et al.*, 1998). The hydrophilic 700 AA C-terminal tail is predicted to reside in the cytoplasm (Shi *et al.*, 2000). *SOS1* expression is up-regulated in both shoots and roots by salt stress but not cold stress or ABA (Shi *et al.*, 2000). Expression patterns of *SOS1* were examined by fusing the ß-glucuronidase (*GUS*) gene to the *SOS1* promoter (Shi *et al.*, 2002). In the root, GUS was localised to the pericycle and parenchyma cells bordering the xylem vessels. In the stem and petiole, GUS was localised to parenchyma cells at the xylem/symplast boundary, suggesting that SOS1 functions in Na^+ loading and unloading from the xylem (Shi *et al.*, 2002). In 100 mM NaCl, the Na^+ concentration in xylem sap increased at a faster rate in *sos1* plants, compared to wild type plants, indicating that SOS1 functions in the pumping of Na^+ out of the xylem (Shi *et al.*, 2002).

The SOS pathway is not limited to the three main proteins as it interacts with other stress related proteins. CIPK24/SOS2 interacts with protein phosphatase 2C ABI2 (Ohta *et al.*, 2003), a negative regulator of ABA signalling (Sheen, 1998). SOS1 interacts with radical induced cell death (RCD1), a regulator of oxidative stress responses (Katiyar-Agarwal *et al.*, 2006), while seven SOS3-interacting (SIP) kinases have been identified, although no functions have been identified for these (Halfter *et al.*, 2000). CIPK24/SOS2 also interacts with the SOS3/CBL4 homolog, CBL10, in a novel Ca^{2+}-dependent salt tolerance signalling pathway, although the recent two studies conflict over whether the CBL10-CIPK24 complex is associated with vacuolar compartments or the plasma membrane (Kim *et al.*, 2007a; Quan *et al.*, 2007).

1.5.5. NHX

The cation proton antiporter 1 (CPA1) family of *A. thaliana* cation/H^+ antiporters contains eight members that have been termed AtNHX (Maser *et al.*, 2001; Ward, 2001) These mediate Na^+/H^+ exchange, with the electrochemical gradient of H^+ driving the influx of Na^+ into the vacuole of cells in the root and shoot (Apse *et al.*, 1999). AtNHX1-5 are equally effective in mediating Na^+/H^+ exchange, as the expression of *AtNHX1-5* in *nhx1* yeast was found to rescue the salt sensitive phenotype in 50-250 mM NaCl at pH 5.5 (Aharon *et al.*, 2003). These proteins are similar to the bacterial Na^+/H^+ antiporter, NhaA, for which the mechanism of cation/proton antiporting was recently identified (Arkin *et al.*, 2007). The electrochemical gradient of H^+ driving NHX is generated by a H^+ ATPase and an inorganic pyrophosphatase, *AtAVP1*, that pump protons into the vacuole (Gaxiola *et al.*, 2001; Rea & Sanders, 1987; Sarafian *et al.*, 1992). Cation transport may not be limited to Na^+, as AtNHX1 catalysed H^+ exchange with Na^+ and K^+ in reconstituted liposomes, with Km's of 45 mM and 42 mM, respectively (Venema *et al.*, 2002). Isolated tomato tonoplast vesicles overexpressing *AtNHX1* also mediated Na^+ and K^+ transport (Zhang & Blumwald, 2001).

Overexpression of *NHX*, the H^+ ATPase or inorganic pyrophosphatase in various plant species, enhances tolerance to NaCl. *AtNHX1* has been overexpressed in *A. thaliana* (Apse *et al.*, 1999), *Brassica napus* (Canola) (Zhang *et al.*, 2001) and tomato (Zhang & Blumwald, 2001). The wheat Na^+/H^+ antiporter (*TNHX1*) and H^+-pyrophosphatase

(*TVP1*) have also been overexpressed in *A. thaliana* (Brini *et al.*, 2005). Wild type *A. thaliana* plants showed growth inhibition at 100 mM NaCl while plants overexpressing *AtNHX1* displayed a normal phenotype and went on to flower at 200 mM NaCl (Apse *et al.*, 1999). Similarly *A. thaliana* overexpressing *TNHX1* or *TVP1* had normal phenotypes at 100-200 mM NaCl, whereas wild type plants became chlorotic and died after ten days of salt treatment (Brini *et al.*, 2005). Analysis of Na^+/H^+ exchange rates in vacuoles isolated from *A. thaliana* overexpressing *AtNHX1* and wild type plants showed that exchange rates were enhanced in transgenics and that H^+ movements were Na^+-dependent (Apse *et al.*, 1999). Overexpression of *AtNHX1* in tomato plants enhanced NaCl tolerance, with Na^+ accumulating in the leaves but not fruit (Zhang & Blumwald, 2001).

Knocking out *AtNHX1* in *A. thaliana* increased the salt sensitivity of the plant. In *AtNHX1* T-DNA insertional mutants, when assessing germination, only 50% of *nhx1* mutants survived on 100 mM NaCl after two weeks, whereas wild type plants required 200 mM to be similarly inhibited. Also, growth in NaCl free media for two weeks, then treatment with 100 mM NaCl for ten days resulted in growth inhibition and a reduction in leaf area in *nhx1* plants relative to wild type plants. Na^+ and K^+-dependent H^+ efflux was higher in wild type plant vacuoles, relative to the *nhx* mutants (Apse *et al.*, 2003).

For *AtNHX1* to effectively function in the sequestration of sodium ions to prevent toxic concentrations building within the cytoplasm of the cell, it must be responsive to certain stimuli. An RNA blot analysis determined that *AtNHX1* was up-regulated by 300 mM NaCl, 100 μM ABA and 300 mM KCl, but not by cold or dehydration in both Ler and Col-0 *A. thaliana* (Shi & Zhu, 2002).

1.6. Improving salinity tolerance in plants

1.6.1. Plant breeding

Plant breeding may be used to generate cereals with enhanced salinity tolerance. This is restricted to plants of the same species that are sexually compatible. Two plants with advantageous traits, such as high yield and salinity tolerance, may be crossed in the hope that some of the progeny will inherit both of the desirable traits. Wild relatives or landraces may also be used to introduce new traits. This process may be accelerated through the use of quantitative trait loci (QTL), employing single nucleotide polymorphisms (SNPs) and molecular markers to track the trait (Torjek *et al.*, 2003). QTL are regions of DNA associated with a particular trait, that may or may not contain the gene responsible for that trait. However, the QTL always cosegregate with the trait. QTL may be identified by the genetic analysis of recombinant inbred populations that are near isogenic. The recombinant inbred population may be subjected to salt stress, the phenotypes analysed and QTL responsible for salinity tolerance identified. This has been used effectively in wheat (Ma *et al.*, 2007), rice (Flowers *et al.*, 2000; Lin *et al.*, 2004), *A. thaliana* (Quesada *et al.*, 2002).

The use of QTL to find the genes responsible for a phenotype is known as forward genetics. This is a promising approach as plants are selected for a specific phenotype such as salt tolerance, then the genes responsible for that phenotype are identified using approaches such as map based cloning. The alternative is reverse genetics, where genes are identified as being responsive to a given stress using techniques such as a microarray. The expression of the identified gene is then altered and the phenotype of the resultant transgenic analysed. The reverse genetics approach has gained less public acceptance due to genetic transformation being involved, even though it has yielded plants with enhanced salt tolerance. A non-transgenic method of reverse genetics does however exist and has been used recently to improve wheat (Slade *et al.*, 2005). This is called TILLING (targeting induced local lesions in genomes), which involves mutagenesis of a target population then high-throughput screening to look for changes in the target gene.

1.6.2. Plant transformation

The salt tolerance of crop species may be enhanced through the introduction of genes from other organisms and plants, or through the altered expression of endogenous genes. Genes may be efficiently introduced into many plant species, including rice (Hiei *et al.*, 1994; Sallaud *et al.*, 2003), wheat (Cheng *et al.*, 1997) and barley (Tingay *et al.*, 1997) using *Agrobacterium*-mediated transformation or by microprojectile bombardment. A criticism of the method is that the transgene is inserted randomly into the genome, and may knockout important genes leading to transcriptional or post-transcriptional gene silencing (Iyer *et al.*, 2000).

A recently developed alternative is homologous recombination, using zinc-finger nucleases to induce double stranded breaks at precise locations, where a transgene in a donor plasmid is used as a template to repair the break (Cai *et al.*, 2009; Katada & Komiyama, 2009; Saika & Toki, 2009; Shukla *et al.*, 2009).

The introduction of a single gene may enhance tolerance to salt or other abiotic stresses, but often there are deleterious effects upon plant development, biomass and yield (Gevaudant *et al.*, 2007; Hsieh *et al.*, 2002a; Hsieh *et al.*, 2002b). The deleterious effects may be the result of transgene expression in inappropriate tissues and at inappropriate developmental stages, which can result in twisted stems and upside down leaves (Gevaudant *et al.*, 2007).

1.7. Transcription factors regulating abiotic stress responses

This thesis concerns six transcription factors from *Oryza sativa*, including transcription factors from the myeloblastosis (MYB), ethylene-responsive-element-binding factors (EREBP/ERF), basic helix-loop-helix (bHLH) and Orphan families. These families have all been extensively reviewed in recent years in rice and *A. thaliana*, as well as some cereal species (Carretero-Paulet *et al.*, 2010; Du *et al.*, 2009; Dubos *et al.*, 2010; Heim *et al.*, 2003; Jin & Martin, 1999; Li *et al.*, 2006; Lin *et al.*, 2007; Nakano *et al.*, 2006; Romero *et al.*, 1998; Rosinski & Atchley, 1998; Toledo-Ortiz *et al.*, 2003; Xu *et al.*, 2008a; Zhang *et al.*, 2008; Zhuang *et al.*, 2011).

Multigene families of transcription factors allow the complex transcriptional regulation of many different cellular processes (Riechmann *et al.*, 2000). Several families of transcription factors have been implicated in regulating plant responses to abiotic stresses. These include; ERF, bZIP, MYB, WRKY, DREB and zinc finger proteins (Hu *et al.*, 2006; Jin & Martin, 1999; Liu *et al.*, 1998; Pandey & Somssich, 2009; Sakamoto *et al.*, 2004; Singh *et al.*, 2002). In the following sections, each family represented in this thesis will be examined for their role in regulating abiotic stress tolerance and specific examples will be given of transcription factors whose altered expression has resulted in plants with enhanced tolerance to abiotic stresses, focused on but not limited to cereals. This is to demonstrate that altered expression of transcription factors can be used to enhance plant tolerance to abiotic stresses.

1.7.1. Myb transcription factors

1.7.1.1. Background

MYB proteins were named from the myeloblast avian oncogene, *c-myb* (Klempnauer *et al.*, 1982). The first MYB protein identified in plants was required for anthocyanin biosynthesis (Paz-Ares *et al.*, 1987). The originally identified c-MYB protein contains three imperfect tandem repeats, each of 51 or 52 AAs, with three conserved tryptophans, 18 or 19 AAs apart and two conserved histidines, which forms a hydrophobic core between three helices that binds DNA (Ogata *et al.*, 1992). The three imperfect repeats, R1, R2 and R3 each form a helix-turn-helix motif (Ogata *et al.*, 1996). The third helix intercalates with the major groove of DNA (Jia *et al.*, 2004). Phylogenetic analysis of the MYB gene family in animals shows a paralogous pattern of gene divergence from a single common ancestor (Rosinski & Atchley, 1998). In plants, only the DNA-binding domain is conserved and the remaining sequence is highly divergent (Rosinski & Atchley, 1998).

Plants contain MYB proteins with between one and four helices. MYB proteins with one repeat will be referred to as 1R-MYB or MYB-related, two as R2R3-MYB, three 3R and four as 4R-MYB.

1.7.1.2. Role in plants

MYB transcription factors are involved in the transcriptional regulation of diverse cellular processes in plants. These include anthocyanin biosynthesis, a component of phenylpropanoid metabolism (Vogt, 2010), in *Rosaceae* (Kui *et al.*, 2010), *Z. mays* (Paz-Ares *et al.*, 1986), *Petunia* spp. (Quattrocchio *et al.*, 1999) and *A. thaliana* (Borevitz *et al.*, 2000; Chen *et al.*, 2006; Matsui *et al.*, 2008).

MYB transcription factors are also involved in cell fate determination (Veit, 2004); *GL1* is required for the initiation of trichome differentiation in *A. thaliana* (Oppenheimer *et al.*, 1991) and *WEREWOLF* (*WER*) is required for position-dependent cell patterning of root epidermal cells and is expressed in root epidermal cells destined to adopt the non-hair fate (Lee & Schiefelbein, 1999). *Asymmetric leaves1* (*AS1*) is involved in stem cell function in *A. thaliana* (Byrne *et al.*, 2000) and *PHANTASTICA* (*PHAN*) gene is

involved in growth and dorsoventrality of lateral organs in *Antirrhinum* (Waites *et al.*, 1998).

MYB transcription factors are also involved in sulphate metabolism (Yatusevich *et al.*, 2010), cell cycle regulation (Dvorackova *et al.*, 2010), circadian rhythms (Lu *et al.*, 2009; Schaffer *et al.*, 1998; Wang *et al.*, 1997), and in responses to cold (Su *et al.*, 2010; Yun *et al.*, 2010; Zhai *et al.*, 2010) and biotic stress (Sonderby *et al.*, 2010).

1.7.1.3. Role in abiotic stress responses in *A. thaliana*

The *A. thaliana* genome contains 198 genes, encoding 126 R2R3-MYB, 5 R3-MYB, 64 MYB-related and 3 atypical MYBs including one 4R-MYB (Stracke *et al.*, 2001; Yanhui *et al.*, 2006). The MYB-related proteins are be divided into 1, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)-like, 2, CAPRICE (CPC)-like, 3, TELOMERIC DNA-BINDING PROTEIN (TBP)-like, 4, I-box-binding-like and 5, R-R-type (Riechmann *et al.*, 2000; Yanhui *et al.*, 2006).

MYB transcription factors in *A. thaliana* have been implicated in responses to drought, salt and cold stresses. Many MYB transcription factors have altered expression in response to abiotic stresses (Kranz *et al.*, 1998; Yanhui *et al.*, 2006), however few have been characterised. The MYB transcription factors in the following section all regulate plant responses to abiotic stresses, through both similar and different downstream pathways. Those that have been characterised are present in Table 1.1 and discussed below.

Table 1.1 MYB transcription factors involved in abiotic stress response in *A. thaliana*

The table lists characterised *A. thaliana* MYB transcription factors involved in abiotic stress responses and specifically, which stresses upregulate transcription. Interacting proteins and the downstream target genes are outlined.

Name	Type	ABA	Ethylene	Jasmonic acid	Drought	Salt	Cold	Phosphate deficiency	Interacts	Regulates	Reference
AtMYB2	R2R3	↑			↑	↑	↑		CaM, AtMYC2	Positively regulates the ABA and stress responsive genes *AtRD22* and *AtADH1*	(Urao *et al.*, 1993) (Abe *et al.*, 1997) (Hoeren *et al.*, 1998)
AtMYB15		↑			↑	↑	???		Binds ICE1	Positively regulates the ABA and stress responsive genes *ABA1*, *ABA2*, *ABI3*, *AtADH1*, *AtEM6*, *RD22* and *RD29B*. Represses CBF genes reducing cold tolerance	(Agarwal *et al.*, 2006a; Ding *et al.*, 2009)
AtMYB41		↑			↑	↑				Regulates genes involved in cell expansion, cuticle deposition and transcription factors	(Cominelli *et al.*, 2008)
AtMYB44		↑	↑	↑	↑	↑	↑			Negatively regulates negative regulators of ABA signalling *ABI1*, *ABI2*, *AtPP2CA*, *HAB1*, and *HAB2*	(Jung *et al.*, 2008)
AtMYBC1		↑			↑	↑					(Zhai *et al.*, 2010)
AtMYBL2									AtMYBL2 repressor binds TT8	Represses *TT8* and *DFR* = anthocyanin biosynthesis	(Matsui *et al.*, 2008)
AtMYBL		↑				↑					(Zhang *et al.*, 2010b)
AtMYB88 AtFLP										Positively regulates stress-responsive genes *AtNAC019*, *WRKY40*, *ERF6*, *ZAT10*, *ZAT12* and *DREB2A*	(Xie *et al.*, 2010b)

1.7.1.3.1. *AtMYB2*

The first identified was the R2R3 transcription factor, *AtMYB2*, a unique MYB as its interacting partners, up- and downstream genes have all been identified. *AtMYB2* is induced by dehydration, salt stress, hypoxia and exogenous ABA (Hoeren *et al.*, 1998; Urao *et al.*, 1993; Yoo *et al.*, 2005). An *AtMYB2 promoter:GUS* reporter construct was induced by dehydration and salt stress. AtMYB2 binds the consensus MYB recognition sequence (TAACTG) (Urao *et al.*, 1993). The C-terminal region of AtMYB2 is sufficient for and acts as a transcriptional activation domain (Urao *et al.*, 1996). AtMYB2 is not strictly an activator however and its function as a transcriptional activator or repressor is determined by its association with different isoforms of CaM (Yoo *et al.*, 2005). Using Western blots and a gel shift assay, AtMYB2 was found to bind CaM via an 18AA CaM binding motif in the R2R3 DNA binding domain in a Ca^{2+}-dependent manner. Using a competition assay, *Glycine max* GmCAM4 was found to have a higher binding affinity than GmCAM1 to the 18AA CaM binding peptide from AtMYB. AtMYB2 was found to form a stable complex with GmCAM1 and GmCAM4 *in vivo* (Yoo *et al.*, 2005). Using an electrophoretic mobility shift assay (EMSA), the Ca^{2+}-dependent DNA binding activity of AtMYB2 was found to be enhanced by its interaction with GmCaM4, but inhibited by GmCaM1 (Yoo *et al.*, 2005). AtMYB2 binds via its DNA binding motif, (PyAACPyPu). In *A. thaliana* protoplasts, a *GUS* reporter driven by a tandem hexamer of the AtMYB2 DNA binding motif and the pDEL minimal promoter, was induced when coexpressed with different combinations of *CaM* and *AtMYB2*. *GUS* expression was increased when coexpressed with *GmCaM4* and *AtMYB2*, but repressed when coexpressed with *GmCaM1* and *AtMYB2*, relative to expression of *AtMYB2* alone. *A. thaliana* plants overexpressing *GmCaM4* had increased expression of *AtMYB2* and the salt- and dehydration-responsive genes *P5CS1*, *rd22*, and *ADH1*, 3-fold higher proline accumulation and increased tolerance to a 100mM NaCl stress, relative to wild type plants and plants overexpressing *GmCaM1* (Yoo *et al.*, 2005).

AtMYB2 regulates several stress-related genes, including *rd22* (Abe *et al.*, 1997), involved in ABA-mediated plant responses to dehydration, and *alcohol dehydrogenase* (*ADH1*) (Hoeren *et al.*, 1998), involved in fermentative carbohydrate metabolism under low oxygen conditions. AtMYB2 acts with the basic helix-loop-helix transcription factor, AtMYC2, which together function as ABA-dependent transcriptional activators

under drought stress (Abe *et al.*, 2003). *AtMYC2*, like *AtMYB2*, is induced by dehydration stress and exogenous ABA (Abe *et al.*, 1997). AtMYC2 and AtMYB2 bind cis-acting elements in the *rd22* promoter to induce expression in response to dehydration and ABA (Abe *et al.*, 1997). *A. thaliana* plants overexpressing *AtMYB2*, *AtMYC2* and *AtMYB2/AtMYC2* had increasing sensitivity to ABA, in that order and all were more sensitive than wild type plants. All transgenics also had increased expression of the stress- and ABA-responsive genes *rd22* and *AtADH1* in response to exogenous ABA, while plants overexpressing *AtMYC2* and *AtMYB2/AtMYC2* had increased expression of *rd22* and *AtADH1* when untreated (Abe *et al.*, 2003). An *AtMYC2* Ds mutant had reduced ABA sensitivity and reduced induction of *rd22* and *AtADH1*. *A. thaliana* plants constitutively overexpressing *AtMYC2/AtMYB2* had less electrolyte leakage than wild type plants when subjected to 0.4M, 0.5M and 0.6M mannitol osmotic stresses (Abe *et al.*, 2003).

AtMYB2 binds the GT-motif (59-TGGTTT-39) in the promoter of *ADH1* and positively regulates expression in response to low oxygen. Expression of *AtMYB2* mirrored *AtADH1*, increasing at 2-4 hours, declining to 15 hours and increasing again to peak at 24 hours. *AtMYB2* was induced by low temperature and hypoxia in the roots only and by dehydration and ABA in both leaves and roots, whereas *ADH1* is induced predominantly in the roots. The protein synthesis inhibitor cyclohexamide prevented *ADH1* mRNA accumulation but increased *AtMYB2* mRNA accumulation 2-9-fold, showing that *AtMYB2* mRNA can accumulate without *de novo* protein synthesis (Hoeren *et al.*, 1998).

Possible regulators of *AtMYB2* include SOS1 and CIPK24/SOS2 as *AtMYB2* expression is altered in the *sos1* and *sos2-1* mutants. (Liu & Zhu, 1997; Zhu *et al.*, 1998). *AtMYB2* expression was higher in the *sos1* mutant in unstressed conditions, relative to wild type plants and was strongly induced in the *sos1* mutant in response to 100mM NaCl treatment but expression did not increase significantly in wild type plants (Liu & Zhu, 1997). *AtMYB2* expression increased to a greater extent in the *sos2-1* mutant in response to NaCl stresses of 100mM, 150mM and 200mM, relative to wild type plants. This suggests that SOS1 and CIPK24/SOS2 are negative regulators of *AtMYB2* (Liu & Zhu, 1997; Zhu *et al.*, 1998).

1.7.1.3.2. *AtMYB15*

Another well characterised *A. thaliana* MYB, is the R2R3 transcription factor AtMYB15, originally identified as being wound inducible (Cheong *et al.*, 2002), but has also been found to be induced strongly induced by ethylene and moderately induced by IAA, JA, $CdCl_2$, NaCl, ABA, drought, salt (Ding *et al.*, 2009; Yanhui *et al.*, 2006) and cold stresses (Agarwal *et al.*, 2006a). AtMYB15 is nuclear localised and contains a C-terminal transcriptional activation domain of 71AAs, from positions 215–285 (Chen *et al.*, 2006).

Overexpression of *AtMYB15* activates genes involved in the phenylpropanoid pathway, responsible for the biosynthesis of aromatic compounds involved in signalling and defence responses, as well as the biosynthesis of the AAs phenylalanine, tyrosine, and tryptophan (Chen *et al.*, 2006). AtMYB15 bound the adenosine- and cytosine-rich (AC) element *in vitro* (Romero *et al.*, 1998) and the phenylpropanoid pathway genes upregulated in *AtMYB15* overexpression plants had AC elements in their promoters (Chen *et al.*, 2006). *AtMYB15* was also induced slightly ahead of these genes in response to wounding, suggesting that AtMYB15 may regulate the genes in the phenylpropanoid pathway (Chen *et al.*, 2006).

A. thaliana plants constitutively overexpressing *AtMYB15* are ABA sensitive, have inhibited root elongation and a greater reduction in stomatal aperture when treated with exogenous ABA, relative to wild type plants (Ding *et al.*, 2009). *AtMYB15* overexpressors treated with exogenous ABA had greater upregulation of the ABA and stress responsive genes *ABA1*, *ABA2*, *ABI3*, *AtADH1*, *AtEM6*, *RD22* and *RD29B*, and when untreated, had greater upregulation of *ABA1*, *ABI3*, *AtADH1* and *RD22*, relative to wild type plants (Ding *et al.*, 2009). Water was withheld from four week old *A. thaliana* plants constitutively overexpressing *AtMYB15* for two weeks and then they were rewatered. The transgenic plants had 60% survival, whereas wild type plants had 20%. Water was withheld from plants at the flowering stage; *A. thaliana* plants constitutively overexpressing *AtMYB15* had more abundant vegetative and reproductive growth. When grown on plate media supplemented with 200mM NaCl, seedlings of *A. thaliana* plants constitutively overexpressing *AtMYB15* had increased chlorophyll retention,

relative to wild type plants (Ding *et al.*, 2009). The role of *AtMYB15* in the dehydration responsive element binding protein (DREB) pathway is discussed later.

1.7.1.3.3. *AtMYB41*

The *A. thaliana* R2R3-MYB transcription factor, *AtMYB41*, is rapidly induced in three week old plants in response to desiccation on filter paper, 100µM exogenous ABA and 200mM NaCl stress, but is undetectable under normal conditions (Cominelli *et al.*, 2008). Plants overexpressing *AtMYB41* had a dwarf phenotype, higher transpiration rates and sensitivity to desiccation and a discontinuous cuticle enhancing leaf surface permeability. They also had smaller cells in the leaf palisade parenchyma and leaf epidermis, due to reduced cell expansion, compared to wild type plants. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed that transgenic plants also had decreased expression of *LACS2* and *ATT1* involved in elongation of fatty acyl chains and increased expression of *WIN1/SHN*, an AP2 transcription factor that positively regulates epidermal wax accumulation and cutin deposition. Analysis of global transcript levels in wild type plants and plants constitutively overexpressing *AtMYB41* did not show the same genes involved in cell expansion and cuticle deposition, as being differentially expressed. In the *AtMYB41* overexpressors, 149 and 28 genes were up- and down-regulated, respectively. Up-regulated genes included genes involved in lipid biosynthesis and transport, two lipase/hydrolase family proteins, a putative acyl CoA reductase as well as membrane proteins with unknown functions. Down-regulated genes were involved in cell expansion, lipid biosynthesis and transport, including cytochrome P450 and a lipid transfer protein. Many transcription factors also had altered expression, from the MYB, NAM, zinc finger, AP2, WRKY, HB and MADS families. Using Genevestigator, the authors also determined that many genes up-regulated in *AtMYB41* overexpressing plants are also upregulated in response to dehydration, ABA and salt treatments. These data suggest that in response to stress, AtMYB41 regulates genes involved in cell expansion, cuticle deposition and cell wall modification, as well as a network of transcription factors, to enhance plant stress tolerance (Cominelli *et al.*, 2008).

1.7.1.3.4. *AtMYB44*

The R2R3 transcription factor, *AtMBY44*, is induced in response to a wide array of biotic and abiotic stress signalling molecules as well as abiotic stresses. *AtMYB44* transcript levels increased within 30 minutes in response to 100µM ABA, 100µM methyl jasmonate or 50µM ethylene. *AtMBY44* transcript levels increased within 30 minutes, then declined over 24 hours in response to drying on filter paper, or 4°C cold treatment. Transcript levels increased incrementally over 24 hours in response to 250mM NaCl. *A. thaliana* plants constitutively overexpressing *AtMYB44* had smaller leaves at early stages of vegetative growth, delayed flowering, then longer and wider leaves after flowering, relative to wild type plants. *A. thaliana* plants constitutively overexpressing *AtMYB44* were hypersensitive to 3µM ABA at germination. In response to 1µM ABA, the stomatal apertures of *A. thaliana* plants constitutively overexpressing *AtMYB44* were reduced to 60-70% of untreated plants, whereas the stomatal apertures of wild type and *atmyb44* knockout plants were only reduced to 80% of untreated plants. (i.e. more rapid ABA-induced stomatal closure.) *A. thaliana* plants constitutively overexpressing *AtMYB44* had reduced fresh weight loss in detached shoots and a higher survival rate (82% and 89%) following 12 days of water deprivation and rewatering, relative to wild type plants (17%) and *atmyb44* knockout plants (8%). In response to watering with up to 300mM NaCl, *A. thaliana* plants constitutively overexpressing *AtMYB44* had a higher survival rate (83%, 87% and 89%), relative to wild type (17%) and *atmyb44* knockout plants (7%). Gene expression was examined in unstressed conditions and in response to 250mM NaCl using the Affymetrix ATH1 genome array. Plants overexpressing *AtMYB44* had reduced expression of the serine/threonine protein phosphatases 2C (*PP2Cs*), *ABI1*, *ABI2*, *AtPP2CA*, *HAB1*, and *HAB2*, that function as negative regulators of ABA signalling, and increased salt and drought tolerance, whereas *atmyb44* mutants had increased expression of those genes and reduced salt and drought tolerance, relative to wild type plants (Jung *et al.*, 2008).

1.7.1.3.5. *AtMYBC1*

The single-repeat R3-MYB transcription factor AtMYBC1 is nuclear localised and has increased expression in response to salt, drought and ABA but reduced expression in response to cold. The *A. thaliana mybc1* mutant and overexpression plants had reduced and increased electrolyte leakage, respectively, when treated with -2°C, -4°C and -6°C stresses. The *mybc1* mutant was more tolerant to cold stress and the *MYBC1*

overexpression lines were significantly more sensitive to cold stress, relative to wild type plants, suggesting MYBC1 negatively regulates freezing tolerance in *A. thaliana*. No significant changes in the *CBF* pathway genes were observed in the *mybc1* mutant or overexpression lines. Analysis of publicly available data showed that *MYBC1* is not regulated by *CBF/DREB* or known *CBF*-independent genes. (Zhai *et al.*, 2010).

1.7.1.3.6. *AtMYBL*

The nuclear MYB-like R-R-type transcription factor, *AtMYBL*, was induced in response to exogenous ABA and salinity stress. *AtMYBL* transactivated a reporter gene in yeast. Overexpression of *AtMYBL*, reduced ABA and salinity sensitivity and RNAi *atmybl* plants had enhanced ABA and salinity sensitivity in germinating *A. thaliana* seeds. However, the salt tolerance of plants overexpressing *AtMYBL* was not maintained with prolonged salt stress during later vegetative growth stages. Overexpression of *AtMYBL* induced chlorotic leaf senescence and *atmybl* RNAi lines had no senescence-related phenotypes (Zhang *et al.*, 2010b).

1.7.1.3.7. *AtFLP and AtMYB88*

Two *A. thaliana* MYB transcription factors, FOUR LIPS (FLP) and MYB88 function in stomatal patterning. The *flp-1/myb88* double mutant has an excess of stomata, increased water loss and is more sensitive to drought and salt stresses, however has a normal phenotype in unstressed conditions. Transcriptome analysis of the *flp-1/myb88* double mutant using the *A. thaliana* ATH1 Affymetrix microarray showed a reduction in abiotic stress gene expression in unstressed conditions and a lower induction upon stress imposition, relative to wild type plants. The over-represented gene ontologies for genes with lower expression in the *flp-1/myb88* double mutant were cellular communication and signal transduction, cell rescue, defence and virulence, interaction with the environment and systemic interaction with the environment (Xie *et al.*, 2010b). The authors conclude that FLP/MYB88 positively regulate stress-related genes and negatively regulate genes associated with stomatal development (Xie *et al.*, 2010a). Expression of these stress-related genes was the same as wild type plants in another stomatal patterning mutant, showing that the altered gene expression was not a result of the stomatal patterning. The stress-responsive genes *WRKY40*, *ERF6*, *ZAT10*, *ZAT12* and *DREB2A* were not induced in seedlings of the *flp-1/myb88* double mutant subjected to 20% PEG, 300mM NaCl or exogenous ABA, however the genes were strongly

induced in wild type plants. Endogenous ABA levels were the same between the *flp-1/myb88* double mutant and wild type plants, suggesting that the deficiency in stress sensing is downstream of ABA (Xie *et al.*, 2010b). Complementation with an *FLP promoter:gene* construct restored responsiveness of the stress-responsive genes to salt stress. Therefore sensitivity of the *flp-1/myb88* double mutant to abiotic stress may be the result of reduced stress-responsive gene expression (Xie *et al.*, 2010b). Using ChIP-chip, *AtNAC019* was found to be a target of FLP/MYB88, which binds to the *NAC019* promoter (Xie *et al.*, 2010b). *AtNAC019* is induced by drought, high salinity and abscisic acid, and overexpression increases stress-inducible gene expression and enhances drought tolerance (Tran *et al.*, 2004). The *flp-1/myb88* double mutants germinated significantly faster than wild type plants when subjected to 1 or 2µM ABA treatment. Endogenous ABA levels did not differ (Xie *et al.*, 2010b).

1.7.1.4. Role in abiotic stress responses in cereals

In cereals and other crop species, there are few examples of well characterised MYB transcription factors involved in abiotic stress tolerance. The better characterised transcription factors from the rice MYB family. The rice genome contains 183 MYB genes, which encode 109 R2R3-MYB proteins, four 3R-MYB proteins and 70 MYB-related and one 4R-MYB proteins (Yanhui *et al.*, 2006).

Characterised MYBs involved in abiotic stress responses include OsMYB4, involved in drought, salt, cold and pathogen signalling responses (Vannini *et al.*, 2006; Vannini *et al.*, 2004), OsMYB3R-*2*, involved in drought, salt and cold signalling responses (Dai *et al.*, 2007) and OsMYBS-*3*, involved in cold and salt signalling through the CBF/DREB pathway (Su *et al.*, 2010).

1.7.1.4.1. *OsMYB4*

The most well characterised transcription factor is OsMYB4, the gene for which *Osmyb4*, was originally cloned from cold stressed rice coleoptiles (Pandolfi *et al.*, 1997). Overexpression of *Osmyb4* enhances abiotic stress tolerance in *A. thaliana* (Mattana *et al.*, 2005; Vannini *et al.*, 2006; Vannini *et al.*, 2004), tomato (Vannini *et al.*, 2007) and apple (Pasquali *et al.*, 2008).

A. thaliana plants constitutively overexpressing *Osmyb4* showed enhanced tolerance to freezing stress (-6°C), cold stress (average 5°C day and lowest -3°C) (Vannini *et al.*, 2004) and enhanced tolerance to drought stress through compatible solute accumulation, relative to wild type plants (Mattana *et al.*, 2005). The stress tolerance also correlates with *Osmyb4* transcript levels, with wild type, low, medium and high *Osmyb4* expression resulting in 10%, 10%, 45% and 60% survival, respectively, following water withholding for three weeks and three days of rewatering. In the salt treatment, plants were watered with 300 mM NaCl every five days for two weeks, after which the wild type and low expressors died, whereas 50% of the medium and high expressors survived. The plants with high and medium expression of *Osmyb4* had enhanced tolerance to tobacco necrosis virus, *Phaseolus vulgaris* cv. *Saxa*, *Pseudomonas syringae* pv. *Tomato* and *Botrytis cinerea*, relative to wild type plants and those with low

expression of *Osmyb4*. The plants with high and medium expression of *Osmyb4* also had enhanced tolerance to UV irradiation, ozone and paraquot exposure (Vannini *et al.*, 2006).

The effect of *Osmyb4* overexpression on global gene expression has been examined in *A. thaliana* in unstressed conditions and in *O. sativa* subjected to cold stress (Park *et al.*, 2010; Vannini *et al.*, 2006). In *A. thaliana* plants constitutively overexpressing *Osmyb4*, 254 and 83 genes were up- and down-regulated, respectively, relative to wild type plants. Of the up-regulated genes, 26% were involved in the control of gene expression and 9% related to signal transduction, many of which were shown to be upregulated by biotic and abiotic stresses on Genevestigator (Vannini *et al.*, 2006).

An *in silico* promoter analysis of first 1000bp promoter region of 167 known up-regulated genes identified four classes of transcription factor binding sites, including; myb, ABA responsive, abiotic stress responsive and biotic stress responsive binding sites. Promoter analysis showed that 43% contained putative Myb4 recognition sites, 72% contained multiple ABA responsive sites, 81% contained sequences involved abiotic stress response and 87% contained sequences involved in UV, biotic stress and hormone responses. The AA profile was altered in plants constitutively overexpressing *Osmyb4*, including AAs acting as osmolytes, scavengers and precursors of phospholipids, polyamines, phytoanticipins and phytoalexins, as well as nitrogen assimilation, transport and storage (Vannini *et al.*, 2006).

Recently, the OsMYB4 regulon was analysed by microarray in *O. sativa* ssp. Nipponbare. In plants overexpressing *Osmyb4*, 10% of the up-regulated genes and 11% of the down-regulated genes were shared with cold stressed Nipponbare, indicating that OsMYB4 controls mostly cold-independent signalling. Analysis of all up- and down-regulated genes showed that OsMYB4 controls a significant proportion of all stress related signalling in Nipponbare. The up-regulated cold-independent transcriptome of *Osmyb4* overexpressing plants was enriched for gene ontology classifications of; transport mechanism and facilitation, protein biosynthesis, growth development and morphogenesis, cell cycle regulation and DNA synthesis. The down-regulated cold-independent transcriptome of *Osmyb4* overexpressing plants was enriched for gene

ontology classifications of; transport mechanism and facilitation, and metabolic process (C-compound and carbohydrate). The up-regulated cold-mediated transcriptome of *Osmyb4* overexpressing plants was enriched for gene ontology classifications of: stress associated transcription, redox homeostasis and detoxification, cellular communication and signal transduction. The down-regulated cold-mediated transcriptome of *Osmyb4* overexpressing plants was enriched for gene ontology classifications of: stress-associated proteins, protein modification, metabolic process (AA and derivative) (Park *et al.*, 2010).

Promoter analysis showed that approximately 60% of the cold-mediated and -independent genes contained MYB-related *cis*-elements in their promoters, suggesting they are targets of OsMYB4 or of MYB transcription factors regulated by OsMYB4. MYB-related motifs did not overlap between the cold-mediated and -independent transcriptomes. Of the up-regulated transcription factors, 80% contained MYB-related *cis*-elements in their promoters, followed by bZIP-related at 60%, suggesting the most up-regulated transcription factors are directly regulated by OsMYB4 and others by OsMYB4-regulated bZIP transcription factors. In their model, under cold stress OsMYB4 activates known ERF, NAC, bZIP, DOF.PHD and ARF transcription factors involved in biotic and abiotic stress responses.

Plants overexpressing *Osmyb4* had increased expression of defence genes involved in redox homeostasis and detoxification. These plants were found to have higher peroxidise activities, higher total antioxidant capacity, based on a 2,2-diphenyl-1-picrylhydrazyl (DPPH) reduction assay, and significantly lower electrolyte leakage when grown at 10°C, relative to wild type plants (Park *et al.*, 2010).

High overexpression of *OsMYB4* produced awnless plants, reduced height and biomass, longer panicles and more spikelets, but with dramatically reduced seed set and consequently, reduced yield (Park *et al.*, 2010).

1.7.1.4.2. *OsMYB2R-2*

Another well characterised MYB is the R1R2R3 MYB, *OsMYB3R-2*, identified as a cold-responsive gene in *O. sativa* cv. Yuedongdao using a cDNA microarray. *OsMYB3R-2* transcript levels continually increased in response to 2 °C cold stress over 72 hours *OsMYB3R-2* was also induced in response 250mM NaCl stress and dehydration on filter paper. Overexpression in *A. thaliana* increased tolerance to freezing stresses of -2 to -10°C, with 42.8% of transgenic plants surviving, relative to 5.6% of wild type plants. Transgenics also had increased tolerance to a drought stress of two weeks of water being withheld, with 85% surviving, relative to 26.7% of wild type plants. Transgenics also had increased tolerance to salt stresses of 150mM and 200mM NaCl, when grown on plates (Dai *et al.*, 2007).

Overexpression of *OsMYB3R-2* in *O. sativa* increased cold stress tolerance. When subjected to a cold stress of 2°C for 72 hours, followed by growth under normal conditions, *O. sativa* plants overexpressing *OsMYB3R-2* had 50% survival and continued to grow, whereas wild type plants had 20% survival following the cold treatment but did not continue to grow. *OsMYB3R-2* antisense lines had reduced survival relative to wild type plants. All plants were also cold stressed for different length treatments. Each time, *OsMYB3R-2* overexpressing plants had increased survival rates, relative to wild type plants. Plants overexpressing *OsMYB3R-2* had increased expression of *OsCPT1*, thought to be involved in the DREB/CBF pathway, because it has a DRE/CRT *cis*-element in its promoter. All other analysed cold responsive genes did not have altered expression. Cold treatment increased free proline levels in plants overexpressing *OsMYB3R-2* to 300µg.g^{-1} relative to 188µg.g^{-1} in wild type plants. OsMYB3R-2 binds the MSA-like element CCCAACGG in the promoter of the cyclin gene *OsCycB1;1*. Plants overexpressing *OsCycB1;1* had increased cold tolerance, with 67% of plants surviving, relative to 58% for wild type plants and 33% for *OsCycB1;1* antisense plants. Cold treatment increased free proline levels in plants overexpressing *OsCycB1;1* to 243µg.g^{-1}, to 197µg.g^{-1} for wild type plants and to 185µg.g^{-1} for *OsCycB1;1* antisense plants (Ma *et al.*, 2009a).

1.7.1.4.3. *OsMYBS-3*

Another well characterised MYB transcription factor, *OsMYBS-3*, regulates genes in the CBF/DREB pathway of cold stress signalling. *OsMYBS-3* is induced in the root and shoot in response to cold stress (4°C) and in the shoot in response to salt stress (200mM NaCl). GFP-tagged OsMYBS3 showed that OsMYBS-3 is constitutively localised to the nucleus. During cold treatment at 4°C, *OsMYBS-3 RNAi* lines showed leaf rolling at 8 hours, wild type plants showed leaf rolling at 24 hours and *OsMYBS-3* overexpression lines remained normal. In the greenhouse, *OsMYBS-3* overexpression lines were 20% shorter, had 30% fewer tillers and heading was delayed by one week, relative to wild type plants. In the field, no differences were apparent. Using the Affymetrix ATH microarray, overexpression of *OsMYBS-3* was found to reduce the expression of the cold responsive genes *DREB1A*, *DREB1B*, and *DREB1C*, and another two *DREB1-like* genes (*ERF#025*) and *ERF#104*), that were up-regulated in cold treated wild type plants. Members of the DREB regulon, *DREB1*, *αAmy3/RAmy3D* and *cytochrome P450*, had reduced expression in the *OsMYBS-3* overexpression plants and increased expression in the *OsMYBS-3 RNAi* plants. Using a transient luciferase assay in rice embryos, MYBS3 was found to repress *DREB1B* promoter and *αAmy3* SRC at 4°C, but induce the *DREB1A* promoter (Su *et al.*, 2010).

Three wheat MYB transcription factors have been identified as either being responsive to abiotic stress (Lee *et al.*, 2007), or differentially expressed between salt tolerant and sensitive cultivars (Rahaie *et al.*, 2010; Zhang *et al.*, 2009a), but their up and downstream pathways have not been investigated.

1.7.1.4.4. *TaMYB1*

Expression of *TaMyb1* in wheat roots increased in response to hypoxia, but not during anoxia. Expression also increased in response to high light under hypoxia and to 200mM NaCl stress, 100µM exogenous ABA and 25% polyethylene glycol. Expression was localised to the epidermis, endodermis and cortex adjacent to the endodermis under hypoxia and absent from the vasculature or cortex (Lee *et al.*, 2007).

1.7.1.4.5. *TaMYB32*

TaMYB32 was identified in a comparison of salt sensitive and salt tolerant cultivars of wheat. *TaMYB32* shared 72.4% and 73.7% AA identity to rice and maize R2-R3 MYB, respectively. *TaMYB32* was expressed in root, shoot, leaf, pistil and anther tissue. In response to 250mM NaCl stress, transcript levels of *TaMYB32* in salt sensitive Chinese Spring increased at 3, 7 and 12 hours, then decreased by 24 hours. There was a larger and more rapid increase in transcript levels in salt tolerant Chadianhong, where transcript levels peaked at 1 hour (6-fold over unstressed), then gradually returned to basal levels by 24 hours (Zhang *et al.*, 2009a).

1.7.1.4.6. *TaMYBsdu1*

The expression of ten MYB transcription factors was examined in two recombinant inbred lines (RILs) of wheat (*Triticum aestivum*) differing in salt tolerance. In response to a 200mM NaCl stress, the R2/R3-type MYB transcription factor *TaMYBsdu1* was found to be differentially expressed between the two cultivars, being 4-fold more highly expressed in the salt tolerant cultivar. *TaMYBsdu1* expression in response to drought stress was examined in a parent of the RILs, cv. Babax. Plants were grown for four weeks and water was withheld until the relative water content of the plant leaves reached 68 to 80%. *TaMYBsdu1* was up-regulated approximately 170-fold in roots and leaves by this long term drought stress (Rahaie *et al.*, 2010). Rahaie *et al.* note that the *A. thaliana* homologs of *TaMYBsdu1* are MYB85, which is also up-regulated in roots by salt or drought treatment ((Winter *et al.*, 2007)) and MYB20 which is down-regulated by salt and drought stresses ((Winter *et al.*, 2007; Zeller *et al.*, 2009)).

The AP2/ERF transcription factor superfamily also has many members involved in abiotic stress response signalling.

1.7.2. AP2/EREBP/ERF transcription factors

1.7.2.1. Background

The AP2/EREBP/ERF superfamily of transcription factors is found only in plants (Riechmann & Meyerowitz, 1998) and can be divided into two groups based upon the number of AP2 domains; AP2 proteins contain two copies and EREBP/ERF proteins contain one copy (Jofuku *et al.*, 1994; Ohme-Takagi & Shinshi, 1995). The AP2 domain was first described in *APETALA2* which functions in the establishment of the floral meristem (Irish & Sussex, 1990; Jofuku *et al.*, 1994). The domain is 68AAs in length with an 18AA core region, which forms an amphipathic α-helix (Jofuku *et al.*, 1994). An ERF protein with a single AP2 domain was first identified in tobacco in proteins designated as ethylene responsive element binding proteins, due to their binding affinity for the 11-bp GCC box (TAAGAGCCGCC), found in the promoters of ethylene responsive pathogenesis-related genes (Büttner & Singh, 1997; Ohme-Takagi & Shinshi, 1995; Zhou *et al.*, 1997). The solution structure of the single copy AP2 domain has been characterised in AtERF1 (At4g17500) in complex with its target GCC box, where recognition of the DNA molecule occurs via a three stranded anti-parallel β-sheet, packed parallel to an α-helix (Allen *et al.*, 1998).

Based upon the number and sequence similarity of the AP2/ERF domains, the family can be further divided into five groups; APETALA (AP2), RAV, DEHYDRATION RESPONSE ELEMENT BINDING PROTEIN (DREB), ETHYLENE RESPONSIVE FACTOR (ERF) and others (Sakuma *et al.*, 2002). As described above, the AP2 group contains two AP2 domains. The DREB and ERF groups contain a single AP2 domain which differs by two conserved AAs at the 14th and 19th position. Valine and glutamic acid are conserved in DREB proteins whereas the alanine and aspartic acid are conserved in ERF proteins (Cao *et al.*, 2001; Sakuma *et al.*, 2002). These small differences mean that DREB proteins bind the dehydration responsive element (DRE) and ERF proteins bind the GCC box. The core sequence of DRE is A/GCCGAC, and the 4th C, 5th G and 7th C are required for specific interaction with DREB proteins (Sakuma *et al.*, 2002). The *rd29A* and *cor15a* promoters contain the DRE, which is involved in rapid responses to dehydration and salt stresses and in responses to low temperature (Baker *et al.*, 1994; Yamaguchi-Shinozaki & Shinozaki, 1994). The core sequence of the GCC box is GCCGCC, and the 1st G, 4th G and 6th C are required for

specific interaction with ERF proteins (Hao *et al.*, 1998). RAV group proteins contain a single AP2 domain and a B3 type domain (Sakuma *et al.*, 2002).

The AP2 subfamily can be further divided into ANT and euAP2 lineages, containing the genes *ANT* and *AP2*, respectively (Kim *et al.*, 2006). Some genes in the euAP2 lineage contain *microRNA172* binding regions, which regulates flowering time and floral organ identity by translational repression (Aukerman & Sakai, 2003).

1.7.2.2. Role of AP2/ERF genes in plants

Transcription factors from the AP2/EREBP family are involved in a diverse range of signalling processes, including abiotic (Kizis *et al.*, 2001), biotic (Gutterson & Reuber, 2004) and floral development (Jofuku *et al.*, 1994).

AP2 group members include AP2 (Jofuku *et al.*, 1994) and AINTEGUMENTA (ANT) (Elliott *et al.*, 1996; Klucher *et al.*, 1996), involved in floral development and Glossy15 (Gl15) (Moose & Sisco, 1996), involved in regulating epidermal leaf cell identity. RAV group genes are involved in biotic and abiotic stress responses (Lee *et al.*, 2010; Li *et al.*, 2011; Sohn *et al.*, 2006) and flower development (Castillejo & Pelaz, 2008; Hu *et al.*, 2004). DREB group genes are involved in biotic and abiotic stress tolerance (Agarwal *et al.*, 2006b; Yamaguchi-Shinozaki & Shinozaki, 2001).

Analysis of 70 ERF group genes from various species determined that there are eight clusters and 14 subclusters according to phylogenetic relationships, gene structure, conserved motifs and biological function (Xu *et al.*, 2008a). Clusters I, II, Va, and VII, integrate the ethylene/jasmonic acid and salicylic acid stress signalling pathways and Clusters II, Va, and VII contain genes involved in abiotic stress response pathways (Xu *et al.*, 2008a). Clusters Ia, II, Va, and VII, are involved in disease resistance responses (Xu *et al.*, 2008a).

1.7.2.3. Role AP2/ERF genes in abiotic stress responses in *A. thaliana*

The best characterised transcription factors involved in abiotic stress responses come from the *A. thaliana* AP2/ERF superfamily. The AP2/ERF transcription factor superfamily is one of the three largest in *A. thaliana* with 122 members (Nakano *et al.*, 2006; Riechmann *et al.*, 2000). The ERF group members include subgroups B-1 through B-6 and the DREB group members include subgroups A-1 through A-5 (Nakano *et al.*, 2006). Many ERF group members are involved in responses to biotic stresses, although some also to abiotic stresses, while the DREB group members are predominantly involved in abiotic stress responses.

1.7.2.3.1. *ERF group*

Some ERF group members may be involved in linking biotic and abiotic stress pathways, as they are induced by hormone and abiotic stress treatments (Fujimoto *et al.*, 2000). Transcripts of *AtERF1*, *AtERF2* and *AtERF5* increase in response to exogenous ethylene, but transcripts of *AtERF3* and *AtERF4* do not. Wounding induced all *AtERFs* except *AtERF3*. *AtERF5* was induced by cold stress, *AtERF4* was induced by cold, NaCl and drought, while *AtERF3* was induced by NaCl and drought stresses. All *AtERFs* were not responsive to exogenous ABA. *AtERF3* and *AtERF4* were not induced by NaCl in ethylene insensitive *ein2* mutants, indicating that they may be regulated by the ethylene signalling pathway mediated by *ein2*, while not being responsive to ethylene (Fujimoto *et al.*, 2000). AtERF1 to AtERF5 all bind the GCC box, with AtERF1, AtERF2 and AtERF5 activating reporter gene expression and AtERF3 and AtERF4 repressing reporter gene expression (Fujimoto *et al.*, 2000).

The GCC and DRE elements are often found in the promoters of abiotic and biotic stress responsive gene promoters, respectively (Ohme-Takagi & Shinshi, 1995; Yamaguchi-Shinozaki & Shinozaki, 2005). AtERF1, AtERF4 and AtEBP bind both the DRE and GCC motifs, but have higher affinity for the latter. AtDREB1B/AtCBF1 (discussed below) is the opposite and has a higher affinity for DRE. Mutations of DRE (ATACTACCGACAT) showed that substitution at C8 or G9 abolished specific recognition of the DRE motif by all four of the AtERFs (Yang *et al.*, 2009). Randomised oligonucleotide selection showed that AtERF1 and AtEBP select GCCGCC, AtERF4 selects G/aCCGCC, with a relaxed A or G at position two and

CBF1 selects AA/cCGAC. All contain a GC step at the core and a conserved C at the last position. The different flanking sequences are proposed to be crucial for regulation by different ERFs with different flanking sequence preferences (Yang *et al.*, 2009).

The GCC-box in the promoter of *PDF1.2* confers jasmonate responsiveness, however additional elements may be involved. The ERF genes *ERF1*, *AtERF2*, *AtERF3*, *AtERF4* and *RAP2.10* are induced by exogenous methyl jasmonate, peaking one hour after treatment, whereas *AtERF4* peaks at four hours. *AtERF2* is more responsive to methyl jasmonate than *ERF1*. *AtERF2*, *AtERF3*, *AtERF4* and *RAP2.10* respond similarly to exogenous ethylene, peaking at two hours and then declining (Brown *et al.*, 2003). *AtERF2, AtERF3, AtERF4,* and *RAP2*.10 are all induced by *A. brassicicola* inoculation (Brown *et al.*, 2003). *A. thaliana* plants constitutively overexpressing *AtERF2* have increased expression of *PDF1.2*, *Thi2.1* and *PR4* (Brown *et al.*, 2003). *AtERF2* and *AtERF4* act as positive and negative regulators, respectively, of jasmonic acid-mediated defence signalling, of resistance to the necrotrophic fungal pathogen *Fusarium oxysporum* and of jasmonic acid inhibition of root elongation (McGrath *et al.*, 2005).

1.7.2.3.2. *DREB group*

The DREB pathway is the best characterised of the abiotic stress response pathways involving ERF/AP2 transcription factors. DREB/C-repeat binding factor (CBF) proteins bind the DRE or C-repeat (CRT) in response to dehydration and low temperature (Stockinger *et al.*, 1997). Characterised DREB group members include the subgroup A-1 members, *DREB1A/CBF3*, *DREB1B/CBF1*, *DREB1C/CBF2* and *DREB1D/CBF4*, the subgroup A-2 members *DREB2A* through to *DREB2H* and the subgroup A-3 member *ABI-4* (Nakano *et al.*, 2006).

The *DREB1* and *DREB2* genes are all induced by various abiotic stresses. *AtDREB1A*, *AtDREB1D* RNA levels are mildly induced and *AtDREB1F* is strongly induced by 250mM salt treatment. *DREB1A* is also strongly induced by 4°C cold treatment. *DREB2A* is strongly induced and *DREB2C*, *DREB2D* and *DREB2F* are mildly induced by 250mM salt treatment. *DREB2A* is also induced by dehydration, excess water, ABA and cold stresses. *DREB2C* and *DREB2E* are mildly induced by ABA and the former

also by excess water (plant transfer to water) (Sakuma *et al.*, 2002). This indicates that the DREB family in *A. thaliana* may play different and partially redundant roles in plant signalling in response to different abiotic stresses.

DREB1A and DREB2A both bind the DRE *in vitro*, however, DREB1A also binds the GCC-box with equal affinity (Hao *et al.*, 2002). *DREB1A* is induced in response to low temperature and *DREB2A* is induced in response to dehydration (Liu *et al.*, 1998). *A. thaliana* plants constitutively overexpressing *DREB1A* are stunted, have improved cold and dehydration stress tolerance and downstream target genes are strongly induced, and plants overexpressing *DREB2A* are stunted, have improved dehydration stress tolerance only but only have mild induction of downstream genes (Liu *et al.*, 1998). Interestingly, constitutive overexpression of *AtDREB1A* in rice enhances cold, drought and salt tolerance, without negative effects on plant growth (Oh *et al.*, 2005). Overexpression in *A. thaliana* using the inducible promoter, *Rd29A*, can enhance tolerance to dehydration, salt and cold stress without negative effects on plants growth (Kasuga *et al.*, 1999). The promoter for *rd29A* is responsive to dehydration, low temperature, salt and osmotic stresses (Yamaguchi-Shinozaki & Shinozaki, 1994).

Global gene expression and metabolite profiles were analysed in cold and dehydration stressed wild type *A. thaliana* plants and in plants constitutively overexpressing At*DREB1A* or At*DREB2A* (Maruyama *et al.*, 2009). The putative functions of *AtDREB1A* and *AtDREB2A* downstream genes were similar, although *AtDREB2A* also induced molecular chaperones and enzymes for toxin catabolic processes while *AtDREB1A* induced genes involved in carbohydrate metabolism. Constitutive overexpression of *AtDREB1A* and the response to cold and dehydration stresses, up-regulated the same genes, including genes encoding starch- degrading enzymes, sucrose metabolism enzymes and sugar alcohol synthases, resulting in the accumulation of different kinds of monosaccharides, disaccharides, trisaccharides and sugar alcohols (Maruyama *et al.*, 2009). These did not accumulate in *AtDREB2A* overexpressing plants (Maruyama *et al.*, 2009). The metabolite profiles in *AtDREB1A* and *AtDREB2A* overexpressing plants resembled cold stressed and dehydration stressed plants, respectively (Maruyama *et al.*, 2009). Analysis of the target gene promoters revealed different forms of DRE. Using gel mobility shift assays with mutated DRE, DREB1A had the highest affinity for A/GCCGACNT, whereas DREB2A preferentially bound

ACCGAC (Maruyama *et al.*, 2004; Sakuma *et al.*, 2006a). Three groups of genes were found to be regulated by DREB2A and/or DREB1A. The first was regulated by both DREB2A and DREB1A, contained ACCGACNT in their promoters and included *RD29A*, *RD17* and *LEA14*. The second was regulated by DREB1A only, contained A/GCCGACNT in their promoters and included *COR15A*, *KIN1*, *KIN2*, *COR15B* and AtCOR413-TM1. The third was regulated by DREB2A only, contained ACCGACNA/G/C in their promoters and included *RD29B*, *MT2A* and other genes (Sakuma *et al.*, 2006a). These different forms of DRE allow *A. thaliana* plants to respond to different stresses through DREB1A and DREB2A.

Cold stress-responsive *DREB1A*/*CBF3* is regulated by the MYC type bHLH transcription factor inducer of *CBF* expression 1 (ICE1). *AtICE1* is a constitutively expressed transcriptional activator which binds Myc recognition sites in the *DREB1A* promoter. *A. thaliana* plants overexpressing *AtICE1* have enhanced cold tolerance and increased expression of *DREB1A/CBF3*, *RD29A* and *COR15A* (Chinnusamy *et al.*, 2003). *AtICE1* overexpression also improves cold tolerance in rice plants (Xiang *et al.*, 2008a). AtICE1 is also involved in the regulation of stomatal development (Hofmann, 2008; Kanaoka *et al.*, 2008). *AtICE1* is positively regulated by the SUMO E3 ligase, SIZ1 and negatively regulated by high expression of osmotically-responsive genes (HOS1). In response to cold stress, the RING E3 ligase, HOS1, a negative regulator of plant cold responses, migrates to the nucleus and mediates the ubiquitination and degradation of ICE1 (Dong *et al.*, 2006; Ishitani *et al.*, 1998; Zhu *et al.*, 2005). SIZ1 facilitates conjugation of SUMO to ICE1, which represses HOS1-mediated polyubiquitination and enhances ICE1 stability (Miura *et al.*, 2007).

AtMYB15 interacts with ICE1 and together they bind the Myb and Myc recognition sequences, respectively, in the promoters of *DREB/CBF* genes (Agarwal *et al.*, 2006a; Chinnusamy *et al.*, 2003). Overexpression of *AtMYB15* reduces *CBF* expression and reduces plant tolerance to freezing stress, whereas the *myb15* mutants have increased *CBF* expression and enhanced freezing tolerance (Agarwal *et al.*, 2006a). *AtMYB15* had increased expression in *siz1* mutants, indicating that SIZ1 represses *MYB15* expression (Miura *et al.*, 2007).

In another study, conserved DNA motifs (CM) were identified in the *DREB1C/CBF2* promoter (Doherty *et al.*, 2009). *DREB1C/CBF2* was found to be regulated by the calmodulin binding transcription activator 3 (CAMTA3), which binds the CM2 motif in the *DREB1C/CBF2* promoter and positively regulates transcription in response to cold stress, linking *DREB1C/CBF2* expression to calcium signalling (Doherty *et al.*, 2009).

Additionally, the DREB pathway is also under circadian control (Harmer, 2000; Kidokoro *et al.*, 2009). This is discussed in a later section of this introduction.

DREB2A and DREB2B are induced by dehydration and salt stresses (Liu *et al.*, 1998) and are negatively regulated by the RING finger E3 ligases, DREB-INTERACTING PROTEIN 1 and 2 (DRIP1 and DRIP2), that mediate ubiquitination and target DREB2 to the proteosome (Qin *et al.*, 2008). AtDREB2A contains a transcriptional activation domain from 254-335AA and a repression domain from 135-165AA, which results in a constitutively active form if deleted (Sakuma *et al.*, 2006a). AtDREB2A is also induced rapidly in response to heat stress (Sakuma *et al.*, 2006b). AtDREB2A, AtDREB2B and to a limited extent AtDREB1B/CBF1, bind DRE1 with the sequence AACCGACAA in the promoter of HsfA3. In response to heat stress, AtDREB2A induces HsfA3 expression, which activates heat stress-inducible genes (Schramm *et al.*, 2008). In a northern analysis *DREB2A* and *DREB2B* were found to be induced by dehydration and 250mM NaCl stresses. Constructs containing the *DREB* promoters fused to the *GUS* reporter gene were expressed in *A. thaliana* in response to dehydration and 250mM NaCl stresses (Nakashima *et al.*, 2000).

The subgroup A-4 DREB-like protein, AtTINY, was isolated in a transposon-tagging screen looking for gain-of-function alleles (Wilson *et al.*, 1996). Using yeast one-hybrid and gel mobility shift assays, a competition assay and atomic force microscopy, AtTINY was found to bind both DRE and ethylene responsive element (ERE) with equal affinity. Ser-15 was found to be crucial for binding the ERE. It has a C-terminal transcriptional activation domain. *AtTINY* expression increased; 2.7-fold after 40 minutes in response to 250mM NaCl, ~60-fold at 4 hours in response to dehydration, slowly increased to ~60-fold at 6 hours in response to 4°C cold stress, ~20-fold after 3 hours in response to 1mM ethephon (ethylene) and ~7-fold after 10 hours in response to

50µM methyl jasmonate. *A. thaliana* plants overexpressing *AtTINY* had a dwarf phenotype and had increased expression of the DRE-regulated genes *COR6*.6, *COR15A* and *COR78* and increased expression of the ERE-regulated genes *PDF1*.2 and *HLS1*. Cross-talk between the DRE- and ERE-mediated signalling pathways was demonstrated by the DRE-regulated genes *COR6*.6 and *ERD10* being induced by ethylene and the ERE-regulated gene *HLS1*, being induced by cold stress. This suggests that TINY may contribute to the cross-talk (Sun *et al.*, 2008).

Abiotic stress responsive AP2/ERF transcription factors have also been investigated in cereals.

1.7.2.4. Role of AP2/ERF genes in abiotic stress response in cereals

Since the discovery of the important role that DREB and other ERF transcription factors play in regulating plant abiotic stress tolerance, much effort has gone into characterising homologous proteins from cereals and other crop species.

AP2/ERF transcription factors involved in abiotic stress responses have been identified in many different species, including wheat (*Triticum* spp.) (Xu *et al.*, 2008b; Xu *et al.*, 2007), barley (*Hordeum vulgare*) (Jung *et al.*, 2007; Xue & Loveridge, 2004), rice (*Oryza sativa*) (Cao *et al.*, 2006; Cao *et al.*, 2005b; Chen *et al.*, 2008a; Dubouzet *et al.*, 2003; Gutha & Reddy, 2008; Li *et al.*, 2005; Qin *et al.*, 2007; Tian *et al.*, 2005; Wang *et al.*, 2008b), xeric (*Hordeum spontaneum* L.) (James *et al.*, 2008), pearl millet (*Pennisetum glaucum*) (Agarwal *et al.*, 2010), soybean (*Glycine max*) (Chen *et al.*, 2007; Chen *et al.*, 2009; Yu *et al.*, 2008), maize (*Zea mays* L. ssp. mays) (Wang *et al.*, 2008a), chickpea (*Cicer arietinum*) (Shukla *et al.*, 2006), tomato (*Solanum lycopersicum*) (Huang *et al.*, 2004; Zhang *et al.*, 2004), capsicum (*Capsicum annuum*) (Yi *et al.*, 2004), tobacco (*Nicotiana tabacum*) (Guo *et al.*, 2004; Lee *et al.*, 2005b; Park *et al.*, 2001), cotton (*Gossypium hirsutum*) (Gao *et al.*, 2009), mustard greens (*Brassica juncea* L.) (Cong *et al.*, 2008), sweet potato (*Ipomoea batatas*) (Kim *et al.*, 2008), perennial ryegrass (*Lolium perenne* L.) (Xiong & Fei, 2006).

1.7.2.4.1. AP2/ERF genes in wheat

A recent analysis of the wheat AP2/ERF family was conducted by doing BLAST searches of EST databases using *A. thaliana* representative members of the DREB/CBF, AP2, RAV and other subfamilies. Phylogenetic analysis separated the wheat AP2/ERF family into the same five groups seen in *A. thaliana* (Zhuang *et al.*, 2011).

Characterised members of the wheat AP2/ERF family involved in abiotic stress responses include *ethylene-responsive factor 1* (*TaERF1*), *abiotic stress induced dehydration response element-binding factor a* (*TaAIDFa*) (Xu *et al.*, 2008b; Xu *et al.*, 2007) and recently, *TaDREB2* and *TaDREB3* (Morran *et al.*, 2011). Other recently characterised AP2/ERF family members are involved in biotic stress responses only

(Chen *et al.*, 2008b; Dong *et al.*, 2010; Zhang *et al.*, 2007b) and will not be discussed here.

TaERF1 is involved in plant responses to both abiotic and biotic stresses. *TaERF1* transcripts increased in response to drought, salt and cold, peaking at 10 hours. In response to ABA, transcripts peaked at 5 hours then declined. *TaERF1* transcripts also increased in response to ethylene and salicylic peaking at five hours, and also increased in response pathogen challenge by wheat powdery mildew fungus (*B. graminis* f. sp. tritici) by 24 hours and increased until 48 hours (Xu *et al.*, 2007). TaERF1 was found to be targeted to the nucleus to interact with TaMAPK1 protein kinase *via* a C-terminal phosphorylation site. TaERF1 was found to bind the GCC-box and CRT/DRE element *in vitro*. A GCC-box driven *GUS* reporter gene was induced 3.2 fold over control plants when coexpressed with *TaERF1* in tobacco seedlings. Expression of *TaMAPK1* enhanced *GUS* expression 2.3 fold and coexpression of *TaERF1* and *TaMAPK1* enhanced *GUS* expression 6.5 fold, over control plants (Xu *et al.*, 2007).

Ectopic expression of *TaERF1* expression in *A. thaliana* and tobacco also changes plant tolerance to abiotic and biotic stresses. Three week old *A. thaliana* plants constitutively overexpressing *TaERF1* were more tolerant to three weeks of water withholding, with 50-60% survival, and to being irrigated with 400 mM NaCl, with 30% survival, whereas all wild type plants died for both stresses. *A. thaliana* plants constitutively overexpressing *TaERF1* were more tolerant to a freezing stress of -10°C for 12 hours, with 65% survival, whereas wild type plants had 50% survival (Xu *et al.*, 2007). Tobacco plants constitutively overexpressing *TaERF1* were more tolerant to growth in MS medium supplemented with 200 mM NaCl and had a reduced rate of bleaching in leaf discs floating in medium containing 400mM NaCl, relative to wild type plants. *A. thaliana* plants constitutively overexpressing *TaERF1* were more resistant to infection by *B. cinerea* and *P. syringae* and had increased expression of the stress-responsive genes *PR-1, PR-2, PR-5, ERD10A* (homolog of *A. thaliana* salt-inducible gene) and a cysteine protease (Xu *et al.*, 2007). Since TaERF1 bound DRE *in vitro* and the *ERD10A* promoter contains DRE-elements in its promoter, it suggests that TaERF1 may also regulate other genes involved in salt and drought tolerance, such as *RD22*.

TaAIDFa is also involved in abiotic stress responses. TaAIDFa was found to be targeted to the nucleus by expressing a fusion of the N-terminal region of *TaAIDFa* and *hGFP*, in onion epidermal cells (Xu *et al.*, 2008b). TaAIDFa bound to the CRT/DRE element *in vitro* and in a yeast one-hybrid assay, activating the *beta-galactosidase* reporter gene. The C-terminal region was found to be necessary for activating reporter genes in yeast (Xu *et al.*, 2008b). *TaAIDFa* was induced in response to; drought and ABA, which both peaked at 2 hours then declined until 48 hours, salt, which peaked at 6 hours then declined up to 24 hours and cold, which peaked at 12 hours then declined until 24 hours. A *TaAIDFa promoter:GUS* fusion was transiently expressed in wheat calli. GUS staining was present in response to 2%PEG, 200mM NaCl, 4ºC and 100µM ABA. *A. thaliana* plants constitutively overexpressing *TaAIDFa* had greater root growth when exposed to 100 mM NaCl, increased germination on MS plates supplemented with 100 mM mannitol and enhanced tolerance to three weeks drought stress, relative to wild type plants. *A. thaliana* plants constitutively overexpressing *TaAIDFa* had increased expression of *RD29A*, *COR15A* and *ERD10* (Xu *et al.*, 2008b). Indicating that TaAIDFa functions similarly to AtDREB genes, as it induces the same downstream genes.

Recently, two *DREB* genes were identified that are involved in cold and drought tolerance. Barley plants constitutively overexpressing the wheat genes *TaDREB2* and *TaDREB3* had enhanced tolerance to 18-21 days of drought stress followed by 1-2 weeks of rewatering with almost 100% survival whereas control plants only had 5%-10% survival (Morran *et al.*, 2011). Transgenic plants also had enhanced tolerance to a cold stress of -6ºC for 12 hours, with 50% survival for the *TaDREB2* overexpressors and 45% survival for the *TaDREB3* overexpressors, and only 12% survival for the control plants. Wheat and Barley plants expressing *TaDREB2* and *TaDREB3* driven by the drought inducible maize *Rab17* promoter had enhanced tolerance to 14 days drought stress, followed by 1-2 weeks of rewatering, with 33%-100% survival, whereas only 14% of control plants survived. Barley plants constitutively overexpressing *TaDREB3* or *TaDREB2* had increased expression of the dehydrins *HvDHN8, HvA22, HvCOR14B* and HvDHN5, and the DREB/CBFs *HvCBF1, HvCBF3, HvCBF6, HvCBF10A, HvCBF11, HvCBF15* and *HvCBF16* and decreased expression of *HvCBF2, HvCBF9*, and *HvCBF14*. (Morran *et al.*, 2011).

1.7.2.4.2. AP2/ERF genes in rice

Many AP2/ERF transcription factors involved in abiotic stress tolerance have also been identified in rice, mostly from the DREB group. There are 163 AP2/ERF genes in rice, including 24 AP2, five RAV, 57 DREB/CBF and 77 ERF group genes (Sharoni *et al.*, 2011). The DREB group is divided into subgroups, A-1 to A-6 and the ERF group into subgroups B-1 to B-7 (Sharoni *et al.*, 2011). Transcription factors from the DREB group are the most studied and form the basis for the following section. The DREB group members isolated from rice include; *OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D, OsDREB1E, OsDREB1F* and *OsDREB1G* from subgroup A-1, *OsDREB2A, OsDREB2B, OsDREB2C* and *OsDREB2E* from subgroup A-2 and *OsABI4* from subgroup A-3 (Chen *et al.*, 2008a; Dubouzet *et al.*, 2003; Ito *et al.*, 2006; Matsukura *et al.*, 2010; Wang *et al.*, 2008b). Other rice DREB members that have been identified but not isolated include *OsDREB1H* and *OsDREB1I* (Yamaguchi-Shinozaki & Shinozaki, 2004).

Like the *A. thaliana DREB* group genes, the rice *DREB* genes are induced in response to a variety of abiotic stresses. The first five *OsDREBs* were isolated using the *A. thaliana* DREB transcription factor AA sequences to query the rice genome database, including *OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D* and *OsDREB2A* (Dubouzet *et al.*, 2003). The proteins shared high sequence similarity within the ERF/AP2 DNA-binding domain, in the nuclear localisation signal (N-terminal) and in the C-terminal region. *OsDREB1A* and *OsDREB1B* were induced by 4ºC cold stress after 40 minutes and *OsDREB1A* was also induced by 250mM NaCl stress after 5 hours and transiently induced by wounding (Dubouzet *et al.*, 2003). In another study, *OsDREB1B* was induced within one hour by 200mM mannitol, 200mM NaCl, 10% PEG6000, 1mM salicylic acid, and 4°C cold, within three hours by 10µM methyl viologen, and only mildly induced by 1mM ABA after 24 hours (Gutha & Reddy, 2008). *A. thaliana* plants transformed with a *OsDREB1B promoter:GUS fusion* showed staining in the vascular tissue in young seedlings, in lower older leaves in response to 100µM ABA and 4°C and showed strong staining in root and stem but not leaves in response to 10% PEG, 200mM NaCl, 200mM mannitol and 10µM methyl viologen. Treatment with 1mM salicylic acid induced strong staining in the stem only (Gutha & Reddy, 2008). *DREB1F* transcript levels increased in response to 150mM NaCl, 7% PEG6000, 4ºC cold and ABA stresses (Wang *et al.*, 2008b). *OsDREB1F* was however previously reported to be

non-responsive to ABA, but still responsive to 4ºC cold, drought and 200mM NaCl (Liu *et al.*, 2007).

Similarly to *AtDREB2A*, *OsDREB2A* is not induced by 4ºC cold stress but is induced 24 hours after dehydration and after 250mM NaCl stress (Dubouzet *et al.*, 2003). *OsDREB2A* had moderate constitutive expression and is induced further by 42ºC heat, drought and 250mM NaCl stresses, but not by 4ºC cold stress (Matsukura *et al.*, 2010). *OsDREB2B* is alternatively spliced, with one active (*DREB2B2*) and one non-functional (*DREB2B1*) splice form. Non-functional *OsDREB2B1* is five-fold more abundant than *OsDREB2B2* in unstressed conditions, but *OsDREB2B2* is equally or more abundant under stressed conditions. Unlike *AtDREB2B*, *OsDREB2B2* is induced by 4ºC cold, but also by 42ºC heat, drought and 250mM NaCl stresses. *OsDREB2B2* is up-regulated to a greater extent than *OsDREB2A*; 20-fold for *OsDREB2B2* and three-fold for *OsDREB2A* during 42ºC heat stress (Matsukura *et al.*, 2010). Similarly to the *DREB* genes in *A. thaliana*, this indicates that the DREB family in rice may play different and partially redundant roles in plant signalling in response to different abiotic stresses.

Most rice DREB proteins have been found to bind DRE, specifically from subgroups A-1 and A-2 including *OsDREB1A* and *OsDREB2A* which bind specifically to DRE in rice protoplasts (Dubouzet *et al.*, 2003), OsDREB1B (Qin *et al.*, 2007), OsDREB1D (Zhang *et al.*, 2009b), OsDREB1E, OsDREB1G, and OsDREB2B (Chen *et al.*, 2008a) which all bind DRE in yeast one-hybrid assays. OsDREB1D also binds the low temperature response element (LTRE) in yeast on-hybrid assays (Zhang *et al.*, 2009b) and DREB1F binds the DRE/CRT but not the ABRE element in a yeast one-hybrid and gel shift assay (Wang *et al.*, 2008b).

Altered expression of many of the rice or *A. thaliana* DREB group transcription factors changes plant abiotic stress tolerance in different plant species, likely due to conservation of DREB protein structure and function between species. Differences in expression of the downstream target genes account for the differences in plant tolerance to particular abiotic stresses.

Transgenic plants overexpressing *OsDREB1A*, *OsDREB1B*, *OsDREB1D* or *OsDREB1F* have enhanced tolerance to cold and salt stresses, and *OsDREB1A, OsDREB1B* and *OsDREB1F* expression also enhances tolerance to drought stress (Dubouzet *et al.*, 2003; Gutha & Reddy, 2008; Ito *et al.*, 2006; Li *et al.*, 2005; Qin *et al.*, 2007; Wang *et al.*, 2008b; Zhang *et al.*, 2009b). Transgenic plants overexpressing *OsDREB2B* have enhanced tolerance to heat and drought stresses while *OsDREB1G* and *OsDREB1E* have enhanced tolerance to drought stress only (Chen *et al.*, 2008a). Uniquely, *OsDREB1B* overexpression in tobacco enhances tolerance to tobacco streak virus and to oxidative stress induced by methyl viologen treatment (Gutha & Reddy, 2008).

Analysis of downstream gene expression in *A. thaliana* plants overexpressing *OsDREB1A* showed that not all *AtDREB1A* target genes are upregulated by *OsDREB1A*. Target gene promoter analysis showed that different forms of DRE were present. OsDREB1A binds GCCGAC more specifically than ACCGAC, whereas AtDREB1A binds both sequences with equal efficiency (Dubouzet *et al.*, 2003). When unstressed, rice plants overexpressing *OsDREB1A* or *AtDREB1A* accumulated similar levels of proline, raffinose, sucrose, glucose and fructose to salt or cold stressed wild type plants. Rice plants overexpressing *OsDREB1A* had increased expression of genes encoding dehydrins, α-amylase, ACC oxidase, cytochrome P450, receptor-like protein kinase, ATPase family, β-1,3-glucanase, glycine-rich protein and a protease inhibitor (Ito *et al.*, 2006). Many of these proteins are known to function in plant responses to stresses (Kosová *et al.*, 2007; Narusaka *et al.*, 2004). The data show there is significant similarity between the *A. thaliana* and rice DREB pathways.

A. thaliana plants constitutively overexpressing *OsDREB1F* have increased expression *RD29A* and *COR15A* which both contain DRE in their promoters and can be induced in an ABA-independent manner. However, the transgenic plants also had increased expression of the ABA-dependent stress inducible genes *RD29B* and *RAB18*, which only contain ABRE in their promoters (Nakashima *et al.*, 2006), suggesting that *OsDREB1F* is involved in an ABA-dependent signalling pathway (Wang *et al.*, 2008b). Therefore, OsDREB1F must activate ABA-dependent gene expression though an intermediary protein, as it cannot bind ABRE (Wang *et al.*, 2008b). Unstressed plants constitutively overexpressing *OsDREB1D* had increased expression of *RD29A*, *COR15A* and *KIN1*, which all contain DRE in their promoters. *A. thaliana* plants

overexpressing *OsDREB1D* were ABA-insensitive, suggesting that *OsDREB1D* is involved in an ABA-dependent signalling pathway (Zhang *et al.*, 2009b).

The constitutive overexpression of genes in the studies above enhances abiotic stress tolerance, however the plants can be stunted, have delayed development and abnormal phenotypes, due to the extra metabolic load associated with being constantly ready to deal with stresses. Similarly to *AtDREB1A*, constitutive overexpression of five rice DREB transcription factors in *A. thaliana* produced varied stunted phenotypes with *OsDREB1A* exhibiting severe, *OsDREB1B* moderate, *OsDREB1C* mild and *OsDREB2A* very mild phenotypic changes (Dubouzet *et al.*, 2003). In another study, rice plants constitutively overexpressing *OsDREB1A*, *OsDREB1B*, *AtDREB1A*, *AtDREB1B* and *AtDREB1C* were stunted, relative to wild type plants (Ito *et al.*, 2006). Unusually, *A. thaliana* and rice plants constitutively overexpressing *OsDREB1F* did not show stunted growth (Wang *et al.*, 2008b) and also, tobacco plants constitutively overexpressing *OsDREB1B* showed no growth inhibition or unusual phenotypes (Gutha & Reddy, 2008).

Stress inducible overexpression can be used to overcome the growth repression seen with constitutive overexpression. Stress inducible overexpression has produced plants with no negative effects on plant growth or phenotype using the *RD29A* promoter to drive expression of *AtDREB1A* in *A. thaliana* (Kasuga *et al.*, 1999), *OsDREB2B2* in rice plants (Matsukura *et al.*, 2010) and using the maize *Rab17* promoter to drive expression of *TaDREB2* and *TaDREB3* in barley and wheat (Morran *et al.*, 2011).

The AP2/ERF superfamily contains many members involved in plant responses to abiotic stresses and therefore represents an attractive target for engineering improved plant fitness. The bHLH family also has many members involved in abiotic stress responses.

1.7.3. bHLH transcription factors

1.7.3.1. Background

The crystal structure of bHLH proteins and bHLH-ZIP proteins are very similar and they bind DNA in exactly the same way (Ferre-D'Amare *et al.*, 1993; Ma *et al.*, 1994). The bHLH domain structure was first determined for the transcription factor, Max, with a truncated form (22-113AA) composed of the basic helix-loop-helix and leucine zipper domains bound to the target sequence, CACGTG. Max forms a symmetric homodimer, with the monomers forming a parallel left-handed four helix bundle, called a coiled coil (Ferre-D'Amare *et al.*, 1993; Lupas, 1996). Each monomer contains two α-helices, each composed of a basic region, helix 1, helix 2 and a leucine repeat. The two N-terminal basic α-helices, one from each monomer, project towards the DNA and bind the major groove on either side of the DNA strand, in opposite directions. The N-terminal 15-20AA are involved in DNA binding. Conserved hydrophobic AAs at the core of the dimer stabilise its structure (Ferre-D'Amare *et al.*, 1993). bHLH proteins form homo- or heterodimers through specific interhelical electrostatic interactions (Lavigne *et al.*, 1995). The E-box minimal DNA element, CANNTG, is required for binding by bHLH proteins (Halazonetis & Kandil, 1991; Kerkhoff *et al.*, 1991; Prendergast & Ziff, 1991). The conserved residues His-5, Glu-9 and Arg-13 are required for E-box recognition and Arg-2 and His-5 are required for recognition of the 3′-flanking bases, GC (Shimizu *et al.*, 1997). Different AAs in the basic α-helix provide different binding specificities to different E-boxes or the G-box (CACGTG), and the flanking bases provide additional binding specificity (Atchley *et al.*, 1999; Fisher & Goding, 1992; Shimizu *et al.*, 1997).

1.7.3.2. Role of *bHLH* genes in plants

First characterised plant bHLH, Lc, was identified in maize and involved in tissue specific anthocyanin biosynthesis (Ludwig *et al.*, 1989). *Lc* is a member of the maize R gene family and encodes a protein containing a *myc*-homology region (Ludwig *et al.*, 1989). *bHLH* genes have been identified that are involved in a wide array of plant processes. These include plant development processes such as the regulation of panicle architecture (Komatsu *et al.*, 2001), dehiscence of fruit (Rajani & Sundaresan, 2001), fruit development (Liljegren *et al.*, 2004), control of petal size (Szecsi *et al.*, 2006), tapetum development (Zhang *et al.*, 2006), female reproductive tract development (Gremski *et al.*, 2007), microspore development (Sorensen *et al.*, 2003), trichome development (Morohashi *et al.*, 2007; Payne *et al.*, 2000), stomata development

(Kanaoka *et al.*, 2008; Pillitteri *et al.*, 2007) root development (Menand *et al.*, 2007; Ohashi-Ito & Bergmann, 2007) and anther development (Zhang *et al.*, 2010a).

bHLH genes have also been identified involving brassinosteroid signalling (Friedrichsen *et al.*, 2002; Yin *et al.*, 2005), gibberellin signalling (Lee *et al.*, 2006), anthocyanin biosynthesis (de Pater *et al.*, 1997), tryptophan metabolism and activation of stress-responsive genes (Smolen *et al.*, 2002), cold stress signalling through AtICE1 (Badawi *et al.*, 2008; Chinnusamy *et al.*, 2003; Lee *et al.*, 2005a; Miura *et al.*, 2007; Xiang *et al.*, 2008a; Xiang *et al.*, 2007), jasmonic acid signalling through RERJ1 (Kiribuchi *et al.*, 2004), ABA-mediated drought response signalling through AtMYC2 (Abe *et al.*, 1997) and finally light signalling through PIF3 (Halliday *et al.*, 1999; Ni *et al.*, 1998), *HFR1* (Fairchild *et al.*, 2000), PIF4 (Huq & Quail, 2002), PIF5 and PIF6 (Khanna *et al.*, 2004), PIF3-like 5 (PIL5) (Oh *et al.*, 2004), KIDARI (Hyun & Lee, 2006), PAR1 and PAR2 (Roig-Villanova *et al.*, 2007) and PIF7 together with PIF3 and PIF4 (Leivar *et al.*, 2008).

A recent analysis of the *bHLH* genes in *A. thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Physcomitrella patens* and five algae species divided the family into 32 subfamilies, most of which were predicted to bind the E- or G-box and 167 did not contain a basic region, so were not predicted to be involved in DNA binding (Carretero-Paulet *et al.*, 2010).

There are 170 and 178 *bHLH* genes in the *A. thaliana* and rice genomes, respectively (Carretero-Paulet *et al.*, 2010). Leu-27 and Leu73, of helix 1 and 2, respectively, are the most highly conserved residues in plants and are essential for dimerisation activity (Carretero-Paulet *et al.*, 2010). Within each subfamily, intron patterns were almost completely conserved (Carretero-Paulet *et al.*, 2010).

1.7.3.3. Role of *bHLH* genes in abiotic stress responses in *A. thaliana*

Several bHLH transcription factors involved in abiotic stress response signalling have been characterised in *A. thaliana*. These include, AtICE1, involved in cold tolerance (Lee *et al.*, 2005a), AtAIB, involved in drought tolerance (Li *et al.*, 2007), AtNIG1, involved in salt and dehydration stress responses (Kim & Kim, 2006), AtbHLH92, involved in salt, dehydration and temperature responses (Jiang *et al.*, 2009) and AtMYC2, involved in dehydration (Abe *et al.*, 2003), jasmonic acid signalling (Chini *et al.*, 2007), defence (Lorenzo *et al.*, 2004), photomorphogenesis (Gangappa *et al.*, 2010) and auxin signalling (Dombrecht *et al.*, 2007).

1.7.3.3.1. *AtICE1*

The bHLH, AtICE1, is involved in the DREB/CBF pathway which regulates plant responses to cold and other abiotic stresses. AtICE1 was discussed in context with its role in the DREB/CBF pathway in Section 1.7.2.3.

1.7.3.3.2. *AtAIB*

The *bHLH*, *AtAIB*, is involved in plant responses to drought stress. *AtAIB* is induced in response to exogenous ABA application and when overexpressed, increases ABA sensitivity and improves drought tolerance in *A. thaliana* (Li *et al.*, 2007). *aib* mutants have reduced ABA sensitivity and reduced drought tolerance. Two week old plants were subjected to water withholding for ten days, followed by two days rewatering. Plants overexpressing *AIB* had an 80% survival rate, wild type plants which had a 40% survival rate and *AIB* antisense plants had 30% survival rate. The stress inducible genes, *RD29A*, *RD22*, *COR47*, and *RAB18* were not induced in plants overexpressing *AtAIB*, suggesting that enhanced dehydration tolerance is mediated through different downstream genes (Li *et al.*, 2007).

1.7.3.3.3. *AtNIG1*

Another bHLH transcription factor involved in abiotic stress responses is *salt inducible gene 1* (*AtNIG1*), which was identified from *A. thaliana* plants stressed with 200mM NaCl using suppression subtractive hybridisation. AtNIG1 is closely related to

AtMYC3. AtNIG1 contained an N-terminal EF-hand calcium binding motif and a C-terminal bHLH domain. AtNIG1 was found to bind Ca^{2+} and the E-box sequence and to be nuclear localised. *atnig1-1* mutants had reduced germination when treated with NaCl, mannitol or exogenous ABA, relative to wild type seeds. *atnig1-1* mutants had enhanced sensitivity to 100mM NaCl, 200mM mannitol, or 0.5µM ABA, relative to wild type plants. *atnig1-1* mutants treated with 100mM NaCl for 7 days had reduced survival, fresh weight, chlorophyll content and protein content, relative to wild type plants (Kim & Kim, 2006). This indicates that *AtNIG1* positively regulates plant tolerance to salt stress.

1.7.3.3.4. *AtbHLH92*

The bHLH AtbHLH92 was studied in wild type plants, ABA-signalling mutants and *SOS* mutants (Jiang *et al.*, 2009). AtbHLH92 was nuclear localised and transcripts increased in six hours in response to 150mM NaCl, 200mM mannitol, dehydration, 10µM methyl viologen, 4ºC and 37ºC but not in response to 50µM ABA. After 24 hours, transcripts were still elevated for NaCl, mannitol, dehydration and cold stresses. Transcripts were highest for NaCl at all time points. The *bHLH92* homologs *bHLH41* and *bHLH42* were identified and transcripts were found to be responsive to NaCl, were non-responsive or decreased in response to ABA and decreased in response to 37ºC. *bHLH42* transcripts increased in response to mannitol treatment (Jiang *et al.*, 2009). *bHLH92* expression is partially dependent on ABA as NaCl induced expression of *bHLH92* was reduced by 39% and 37%, compared to wild type plants, in the ABA biosynthesis-deficient mutant, *aba1-5* and the ABA signalling-deficient mutant, *abi4-1*, respectively. Induction of *bHLH41* and *bHLH42* was unaffected. SOS2 is a possible regulator of *bHLH92* as NaCl induced expression of *bHLH92* was significantly higher in *sos2-1* and unaffected in the *sos1-1* and *sos3-1* mutants. NaCl induced expression of *bHLH42* was significantly lower in *sos2-1* (Jiang *et al.*, 2009). *bHLH92* expression was however up-regulated two-fold in *sos3* mutants treated with 250mM NaCl (Kamei *et al.*, 2005).

The *bhlh92-1* and *bhlh92-2* mutants had shorter roots from 100mM and 150mM NaCl treatments, less lateral roots from 150mM NaCl and significantly shorter roots from 100mM and 150mM mannitol treatments, relative to wild type plants. Relative

electrolyte leakage was significantly higher in the mutants than in wild type plants. *A. thaliana* plants constitutively overexpressing *bHLH92* had normal morphology, with 25% and 28% longer roots for 100mM NaCl and 200mM mannitol treatments, respectively, more lateral roots for 100mM and 150mM NaCl treatments and slightly shorter roots for 0.5µM ABA, relative to wild type plants (Jiang *et al.*, 2009). *A. thaliana* plants constitutively overexpressing *bHLH92* had increased expression of *COR47* and the class III peroxidases *PER10*, *PER52* and *PER59* and reduced expression of *KIN1* (Jiang *et al.*, 2009). Gene expression was analysed using a microarray, in *A. thaliana* plants constitutively overexpressing *bHLH92* and in wild type plants, following a 150mM NaCl treatment for six hours. Differentially expressed genes included ten down-regulated and nice up-regulated genes. Up-regulated genes included the LEA-like genes, *ERD10* and *KIN1*, two transcription factors, the homeobox-leucine zipper family protein gene, HB53, and the WRKY gene, WRKY2, and Auxin-regulated protein, a protease inhibitor/seed storage/lipid transfer protein and other uncharacterised genes. Down-regulated genes included the three peroxidases, *PER10*, *PER52* and *PER59*, which suggests there was an oxidative burst under salt stress. H_2O_2 levels were comparable between wild type and *bHLH92* overexpresssors and the *bhlh92-1* and *bhlh92-2* mutants. Twelve copies of the canonical G-box element (CACGTG) were found in the promoters of four of the nine up-regulated genes. None of the down-regulated genes contained G-box elements (Jiang *et al.*, 2009). These results suggest that *bHLH92* is negatively regulated by SOS2 and possibly SOS3, and that bhLH92 positively regulates plant responses to salt and osmotic stresses by binding to the G-box element in the promoters of LEA-like and transcription factor genes.

1.7.3.3.5. *AtMYC2*

Another well characterised bHLH, AtMYC2, is involved in jasmonic acid, ABA and light signalling to control biotic and abiotic stress responses and photomorphogenic growth(Abe *et al.*, 2003; Anderson *et al.*, 2004; Gangappa *et al.*, 2010; Lorenzo *et al.*, 2004; Yadav *et al.*, 2005). AtMYC2 has 30% AA identity with AtAIB1 (Li *et al.*, 2007). AtMYC2 acts together with the myb transcription factor, AtMYB2 (discussed in Section 1.7.1.3), as ABA-dependent transcriptional activators under drought stress (Abe *et al.*, 2003). *AtMYC2*, like *AtMYB2*, is induced by dehydration stress and exogenous ABA (Abe *et al.*, 1997). AtMYC2 and AtMYB2 bind cis-acting elements in the *rd22* promoter to induce expression in response to dehydration and ABA (Abe *et al.*, 1997).

A. thaliana plants overexpressing *AtMYB2*, *AtMYC2* and *AtMYB2/AtMYC2* had increasing sensitivity to ABA, in that order and all were more sensitive than wild type plants. *A. thaliana* plants overexpressing *AtMYC2* had increased expression of the stress- and ABA-responsive genes *rd22* and *AtADH1* when untreated and in response to exogenous ABA (Abe *et al.*, 2003). An *AtMYC2* Ds mutant had reduced ABA sensitivity and reduced induction of *rd22* and *AtADH1*. *A. thaliana* plants constitutively overexpressing *AtMYC2/AtMYB2* had less electrolyte leakage than wild type plants osmotic stresses (Abe *et al.*, 2003). This shows that *AtMYC2* is involved the ABA-dependent regulation of plant tolerance to dehydration stress, through the action of *rd22* and *AtADH1*.

AtMYC2 has also been shown to be involved in jasmonate responses (Chini *et al.*, 2007). The jasmonate ZIM-domain (JAI3/JAZ) proteins act as repressors of MYC2 in a negative regulatory feed-back loop. In response to jasmonate, the jasmonate receptor CORONATINE-INSENSITIVE1 (COI1) binds jasmonate-isoleucine (Yan *et al.*, 2009), interacts with the JAI3 N terminus (containing the ZIM domain) and targets it for degradation by the 26S proteosome. JAI3 is a negative regulator of MYC2. *In vitro* pull-down assays demonstrated a direct MYC2–JAI3 interaction, where the C-terminal domain of JAI3 interacts with the N-terminal domain of MYC2 (Chini *et al.*, 2007). Redundancy amongst JAZ proteins keeps MYC2 repressed in the *jai3-1* mutant, where the JAI3-1 protein keeps the SCF^{COI1} complex in an inactive state as it is resistant to degradation. Ten JAZ proteins are rapidly up-regulated in response to exogenous jasmonate. Eight of those are regulated by MYC2, as they were constitutively overexpressed in *35S:MYC2* plants. Seven of those have reduced expression in jasmonate treated *atmyc2* mutants. The promoter target binding motifs of MYC2 are the G-box and G/T box, which were the most significantly over-represented in the promoters of the *JAZ* genes (Chini *et al.*, 2007). So, in response to jasmonate, MYC2 repression is relieved by JAI3 degradation and MYC2 can bind the G-box and G/T box of target genes to activate or repress expression.

Two MYC2 homologs, MYC3 and MYC4 also interact with JAZ repressors *in vitro* and *in vivo* and are able to form homo- and heterodimers with themselves and MYC2. GFP fusion constructs demonstrated that both MYC3 and MYC4 were nuclear localised. MYC2 and MYC3 had almost identical DNA-binding specificities for the G-box and G-

box variants, whereas MYC4 had a similar by slightly different binding affinity, suggesting that it may target different downstream genes. MYC3 and MYC4 expression was weakly induced by exogenous jasmonic acid were expressed in most aerial portions of the plant, whereas MYC2 is more highly expressed in the roots. Analysis of early (*JAZ10*), middle (*VSP2*) and late (*PDF1.2*) jasmonate-responsive expressed genes in *myc* mutants, suggest that *JAZ10* is positively regulated by all three MYCs, *VSP2* is positively regulated by all MYC2 and MYC4 but may be negatively regulated by MYC3, whereas *PDF1.2* is negatively regulated by MYC2 and MYC3 but may by positively regulated by MYC4. They also suggest that each MYC transcription factor has an additive effect on reducing the expression of *JAZ10* and *VSP2*, where the triple mutant has almost abolished expression, which is comparable to the jasmonate insensitive mutant, *coi1-1*. Root growth inhibition assay show that MYC2 is more important in root jasmonate responses, than MYC3 and MYC4, which is consistent with MYC2 having higher expression in the roots. MYC2, 3 and 4 also regulate jasmonic acid-mediated defence responses to the herbivore, *Spodoptera littoralis* and the hemibiotrophic pathogen, *Pseudomonas syringae* pv *tomato* DC3000 (Fernández-Calvo *et al.*, 2011).

Another recent study analysed MYC3, which was isolated in a yeast two-hybrid screen using JAZ1 as bait (Cheng *et al.*, 2011). *A. thaliana* plants constitutively overexpressing *AtMYC3* were hypersensitive to 1μM methyl-jasmonate-mediated root growth inhibition and were reduced to 27.6% and 30% of uninhibited root growth, whereas wild type roots were only reduced to 39.5%. Growth of the aerial portions of the transgenic plants was severely inhibited by 10μM methyl-jasmonate. *MYC3-RNAi* knockdown plants had similar responses to wild type plants when treated with methyl-jasmonate (Cheng *et al.*, 2011). Microarray analysis of gene expression showed that *MYC3* may be involved in regulating genes involved in jasmonic acid biosynthesis, salt stress, wounding and defence responses. In response to methyl jasmonate, *MYC2* had a rapid and strong response, whereas *MYC3* had a slow and mild response. MYC3-GFP was nuclear localised in *Nicotiana benthamiana* leaves (Cheng *et al.*, 2011).

In another study, *JAI1/JIN1* was identified, that encodes AtMYC2 and differentially regulates two groups of JA-induced genes. AtMYC2 represses the genes *PR1*, *PDF1.2*, *CHIB/PR3* and *HEL/PR4*, involved in defence responses against pathogens, accordingly

the *jin1* mutant has increased resistance to the necrotrophic pathogens *Plectosphaerella cucumerina*, *Botrytis cinerea*, *Fusarium oxysporum* and *P. syringae* (Anderson *et al.*, 2004; Laurie-Berry *et al.*, 2006; Lorenzo *et al.*, 2004). AtMYC2 also activates the genes involved in JA-mediated systemic responses to wounding *AtVSP2*, *AtLOX3*, and *AtTAT* (Lorenzo *et al.*, 2004). AtERF1 has the opposite effect, so they work antagonistically, to alternatively regulate genes associated with pathogen attack and wounding (Lorenzo *et al.*, 2004). *AtMYC2* also suppresses SA-dependent defences during *P. syringae* infection (Laurie-Berry *et al.*, 2006).

When treated with methyl jasmonate, the *jin1-9* mutant had increased expression of tryptophan biosynthesis and tryptophan-derived secondary metabolism genes, including those for indole-glucosinate (IG) biosynthesis, which are involved in plant defence and decreased expression of phenylpropanoid and flavonoid metabolism genes, indicating that MYC2 differentially regulates both sets of genes (Dombrecht *et al.*, 2007).

AtMYC2 also regulates terpenoid indole alkaloid biosynthesis genes through ORCA3. In C*atharanthus roseus*, the ERF ORCA3 is induced by jasmonic acid and activates terpenoid indole alkaloid biosynthesis genes. The *ORCA3* promoter contains an autonomous jasmonate-response element (JRE). A *JRE tetramer-GUS reporter* was induced in response to jasmonic acid, but not ethephon, salicylic acid or ABA. JRE responsive expression did not require de novo protein synthesis. The JRE tetramer is bound by AtMYC2 in EMSAs and *A. thaliana* protoplasts. JRE from the ORCA3 promoter is also bound by AtMYC2 in *A. thaliana* protoplasts. A *JRE tetramer-GUS reporter* was not induced in *atmyc2-1* mutants, but was when coexpressed with *AtMYC2* (Montiel *et al.*, 2011).

AtMYC2 is also involved in photomorphogenesis, the process following germination where a plant is exposed to light, so hypocotyl elongation ceases, chloroplasts differentiate, chlorophyll accumulates and the cotyledons expand (Gangappa *et al.*, 2010; Yadav *et al.*, 2005). COP1, a negative regulator of photomorphogenic growth, is an E3 ubiquitin ligase and its activity is stimulated by SPA1 (Hoecker & Quail, 2001; Saijo *et al.*, 2003; Seo *et al.*, 2003; Yang & Wang, 2006). In response to far-red light, Phytochrome A (PhyA) is activated and the signal is then transduced through the

positive regulators of photomorphic growth, HY5, LAF1 and HFR1 (Jang *et al.*, 2005; Osterlund *et al.*, 2000; Saijo *et al.*, 2003; Seo *et al.*, 2003; Yang *et al.*, 2005). COP1 attenuates the signal by ubiquitinating PhyA and the downstream proteins HY5, LAF1 and HFR1, targeting them for degradation (Jang *et al.*, 2005; Saijo *et al.*, 2003; Seo *et al.*, 2004; Seo *et al.*, 2003; Yang *et al.*, 2005; Yang & Wang, 2006). SPA1 also physically interacts with HFR1, HY5 and COP1 (Hoecker & Quail, 2001; Osterlund *et al.*, 2000; Yang *et al.*, 2005).

Z-box binding factor 1, encoding AtMYC2, interacts with the Z- and G-box light-responsive elements of light-regulated gene promoters (Puente *et al.*, 1996; Yadav *et al.*, 2002; Yadav *et al.*, 2005). AtMYC2 also binds the G-box in the promoter of *SPA1* and together with COP1, negatively regulates *SPA1* expression (Gangappa *et al.*, 2010). a*tmvc2* mutants have enhanced inhibition of hypocotyl elongation in constant blue light suggesting that AtMYC2 acts as a negative regulator of blue light-mediated photomorphogenic growth (Yadav *et al.*, 2005). *atmyc2 spa1* double mutants have shorter hypocotyls than the *spa1* mutants in far red light and shorter hypocotyls than both single mutants in blue light and white light. *atmyc2* mutants also have no change in hypocotyl length in red light, whereas the *spa1* mutant and *atmyc2 spa1* double mutant have comparable reductions in hypocotyls length, suggesting that AtMYC2 does not function in SP1-mediated control of hypocotyl length in red light (Gangappa *et al.*, 2010). The *atmyc2 spa1* double mutant accumulates more anthocyanin than either of the single mutants (Gangappa *et al.*, 2010; Yadav *et al.*, 2005). The *atmyc2* mutant also had increased expression of the light responsive genes, *CAB*, *RBCS*, and *CHS* under blue and far-red light, suggesting that AtMYC2 negatively regulates blue and far-red-light-regulated gene expression (Yadav *et al.*, 2005). *CAB* is down-regulated and *RBCS* is up-regulated in *spa1*, while *RBCS* is not upregulated in the double mutant, suggesting that AtMYC2 and SPA1 act antagonistically to regulate CAB (Gangappa *et al.*, 2010).

The regulation of light signalling is integrally linked with the circadian clock, which influences a third of all genes in *A. thaliana* (Covington *et al.*, 2008). The circadian clock will be explored following a discussion of *bHLH* genes in cereals.

1.7.3.4. Role of *bHLH* genes in abiotic stress responses in cereals

Several bHLH transcription factors have been identified in cereals that are involved in plant responses to abiotic stresses. These include the *AtICE1* homologs, *OrbHLH2*, involved in salt stress responses (Zhou *et al.*, 2009), *OrbHLH001*, involved in cold and salt stress responses (Li *et al.*, 2010) and *TaICE41* and *TaICE87*, involved in cold tolerance (Badawi *et al.*, 2008). *AtICE1* homologs have also been identified in Barley, including *HvICE1*, which mapped to 7H (Tondelli *et al.*, 2006) and *HvICE2*, which mapped to H3 (Skinner *et al.*, 2006), but these have not been functionally characterised. Other bHLH transcription factors include *OsbHLH1*, involved in cold stress responses (Wang *et al.*, 2003) and *OsbHLH148*, involved in biotic and abiotic stress responses (Seo *et al.*, 2011).

1.7.3.4.1. *OrbHLH2*

An *A. thaliana ICE1* homolog, designated *OrbHLH2*, was cloned from Dong Xiang wild rice (*Oryza rufipogon* Griff.) and the ORF has three nucleotides different to *OsbHLH002* (Li *et al.*, 2006; Zhou *et al.*, 2009). OrbHLH2 is nuclear localised and has transactivation activity in yeast. *A. thaliana* plants overexpressing *OrbHLH2* had longer roots than wild type plants when stressed with 150mM NaCl for five days, had normal green leaves whereas wild type plants had discoloured leaves, when stressed with 200mM NaCl for six days and had a higher germination rate of 82% on 100mM NaCl, whereas wild type plants had a germination rate of 68%. The transgenic plants also had increased expression of *DREB1A*/*CBF3*, *COR15A*, *RD29A* and *KIN1* when treated with 100mM NaCl, relative to wild type plants (Zhou *et al.*, 2009). *A. thaliana* overexpressing *OrbHLH2* also had increased tolerance to 200mM mannitol, but were not more tolerant to a freezing stress of -6°C for six hours (Zhou *et al.*, 2009). Increased tolerance to freezing stress may not have been evident as the plants were not cold acclimated. The transgenic plants subjected to 4ºC cold treatment also did not have increased expression of the cold tolerance gene, *KIN1* (Wang *et al.*, 1994), relative to wild type plants. The ABA-responsive genes, *KIN1* and *COR15A* had similar transcript levels in transgenic and wild type plants in unstressed conditions and following 50µM ABA treatment, indicating that *OrbHLH2* is ABA-independent (Zhou *et al.*, 2009).

1.7.3.4.2. *OrbHLH001*

The Dongxiang wild rice bHLH protein, OrbHLH001, an OsbHLH001 (AK102594.1) homolog (Li *et al.*, 2006), shares 39.66% AA identity with AtICE1 (Li *et al.*, 2010). In 12 day old rice plants treated with 250mM NaCl, *OrbHLH001* expression increased 3-fold by one hour and >4-fold after 24 hours. After 80 hours, expression was still 3.8-fold higher in leave blades, 2.7-fold higher in the sheaths but undetectable in the roots. *OrbHLH001* expression was not responsive to cold stress. Cold acclimated *A. thaliana* plants constitutively overexpressing *OrbHLH001* had increased tolerance to -12 ºC freezing stress, with 45% and 23.5% survival after 25 days recovery, whereas all wild type plants died. The transgenic plants also had increased root growth when grown on MS agar plates supplemented with 50, 100 or 200mM NaCl, compared to wild type plants, and remained bright green and maintained growth when grown in vermiculite and watered with 300mM NaCl, relative to wild type plants. Expression of the cold-responsive genes *ICE1*, *CBF1*, *CBF2*, *CBF3*, *RD29A* and *COR47* was the same in *A. thaliana* plants constitutively overexpressing *OrbHLH001* and in wild type plants, suggesting that OrbHLH001 enhances plant cold tolerance independently of the CBF/DREB pathway (Li *et al.*, 2010).

1.7.3.4.3. *TaICE41 and TaICE87*

Two wheat *ICE* genes, *TaICE41* and *TaICE87*, were isolated from cDNA from cold-treated wheat and share 50% and 47% AA identity, respectively, with AtICE1. Both were not induced by cold treatment. In gel mobility shift assays, both bound MYC elements in the *TaCBFIVd-B9* promoter and could activate *TaCBFIVd-B9* expression in *Nicotiana benthamiana*. TaICE41 and TaICE87 had different affinities for different MYC elements. *A. thaliana* plants constitutively overexpressing *TaICE41* or *TaICE87* had enhanced tolerance to -10.5ºC for six hours, but only following cold acclimation and had enhanced expression of *AtCBF2*, *AtCBF3*, *COR15a* and *COR47*. Plants overexpressing *TaICE87* but not *TaICE41* had increased expression of *AtCOR6.6* (Badawi *et al.*, 2008). This indicates that *TaICE87* and *TaICE41* enhance cold tolerance through induction of the CBF/DREB pathway in *A. thaliana*.

The data show that despite the AtICE1 homologs having significant sequence similarity, they do not function identically when expressed in *A. thaliana*. *OrbHLH2* induces cold-

responsive genes, but does not enhance cold tolerance, whereas *OsbHLH001* is the opposite, not inducing cold-responsive genes from the CBF regulon, but still enhancing cold tolerance. Both of the rice homologs also enhance salt tolerance. The wheat homologs act similarly to AtICE1.

1.7.3.4.4. *OsbHLH1*

Another rice *bHLH*, *OsbHLH1*, is responsive to cold stress only. In whole plants treated with 4ºC cold stress, *OsbHLH1* expression increased after 30 minutes, peaked at one hour and then declined after five hours. Expression was not induced by ABA, NaCl or PEG. In *japonica* rice JX17 and *indica* rice 9311 treated with 4ºC cold stress, transcripts were only detectable in the roots. *OsbHLH1* is nuclear localised, has transactivation activity and will homodimerise in yeast, indicating it is a transcription factor. It is not known whether *OsbHLH1* is involved in cold tolerance (Wang *et al.*, 2003).

1.7.3.4.5. *OsbHLH148*

The rice bHLH *OsbHLH148* is similar to AtMYC2 in its responsiveness to multiple abiotic stresses, protein-protein interactions and downstream gene expression. *OsbHLH148* expression rapidly increases in response to 100µM methyl jasmonate, 200µM ABA, wounding, dehydration, 200mM high salinity and more slowly in response to 4ºC. Rice plants constitutively overexpressing *OsbHLH148* have increased drought tolerance and have increased expression of *OsDREB1A*, *B*, *C*, *E* and *G* and *OsJAZ1*, *2*, *3*, *4*, *5*, *6*, *10* and *11* genes. In a yeast two-hybrid assay, OsJAZ1, 8 and 9 interacted with putative OsCOI1 in the presence of 120µM coronatine, a jasmonic acid analogue, but not in the presence of jasmonic acid or methyl jasmonate. OsbHLH148 interacted with OsJAZ1 *in vitro* and *in vivo* and interacted with OsCOI1 in the presence of coronatine. In an *in vitro* degradation assay, OsJAZ1 was degraded in whole cell extracts from rice and *A. thaliana* when coronatine was present and degradation was inhibited by the 26S proteosome inhibitor MG132. OsJAZ1 was not degraded in extracts from the jasmonate-isoleucine deficient mutant, *atjar1*, but was in the presence of coronatine or MG132. OsJAZ was not degraded in extracts from the *atcoi1* mutant even in the presence of coronatine. This suggests that OsbHLH148 regulates the jasmonate signalling pathway through SCF^{OsCOI1} complex-mediated degradation of OsJAZ1, to enhance drought tolerance in rice. This required jasmonate-isoleucine *in*

vivo and coronatine *in vitro*. Transcripts for the *OsJAZ* genes *OsJAZ1, 2, 4* and *5* increased in plants treated with methyl jasmonate, to a greater extent with methyl jasmonate and ABA but not with ABA alone. This suggests that the induction of *bHLH148* in response to ABA occurs independently of methyl jasmonate and the OsJAZ genes. Transcripts for *OsJAZ1, 2, 4* and *5* rapidly increased in response to drought and salt stresses, *OsJAZ5* transcripts rapidly increased in response to wounding and *OsJAZ1*, *4* and *5* transcripts increased in response to cold treatment. (Seo *et al.*, 2011). These results suggest that OsbHLH148 is an upstream regulator of jasmonate-mediated responses leading to drought stress tolerance through increased expression of *OsDREB1* genes. The similarities between AtMYC2 and OsbHLH148, including the interaction with putative OsCOP1, suggest that OsbHLH148 could function in the regulation of photomorphic growth and light signalling, which is integrally linked with the circadian clock.

1.7.4. The circadian clock and abiotic stress

Modulation of the circadian clock is an attractive strategy for enhancing plant tolerance to salt and other abiotic stresses. The timing of the circadian clock affects chlorophyll accumulation, photosynthesis and stomatal opening which has a direct impact upon plant biomass, plant fitness and subsequent yield (Dodd *et al.*, 2005). Analysis of global gene expression in *A. thaliana* determined that a third of all expressed genes are controlled by the circadian clock (Covington *et al.*, 2008). This includes genes involved in the synthesis of geranylgeranyl diphosphate, a precursor for isoprenoids such as chlorophylls, carotenoids, tocopherols, ABA and gibberellic acid (Covington *et al.*, 2008). A large number of ABA- and jasmonic acid-responsive genes have also shown to be circadian regulated (Mizuno & Yamashino, 2008), or at least ABA levels are clock regulated, which means that ABA-responsive genes are effectively clock regulated. Since both these hormones also mediate plant stress response signalling, it suggests that the circadian clock can be used to alter plant stress tolerance.

The *A. thaliana* circadian clock is proposed to consist of three interacting feedback loops, detailed in recent reviews (de Montaigu *et al.*, 2010; Harmer, 2009). The central negative feedback loop consists of morning phased partially redundant *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) that bind the *TIMING OF CAB1* (*TOC1*)/*PSEUDO-RESPONSE REGULATOR 1* (*PPR1*) promoter and negatively regulate its expression (Alabadi *et al.*, 2001). In the afternoon, *CCA1* and *LHY* expression decreases causing *TOC1* expression to increase. TOC1 closes the main loop by indirectly positively regulating *CCA1* and *LHY* expression. CCA1 HIKING EXPEDITION (CHE) binds the *CCA1* promoter to negatively regulate its expression and TOC1 interacts with CHE to inhibit binding to the *CCA1* promoter, while CCA1 and LHY negatively regulate *CHE* expression (Pruneda-Paz *et al.*, 2009). An unknown factor, termed X, is also involved in *CCA1* and *LHY* regulation and could be *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4* (*ELF4*) (Kikis *et al.*, 2005), *LUX ARRHYTHMO* (*LUX*) (Hazen *et al.*, 2005; Helfer *et al.*, 2011; Onai & Ishiura, 2005) or *TIME FOR COFFEE* (*TIC*) (Ding *et al.*, 2007).

The morning loop involves *PPR7* and *PPR9*, which are positively regulated by CCA1 and LHY and then negatively regulate *CCA1* and *LHY* expression (Farré *et al.*, 2005;

Nakamichi *et al.*, 2005; Salomé & McClung, 2005; Zeilinger *et al.*, 2006). The evening loop involves another unknown factor Y, which may be *GIGANTEA* (*GI*) or *PPR5* (Locke *et al.*, 2006), which is negatively regulated by CCA1, LHY and TOC1, and which positively regulates *TOC1*. The F-box protein, ZEITLUPE (ZTL), stabilised by GI (Kim *et al.*, 2007b), also targets TOC1 for degradation, which is essential for clock resetting (Han *et al.*, 2004; Mas *et al.*, 2003). ZTL also targets PRR5 for degradation (Kiba *et al.*, 2007).

The DREB stress responsive genes have been shown to be regulated by the circadian clock. At warm temperatures, *DREB1A/CBF3* cycles with the circadian clock, with a peak in transcript levels at Zeitgeber time (ZT) ZT4 and a trough at ZT16 (Harmer, 2000). The DREB1A-regulated genes *COR15b* and *COR6.6* follow the same rhythm as *DREB1A*, although delayed by several hours (Harmer, 2000). *DREB1A*, *B* and *C* transcript accumulation depends on which time of the day cold stress is imposed, with highest and lowest transcript levels at 4 ZT and 16 ZT (Fowler *et al.*, 2005). *A. thaliana* plants constitutively overexpressing *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) did not exhibit cycling of *DREB1A* in response cold stress, suggesting *DREB1A* is regulated by CCA1 (Harmer, 2000). Circadian control of *DREB1A* involves transcriptional regulation (Harmer, 2000). The cold responsive genes, *RAV1* and *ZAT12* were also circadian regulated, with *RAV1* cycling with the *DREB* genes and *ZAT12* cycling in an opposite pattern, with highest transcript accumulation at 16 ZT (Fowler *et al.*, 2005).

The promoter of cold-responsive *DREB1C/CBF2* was found to contain six conserved motifs, numbered box I to VI. Box V is a G-box sequence that negatively regulates *DREB1C* expression under circadian control and sequence surrounding box VI contains positive regulatory elements for low-temperature-induced expression (Kidokoro *et al.*, 2009). Phytochrome-Interacting Factor7 (PIF7) was identified in a yeast one-hybrid assay using the G-box as bait. PIF7 bound the G-box present in the *DREB1B* and *DREB1C*, but bound the *DREB1A* promoter less efficiently (Kidokoro *et al.*, 2009). PIF7 protein was nuclear localised and functioned as a transcriptional repressor of *DREB1C* expression in transactivation experiments in *A. thaliana* protoplasts. PIF7 activity is regulated by TIMING OF CAB EXPRESSION1 and Phytochrome B (PhyB) (Kidokoro *et al.*, 2009). This shows that *DREB* genes are regulated by components of the circadian clock.

The *PRR* genes *PRR9*, *PRR7* and *PRR5* have dual roles, both as components of the circadian clock and also as negative regulators of abiotic stress responsive genes (Nakamichi *et al.*, 2009). The *prr9-11/prr7-10/prr5-10* triple mutant (d975) had increased expression of *DREB1*, increased raffinose and proline accumulation and increased tolerance to cold, salt and drought stresses (Nakamichi *et al.*, 2009). It is not known whether *DREB1* expression is directly or indirectly regulated by the *PRR* genes. Other clock mutants have also been found to have altered stress resistance, including the *cca1/lhy* double mutant that was more susceptible to salt, osmotic and heat stress (Kant *et al.*, 2008) and the *GI* mutant *gi-3* that was more susceptible to cold stress (Cao *et al.*, 2005a).

The circadian clock components may regulate stress-responsive genes through the recognition of *cis*-elements in the promoters of stress-responsive genes. The evening element (EE) is overrepresented in the promoters of evening phased genes (Harmer & Kay, 2005) and is also found in the promoters of cold-responsive genes (Kreps *et al.*, 2003). Promoter deletion experiments showed that the EE motif in the promoters of the cold-responsive genes, *CONSTANS-like 1* (*COL1*) and *COLD-REGULATED GENE 27* (*COR27*), was required for cold-induced expression. An artificial promoter containing four EE motifs and three ABA response element-like (ABREL) motifs was sufficient for cold-induced expression (Mikkelsen & Thomashow, 2009). Bioinformatic analysis showed that coupled EE and ABREL motifs are overrepresented in cold-induced gene promoters (Mikkelsen & Thomashow, 2009). The clock transcription factors CCA1 and LHY belong to a Myb-like family containing ten members, including CCA1, LHY and RVE1-8 (Yanhui *et al.*, 2006). Both CCA1, LHY and RVE1 have been found to bind EE (Gong *et al.*, 2008; Harmer & Kay, 2005). Together, these data suggest that the circadian clock is an attractive target for modifying plant abiotic stress tolerance.

Transcription factors from other families have also been shown to be involved in abiotic stress responses, including the NAC family (Gao *et al.*, 2010; Hu *et al.*, 2006; Hu *et al.*, 2008a; Nakashima *et al.*, 2007; Tran *et al.*, 2004; Yokotani *et al.*, 2009; Zheng *et al.*, 2009), WRKY family (Jiang & Deyholos, 2009; Zhou *et al.*, 2008) and bZIP family (Hossain *et al.*, 2010; Xiang *et al.*, 2008b; Zou *et al.*, 2008), however these are beyond the scope of this thesis.

Thus, previous studies strongly indicate that several plant transcription factors have key roles in abiotic stress in general and salt stress, in particular and altering the expression of one or more of these transcription factors could be a viable strategy to engineer salt tolerance in rice. The main aims of this study were therefore to:

a. Identify a set of transcription factors which are differentially regulated in salt tolerant and salt sensitive rice cultivars as those would be the prime candidates for imparting salt tolerance.
b. Alter the expression of these transcription factors individually by a transgenic approach and study their effect on plant growth and performance under salt stress.

Chapter 2. Gene expression analysis of salt responsive rice transcription factors

2.1. Introduction

Transcription factors regulate important cellular processes, including responses to abiotic stresses, such as high soil salinity. In this chapter, transcription factors are identified, that are responsive to the imposition of moderate salinity stress at the transcript level. These are of particular interest, as the changes in transcript levels in salt tolerant rice cultivars are often not seen in salt sensitive cultivars. These transcription factors could therefore be important for salinity tolerance.

2.2. Materials and methods

2.2.1. Materials

TRIzol®, RNaseOUT™, Superscript® III Reverse Transcriptase, Platinum® Taq and 1Kb plus DNA ladder were purchased from Invitrogen, San Diego, California, USA. The chloroform, ethidium bromide was purchased from Sigma-Aldrich, St. Louis, Missouri, USA. EDTA disodium salt, glucose, isopropanol and ethanol were purchased from Merck, Darmstadt, Germany. The $MgSO_4$ and microfuge tubes were supplied by BDH Laboratory Supplies, Victoria, Australia. Liquid nitrogen was purchased from BOC, Sydney, Australia. DEPC treated water was purchased from Biorad, Hercules, California, USA. Primers were purchased from Geneworks, Adelaide, Australia. DNA-*free*™ was purchased from Ambion, Austin, Texas, USA. The NanoDrop™ ND-1000 spectrophotometer was purchased from Thermo Scientific, Wilmington, Delaware, USA. The agarose was purchased from Bio-rad, Hercules, California, USA. The deoxyribonucleotide triphosphates and agarose were purchased from Bioline, London, United Kingdom. ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit was purchased from Applied Biosystems, Foster City, California, USA. MilliQ water generated using a MilliQ Plus system purchased from Millipore, Billerica, Massachusetts, USA. The Ms2 Minishaker Vortex Mixer was purchased from IKA, Petaling Jaya, Malaysia. The Savant SC110 Speedvac vacuum concentrator was purchased from Biolab, Victoria, Australia. The Gene Flash UV transilluminator was supplied by Syngene Bio Imaging, Frederick, Maryland, USA. Eppendorf centrifuge was supplied by Adelab Scientific, Adelaide, South Australia, Australia. DNA Engine TETRAD® 2 thermal cycler and DNA primers were purchased from Geneworks, Adelaide, Australia.

2.2.2. Bioinformatic analysis of microarray data

Global gene transcript levels were analysed in three salt tolerant cultivars and one salt sensitive cultivar of *O. sativa* ssp. *indica* rice, using the Affymetrix Rice 57K GeneChip® to identify transcripts that were differentially regulated in response to salt. The experiment was conducted in collaboration between The Australian Centre for Plant Functional Genomics, Adelaide and The University of California, Riverside. The shoot data for one salt tolerant cultivar, FL478 and the salt sensitive cultivar, IR29, was published in Walia *et al.* (2005). The root data for the three salt tolerant cultivars, FL478, Pokkali and IR63731 and the salt sensitive cultivar, IR29, was published in Cotsaftis *et al.* (2010). Plant growth conditions were outlined in Walia *et al.* (2005). Briefly, plants were grown in sand with nutrient solution until day 22, when NaCl was added until the grown solution reached an EC of 4.8dS.m^{-1}. On day 24, NaCl was added until the EC reached 7.4dS.m^{-1}. The NaCl stress was maintained until the plants were harvested on day 30.

Data from both experiments were used to identify transcription factors differentially expressed in the different rice cultivars under salt stress. Microarray signal intensity levels were compared between the salt tolerant rice cultivars FL478, Pokkali and IR63731, and the salt sensitive cultivar IR29. Transcription factors were selected that were either two-fold up or down-regulated under salt stress, in one or more of the salt tolerant cultivars but not in salt sensitive IR29. Statistical analysis used to identify the transcription factors is described in Cotsaftis *et al.* (2010). Locus numbers were used to obtain the coding sequence (CDS) of each transcription factor from the Rice Transcription Factor Database (http://ricetfdb.bio.uni-potsdam.de/v2.1/), which were aligned with the probeset sequences from the microarray to confirm sequence identity. The mRNA and AA sequences were used in BLASTN and BLASTP searches (Altschul *et al.*, 1997; Dereeper *et al.*, 2008; Zhang *et al.*, 2000), respectively, of the nr and refseq databases, to identify accessions for each transcription factor on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The mRNA sequences were also used in BLASTN searches of the Rice Expressed Sequence Tag Database (REDB) (http://bioinfo.hzau.edu.cn/), to determine whether the transcription factors had known tissue- or developmental-specific expression. The UniGene database (http://www.ncbi.nlm.nih.gov/unigene/) was also used to examine tissue or developmental specific expression.

2.2.3. Quantitative RT-PCR analysis of transcription factor mRNA accumulation

Two Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) experiments were conducted. The first was performed to verify the results from the microarray, using the same cDNA set from the microarray experiment. The second was performed to check that changes in transcription factor expression were consistent between the rice lines used in the microarray experiment and in the rice line used in the established transformation system in our laboratory; *O. sativa* ssp. *japonica* cv. Nipponbare.

2.2.3.1. Primer design

Primers were designed to Affymetrix probeset sequences. Primer candidates were obtained using the web based primer design program, Primer3 (Rozan & Skaletsky, 2000). Candidates were validated using the web based primer validation applet, NetPrimer (http://www.PremierBiosoft.com). The primers obtained were 17 to 27 nucleotides in length, had hairpin values below a ΔG of –0.5 kcal.mol^{-1} and had self- and cross-dimer values below a ΔG of –6 kcal.mol^{-1}. The primers are present in Table 5.1.

2.2.3.2. RNA extraction

cDNA for primer validation and for the second qRT-PCR experiment was prepared from a rice tissue series by Miss Joanna Sundstrom at The University of Adelaide. In her experiment, the rice lines FL478, IR63731 and Nipponbare were germinated on plates, transferred to soil and watered with ACPFG nutrient solution (Table 5.8) until week three, when a 30mM NaCl stress was applied for the last 10 days of growth before harvest. Root material, the sheath and the last fully emerged leaf blade at the time of harvest were snap frozen in liquid nitrogen.

Total RNA was extracted using TRIzol. Since the plant material was already ground, the addition of TRIzol was not carried out in liquid nitrogen. Frozen plant material was placed in a 1.5mL microfuge tube with 500µL of TRIzol and mixed with a sterile plastic pestle. An additional 500µL of TRIzol was added and samples were stored at RT until all samples were ready. All samples were incubated at RT for five minutes, 200µL of chloroform was added and the tubes were vortexed for 15 seconds. All samples were incubated at RT for three minutes, and then centrifuged at 12,000*g* for 15 minutes at 4ºC. The top aqueous layer was transferred to a clean 1.5mL microfuge tube, 500µL of

isopropanol was added and the tube was inverted four times. The tubes were incubated at RT for ten minutes, and then centrifuged at 12,000*g* for ten minutes at 4ºC. The supernatant was removed and the pellet was washed with 70% ethanol. The tubes were centrifuged at 9,000*g* for five minutes at 4ºC. The ethanol was removed and the pellet allowed to air dry in a fume hood for 20 minutes. The pellet was resuspended in 30µL of DEPC treated water and all samples were stored on ice.

2.2.3.3. Removal of contaminating DNA

Contaminating DNA was removed from total RNA using a DNA-*free* kit, by adding 3µL 10X DNaseI buffer and 1µL DNaseI, to each sample. The tubes were inverted twice and incubated at 37ºC for 30 minutes, then 5µL DNase inactivation reagent was added and mixed. The tubes were incubated at RT for two minutes with intermittent mixing. The tubes were centrifuged at 10,000*g* for one minute. The supernatant was transferred to a clean 1.5mL microfuge tube and stored on ice.

RNA concentration and integrity were checked using a Nanodrop spectrophotometer and by separating the RNA on a 1% agarose 1X TAE gel containing 0.5µg/mL ethidium bromide at 80 volts for one hour and examining the separated RNA using a UV transilluminator.

2.2.3.4. cDNA synthesis

cDNA was synthesised using Superscript III RT. Reactions contained the following; RNA (2µg), 1µL 50µM oligo(dT) primer, 2µL 5mM dNTP mix and sterile MilliQ water to 13µL. The samples were incubated at 65ºC for five minutes, placed on ice and 7µL of master mix was added to each tube. The master mix contained; 4µL 5X first stand buffer, 1µL 0.1M DTT, 0.5µL RNaseOUT, 0.25µL Superscript III RT enzyme, 1.25µL sterile MilliQ water.

2.2.3.5. PCR check of primer specificity

PCR was performed using the primers in Table 5.1 with cDNA template from *O. sativa* cv. IR63731 plant roots grown in unstressed conditions. For each primer pair the following was performed. A PCR master mix was made containing; 2.5µL 10X PCR Buffer, 0.75µL $MgCl^2$, 1µL 10mM dNTPs, 1µL 10µM Primer 1, 1µL 10µM Primer 2, 1µL cDNA (1:10 dilution), 0.1µL Platinum Taq and 17.65µL sterile MilliQ water. The thermocycler was programmed as follows; 1, 94ºC for 2 minutes; 2, 94ºC for 30

seconds; 3, 55ºC for 30 seconds; 4, 72ºC for 30 seconds; 5, cycle to step 2 for 35 more times; 6, hold at 15ºC.

2.2.3.6. Agarose gel electrophoresis

PCR products were electrophoresed on a 1% agarose 1X TAE gel containing 0.5µg/mL ethidium bromide at 110 volts for 17 minutes. The DNA marker used was 1Kb plus. The DNA was visualised in the gel using a UV transilluminator and photographed.

2.2.3.7. Confirmation of specific product formation

PCR was performed using primer pairs Table 5.1 and appropriate cDNA. See Table 5.2 for primer:cDNA combinations. For each primer pair the following was performed. A PCR master mix was made containing; 15µL sterile MilliQ water, 15µL 4µM Primer 1, 15µL 4µM Primer 2, 50µL qRT-PCR mix. Aliquots of 19µL were transferred to 100µL PCR tubes and 1µL cDNA added. The PCR products were purified using HPLC (Burton *et al.*, 2004).

2.2.3.8. Sequencing PCR products

Capillary sequencing was performed by the Australian Genome Research Facility (AGRF) in Adelaide. Samples were amplified using ABI Prism BigDye Terminator Sequencing Reaction Kits (v3.1) as per the manufacturer's instructions. Reaction products were dried and sent to the AGRF for resuspension and capillary separation sequencing. The primers used were the same as those used for initial product amplification.

For each sequencing reaction the following was performed: the total volume of template, primer and MilliQ water equalled 8µL. For each reaction the following was used; 1µL of plasmid mini-prep DNA (300ng/µL), 0.5µL 10µM primer, 6.5µL MilliQ water, 3µL 5X buffer and 1µL Big Dye enzyme 2. The thermocycler was programmed as follows; 1, 94ºC for 5 minutes; 2, 96ºC for 10 seconds; 3, 50ºC for 5 seconds; 4, 60ºC for 4 minutes; 5, cycle to step 2 for 24 more times; 6, hold at 15ºC.

The samples were cleaned by adding 75µL of 0.2mM $MgSO_4$ in 70% ethanol to each RT equilibrated reaction tube. The tubes were shaken on a Vortex Mixer and incubated at RT for 25 minutes. The tubes were centrifuged at 4000*g* for 15 minutes, inverted over paper towels to remove the supernatant and dried in a vacuum concentrator for 10 minutes. The samples were wrapped in aluminium foil and stored at –20ºC before being sent to the AGRF in Adelaide for capillary separation sequencing.

2.2.3.9. qRT-PCR

The results of the microarray were tested using qRT-PCR, which was performed as described in Cotsaftis *et al.* (2010) using the primer combinations from Table 5.1. If there was a good correlation between the qRT-PCR and microarray data sets, the transcription factor was selected for further study.

A second qRT-PCR experiment was also conducted on *O. sativa* ssp. *japonica* cv. Nipponbare cDNA from the root, sheath and leaf blade of control and salt treated plants. The experiment was performed as in Cotsaftis *et al.* (2010) using the primer combinations from Table 5.1. Due to variations in control gene expression between the different tissues, the root data was normalised with α-tubulin, glyceraldehyde 3-phosphate dehydrogenase (GAP) and actin, whereas the sheath and leaf blade data was normalised with tubulin, GAP and elongation factor 1. Therefore the root data cannot be directly compared with the sheath and leaf blade data.

2.2.4. Bioinformatic analysis of candidate transcription factors

The predicted molecular weight, isoelectric point (pI), extinction coefficient, estimated half life, instability index, aliphatic index and grand average of hydropathicity were calculated using the ExPASy ProtParam Tool (Gasteiger *et al.*, 2005). BLAST searches were performed on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov), using the BLASTP (Altschul *et al.*, 1997; Dereeper *et al.*, 2008) and BLASTN algorithms (Zhang *et al.*, 2000).

The multiple sequence alignment using MAFFT was performed on the Computational Biology Research Center website using the E-INS-i option for sequences with long unalignable regions (Katoh *et al.*, 2005). Coiled-coil prediction was performed on the Coils Server using a MTIDK matrix (Lupas *et al.*, 1991). ClustalW analysis was performed on the European Bioinformatics Institute website using the ClustalW algorithm (Thompson *et al.*, 1994). Phosphorylation site prediction was performed using NetPhos 2.0 (Blom *et al.*, 1999). Subcellular location and signal peptide cleavage site prediction was performed using TargetP 1.1 Server (Dyrløv Bendtsen *et al.*, 2004; Emanuelsson *et al.*, 2000).

2.3. Results

2.3.1. Comparison of microarray and qRT-PCR data

Global gene transcript levels were examined under salt stress in the salt tolerant *O. sativa* ssp. *indica* rice genotypes FL478, Pokkali and IR63731, and salt sensitive IR29. Probesets were selected representing 12 different transcription factors with differential expression between the salt sensitive and salt tolerant genotypes. The microarray results for the 12 transcription factors were tested using qRT-PCR. There was a good correlation between the microarray data and qRT-PCR data for six of the 12 transcription factors. These can be seen in Figure 2.1, Figure 2.2, Figure 2.3, Figure 2.4, Figure 2.5 and Figure 2.6. The remaining transcription factors were not investigated further, as the qRT-PCR data did not confirm the microarray data (data not shown). Each figure is divided into two panels. The left panel has the microarray data and qRT-PCR data for the ssp. *indica* cultivars. The right panel has the qRT-PCR data for the ssp. *japonica* cv. Nipponbare.

In the microarray data for Os.3397.1.S1_at (Figure 2.1), the signal intensity did not change for IR29 in response to salt stress, whereas there was a reduction in signal intensity in all salt tolerant genotypes. There was also a higher basal level of signal intensity for the salt tolerant genotypes FL478 and Pokkali. The qRT-PCR data correlated with the microarray data, with some exceptions. In the qRT-PCR data, for the shoot of IR29, there was a reduction in transcript abundance in response to salt stress. For the shoot of IR63731, there was an increase in transcript abundance and for the shoot of FL478, there was no change in transcript abundance in response to salt stress. In the qRT-PCR data for Nipponbare, transcript levels were very low and there was little change in transcript number in response to salt treatment.

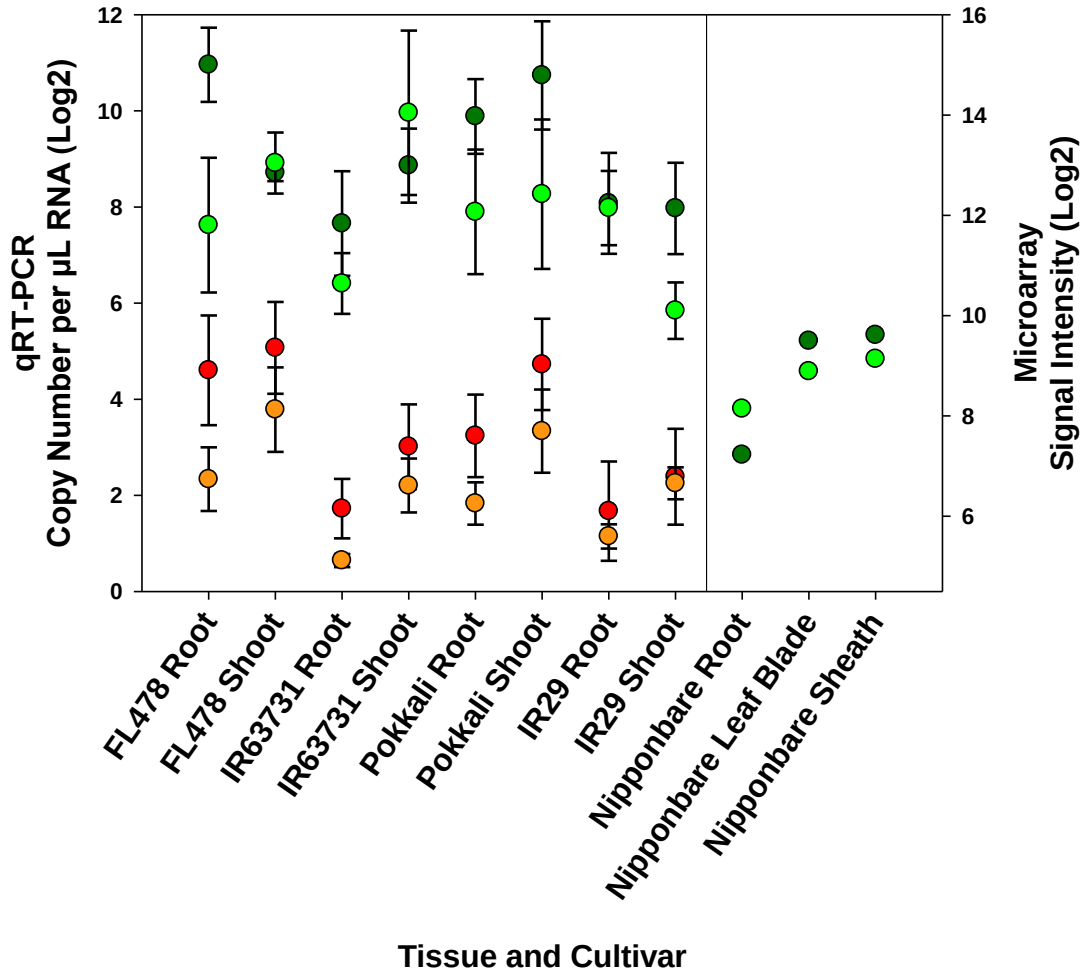


Figure 2.1 Graph comparing microarray and qRT-PCR data for Os.3397.1.S1_at

Data is present from three separate experiments for the transcription factor represented by the probeset Os.3397.1.S1_at. In the left panel, there is microarray signal intensity data from control (●) and salt (●) stressed plants and qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *indica* cv.s FL478, IR63731, Pokkali and IR29. There were three biological replicates for each cultivar and treatment, except IR29 control roots for which there were two replicates. In the right panel there is qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *japonica cv.* Nipponbare, for which there was only one biological replicate for each tissue and treatment. Error bars represent standard error of the mean (SEM).

In the microarray data for Os.11094.1.A1_at (Figure 2.2), there was an increase in signal intensity in IR29 in response to salt stress and 4-fold higher basal signal intensity in the root. There was a similar pattern in IR63731, with slightly higher basal levels of signal intensity and slightly greater induction in response to salt stress. The two other salt tolerant cultivars, FL478 and Pokkali, have no change in signal intensity in the root and a decrease in signal intensity in the shoot. The qRT-PCR data correlate with the microarray data. In the qRT-PCR data for Nipponbare, there was a 2-fold increase in transcript number in the root and sheath, and no change in the leaf blade.

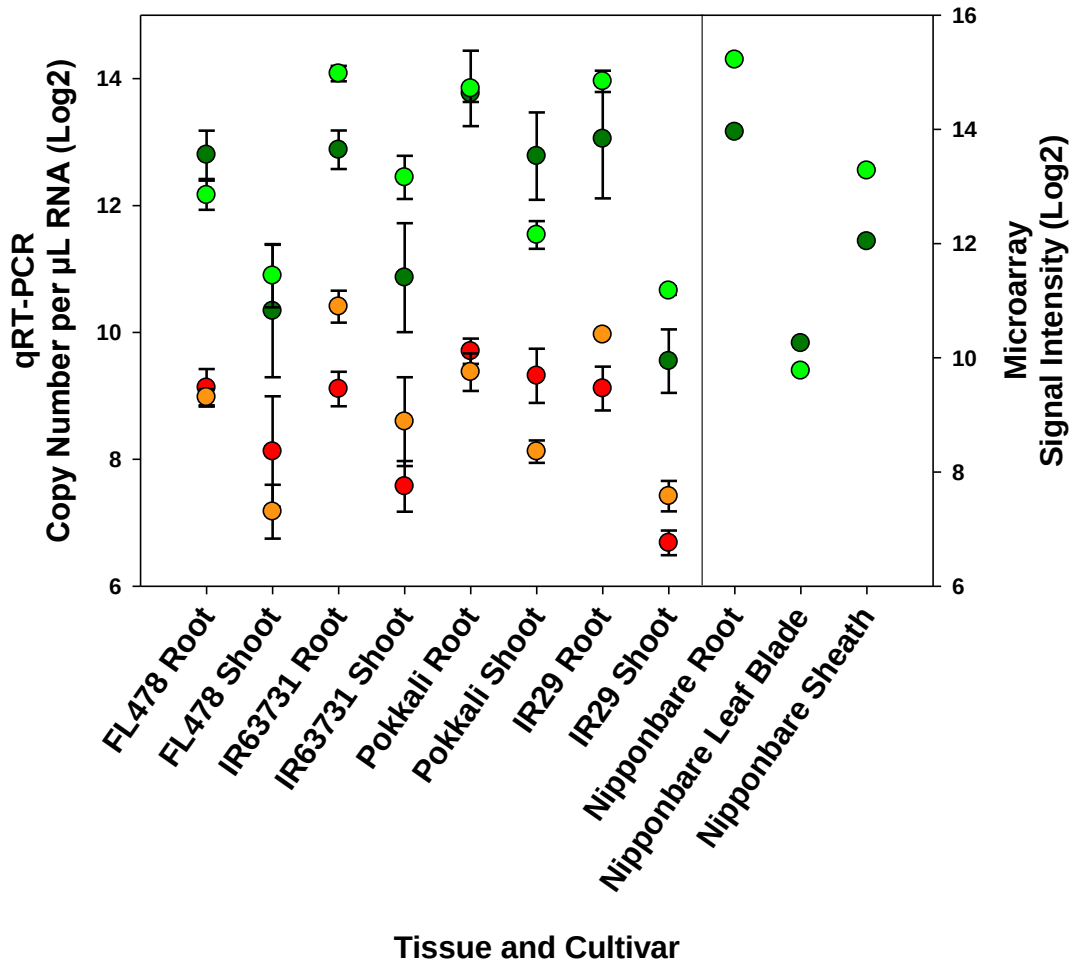


Figure 2.2 Graph comparing microarray and qRT-PCR data for Os.11094.1.A1_at

Data is present from three separate experiments for the transcription factor represented by the probeset Os.11094.1.A1_at. In the left panel, there is microarray signal intensity data from control (●) and salt (●) stressed plants and qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *indica* cv.s FL478, IR63731, Pokkali and IR29. In the right panel there is qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *japonica cv.* Nipponbare. See the legend of Figure 2.1 for replicates. Error bars represent SEM.

In the microarray data for Os.18955.1.S1_at (Figure 2.3), there was a decrease in signal intensity in all genotypes under salt stress, with slightly lower basal levels in IR29 and a smaller decrease in signal intensity under salt stress. The qRT-PCR data correlate with the microarray data. In the qRT-PCR data for Nipponbare, there was 8-fold greater transcript abundance in the leaf blade relative to the sheath, but no change in transcript abundance in response to salt in any tissue.

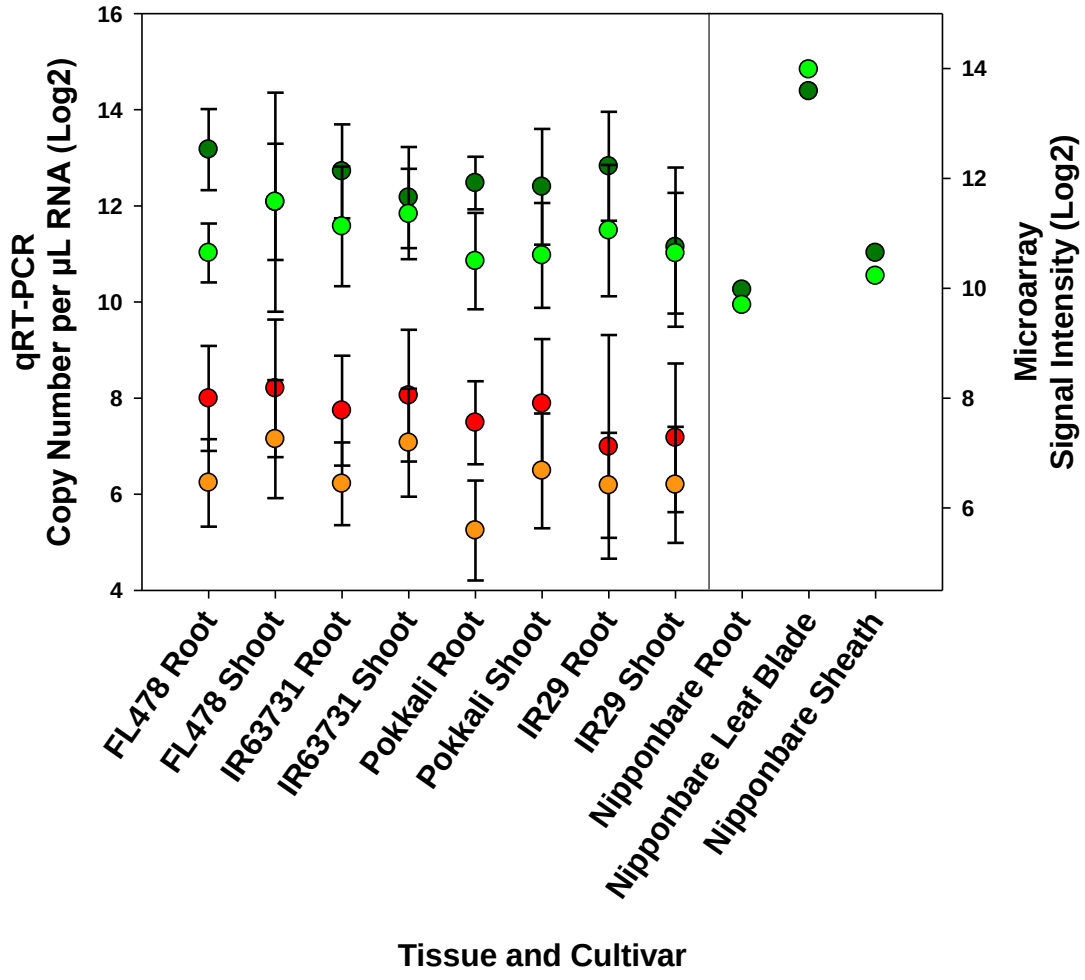


Figure 2.3 Graph comparing microarray and qRT-PCR data for Os.18955.1.S1_at

Data is present from three separate experiments for the transcription factor represented by the probeset Os.18955.1.S1_at. In the left panel, there is microarray signal intensity data from control (●) and salt (●) stressed plants and qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *indica* cv.s FL478, IR63731, Pokkali and IR29. In the right panel there is qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *japonica cv.* Nipponbare. See the legend of Figure 2.1 for replicates. Error bars represent SEM. The FL478 Shoot control data point is hidden by the salt data point.

In the microarray data for Os.46956.1.S1_at (Figure 2.4), there was an increase in signal intensity in IR29 in response to salt stress, with higher basal intensity in the shoot. In IR63731, there was a greater increase in signal intensity in response to salt stress. In Pokkali, basal signal intensity was approximately 4-fold greater in both shoot and root, relative to IR29. In Pokkali, in response to salt stress, there was a decrease in signal intensity to levels comparable to IR29 under salt stress. The qRT-PCR data correlate with the microarray data, with a strong increase in signal intensity in the shoot of IR63731 under salt stress. In the qRT-PCR data for Nipponbare, there was 12-fold higher transcript abundance in the sheath relative to the leaf blade, but no change in transcript abundance in response to salt. There was a 2-fold increase in transcript abundance in the root in response to salt stress.

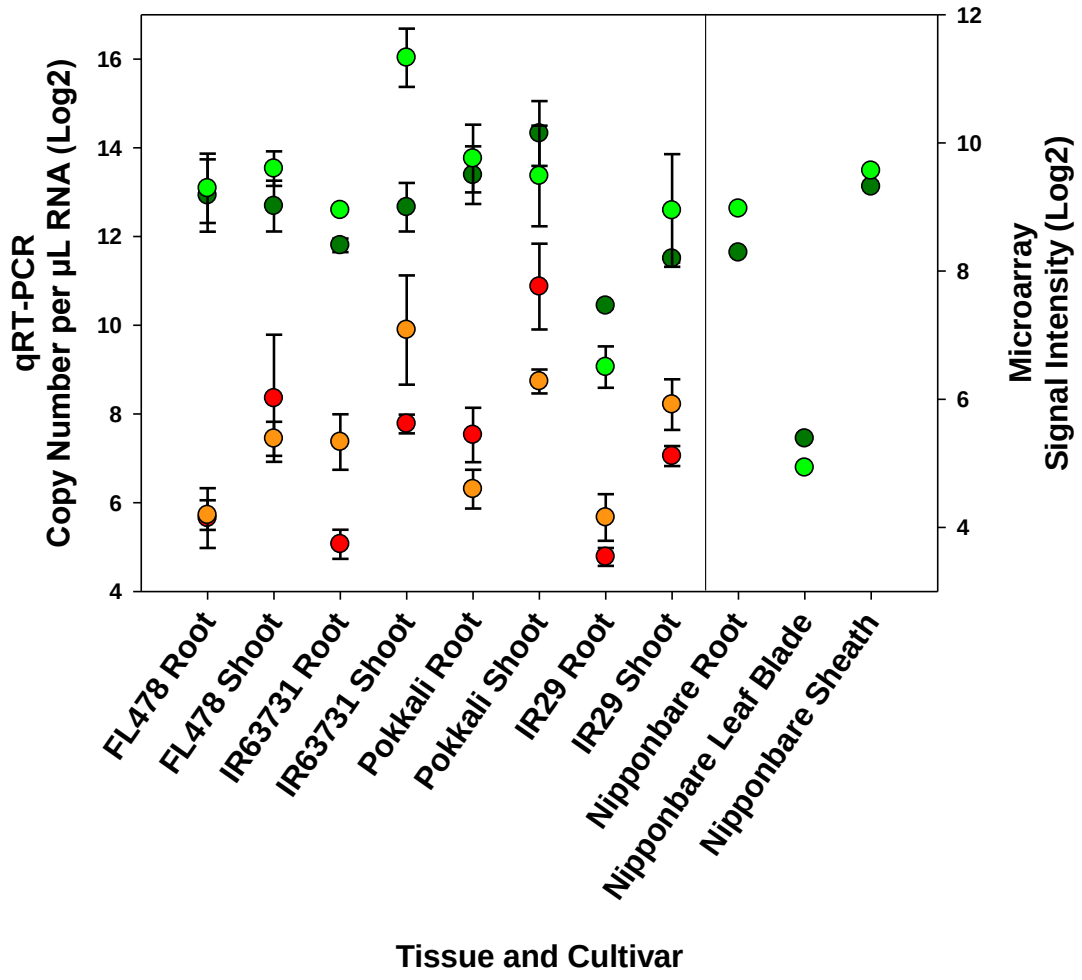


Figure 2.4 Graph comparing microarray and qRT-PCR data Os.46956.1.S1_at

Data is present from three separate experiments for the transcription factor represented by the probeset Os.46956.1.S1_at. In the left panel, there is microarray signal intensity data from control (●) and salt (●) stressed plants and qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *indica* cv.s FL478, IR63731, Pokkali and IR29. In the right panel there is qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *japonica cv.* Nipponbare. See the legend of Figure 2.1 for replicates. Error bars represent SEM.

In the microarray data for Os.49746.1.S1_at (Figure 2.5), there was an increase in signal intensity in all genotypes in response to salt stress, but less in salt sensitive IR29. The qRT-PCR data correlate with the microarray data, with a greater increase in signal intensity in the shoot of Pokkali and IR63731, relative to IR29. In the qRT-PCR data for Nipponbare, there were high transcript levels in the leaf blade and sheath, but no change in transcript abundance in response to salt stress.

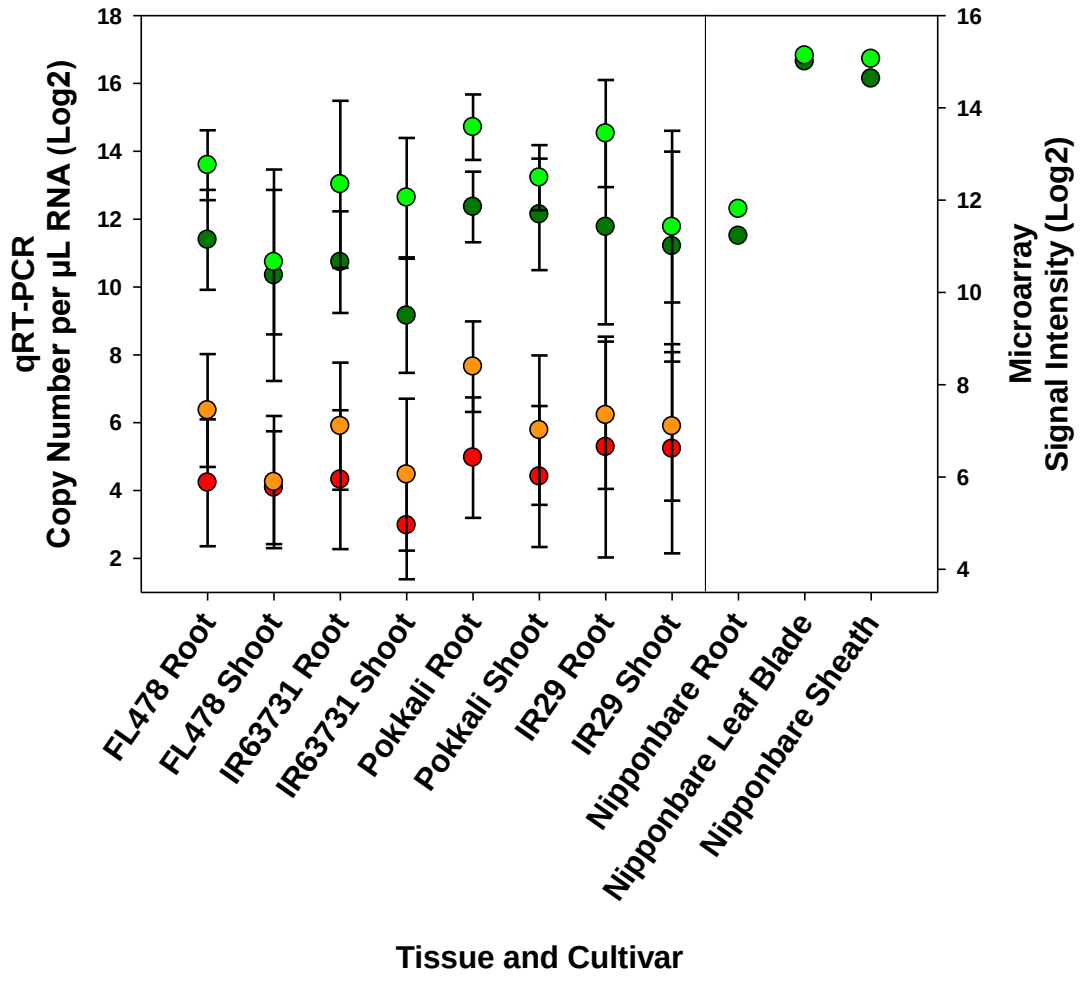


Figure 2.5 Graph comparing microarray and qRT-PCR data for Os.49746.1.S1_at

Data is present from three separate experiments for the transcription factor represented by the probeset Os.49746.1.S1_at. In the left panel, there is microarray signal intensity data from control (●) and salt (●) stressed plants and qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *indica* cv.s FL478, IR63731, Pokkali and IR29. In the right panel there is qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *japonica cv.* Nipponbare. See the legend of Figure 2.1 for replicates. Error bars represent SEM.

In the microarray data for Os.56210.1.S1_at (Figure 2.6), there was an increase in signal intensity in all genotypes in response to salt stress, with a greater increase in signal intensity in the shoot of IR63731, relative to IR29. The qRT-PCR data correlate with the microarray data. In the qRT-PCR data for Nipponbare, was a 2-fold increase in transcript abundance in the root and a smaller increase in the sheath. There was no change in the leaf blade.

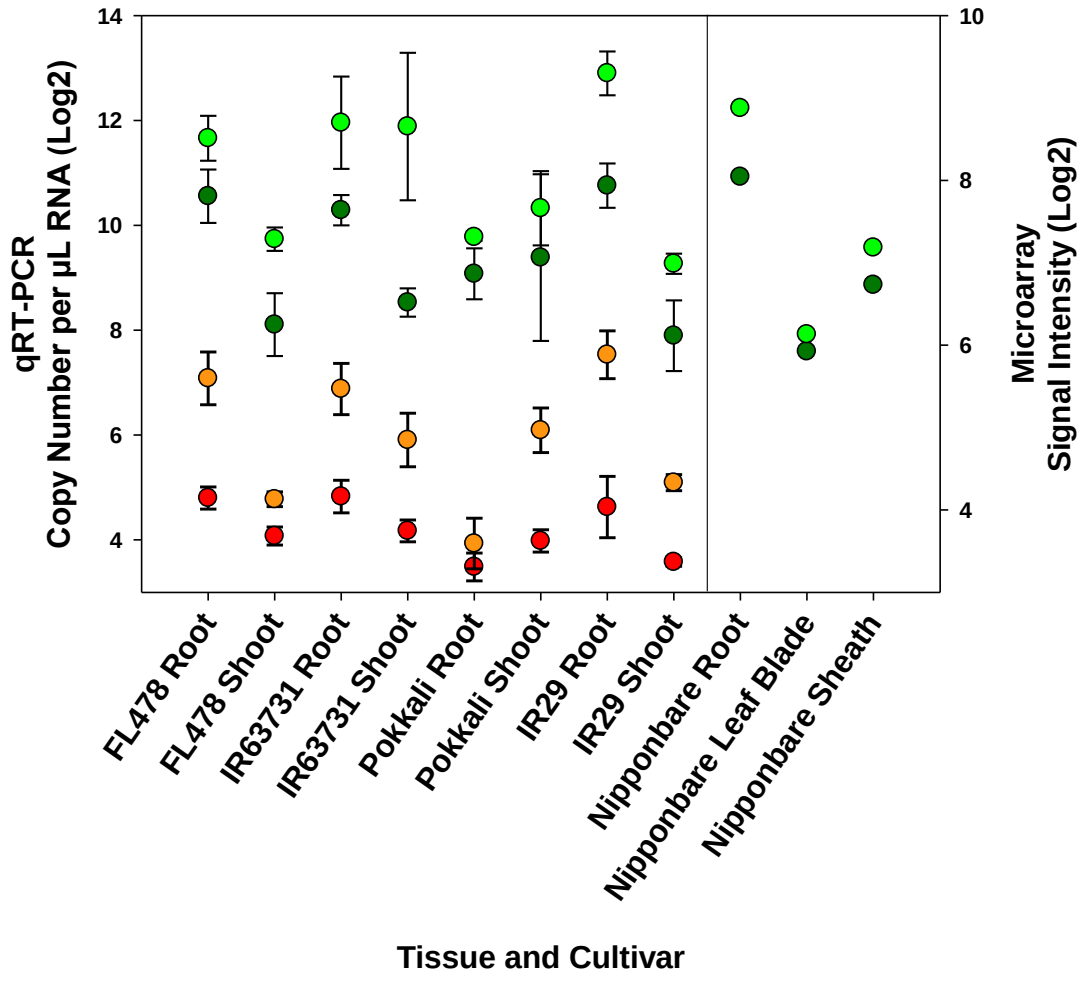


Figure 2.6 Graph comparing microarray and qRT-PCR data for Os.56210.1.S1_at

Data is present from three separate experiments for the transcription factor represented by the probeset Os.56210.1.S1_at. In the left panel, there is microarray signal intensity data from control (●) and salt (●) stressed plants and qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *indica* cv.s FL478, IR63731, Pokkali and IR29. In the right panel there is qRT-PCR data from control (●) and salt stressed (●) plants for the *O. sativa* ssp. *japonica cv.* Nipponbare. See the legend of Figure 2.1 for replicates. Error bars represent SEM.

2.3.2. Transcription factors selected for further study

The transcription factors with expression patterns that were consistent across the microarray and qRT-PCR platforms were selected for further study. These are outlined below in the format of; Affymetrix probeset number, locus number, gene name (Yilmaz *et al.*, 2009), alternative gene name(s).

Os.3397.1.S1_at, LOC_Os02g39360.1, *OsOrphan19* (Yilmaz *et al.*, 2009).

Os.11094.1.A1_at, LOC_Os04g46220.1, *OsEREB67* (Yilmaz *et al.*, 2009), *OsERF#093* (Nakano *et al.*, 2006), *OsERF1* (Hu *et al.*, 2008b).

Os.18955.1.S1_at, LOC_Os06g51260.1, *OsMYBR63* (Yilmaz *et al.*, 2009).

Os.46956.1.S1_at, LOC_Os01g50940.1, *OsbHLH17* (Yilmaz *et al.*, 2009), *OsbHLH010* (Li *et al.*, 2006).

Os.49746.1.S1_at, LOC_Os01g74020.1, *OsLUX*

Os.56210.1.S1_at, LOC_Os04g42950.1, *OsMYB54* (Yilmaz *et al.*, 2009).

The genes will be hereafter referred to according to the systematic naming convention proposed by Yilmaz *et al.* (2009). The gene for locus LOC_Os01g74020.1 has not yet been named by Yilmaz *et al.* (2009), however, the gene is called *OsPCL1* in accession (AB206578) in the NCBI database in an entry from 8/3/2005 entitled “Cloning of the circadian clock gene in Rice” by Onai, K. and Ishiura, M. The protein is called “Induced protein Mgl1” in another accession (AY581256) entitled “Characterisation of two downstream proteins in a possible two-component system in the early responses of rice to infection by a fungal pathogen”, by Qu, L. and Gu, H. Neither accession has an associated publication. The *A. thaliana* ortholog is named *LUX*, so the rice gene will be called *OsLUX*.

2.3.2.1. Further characterisation of selected transcription factors

2.3.2.1.1. OsOrphan19

OsOrphan19 is located on chromosome two and is composed of two exons of 684bp and 556bp, respectively, separated by a single 104bp intron. The 816bp CDS is predicted to encode a protein having 271AAs, a molecular weight of 29148.4kDa and a pI of 4.93. *OsOrphan19* encodes a B-box zinc finger family transcription factor containing two zinc finger B-box-type domains, from 1-47AA and 52-99AA (Figure 2.7).

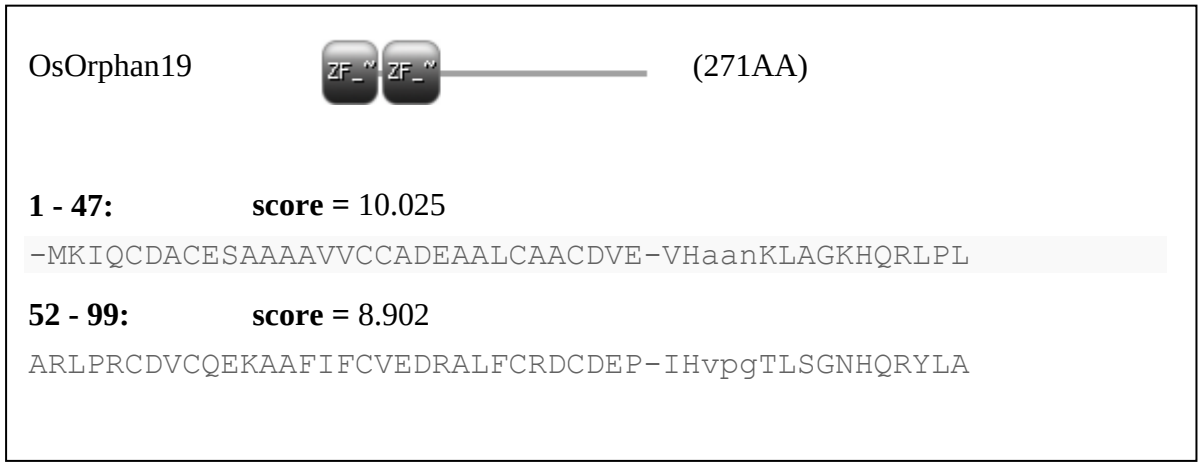


Figure 2.7 Schematic diagram representing the zinc finger B-box-type domains in OsOrphan19

The OsOrphan19 peptide with two zinc finger B-box type DNA-binding domains. Generated by querying the PROSITE database (Hulo *et al.*, 2006).

OsOrphan19 was predicted to be targeted to a secretory pathway by the subcellular location prediction program TargetP, with a 27AA cleaved presequence (Table 5.3). This was not confirmed by the plastid targeting prediction program Predotar. A search of the REDB EST database gave 173 matches but did not show any tissue specific expression patterns. The UniGene entry had 78 matches and confirmed that there were no tissue specific expression patterns.

Using the OsOrphan19 full length peptide as a query, a BLASTP search of the NCBI refseq_protein database showed that the closest homologs include hypothetical B-box zinc finger proteins from *Sorghum bicolour* SORBIDRAFT_04g025400 with 77% identity and 100% coverage (6e-108) and *Zea mays* salt tolerance-like protein (LOC100284784) with 77% identity and 100% coverage (2e-106) as sequence 15636 in US patent 7569389, "Nucleotide sequences and polypeptides encoded thereby useful for

modifying plant characteristics" and *Z. mays* salt tolerance-like protein (LOC100273793) with 74% identity and 100% coverage (5e-95) as sequence 60027 from the same patent. Published homologs include *A. thaliana* SALT TOLERANCE HOMOLOG (STH), with 49% identity (2e-55) over the full length peptide (Holm *et al.*, 2001) and present as sequence 12479 from patent US 7569389 and *A. thaliana* SALT TOLERANCE (STO) protein NP_172094 with 48% identity (4e-55) over the full length peptide (Indorf *et al.*, 2007; Lippuner *et al.*, 1996) and present in US patent 7569389 as sequence 13525.

A CLUSTALW alignment of the AA sequences of OsOrphan19, the closest three homologs and the two published homologs, AtSTH and AtSTO, showed that there was high sequence identity in the two N-terminal zinc-finger domains and at the end C-terminus (Figure 2.8).

```
OsOrphan19                      MKIQCDACESAAAAVVCCADEAALCAACDVEVHAANKLAGKHQRLPLEAL
S.bicolor_SORBIDRAFT_04g025400  MKIQCDACEGAAATVVCCADEAALCARCDVEIHAANKLASKHQRLPLEAL
Z.mays_LOC100284784             MKIQCDACEGAAATVVCCADEAALCARCDVEIHAANKLASKHQRLPLEAL
Z.mays_LOC100273793             MKIQCDACEGAAATVVCCADEAALCARCDVEIHAANKLASKHQRLPLEAL
AtSTH                           MKIQCDVCEKAPATLICCADEAALCAKCDVEVHAANKLASKHQRLFLDSL
AtSTO                           MKIQCDVCEKAPATVICCADEAALCPQCDIEIHAANKLASKHQRLHLNSL
                                ******.** *.*:::********** **:*:*******.***** *::*

OsOrphan19                      SARLPRCDVCQEKAAFIFCVEDRALFCRDCDEPIHVPGTLSGNHQRYLAT
S.bicolor_SORBIDRAFT_04g025400  SARLPRCDVCQEKAAFIFCVEDRALFCRDCDEPIHVPGTLSGNHQRYLAT
Z.mays_LOC100284784             SAKLPRCDVCQEKAAFIFCVEDRALFCQDCDEPIHVPGTLSGNHQRYLAT
Z.mays_LOC100273793             SASLPRCDVCQEKAAFIFCVEDRALFCRDCDEPIHVPGTLSGNHQRYLAT
AtSTH                           STKFPPCDICLEKAAFIFCVEDRALLCRDCDEATHAPNTRSANHQRFLAT
AtSTO                           STKFPRCDICQEKAAFIFCVEDRALLCRDCDESIHVANSRSANHQRFLAT
                                *: :* **:* **************:*:****. *...: *.****:***

OsOrphan19                      GIRVGFASASPCD--GGSDAHDSDHHAPPMGSSEHHHHHQQPAPTVAVDT
S.bicolor_SORBIDRAFT_04g025400  GIRVGFASASACSSDGACDAHDSDHHAPPKATVETPQAQAAVSAAAAAQQ
Z.mays_LOC100284784             GIRVGLASASACS--DACDAHDSDHHAPPKATIEP----PHAAVSAAVQQ
Z.mays_LOC100273793             DIRVGFASASSACSDACDAHDDSDHHAPPKAAVSS-----------AAQQ
AtSTH                           GIRVALSSTSCNQEVEKNHFDPSNQQ-------------------SLSKP
AtSTO                           GIKVALTSTICSKEIEKNQPEPSNNQQKANQIPAK----------STSQQ
                                .*:*.::*:           . *:::                     .

OsOrphan19                      PS--PQFLPQGWAVDELLQFSDYETGDKLQKESSPPLGFQELEWFADIDL
S.bicolor_SORBIDRAFT_04g025400  VPSPPQFLPQGWAVDDLLQFSDYESSDKLHKES--PLGFKELEWFADIDL
Z.mays_LOC100284784             VPSPPQFLPQGWAVDELLQFSDYESSDKLHKEP--TLGFKELEWFADIDL
Z.mays_LOC100273793             VPSPPQFLPQGWAVDELLQFSDCESSDKLHKES--PLGFKELEWFTDIDL
AtSTH                           PTQQPAAPSPLWATDEFFSYSDLDCSNKE-KEQ---LDLGELDWLAEMGL
AtSTO                           QQQPSSATPLPWAVDDFFHFSDIESTDKKGQLD---LGAGELDWFSDMGF
                                    .   .  **.*::: :** :  :*  :     *.  **:*::::.:

OsOrphan19                      FHNQAPKGGAAAGRTTAEVPELFASQAANDVAYYRPPTRTAAAAFTAATG
S.bicolor_SORBIDRAFT_04g025400  FHEQQAP---KAGRTLAEVPELFGSQAANDAAYYRP----AKAAAG--AG
Z.mays_LOC100284784             FHEQAP----KASRTLAEVPELFGYQAANDAAYYRP----AKAAGGGGAG
Z.mays_LOC100273793             FHEQTP----KAGRRLAEVPELSGTQAANDAAYYRP----AKATATAGAG
AtSTH                           FGDQPDQ----EALPVAEVPELSFSHLAHAHSYNRP---------MKSNV
AtSTO                           FGDQIND----KALPAAEVPELSVSHLGHVHSY-KP---------MKSNV
                                * :*        .   ******   : .:  :* :*

OsOrphan19                      FRQSKKARVELPDDEEDYLIVPDLG
S.bicolor_SORBIDRAFT_04g025400  VRQSKKARIEVTDDE-DYLIVPDLG
Z.mays_LOC100284784             VRQSKKARIEVTDDE-DYLIVPDLG
Z.mays_LOC100273793             VRQSKKARTEVTDDE-DHLIVPDLG
AtSTH                           PNKKQRLEYRYDDE-EEHFLVPDLG
AtSTO                           SHKKPRFETRYDDDDEEHFIVPDLG
                                 .:. : . .  *:  ::::*****
```

Figure 2.8 Comparison of multiple amino acid sequence of OsOrphan19 and its homologs

The AA sequence of OsOrphan19 was compared with its closest homologs. There is sequence identity in the zinc finger domains (□) and at the C-terminus (□ □).

2.3.2.1.2. OsEREB67

OsEREB67 is located on chromosome four and is composed of a single exon 2,768bp in length, containing a CDS of 957bp and is predicted to encode a protein having 318AAs, a molecular weight of 33140.7kDa and a pI of 5.01. *OsEREB67* encodes a transcription factor from the AP2/ERF transcription factor superfamily containing a single AP2 domain from 152-210AA (Figure 2.9). A search of the REDB EST database gave 169 matches but did not show any tissue specific expression patterns. The UniGene entry had 175 matches and also indicated that there were no tissue specific expression patterns. In the Rice Annotation Project Database, it is annotated as a pathogenesis-related transcription factor, which may bind the GCC-box pathogenesis-related promoter element.

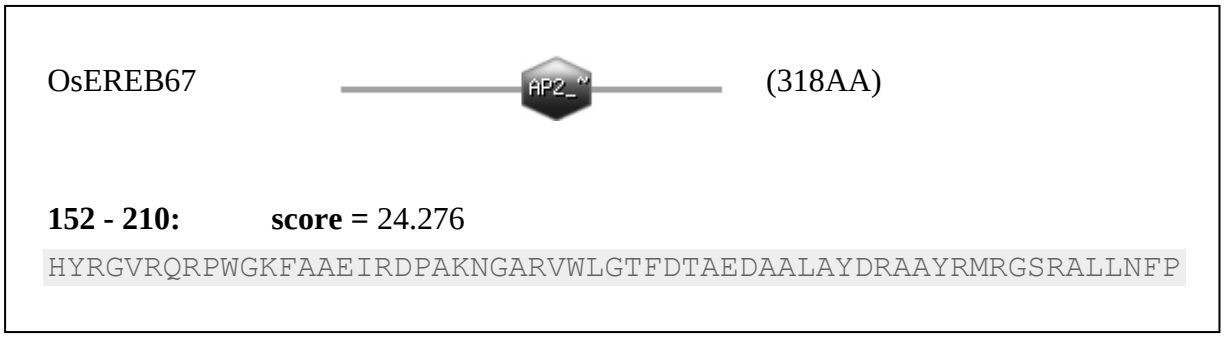


Figure 2.9 Schematic diagram representing the AP2 domain in OsEREB67

A diagram of the OsEREB67 peptide and AP2 domain, generated by querying the PROSITE database (Hulo *et al.*, 2006).

OsEREB67, designated as OsERF#093, was placed in group IXa, characterised by the motif CMIX-3, however OsEREB67 also contains the motif CMIX-2 (Nakano *et al.*, 2006). CMIX-2 and CMIX-3 are putative acidic domains that might function as transcriptional activation domains (Fujimoto *et al.*, 2000; Nakano *et al.*, 2006).

Characterised members of the IXa group include, *Pti4*, which binds the PR-box in defence genes, regulating their expression in tomato (*Lycopersicon esculentum*) (Gu *et al.*, 2002; Zhou *et al.*, 1997), *OPBP1*, involved in abiotic and biotic stress responses in tobacco (*Nicotiana tabacum*) (Guo *et al.*, 2004) and *ORCA3*, involved in the regulation of terpenoid indole alkaloid synthesis in Madagascar Periwinkle (*Catharanthus roseus*) (van der Fits & Memelink, 2000). The *A. thaliana* homologs in group IXa are *AtERF1* and *AtERF2* (Nakano *et al.*, 2006). The AP2/ERF domain in these proteins recognises a

GCC-box in the promoter of genes involved in pathogen defence via a three-stranded beta-sheet and almost parallel alpha-helix (Allen *et al.*, 1998; Brown *et al.*, 2003; Lorenzo *et al.*, 2004).

Using the OsEREB67 full length peptide as a query, a BLASTP search of the refseq_protein database showed that the closest homologs include *S. bicolor* SORBIDRAFT_06g024355 with 69% identity and 98% coverage (4e-89) and *Z. mays* LOC100274398 with 64% identity and 98% coverage (1e-88). The closest published homologs include *A. thaliana* ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 2 (ERF2) with 48% identity and 63% query coverage (4e-39) and *A. thaliana* ERF1A (At4g17500) with 45% identity and 62% query coverage (1e-37). Both of which are involved in pathogen defence signalling (Brown *et al.*, 2003; Oñate-Sánchez & Singh, 2002).

2.3.2.1.3. OsMYBR63

OsMYBR63 is located on chromosome six and has two alternative splice forms. The first (Os06g51260.1) is composed of six exons, with a transcript of 2,113bp in length, containing a CDS of 1356bp and is predicted to encode a protein having 451AAs, a molecular weight of 49486.6kDa and a pI of 5.20. The second (Os06g51260.2) is composed of three exons, with a transcript of 1748bp, containing a CDS of 1047bp and is predicted to encode a protein having 348AA, a molecular weight of 37627.1kDa and a pI of 4.96. *OsMYBR63* encodes a homeodomain-like containing protein, with a myb-type helix-turn-helix DNA-binding domain. EST data showed that the most matches were from leaf tissue, followed by callus and flower, but there were none from root tissue. A fragment of the *OsMYBR63* mRNA sequence that codes for the first eight amino acids of the second splice form, Os06g51260.2, was used as a query in a BLASTN search of the GenBank est_others database. The query mRNA sequence is not present in the first splice form. There were only two hits, both from *japonica* rice.

Queries against the PROSITE database indicated that the Myb-type Helix-Turn-Helix DNA-binding domain is only present in the first splice form and that the second splice form contains no known protein domains (Figure 2.10). A ClustalW alignment of the two splice forms showed that the second splice form is truncated just prior to the Myb-type HTH motif (Figure 2.11).

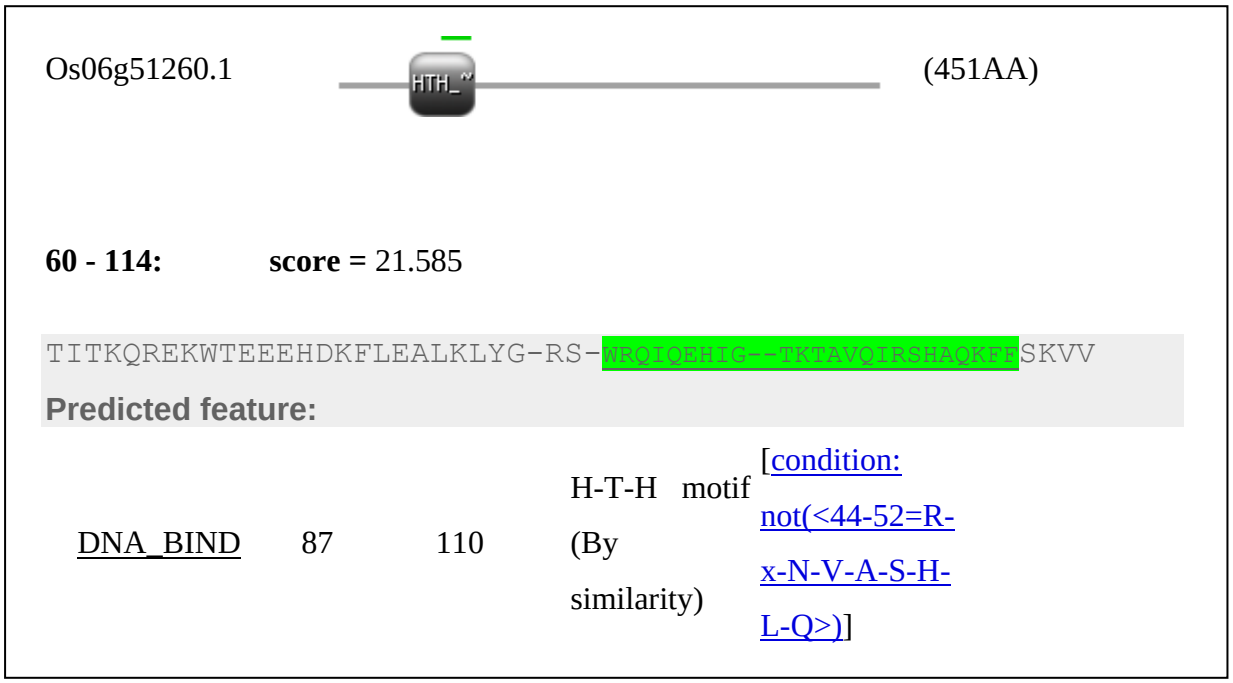


Figure 2.10 Schematic diagram representing the Myb-type HTH domain in OsMYBR63

A diagram of the OsMYBR63 peptide with HTH motif and DNA-binding domain (□). Generated by querying the PROSITE database (Hulo *et al.*, 2006).

```
Os06g51260.1      MASMPQLEEKDSSDLAINKGPSLDLVKSPLMMNDASATVTAMQPNEGMEEFPVKVRKPYT 60
Os06g51260.2      ------------------------------------------------------------

Os06g51260.1      ITKQREKWTEEEHDKFLEALKLYGRSWRQIQEHIGTKTAVQIRSHAQKFFSKVVREPGSN 120
Os06g51260.2      -----------------------------------MVALGVR-------YKVVREPGSN 17
                                                    .*: :*        *********

Os06g51260.1      NAIEIPPPRPKRKPLHPYPRKCANSGSDANPATAQLKLAPGSSSSGSDQENGSPISVLSA 180
Os06g51260.2      NAIEIPPPRPKRKPLHPYPRKCANSGSDANPATAQLKLAPGSSSSGSDQENGSPISVLSA 77
                  ************************************************************

Os06g51260.1      MQSDAFGSSVSNPSTRCTSPASSDDGNNIPTFTSGEDNNVPCEPTVIDPSQSHKEIDQDR 240
Os06g51260.2      MQSDAFGSSVSNPSTRCTSPASSDDGNNIPTFTSGEDNNVPCEPTVIDPSQSHKEIDQDR 137
                  ************************************************************

Os06g51260.1      KDVNNMSEEDSSEEEVQETSLKLFGRTVVIPDPRKRSSSDPKHESEEQISQPSNEEMLQA 300
Os06g51260.2      KDVNNMSEEDSSEEEVQETSLKLFGRTVVIPDPRKRSSSDPKHESEEQISQPSNEEMLQA 197
                  ************************************************************

Os06g51260.1      SSSVGEIPAAYCAPNGWFMSYNSFPFQFGESAADARIPPLHVWWPYYGFAPISHPRGLST 360
Os06g51260.2      SSSVGEIPAAYCAPNGWFMSYNSFPFQFGESAADARIPPLHVWWPYYGFAPISHPRGLST 257
                  ************************************************************

Os06g51260.1      VMQQTEGSDESDGVKSHSSESSSDSGENVQMTAPQSSRIVESLGAIYVRDSGSSFELKPS 420
Os06g51260.2      VMQQTEGSDESDGVKSHSSESSSDSGENVQMTAPQSSRIVESLGAIYVRDSGSSFELKPS 317
                  ************************************************************

Os06g51260.1      ANSAFVRVKPSNSGDEEVIRGFVPYKRCKFQ 451
Os06g51260.2      ANSAFVRVKPSNSGDEEVIRGFVPYKRCKFQ 348
                  ********************************
```

Figure 2.11 Pairwise sequence alignment of splice forms of OsMYBR63

A ClustalW alignment of the two splice forms shows that the critical Myb-type HTH DNA-binding domain ( ) is absent from the second splice form.

Querying the Coils Server with the full length peptide, OsMYBR63 was predicted to contain a coiled-coil domain from I61 to Y83, using a 14AA window and a coiled-coil domain from Q231 to F264, using either a 14 or 21AA window (Figure 2.12) (Lupas *et al.*, 1991). The coiled-coil domains may function in dimerisation (Lupas, 1996).

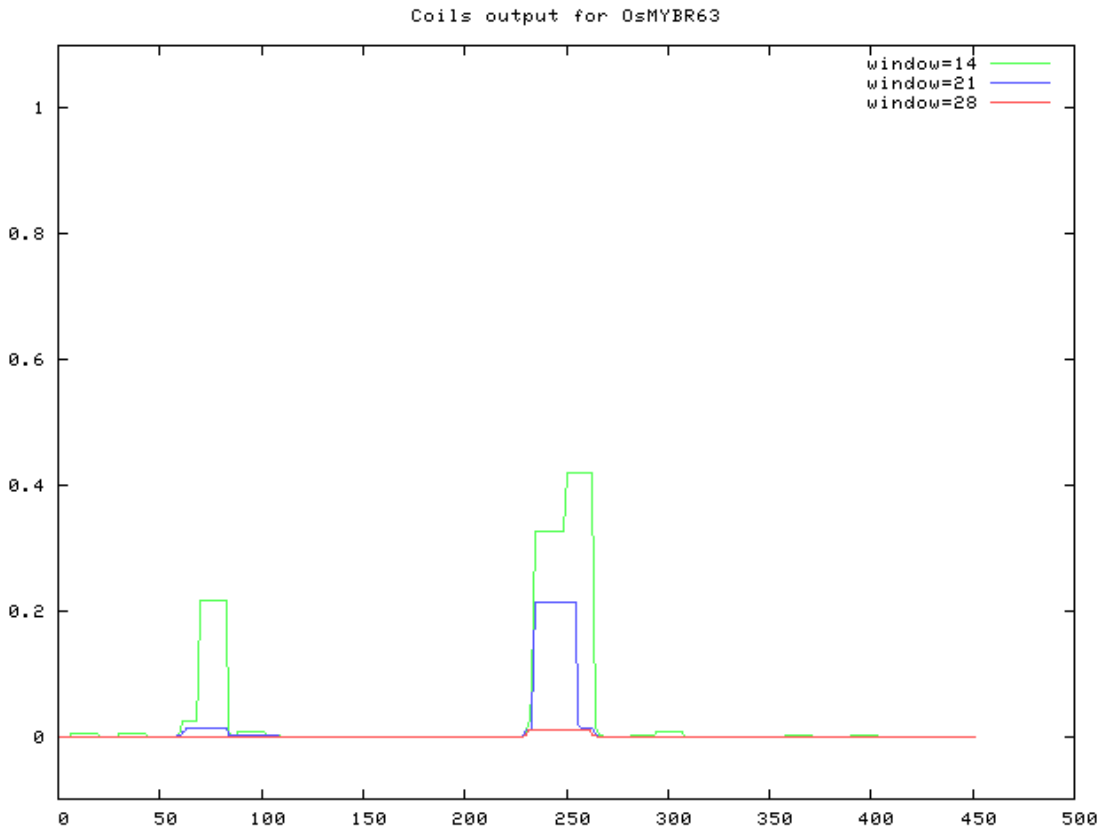


Figure 2.12 Coiled-coil domain prediction in OsMYBR63

Using the OsMYBR63 full length peptide as a query, a BLASTP search of the refseq_protein database showed that the closest homologs include *Z. mays* myb-like protein E1 (LOC100280442) with 61% identity and 100% coverage (3e-127) and a hypothetical myb-like protein from *V. vinifera* (LOC100263252) with 37% identity and 99% coverage (2e-51). The closest published homologs include *A. thaliana* REVEILLE 2 (RVE2)/Circadian 1 (CIR1) with 45% identity and 56% coverage (5e-39) involved in regulation of the circadian clock (Zhang *et al.*, 2007a), *Z. mays* LATE ELONGATED HYPOCOTYL (LHY) with 67% identity and 22% coverage (2e-35), present in US patent 7569389 as sequence 59261 and *A .thaliana* RVE7/EARLY PHYTOCHROME RESPONSIVE 1 (EPR1) with 48% identity and 50% coverage (1e-34), a component of a slave circadian oscillator (Kuno *et al.*, 2003). Querying the same database with the 348AA peptide gave the same hypothetical myb-like homologs, however *A. thaliana* RVE2 was the only published homolog.

2.3.2.1.4. OsbHLH17

OsbHLH17 is located on chromosome 1 and is composed of a single exon of 2,113bp in length, containing a 1,422bp CDS and is predicted to encode a protein having 473AA, a molecular weight of 50668.3kDa and a pI of 8.36. *OsbHLH17* encodes a transcription factor with a single myc-type helix-loop-helix (HLH) domain (Figure 2.13). EST data showed that the most matches were from stem tissue, followed by mixed tissue, whole plant and callus, but there were not matches from root tissue.

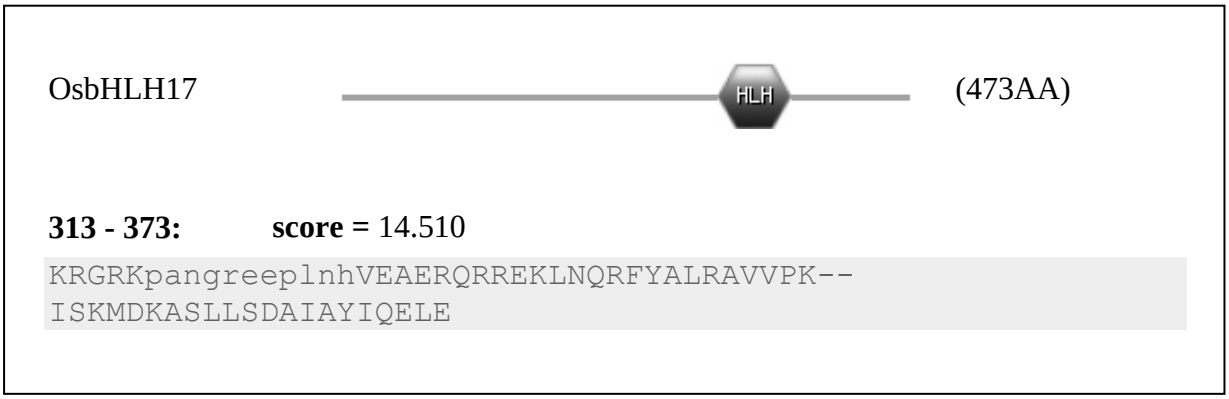


Figure 2.13 Schematic diagram representing the myc-type HLH domain in OsbHLH17

Diagram of the OsbHLH17 peptide with HLH domain. Generated by querying the PROSITE database (Hulo *et al.*, 2006).

Querying the Coils Server with the full length peptide, OsbHLH17 was predicted to contain coiled-coil domains from L326 to F343 and L362 to R377, using a 14AA window, but the probabilities were lower if a 21AA window was used (Figure 2.14) (Lupas *et al.*, 1991). The coiled-coil domains may function in dimerisation (Lupas, 1996).

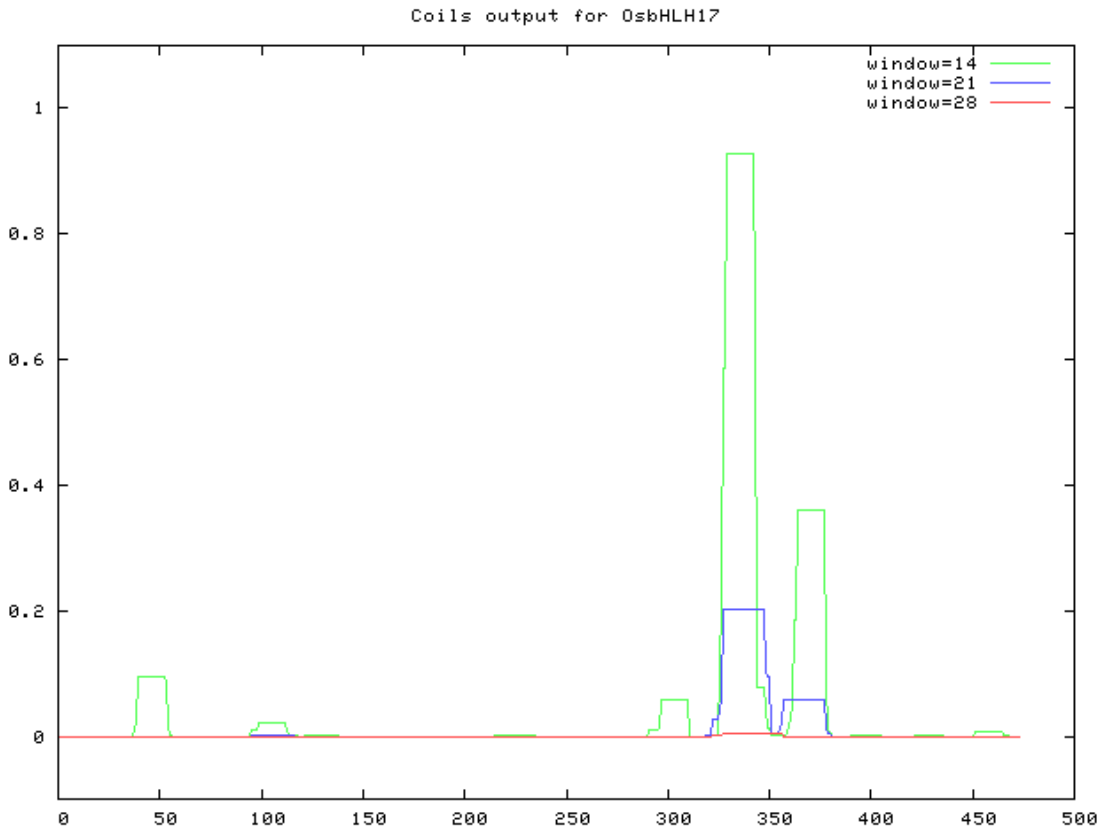


Figure 2.14 Coiled-coil domain prediction in OsbHLH17

The bHLH superfamily has been analysed in two recent reviews (Carretero-Paulet *et al.*, 2010; Pires & Dolan, 2010). In the review by Carretero-Paulet *et al.* (2010) OsbHLH17, designated as OsbHLH010, was placed in subfamily 2, had no intron and contained a G box (5′-CACGTG-3′) DNA-binding motif. Compared to the plant bHLH consensus sequence, *OsbHLH17* matched the following residues in the order of: actual # (expected #); basic 5(5), helix I 6(6), loop 2(2), helix II 9(12). The basic region contained 4 basic residues, 1 acidic residue, 1 glycine, 0 prolines and 7 serines. The loop length was 6 residues. The architecture of conserved protein motifs was 11, 14, 34, 8, 20, 12, 10, 2, 1, 7, 37 (Carretero-Paulet *et al.*, 2010). These are present in Table 2.1 and aligned with OsbHLH17 in Figure 2.1. Motifs 1 and 2 were helix 2 and 1 of the bHLH domain, respectively (Carretero-Paulet *et al.*, 2010).

Table 2.1 Motif consensus sequences from *A. thaliana*, Poplar, Rice, Moss and Algae

An extract from supplemental table 4 in Carretero-Paulet (Carretero-Paulet *et al.*, 2010), showing the consensus sequences of common motifs in bHLH proteins from *A. thaliana*, Poplar, Rice, Moss and Algae. The motifs present in the table are the consensus sequences for those in OsbHLH17.

```
CLUSTAL format alignment by MAFFT (v6.851b)

Os01g50940      MSWSETDAALFAAVLGHDAAHHLATTPPHLDAPEGSPSSAELQASLHDLVERQGGAWTYG
11              ----------------------------------------------------CRNVWWTYA
14              ------------------------------------------------------------
34              ------------------------------------------------------------
8               ------------------------------------------------------------
20              ------------------------------------------------------------
12              ------------------------------------------------------------
10              ------------------------------------------------------------
2               ------------------------------------------------------------
1               ------------------------------------------------------------
7               ------------------------------------------------------------
37              ------------------------------------------------------------

Os01g50940      IFWQESRGAGAASGRAARAVLGWGDGHCRDGAGHGEVGAAERSVARKRVLLRLHALYGGG
11              IFWQIS--------------------------------------------------------
14              --------------SGQGVLGWGDGYYNGPKKTRK-------------------------
34              ------------------------------------------EEWQEMRKRVLRKLHSLFGGP
8               ------------------------------------------------------------
20              ------------------------------------------------------------
12              ------------------------------------------------------------
10              ------------------------------------------------------------
2               ------------------------------------------------------------
1               ------------------------------------------------------------
7               ------------------------------------------------------------
37              ------------------------------------------------------------

Os01g50940      DEDGADYALRLDRVTGAEMYFLASMYFSFPEGSGGPGRALASGRHAWADVDPHPSGSGSA
11              ------------------------------------------------------------
14              ------------------------------------------------------------
34              DEDNYDYV------------------------------------------------------
8               -------------------FYLVCMTFSFPPGVGLPGRAYANGKHVWL-------------
20              ------------------------------------------------------CGAHEC
12              ------------------------------------------------------------
10              ------------------------------------------------------------
2               ------------------------------------------------------------
1               ------------------------------------------------------------
7               ------------------------------------------------------------
37              ------------------------------------------------------------

Os01g50940      PGWYV-RSSLAQSAGLRTVVFLPCKGGVLELGSVVAIRETPEVLRAIQSAMRAVPAPPED
11              ------------------------------------------------------------
14              ------------------------------------------------------------
34              ------------------------------------------------------------
8               ------------------------------------------------------------
20              DSYYCERAFLAKMAGIQTVVCIP---------------------------------------
12              ------------------------MHGVVELGSTDHIPEDWDFVN----------------
10              ------------------------------------------------------------
2               ------------------------------------------------------------
1               ------------------------------------------------------------
7               ------------------------------------------------------------
37              ------------------------------------------------------------

Os01g50940      FMRIFGKDLSPGRPSQPMGCDAPWTPRLVVQTTPVRPAKKEVVKAKPAEPPKSLDFSKAN
11              ------------------------------------------------------------
14              ------------------------------------------------------------
34              ------------------------------------------------------------
8               ------------------------------------------------------------
20              ------------------------------------------------------------
12              ------------------------------------------------------------
10              ------------------------------------------------------------
2               ------------------------------------------------------------
1               ------------------------------------------------------------
7               ------------------------------------------------------------
37              ------------------------------------------------------------
```

```
Os01g50940      VQEQAGGQERRPRKRGRKPANGREEPLNHVEAERQRREKLNQRFYALRAVVPKISKMDKA
11              ------------------------------------------------------------
14              ------------------------------------------------------------
34              ------------------------------------------------------------
8               ------------------------------------------------------------
20              ------------------------------------------------------------
12              ------------------------------------------------------------
10              -------AEKKPKRRNVRICKDPEEVEN--------------------------------
2               ---------------------------------RRRREKINERFKALQSLVPNC------
1               ------------------------------------------------------------
7               ------------------------------------------------------------
37              ------------------------------------------------------------


Os01g50940      SLLSDAIAYIQELEARLRGDAPVPARADGPAVEVKAMQDEVVLRVTTPLDEHPISRVFHA
11              ------------------------------------------------------------
14              ------------------------------------------------------------
34              ------------------------------------------------------------
8               ------------------------------------------------------------
20              ------------------------------------------------------------
12              ------------------------------------------------------------
10              ------------------------------------------------------------
2               ------------------------------------------------------------
1               -MLDEAINYIKFLQRQVQFLEM----------------------------------------
7               ----------------------------PDIEVKIIGWHVMIKIHCRWR----------
37              ------------------------------------------------GWPLSRVLQV


Os01g50940      MRESQISVVASDVAVSDDAVTHTLMVRSAGPERLTAETVLAAMSRGVSVTTPSP
11              ------------------------------------------------------
14              ------------------------------------------------------
34              ------------------------------------------------------
8               ------------------------------------------------------
20              ------------------------------------------------------
12              ------------------------------------------------------
10              ------------------------------------------------------
2               ------------------------------------------------------
1               ------------------------------------------------------
7               ------------------------------------------------------
37              LLEEGFCVINCISTKVNERLYHTIQCEVNDFNCI--------------------
```

Figure 2.15 Multiple sequence alignment of OsbHLH17 and bHLH consensus sequences

Consensus sequences of common motifs in bHLH proteins from *A. thaliana*, Poplar, Rice, Moss and Algae from supplemental table 4 in Carretero-Paulet (Carretero-Paulet *et al.*, 2010) were aligned with the AA sequence of OsbHLH17 in a CLUSTAL format alignment using MAFFT (v6.851b) (Katoh *et al.*, 2005).

In the review by Pires *et al.* (2010), OsOrphan19, designated as OsbHLH010, was placed in subfamily III(d+e). Characterised members of subfamily III(d+e) include two proteins from *A. thaliana* MYC2/JASMONATE INSENSITIVE 1 (MYC2/JAI1/JIN1) (Abe *et al.*, 2003; Dombrecht *et al.*, 2007; Lorenzo *et al.*, 2004; Pozo *et al.*, 2008; Yadav *et al.*, 2005) and AIB (Li *et al.*, 2007), involved in ABA signalling and the former also in jasmonic acid and light signalling. One other characterised member of

the subfamily is from pea, PsGBF, which is involved in phenylpropanoid biosynthesis regulation (Qian *et al.*, 2007). Members of subfamily III(d+e) have a conserved motif towards the N-terminus, designated as motif 7 (Pires & Dolan, 2010) (Table 2.2).

Table 2.2 The conserved AA motif 7 from bHLH subfamily III(d+e) and various plant species

An extract from supplementary table S2 in Pires (Pires & Dolan, 2010), showing the conserved non-bHLH AA motif 7, from *A. thaliana*, *O. sativa* and *Physcomitrella patens*. OsbHLH17 is designated as OsbHLH010.

NOTE:
This table is included on page 98 of the print copy of the thesis held in the University of Adelaide Library.

Using the OsbHLH17 full length peptide as a query, a BLASTP search of the refseq_protein database showed that the closest homologs were *Z. mays* LOC100284359 with 70% identity and 100% coverage (3e-164) and *S. bicolor* SORBIDRAFT_03g032420 with 72% and 100% coverage (3e-163). The published homologs were *A. thaliana* AIB, with 37% identity over the full length peptide (1e-88) (Li *et al.*, 2007), and *A. thaliana* MYC2, with 44% identity (3e-32), but only 70% query coverage (Abe *et al.*, 2003; Dombrecht *et al.*, 2007; Lorenzo *et al.*, 2004; Pozo *et al.*, 2008; Yadav *et al.*, 2005).

2.3.2.1.5. OsLUX

OsLUX is located on chromosome one and is composed of a single exon of 1,309bp, containing a CDS of 717bp, which is predicted to encode a protein having 238AA, a molecular weight of 25617.3 and a pI of 6.48. *OsLUX* encodes a protein containing a myb-type HTH DNA-binding domain, from the SANT superfamily and GARP-G2-like family of transcription factors (Figure 2.16). It is annotated as being similar to Two-component response regulator ARR11 (Receiver-like protein 3), in the RAP-DB. EST data showed that OsLUX transcripts were present in the panicle (nine matches), root (six matches), leaf (two matches) and shoot (one match). OsLUX expression is low at dawn, increases in the afternoon, peaks at dusk and then falls throughout the night (Murakami *et al.*, 2007).

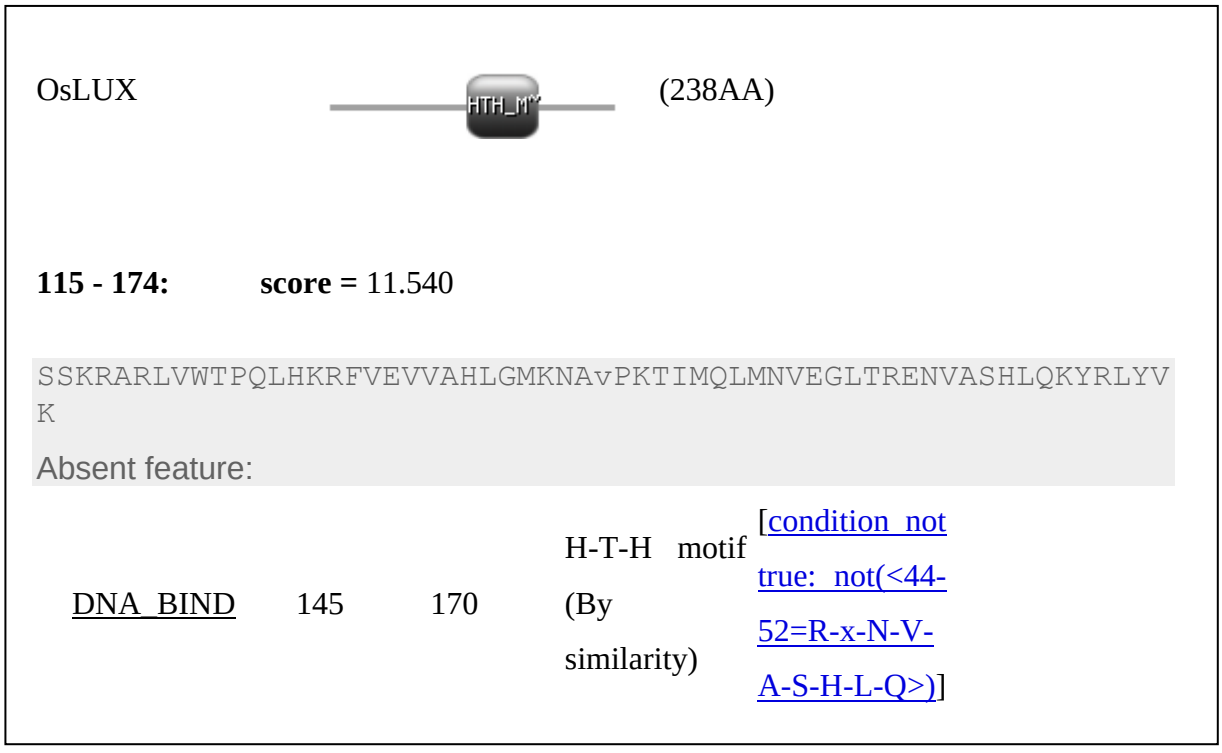


Figure 2.16 Schematic diagram representing the Myb-type HTH DNA-binding domain

A diagram of the OsLUX peptide and HTH DNA-binding domain. Generated by querying the PROSITE database (Hulo *et al.*, 2006).

Using the OsLUX full length peptide as a query, a BLASTP search of the refseq_protein database showed that the most similar homolog was *S. bicolor* SORBIDRAFT_03g047330 with 71% identity and 87% coverage (2e-76) and the published homologs were *Z. mays* (ARABIDOPSIS RESPONSE REGULATOR 1)

ARR1 protein-like (ACX02581) with 67% identity and 92% coverage (5e-70) from US Patent 7569389 titled "Nucleotide sequences and polypeptides encoded thereby useful for modifying plant characteristics" as sequence 24729, *A. thaliana* PHYTOCLOCK 1/LUX ARRYTHMO (PCL1/LUX) with 56% identity and 85% query coverage (9e-55) involved in circadian clock regulation (Hazen *et al.*, 2005; Onai & Ishiura, 2005) and *A. thaliana* ARR12 with 60% identity and 28% coverage (3e-18) involved in cytokinin signalling (Argyros *et al.*, 2008; Ishida *et al.*, 2008; Yokoyama *et al.*, 2007).

OsLUX has 53% AA identity with the B motif DNA-binding domain of *A. thaliana* ARR10, which has a HTH structure (Hosoda *et al.*, 2002). The B-motif contains a nuclear localisation signal and binds the consensus sequence AGAT(ACG/TCG/CTT). The AA sequence of the B motif of OsLUX was aligned with all published homologs (Figure 2.17). The conserved AAs are highlighted.

```
OsLUX !115-177      1 SSKRARLVWTPQLHKRFVEVVAHLGMK.NAVPKTIMQLMN....VEGLTRENVASHLQKYRLYVKRMQ
AtPCL1!141-203      1 TLKRPRLVWTPQLHKRFVDVVAHLGIK.NAVPKTIMQLMN....VEGLTRENVASHLQKYRLYLKRMQ
AtARR1!234-296      1 NLKKPRVVWSVELHQQFVAAVNQLGVE.KAVPKKILELMN....VPGLTRENVASHLQKYRIYLRRLG
AtARR12!192-254     1 AQKKQRVVWTVELHKKFVAAVNQLGYE.KAMPKKILDLMN....VEKLTRENVASHLQKFRLYLKRIS
AtARR2!213-275      1 SLKKPRVVWSVELHQQFVAAVNQLGVD.KAVPKKILEMMN....VPGLTRENVASHLQKYRIYLRRLG
AtARR14!197-259     1 NSKKSRVVWSIELHQQFVNAVNKLGID.KAVPKRILELMN....VPGLSRENVASHLQKFRLYLKRLS
AtARR18!174-236     1 TRKKPRVVWSQELHQKFVSAVQQLGLD.KAVPKKILDLMS....IEGLTRENVASHLQKYRLYLKKID
AtARR10!180-242     1 AQKKPRVLWTHELHNKFLAAVDHLGVE.RAVPKKILDLMN....VDKLTRENVASHLQKFRVALKKVS
AtARR21!227-289     1 PAKKKKIQWTDSLHDLFLQAIRHIGLD.KAVPKKILAFMS....VPYLTRENVASHLQKYRIFLRRVA
AtGPRI1!150-212     1 GKRKVKVDWTPELHRRFVEAVEQLGVD.KAVPSRILELMG....VHCLTRHNVASHLQKYRSHRKHLL
AtARR13!222-284     1 PPKKKKIWWTNPLQDLFLQAIQHIGYD.KVVPKKILAIMN....VPYLTRENVASHLQKYRLFVKRVV
AtGLK2!144-206      1 IKKKPKVDWTPELHRKFVQAVEQLGVD.KAVPSRILEIMN....VKSLTRHNVASHLQKYRSHRKHLL
AtAPRR2!293-355     1 KTSRKKVDWTPELHKKFVQAVEQLGVD.QAIPSRILELMK....VGTLTRHNVASHLQKFRQHRKNIL
AtARR11!190-253     1 SSKKARVVWSFELHHKFVNAVNQIGCDHKAGPKKILDLMN....VPWLTRENVASHLQKYRLYLSRLE
AtARR20!208-275     1 PTKKPRMQWTPELHHKFEVAVEKMGSLEKAFPKTILKYMQEELNVQGLTRNNVASHLQKYRQSSKKTC
AtPHR1!222-285      1 GTGKARMRWTPELHEAFVEAVNSLGGSERATPKGVLKIMK....VEGLTIYHVKSHLQKYRTARYRPE
AtAPRR4!220-282     1 SAKKRRVVWDEELHQNFLNAVDFLGLE.RAVPKKILDVMK....VDYISRENVASHLQVTFLIYNIIV
```

Figure 2.17 Multiple sequence alignment of the myb-type DNA binding domain and B motif

The myb DNA-binding domain of OsLUX was aligned with all published homologs. Conserved AAs are highlighted.

2.3.2.1.6. OsMYB54

OsMYB54 is located on chromosome four and is composed of three exons, with a transcript of 1563bp. The 720bp CDS is predicted to encode a protein having 239AAs, a molecular weight of 26305.6kDa and a pI of 9.61. *OsMYB54* is an R2R3 type MYB transcription factor, with the R2 imperfect repeat from position 20 to 72 and the R3 imperfect repeat from position 73 to 127. EST data did not show any tissue specific expression patterns.

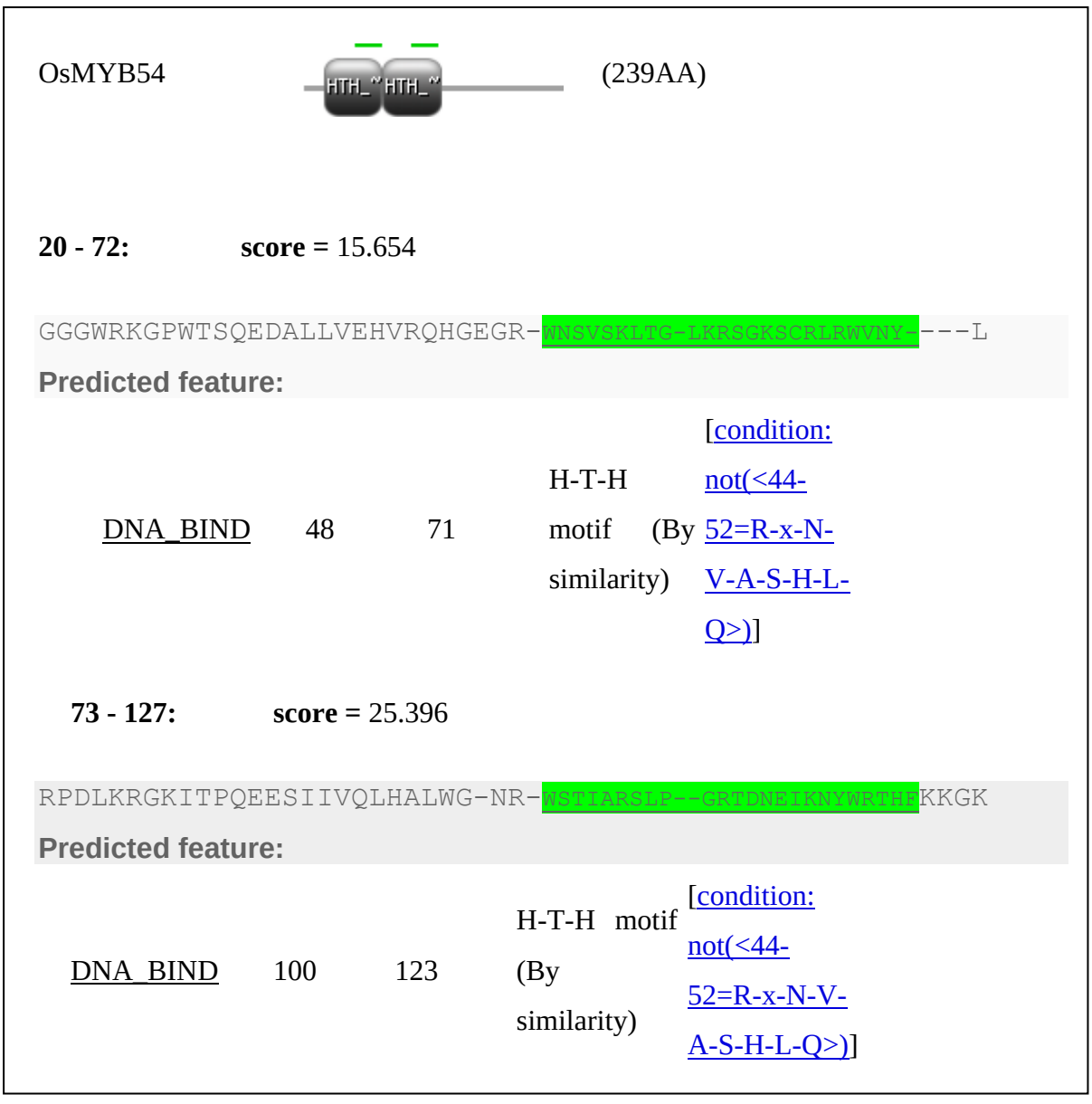


Figure 2.18 Myb-type HTH DNA-binding domains

A diagram of the OsMYB54 peptide and two HTH motifs and DNA-binding domains (□). Generated by querying the PROSITE database (Hulo *et al.*, 2006).

Querying the Coils Server with the full length peptide, OsMYB54 was predicted to contain a coiled-coil domain from S129 to Q153, using a 21AA window (Figure 2.19) (Lupas *et al.*, 1991). The coiled-coil domain may function in dimerisation (Lupas, 1996).

Using the OsMYB54 full length peptide as a query, a BLASTP search of the refseq_protein database showed that the most similar homologs in were the *S. bicolor* hypothetical protein SORBIDRAFT_04g026210 with 63% identity and 97% coverage (4e-73), the homolog *O. sativa* LOC_Os02g40530.1 with 67% identity with 87% coverage (7e-70), present as sequence 1572 from US patent 7659446 (GenBank ADF07733.1), in which transgenic plants were generated with enhanced tolerance to cold treatment at 8°C or to media supplemented with 9.4% sucrose and also present as sequence 1572 from US patent 7511190 (GenBank ACQ21125.1), in which plants were generated with enhanced tolerance to abiotic stresses. The next most similar homolog was AtMYB79 with 76% identity and 53% coverage (2e-52), present as sequence 534 from US patent 7663025 titled "Plant transcriptional regulators", in which transcription factors were used to produce transgenic plants with improved tolerance to drought, shade and low nitrogen conditions and also present as sequence 264 from US patent 7659446 (GenBank ADF07330.1), which is mentioned above.

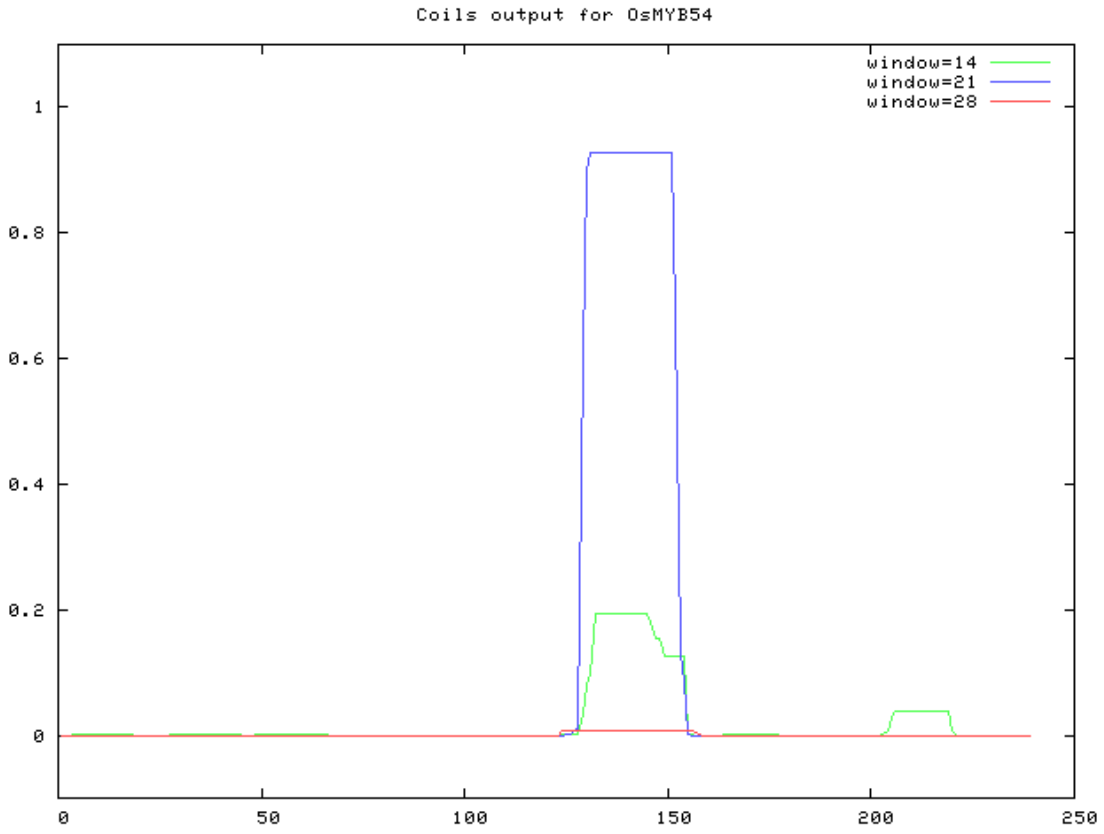


Figure 2.19 Coiled-coil domain prediction in OsMYB54

The full length AA sequence of OsMYB54 was submitted to the Coils Server (Lupas *et al.*, 1991). A coiled-coil domain was predicted from 132-152AA, immediately following the second myb domain.

2.4. Discussion

Salt responsive transcription factor expression was analysed in rice cultivars differing in salinity tolerance. There were minimal changes in transcription factor transcript levels between the salt tolerant and salt sensitive cultivars for all analysed transcription factors. This may be due to the mild and extended salt stress treatment. Differences in gene expression between salt stressed and control plants are known to become less pronounced as plants adapt (Kawasaki *et al.*, 2001). Traditionally, salt stress treatments are high salt shock treatments that are maintained for short periods of time and the genes identified as responsive are those rapidly induced (Chen *et al.*, 2002; Dubouzet *et al.*, 2003; Kreps *et al.*, 2002; Yamaguchi-Shinozaki & Shinozaki, 1993). This project aimed to identify transcription factors involved in maintaining long term salt tolerance. Each identified transcription factor will be discussed individually below.

2.4.1. OsOrphan19

The protein encoded by *OsOrphan19* is a member of the zinc finger B-box family of transcription factors. It has two zinc finger domains, each of which contains two cysteines and two histidines that coordinate one zinc ion (Brown *et al.*, 1985), in a structure containing an α-helix packed against two antiparallel β-strands (Lee *et al.*, 1989). The transcripts for the transcription factor *OsOrphan19* decreased under salt stress. *OsOrphan19* transcripts were higher in the salt tolerant cultivars and there was a greater downregulation under salt stress, relative to the salt sensitive cultivar Nipponbare.

OsOrphan19 displays homology to *A. thaliana* STH (Holm *et al.*, 2001) and STO (Indorf *et al.*, 2007; Lippuner *et al.*, 1996). Heterologous expression of *STO* in yeast deficient in the phosphoprotein phosphatase, calcineurin, conferred enhanced tolerance to Li^+ and Na^+ ions. *STO* expression partially rescued yeast from other calcineurin deficient phenotypes including Mn^{2+} sensitivity, Ca^{2+} tolerance and the inability to recover from α factor mating pheromone, suggesting that STO may function as a phosphoprotein phosphatase, despite having no homology to known phosphoprotein phosphatases (Lippuner *et al.*, 1996). *STO* is also able to complement another salt sensitive yeast mutant, deficient in a L-type calcium channel (Rodriguez-Franco *et al.*, 2008). In *A. thaliana, STO* is not induced by salt stress (Lippuner *et al.*, 1996), however constitutive overexpression in *A. thaliana* plants treated with 50mM or 100mM NaCl enhanced the rate of plant growth and increased root length, relative to wild type plants (Nagaoka & Takano, 2003). This result was not confirmed in other *STO* overexpression

plants and loss-of-function mutants, which did not display altered salt sensitivity (Indorf *et al.*, 2007; Rodriguez-Franco *et al.*, 2008). These results suggest that *OsOrphan19* may be a good target gene to alter plant salt tolerance.

Both STO and STH are also involved in light signalling pathways. The WD40 domains of COP1 form a β-propeller that mediates the interaction with STH and STO. STO and STH interact with COP1 via a motif at the C-terminus, consisting of five negatively charged AAs D-D-D-E-E, a spacer and the motif V-P-E/D-Φ-G, where Φ is a hydrophobic AA (Holm *et al.*, 2001). The interactions have been demonstrated using yeast two-hybrid assays and bioluminescence resonance energy transfer (Holm *et al.*, 2001; Subramanian *et al.*, 2006). OsOrphan19 displays homology to this novel motif at the STO and STH C-terminus, so may interact with COP1 (Holm *et al.*, 2001). However, shared sequence identity over the full length peptide was only 48-49%, which would suggest structural similarity, but not necessarily functional similarity.

STO functions as a negative regulator of phytochrome and blue-light signalling (Indorf *et al.*, 2007). In red, far-red and blue light, *STO* RNAi and T-DNA mutants had inhibited hypocotyl growth and larger cotyledons, whereas *STO* overexpresssors had increased hypocotyl growth and smaller cotyledons, relative to wild type plants, suggesting STO functions as a negative regulator in the light-mediated inhibition of hypocotyl elongation (Indorf *et al.*, 2007). In the light, STO accumulates and negatively regulates chalcone synthase (*CHS*). *STO* induction is PhyA- and B-dependent. In the dark, COP1 interacts with STO and targets it for degradation. *STO* is also under circadian control, as transcripts continued to cycle in continuous light (Indorf *et al.*, 2007). *STO* transcripts cycle similarly to *SPA1*, increasing at end of the dark phase, remaining high during the light phase and then decreasing at the end of the light phase. Both genes are suggested to be part of a negative regulatory network controlled by the circadian clock that prevents an exaggerated light response (Indorf *et al.*, 2007). *A. thaliana* STH displays high homology to STO and loss-of-function mutants display a short hypocotyl under red and far-red light, however the cotyledon area is similar to wild type plants, suggesting that STH plays a smaller role is photomorphogenesis (Khanna *et al.*, 2006). OsOrphan19 may play a role in photomorphogenesis and be circadian regulated, as homology to STO and STH make this a possibility, however, investigating the role of OsOrphan19 in regulating plant salt tolerance is the aim of this work and will be the focus hereon.

2.4.2. OsEREB67

OsEREB67 encodes a single AP2 domain containing transcription factor. The transcripts for the transcription factor *OsEREB67* increased under salt stress in the cultivar IR63731 but not in the other salt tolerant cultivars.

OsEREB67 transcript levels were examined in *O. sativa* L. ssp. *japonica* cv. Zhonghua. *OsEREB67* had comparable transcript levels in roots, leaves, inflorescences and buds (Hu *et al.*, 2008b). Transcripts increased gradually 0.7-fold over four hours in rice in response to exogenous ethylene (0.1mM ethrel) (Hu *et al.*, 2008b). Constitutive overexpression in *A. thaliana* up-regulated the expression of the ethylene responsive genes *PDF1.2* and *b-chitinase* (Hu *et al.*, 2008b). Therefore, *OsEREB67* may be involved in diverse stress responses, as transcripts increased in this project in response to the abiotic stress, salt, and in Hu *et al.* (Hu *et al.*, 2008b), transcripts increased in response to the biotic stress signalling molecule, ethylene.

The closest published homologs include AtERF2 and AtERF1. Both of which are involved in jasmonic-acid mediated pathogen defence signalling (Brown *et al.*, 2003; Oñate-Sánchez & Singh, 2002), are induced by exogenous ethylene and wounding and function as transcriptional activators (Fujimoto *et al.*, 2000). Both have been shown not to be involved in drought or salt responses (Fujimoto *et al.*, 2000). *AtERF1* and *AtERF2* had increased expression in response to chitooctoase, an elicitor of the plant defence response against pathogens (Libault *et al.*, 2007). *AtERF1* and *AtERF2* expression increased in response to mosaic virus species, *Botrytis cinerea*, *Pseudomonas syringae* pv. tomato DC3000, methyl jasmonate and reduced in response to wounding (Chen *et al.*, 2002). *AtERF1* and *AtERF2* are both induced in response to *Alternaria brassicicola* infection and methyl jasmonate (McGrath *et al.*, 2005).

A. thaliana plants constitutively overexpressing *AtERF2* have increased expression of *PDF1.2, Thi2.1* and *PR4* (Brown *et al.*, 2003). *AtERF2* overexpression in *A. thaliana* enhanced resistance to *F. oxysporum*, increased *PDF1.2* expression 70- to 350-fold and *CHIB* expression 1.5- to 4-fold, above wild type plants (McGrath *et al.*, 2005). *AtERF2* and *AtERF4* act as positive and negative regulators, respectively, of jasmonic acid-mediated defence signalling, of resistance to the necrotrophic fungal pathogen *Fusarium oxysporum* and of jasmonic acid inhibition of root elongation (McGrath *et al.*, 2005).

AtER1 was induced by both salicylic acid and methyl jasmonate. AtERF1 was induced by methyl jasmonate in the *jar1-1* mutant to a similar level observed in wild type plants. *AtERF1* was induced by salicylic acid in wild type plants and in the *pad4-1* mutant but to a lesser extent in the *npr1*-5 mutant. Induced by pathogen infection (Oñate-Sánchez & Singh, 2002). *AtERF1* may be regulated by AP2, which is involved in floral organ development (Jofuku *et al.*, 1994; Ogawa *et al.*, 2007). There was increased expression of *AtERF1* in *ap2* mutant plants, suggesting that AP2 negatively regulates *AtERF1* (Ogawa *et al.*, 2007). *AtERF1* may also be involved in ethylene signalling, as there was reduced expression of *AP2* and *AtERF1* in *ein2-1* mutants, suggesting that EIN2 positively regulates *AP2* and *AtERF1* (Ogawa *et al.*, 2007). There was increased expression of *AP2* and *AtERF1* in *AtEBP* overexpressing plants, suggesting that AtEBP positively regulates *AP2* and *AtERF1* (Ogawa *et al.*, 2007).

Another homolog, tobacco *OPBP1*, is involved in salt and defence responses as overexpression enhanced salt tolerance and disease resistance in rice (Chen & Guo, 2008). Rice plants were shock treated with 250mM NaCl for 18 hours, then allowed to recover in ½ MS. All control plants died, whereas half of the transgenic plants survived (Chen & Guo, 2008). OsEREB67 does however only have sequence identity with OPBP1 in the AP2 domain. The study on OsEREB67 and those on the homologs suggest that OsEREB67 is involved in defence signalling and may be involved in salt stress responses.

2.4.3. OsMYBR63

OsMYBR63 encodes a myb-type HTH domain containing transcription factor. Transcripts for the transcription factor *OsMYBR63* decreased under salt stress. EST data suggest that the second splice form, Os06g51260.2, may not be expressed in *indica* rice. OsMYBR63 has sequence identity with two *A. thaliana* proteins involved in regulation of the circadian clock, including RVE2 and RVE7/EPR1. There is only moderate sequence identity however, with 45% identity and 56% coverage (5e-39) for RVE2 and 48% identity and 50% coverage (1e-34) for REV7/EPR1, which is restricted to the myb-type HTH domain and some surrounding sequence.

The circadian cycling of *RVE2* is controlled by the central clock oscillator CCA1. *RVE2* overexpression altered endogenous *RVE2* expression, reduced expression of the central clock components *CCA1* and *LHY*, affected the rhythm of the slave clock component *RVE7/EPR1* and downstream genes *Lhcb* and *CAT3* (Zhang *et al.*, 2007a). *RVE2* overexpression also delayed flowering, reduced expression of flowering promoting genes CONSTANS (CO) and FLOWERING LOCUS T (FT), increased hypocotyl elongation 2.5-fold and inhibited seed germination in the dark to <50% of wild type plants. *rve2* mutants had early flowering and increased FT expression (Zhang *et al.*, 2007a).

RVE2 is also involved in abiotic stress response. *RVE2* expression increased 2-fold under cold and light treatment and 3.5-fold under cold and dark treatment (Soitamo *et al.*, 2008). *RVE2* expression was up-regulated in O-acetylserine(thiol)lyase deficient mutants that were oxidatively stressed through increased H_2O_2 production (López-Martin *et al.*, 2008). *RVE2* expression was up-regulated 3.6-6.3-fold in *A. thaliana* plants overexpressing *Zinc finger homeodomain 1* (*ZFHD1*) that had improved drought tolerance, relative to wild type plants (Tran *et al.*, 2007). *RVE2* expression was up-regulated 2.23-fold in cold treated *A. thaliana* plants overexpressing spermidine synthase that had enhanced tolerance to multiple stresses, relative to wild type plants (Kasukabe *et al.*, 2004). Together, these studies show that RVE2 is involved in plant responses to a range of abiotic stresses.

RVE7/EPR1 has not been reported to be involved in plant abiotic stress responses. *RVE7/EPR1* negatively regulates itself, creating its own circadian rhythm and cycles similarly to *CCA1* and *LHY* (Kuno *et al.*, 2003). *RVE7/EPR1* is also regulated by PhyA and PhyB. Overexpression of *RVE7/EPR1* did not change *CCA1* or *LHY* expression, however, constitutive overexpression of *LHY* or *CCA1* disrupted *RVE7/EPR1*

rhythmicity. Overexpression of *RVE7/EPR1* abolished the rhythmicity of *Lhcb*, a clock target gene. Overexpression of *RVE7/EPR1* in *A. thaliana* did not change hypocotyl elongation, enhanced far-red light-induced cotyledon opening and delayed flowering by five to six days (Kuno *et al.*, 2003). Enhanced cotyledon opening was *PhyA* dependent (Kuno *et al.*, 2003). *RVE7/EPR1* T-DNA insertion mutants displayed no aberrant growth phenotypes in response to different light conditions (Khanna *et al.*, 2006).

Sequence similarity of OsMYBR63 to RVE2 and RVE7/EPR1 suggest that OsMYBR63 may be involved in plant abiotic stress responses and may be regulated by clock components.

2.4.4. OsbHLH17

OsbHLH17 encodes a bHLH transcription factor containing a single myc-type HLH domain. Transcripts for the transcription factor *OsbHLH17* increased in the shoot of IR63731, but there were only small changes in mRNA levels for the other salt tolerant cultivars. OsbHLH17 contains a G box DNA-binding motif, a specific type of E-box hexanucleotide consensus sequence (5′-CANNTG-3′) recognised by bHLH proteins (Carretero-Paulet *et al.*, 2010). The bHLH motif functions as a dimerisation domain (Ferre-D'Amare *et al.*, 1993), so OsbHLH17 may form a homo- or heterodimers *in planta*.

The two most similar published homologs include AIB and MYC2 from *A. thaliana*, which are both involved in abiotic stress responses and the latter also in biotic signalling and photomorphogenesis (Abe *et al.*, 2003; Dombrecht *et al.*, 2007; Li *et al.*, 2007; Lorenzo *et al.*, 2004; Pozo *et al.*, 2008; Yadav *et al.*, 2005). *AtAIB* is induced by exogenous ABA. *aib* mutants have reduced ABA sensitivity and reduced drought tolerance, whereas *AIB* overexpressing plants have improved drought tolerance, relative to wild type plants (Li *et al.*, 2007). Similarly, *AtMYC2* is also induced by exogenous ABA and drought stress, and enhances osmotic stress tolerance when overexpressed (Abe *et al.*, 2003). AtAIB and AtMYC2 induce different downstream genes, as *RD22* was induced in *AtMYC2* but not *AtAIB* overexpressing plants (Abe *et al.*, 2003; Li *et al.*, 2007). AtMYC2 is also involved in jasmonate-mediated responses to wounding and pathogen attack (Chini *et al.*, 2007; Fernández-Calvo *et al.*, 2011; Lorenzo *et al.*, 2004), terpenoid indole alkaloid biosynthesis (Montiel *et al.*, 2011) and photomorphogenesis (Gangappa *et al.*, 2010; Yadav *et al.*, 2005).

Sequence identity to OsbHLH17 is moderate at 37% identity over the full length peptide (1e-88) for AIB and 44% identity (3e-32) with 70% query coverage for MYC2. Therefore the function of OsbHLH17 cannot really be inferred from these homologs.

The significance of N-terminal motif 7 found in *A. thaliana*, *O. sativa* and *Physcomitrella patens* subfamily III(d+e) bHLH proteins is unknown and it is not present in the PFAM database (Pires & Dolan, 2010). Subfamily members are however involved in stress response signalling (Abe *et al.*, 2003; Abe *et al.*, 1997; Anderson *et al.*, 2004; Cheng *et al.*, 2011; Chini *et al.*, 2007; Fernández-Calvo *et al.*, 2011; Laurie-Berry *et al.*, 2006; Li *et al.*, 2007; Lorenzo *et al.*, 2004).

Regarding the significance of consensus sequences of common motifs in bHLH proteins from *A. thaliana*, Poplar, Rice, Moss and Algae; motifs 1 and 2 were helix 2 and helix 1 of the bHLH domain (Carretero-Paulet *et al.*, 2010). In a different bHLH subfamily, motif 7 forms part of a domain affecting stomata phenotype (Carretero-Paulet *et al.*, 2010; Pillitteri *et al.*, 2007), however OsbHLH17 does not contain the other motifs required to form the domain.

2.4.5. OsLUX

OsLUX encodes a myb-like transcription factor and transcripts for the transcription factor *OsLUX* increased in response to salt stress. OsLUX likely binds DNA as a monomer, due to the AA identity with ARR10 and as it lacks the coiled-coil (CC) domain of other members of the GARP family, such as PHR1 and PSH1, which bind as dimers (Hosoda *et al.*, 2002; Rubio *et al.*, 2001).

The residues involved in DNA binding in ARR10 include R185, L187, W188, E225, A228, S229, L231, Q232, K233, A237 and K239. All residues except R185, L187 and W188 form part of the α3 helix which binds the major groove of DNA. The three aforementioned residues are part of an N-terminal flexible arm that binds the minor groove (Hosoda *et al.*, 2002). OsLUX shares all except L187 and A237, which are involved in binding with the minor groove and a nucleotide outside the consensus sequence (AGATT), respectively (Hosoda *et al.*, 2002). This would give OsLUX different DNA-binding specificity for residues around the consensus sequence. Similarity to ARR10 suggests that OsLUX contains a HTH motif from the second to third helices. Amino acid sequence similarity is mainly restricted to the MYB DNA binding domain, suggesting that OsLUX binds DNA, however function cannot be inferred.

OsLUX shares greater sequence identity with AtPCL1/AtLUX. The *A. thaliana PHYTOCLOCK 1* (*PCL1*) gene was identified from ethylmethanesulfonate mutagenised *A. thaliana* plants with altered circadian rhythms. *PCL1* encodes a golgi associated retrograde protein (GARP) family transcription factor which functions as a clock oscillator gene (Onai & Ishiura, 2005). A *PCL1 promoter:luciferase gene* fusion was expressed in wild type plants, which showed bioluminescence in a circadian rhythm with a sharp increase and slow decrease. In the model proposed, *PCL1* upregulates *CCA1* and *LHY* in the morning and downregulates *GI*, *TOC1*, *ELF4* and *PCL1* itself (Onai & Ishiura, 2005).

In *lux-1* and *lux-2* mutants, *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* and *LATE ELONGATED HYPOCOTYL (LHY)* are repressed and *TIMING OF CAB2 EXPRESSION (TOC1)* is activated (Hazen *et al.*, 2005). An electrophoretic mobility shift assay confirmed that LHY and CCA1 bind the evening element in the *LUX*/*PCL1* promoter, and repress its expression (Hazen *et al.*, 2005). An EMSA confirmed that *LHY* and *CCA1* also act directly on the evening element in *TOC1* to repress expression, while *TOC1* positively regulates *LHY* and *CCA1* as their transcript levels were reduced

in the *toc1-1* mutants (Alabadi *et al.*, 2001). Therefore *LUX/PCL1* and *TOC1* may be part of the same regulatory feedback loop.

Recently, a LUX binding site (LBS) motif was identified; represented as GATWCG (where W indicates A or T) (Helfer *et al.*, 2011). The LBS is found in the *PRR9* and *PRR7* promoters, however, LUX only binds LBS in the *PRR9* promoter but not *PRR7 in vivo*. LUX also binds LBS in the *LUX* promoter *in vivo*, to repress its own expression. LUX represses *PRR9* expression *in vivo* (Helfer *et al.*, 2011).

The circadian clock has also been shown to regulate genes involved in responses to salt, dehydration, cold, heat and reactive oxygen species (Covington *et al.*, 2008; Harmer, 2000; Kreps *et al.*, 2002). Some circadian clock genes have been identified as cold responsive, however this effect was not observed with LUX/PCL1, which maintained the same amplitude in cold and control conditions (Bieniawska *et al.*, 2008; Espinoza *et al.*, 2008). It is possible however, that *OsLUX* is involved in plant responses to salt stress and that altered expression of *OsLUX* could alter plant salt tolerance.

OsLUX should not have been accidentally isolated in this study as plant tissue samples were taken from control treatment and salt stressed plants at the same time of the day.

2.4.6. OsMYB54

OsMYB54 is an R2R3-MYB transcription factor with a predicted coiled-coil domain on the C-terminal side of the R3 domain that may function in protein dimer formation (Lupas, 1996). Transcripts for the transcription factor OsMYB54 increased to a greater extent in the shoot of IR63731 and Pokkali, relative to IR29. OsMYB54 is also rapidly responsive to high salt stress and has increased expression after three hours of 200mM NaCl (Jain *et al.*, 2007).

Two homologs were identified that function in plant abiotic stress tolerance. The closest homolog with a known function was *O. sativa* LOC_Os02g40530.1, which was not in any publications but reported in two patents where plants were generated with enhanced tolerance to cold treatment, osmotic stress and other abiotic stresses. The second was *A. thaliana* AtMYB79, also not reported in any publications, but present two patents where plants were generated with enhanced tolerance to drought, shade, low nitrogen, cold and osmotic stresses. AtMYB79 is an R2R3-MYB protein, but lacks any other distinguishing features and therefore is not categorised in a subgroup (Stracke *et al.*, 2001). It is also not induced by plant stress-related hormones, $CdCl_2$ or NaCl (Yanhui *et al.*, 2006).

Six transcription factors were identified that were responsive to salt stress, which makes them interesting candidates as they may be involved in regulating the salinity tolerance of a rice plant.

Chapter 3. Generation of transgenic rice

3.1. Introduction

The modification of gene expression patterns in the plant is a promising technique for overcoming the biotic and abiotic obstacles that impact on crop health and reduce productivity. Modifying transcription factor expression is an effective approach as a single transcription factor may regulate the expression of many downstream genes and have a greater effect upon the plant physiologically, than the modification of a single transporter. This chapter outlines the process of generating transgenic rice plants with altered transcription factor expression levels.

3.2. Materials and methods

3.2.1. Materials

The NucleoSpin Extract II Kit was purchased from Macherey-Nagel, Dueren, Germany. The pCR8®/GW/TOPO® TA Cloning® Kit, Mach1™ chemically competent *E. Coli*, Platinum® Taq, LR Clonase™ Plus II Enzyme Mix, Proteinase K, T4 DNA ligase and RNaseA in R40 were purchased from Invitrogen, San Diego, California, USA. The Phusion DNA polymerase, BSA and restriction endonucleases *Acc*I, *Apa*I, *Asc*I, *Hpa*I, *Kpn*I, *Pme*I, *Sac*II and *Sex*AI were purchased from New England Biolabs, Ipswich, Massachusetts, USA. The Tween20 and restriction endonucleases *Eco*RI, *Mlu*I and *Hind*III were purchased from F. Hoffmann-La Roche AG, Basel, Switzerland. The QiaPrep Spin Miniprep Kit was purchased from Qiagen, Venlow, Netherlands. *A. tumefaciens* AGL1 was supplied by Dr. Alex Johnson, ACPFG. The DH5α chemically competent cells were supplied by Dr. Andrew Jacobs, ACPFG. The bleach was purchased from White King, Pymble, Australia. The Jiffy Peat Pots were purchased from Novosel Enterprises, Oberlin, Pennsylvania, USA. The Parafilm was purchased from the Pechiney Plastic Packaging Company, Chicago, Illinois, USA. The sodium hydroxide, sodium dodecyl sulphate, potassium acetate, ethanol, glycerol and DMSO were supplied by BDH Laboratory Supplies, Victoria, Australia. Yeast extract was supplied by US Biological, Swampscott, Massachusetts, USA. Tryptone was supplied by Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA. Tris base, rifampicin, kanamycin, spectinomycin, cefotaxime, vancomycin, ethidium bromide and plant growth media chemicals were purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

3.2.2. Cloning transcription factor coding sequences

3.2.2.1. Nested PCR to amplify transcription factor coding sequences

Transcription factor CDSs were amplified from *O. sativa* cv. FL478 cDNA or *O. sativa* cv. Nipponbare genomic DNA using Platinum Taq DNA polymerase. Nested primer sets were designed with the internal primers ending at the start and stop codons. The outside primers were positioned in the 5′ and 3′ untranslated regions. For the primers used, see Table 5.4. The first round of amplification used the above mentioned templates. The reactions for the second round of amplification were spiked with the product from the first round of amplification. Each PCR reaction contained the following; 1µL 10µM forward primer, 1µL 10µM reverse primer, 0.5µL 500ng/µL template DNA, 2.5µL 10X buffer, 0.75µL 50mM $MgCl_2$, 0.1µL Platinum Taq, 14.15µL MilliQ water and 2.5µL DMSO. The thermocycler was programmed as follows: 1, 94ºC for 2 minutes; 2, 94ºC for 30 seconds; 3, 50ºC for 30 seconds; 4, 72ºC for 1 minute 30 seconds; 5, repeat from step 2 for 39 times; 6, 15ºC forever.

3.2.2.2. Gel purification of transcription factor coding sequences

The PCR products were separated on a 1% agarose 1X TAE gel containing ethidium bromide, at 110 volts for 20 minutes. The DNA was visualised in the gel using a transilluminator and the bands of appropriate sizes excised. The DNA was separated from the agarose gel and other impurities using a NucleoSpin Extract II Kit as per the manufacturer's instructions.

3.2.2.3. Cloning transcription factor coding sequences

The transcription factor CDSs were cloned using a pCR8®/GW/TOPO® TA Cloning® Kit as per the manufacturer's instructions. See Figure 3.1 for pCR8/GW/TOPO entry vector. The entry vectors produced were; pCR8/OsOrphan19, pCR8/OsEREB67, pCR8/OsMYBR63, pCR8/OsbHLH17, pCR8/OsLUX and pCR8/OsMYB54.

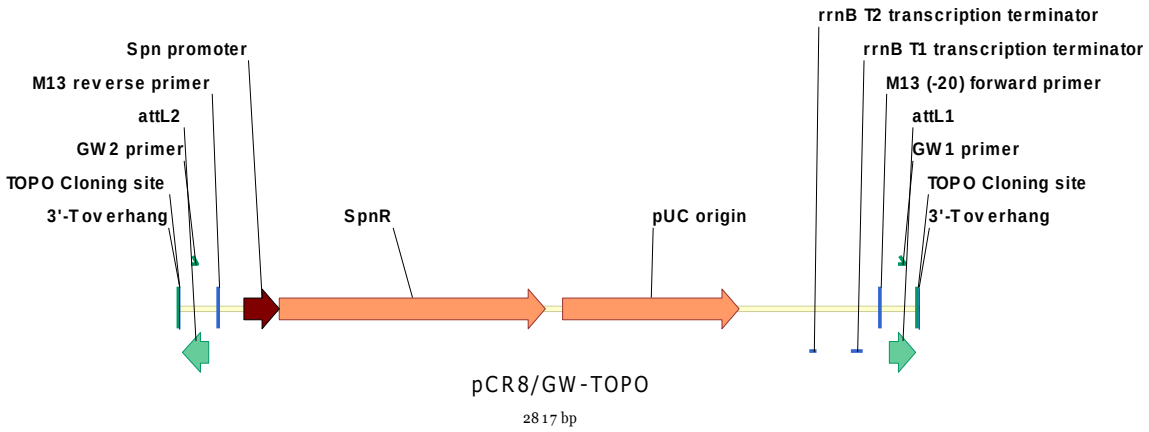


Figure 3.1 Schematic diagram representing the pCR®8/GW/TOPO® entry vector

Vector features taken from the pCR®8/GW/TOPO® TA Cloning® kit.

Feature	Description
Spectinomycin resistance gene (SpnR)	Allows selection of the plasmid in *Escherichia coli*.
GW2 priming site	Permits sequencing in the anti-sense orientation.
M13 reverse priming site	Permits sequencing in the anti-sense orientation.
GW1 priming site	Allows sequencing in the sense orientation.
M13 forward (-20) priming site	Allows sequencing in the sense orientation.
TOPO Cloning Sites	Allows rapid cloning of the *Taq*-amplified PCR product.
pUC origin	Permits high-copy replication and maintenance of the plasmid in *E. coli*.
*rrn*B T1 and T2 transcription terminators	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product.
attL1 and attL2 sites	Bacteriophage λ-derived recombination sequences that have been optimized to allow recombinational cloning of a DNA fragment in the entry construct with a Gateway® destination vector.

3.2.2.4. Transformation of Mach1 Competent Cells

Mach1™ chemically competent *E. coli* were removed from –80ºC storage and allowed to thaw on ice for 5 minutes. For each transformation the following was performed: the entire contents of the ligation was added to a vial containing 50µL of Mach1 chemically competent *E. coli*. The vial was incubated on ice for 20 minutes. The cells were heat shocked in a 42ºC water bath for 30 seconds, incubated on ice for 2 minutes then 250µL RT LB broth was added to the cells. The cells were incubated at 37ºC for 1 hour at 220rpm. The entire contents of the vial was spread over two LB (Section 5.2) 1% agar plates supplemented with 100µg/mL spectinomycin and incubated at 37ºC overnight.

3.2.2.5. Colony PCR to screen for entry clones

Colony PCR was used to identify entry clones containing the transcription factor CDS. PCR was performed using the reverse primer specific for the CDS of interest (Table 5.4) and the GW1 primer (Table 5.7), which binds within the vector backbone. The presence of a product therefore indicates that the CDS is present and in the correct orientation.

Each PCR reaction contained the following; 1µL GW1 primer, 1µL reverse primer, bacterial colony (template DNA), 2.5µL 10X buffer, 0.75µL 50mM $MgCl_2$, 0.1µL Platinum® Taq, 15.65µL MilliQ water and 1µL DMSO. The thermocycler was programmed as follows: 1, 94ºC for 4 minutes; 2, 94ºC for 30 seconds; 3, 50ºC for 30 seconds; 4, 72ºC for 1 minute 30 seconds; 5, repeat from step 2 for 39 times; 6, 15ºC forever. The PCR products were separated by agarose gel electrophoresis and visualised using a transilluminator as per Section 2.2.3.6.

3.2.2.6. Alkaline lysis DNA mini-prep

PCR positive entry clones were cultured overnight in 3.5mL LB broth supplemented with 100µg/mL spectinomycin at 37ºC on a rotary shaker at 180rpm. For each entry clone broth culture, the following was performed.

A 2mL microfuge tube was filled with culture, spun for 2-3 minutes at 13,000*g* and the supernatant discarded. The pellet was resuspended in 100µL GTE buffer (Section 5.2) and placed on ice, then 200µL of NaOH/SDS solution (Section 5.2) was added and the microfuge tube rolled gently to break open the cells. To neutralise the solution, 150µL 3M potassium acetate was added and the tube was rolled to precipitate the DNA. The sample was placed at –20ºC for 10 minutes then centrifuged at 13,000*g* for 15 minutes. The supernatant was transferred to a clean 1.5mL microfuge tube and 1mL 100% ethanol added. The sample was placed on ice for a minimum of 5 minutes and centrifuged at 13,000*g* for 10 minutes. The supernatant was poured off and 200µL 70% ethanol was added prior to centrifugation at 13,000*g* for 2 minutes. The supernatant was removed using a pipette and the DNA was allowed to dry fully in a 37ºC incubator. The pellet of plasmid DNA was resuspended in 30µL of R40 (Section 5.2).

3.2.2.7. Restriction endonuclease digests

Restriction endonuclease digests were performed to confirm the results of the Colony PCR. The following generic procedure was applied to each putative entry vector varying only the restriction enzymes and their cognate buffers. Different enzyme combinations were used for each entry vector; pCR8/OsOrphan19, *Mlu*I, buffer H; pCR8/OsEREB67, *Mlu*I, buffer H, pCR8/OsMYBR63, *Eco*RI, H; pCR8/OsbHLH17, *Eco*RI, buffer H; pCR8/OsLUX, *Hpa*I, *Sex*AI, buffer 4, BSA; pCR8/OsMYB54, *Apa*I, *Acc*I, buffer 4.

Each restriction endonuclease digest contained; 5µL template DNA, 2µL 10X Buffer, 2µL bovine serum albumin (BSA) as necessary, 0.3µL of each restriction endonuclease and MilliQ water to 20µL. The restriction digests were incubated in a 37ºC water bath for 3 hours. The restriction digest products were separated by agarose gel electrophoresis and visualised in the gel using a transilluminator as per Section 2.2.3.6.

3.2.2.8. Glycerol culture stocks

Glycerol culture stocks were made for each entry vector. Each glycerol stock contained; 500µL overnight culture and 500µL of 80% glycerol. These were vortexed, frozen in liquid nitrogen and stored at –80ºC.

3.2.2.9. Sequencing of entry vectors

Entry vectors were sequenced as per Section 2.2.3.8 to confirm that the transcription factor CDSs were correct. Primers used were GW1 and GW3 (Table 6.4) which were positioned within the pCR8 vector, facing the insert.

3.2.3. Construction of expression vectors

3.2.3.1. 35S constitutive overexpression constructs

3.2.3.1.1. LR recombination reaction for the construction of expression vectors

LR recombination reactions were performed to recombine the transcription factor CDSs from the entry vectors into a destination vector suitable for plant transformation. Each LR recombination reaction contained an entry vector and a destination vector. The destination vector was generic, pMDC32 (Figure 3.2) and contained a dual 35S promoter to drive expression of the transcription factor CDS *in planta*. The entry vector was specific to each LR recombination reaction. The entry vectors were

pCR8/OsOrphan19, pCR8/OsEREB67, pCR8/OsMYBR63, pCR8/OsbHLH17, pCR8/OsLUX and pCR8/OsMYB54. The expression vectors produced were pMDC32/OsOrphan19, pMDC32/OsEREB67, pMDC32/OsMYBR63, pMDC32/OsbHLH17, pMDC32/OsLUX and pMDC32/OsMYB54.

The entry vector and destination vector DNA was diluted to 300ng/µL using sterile MilliQ water. Each reaction contained; 1µL entry vector 1 DNA (pCR8/OsCDS), 1µL destination vector DNA (pMDC32), 2µL Tris-EDTA, 1µL LR Clonase™ Plus II Enzyme Mix. The reaction mixture was incubated for 16 hours at 25ºC. To terminate the reaction, 0.5µL 2µg/µL Proteinase K was added to each reaction and incubated at 37ºC for 20 minutes.

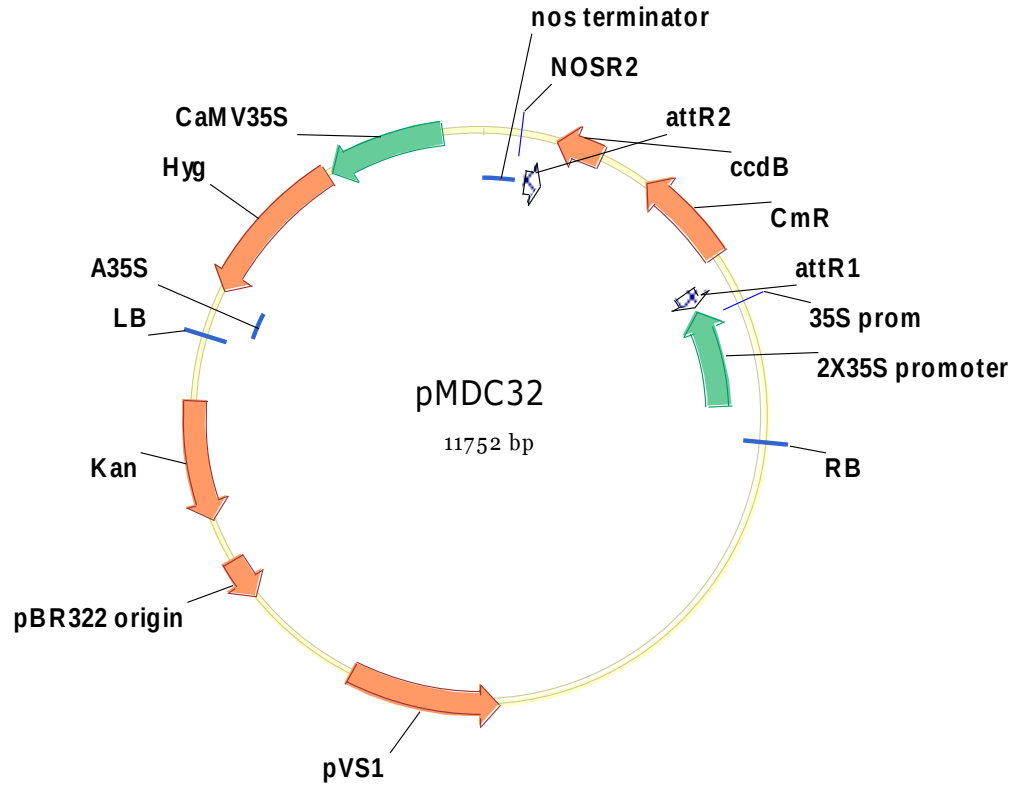


Figure 3.2 Schematic diagram representing the pMDC32 expression vector

Feature	**Description**
2X35S promoter	Tandem repeat of cauliflower mosaic virus 35S constitutive promoter
35S prom	Primer binding site in 35S promoter
CmR	Chloramphenicol resistance gene
attR2 and attR1	Bacteriophage λ-derived DNA recombination sequences that have been optimized to permit recombinational cloning of DNA fragments with specific *attL* sites
*ccd*B gene	Permits negative selection of the plasmid
Nos terminator	Nopaline synthase (Nos) terminator
NOSR2	Nos reverse primer site
CaMV35S	Cauliflower mosaic virus 35S promoter
Hygromycin resistance gene (Hyg)	Allows selection of the T-DNA *in planta*
A35S	Cauliflower Mosaic Virus 35S terminator
Kanamycin resistance gene (Kan)	Allows selection of the plasmid in *E. Coli*
pBR322 origin	Origin of replication for *E. Coli*
pVS1	Minimal replicon necessary for stable maintenance in *E. coli*
RB and LB	Right and left border sequences for excision of T-DNA

3.2.3.1.2. Transformation of DH5α competent cells

DH5α competent cells were transformed with the LR recombination reactions as per Section 3.2.2.5.

3.2.3.1.3. Restriction endonuclease digests to screen expression clones

Colonies were selected from the LB plates and grown overnight in 3.5mL of LB broth supplemented with 50µg/mL kanamycin, at 37ºC, on a rotary shaker at 180rpm. Plasmid DNA was isolated using the alkaline lysis DNA mini-prep procedure as per Section 3.2.2.6. Putative expression vector DNA was subjected to restriction endonuclease digestion to identify correct expression vectors as per Section 3.2.2.7 using the following enzymes and buffers; pMDC32/OsOrphan19 and pMDC32/OsEREB67, *Eco*RV, *Mlu*I, buffer H; pMDC32/OsMYBR63 and pMDC32/OsMYB54, *Eco*RV, *Hind*III, B; pMDC32/OsbHLH17, *Eco*RV, *Pst*I, buffer H; pMDC32/OsLUX, *Eco*RV, *Apa*I, buffer 4, BSA and *Eco*RV, *Sac*I, buffer A. The restriction digest products were separated by agarose gel electrophoresis and visualised in the gel using a transilluminator as per Section 2.2.3.6.

3.2.3.2. Salt inducible expression constructs

The salt inducible promoter JRC0470 (NCBI: BP432960), identified by (Rabbani *et al.*, 2003), was cloned from *O. sativa* cv. Nipponbare genomic DNA by Ms. Natalia Tikhomirov, ACPFG and provided in the vector pMDC164/JRC0470 (Figure 3.10).

3.2.3.2.1. Restriction endonuclease digests

Restriction endonuclease digests were performed on pMDC164/JRC0470 and each of the six pMDC32 based expression vectors (Section 3.2.1.1.1) using *Asc*I and *Pme*I. Each reaction contained; 10µL vector DNA, 2µL buffer 4, 2µL BSA, 0.5µL *Asc*I, 0.5µL *Pme*I and 5µL MilliQ water. Each reaction was incubated at 37°C for 16 hours.

3.2.3.2.2. Gel purification

The restriction digest products were separated on a 0.8% agarose 1X TAE gel containing ethidium bromide, at 90 volts for 60 minutes. The DNA was visualised in the gel using a transilluminator and the bands of appropriate sizes excised. The DNA was purified as per Section 3.2.2.2.

3.2.3.2.3. Ligation

The products from the gel purification were ligated together. Gel electrophoresis was used to ensure equal loading of the digested vector backbone and digested transcription factor CDS fragment. Each ligation included; 1µL pMDC32/JCR0470 cut with *Asc*I and *Pme*I, 1µL transcription factor CDS cut with *Asc*I and *Pme*I, 2µL T4 DNA ligase buffer, 0.5µL T4 DNA ligase and 5.5µL MilliQ water. The ligation reactions were incubated at 4°C overnight.

3.2.3.2.4. Transformation of competent cells

DH5α competent cells were transformed with the ligations as per Section 3.2.2.4.

3.2.3.2.5. Restriction endonuclease digests

Colonies of putative entry clones were cultured overnight, the plasmid DNA isolated as per Section 3.2.2.6 and screened using restriction endonuclease digests as per 3.2.2.7 using the enzymes *Kpn*I and *Sac*II, with Buffer 2 and BSA. The digest products were separated by agarose gel electrophoresis and visualised in the gel using a transilluminator as per Section 2.2.3.6.

3.2.3.2.6. Glycerol culture stocks

Glycerol culture stocks were made for each expression vector as per Section 3.2.2.8.

3.2.3.2.7. DNA sequencing

The expression vectors were sequenced as per Section 2.2.3.8 using primers pMDC32_3165R and JRC0470_3′F (Table 5.7).

3.2.3.3. JRC0470 reporter constructs

The reporter construct pMDC164/JRC0470 and the entry vector pENTR/JRC0470 were supplied by Ms. Natalia Tikhomirov, ACPFG. The reporter construct had the promoter, JRC0470, functionally linked to the reporter gene, *β-glucuronidase* (*uidA*). The JRC0470 promoter in pENTR/JRC0470 was recombined into the GFP reporter construct pMDC107 as per Section 3.2.3.1.1. DH5α competent cells were transformed with the LR recombination reactions as per Section 3.2.2.4. Colonies of putative entry clones were cultured overnight, the plasmid DNA isolated as per Section 3.2.2.6 and screened using restriction endonuclease digests as per 3.2.2.7 using the enzyme *Pvu*II

with Buffer 2. The digest products were separated by agarose gel electrophoresis and visualised in the gel using a transilluminator as per Section 2.2.3.6.

3.2.3.4. Artificial microRNA knockdown constructs

Artificial microRNA (amiRNA) constructs were created to reduce expression of the transcription factors as per Schab *et al.* (Ossowski *et al.*, 2008; Schwab *et al.*, 2006; Warthmann *et al.*, 2008). The locus identifiers for the genes of interest were submitted to Weigelworld WMD2 Web MicroRNA Designer (http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl). The program identified unique 21-nucleotide target sites for amiRNAs within the gene of interest to ensure single gene silencing. The 21-nucleotide sequence was submitted to the oligo design program on the website, which returned four oligo sequences for the site directed mutagenesis of pNW55.

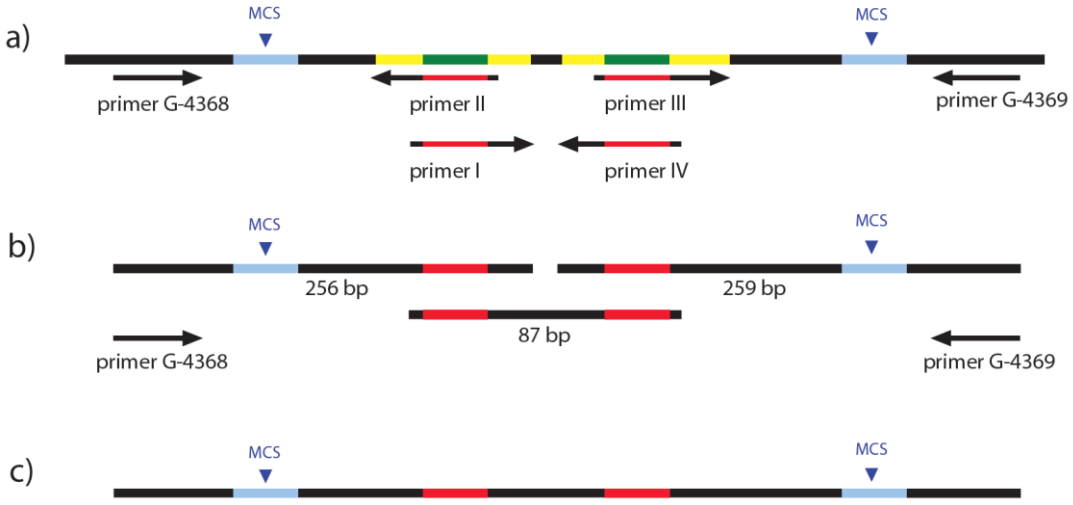


Figure 3.3 Schematic diagram representing the artificial microRNA construction using PCR

The amiRNA constructs were generated by modifying the vector pNW55 with specific primers using PCR. a) The original amiRNA sequences of pNW55 (green segments) are replaced by the new amiRNA sequences (red segments) in three PCR reactions using the primers I, II, III and IV. b) The three PCR products are combined in a fusion PCR. c) The modified pNW55 vector containing the new amiRNA sequences. Diagram from http://wmd2.weigelworld.org.

The site directed mutagenesis of pNW55 was performed using three PCR reactions that introduced the 21-nucleotide amiRNA sequences. A final fusion PCR then joined the three fragments to form the complete sequence that was introduced into the plant. See Figure 3.3. The following was performed to create six amiRNA constructs to knock down each of the six rice transcription factors. The primers used are in Table 5.5.

The PCRs were performed as follows: PCR1 contained primer G-4368 + primer II (product size 256bp), PCR2 contained primer I + primer IV (product size 87bp) and PCR3 contained primer III + primer G-4369 (product size 259bp). Each PCR reaction contained: 1µL 10µM primer A, 1µL 10µM primer B, 1µL 5mM dNTPs, 4µL 5X Phusion HF buffer, 50nM pNW55 vector template, 0.3µL Phusion DNA polymerase and 11.8µL MilliQ water. The thermocycler was programmed as follows: 1, 95°C for 2 minutes; 2, 95°C for 30 seconds; 3, 55°C for 30 seconds; 4, 72°C for 30 seconds; 5, repeat from step 2 for 34 times; 6, 72°C for 7 minutes.

All PCR products were separated on an agarose gel and purified as per Section 3.2.2.2.

The fusion PCR was used to join the three gel purified fragments together. Each reaction contained; 0.5µL PCR reaction 1, 0.5µL PCR reaction 2, 0.5µL PCR reaction 3, 1µL 5µM dNTPs, 2.5µL buffer A, 2.5µL buffer B, 0.5µL Elongase DNA polymerase, 1µL 10µM primer G-4368, 1µL 10µM primer G-4369. The thermocycler was programmed as follows: 1, 94°C for 30 seconds; 2, 94°C for 30 seconds; 3, 55°C for 30 seconds; 4, 68°C for 45 seconds; 5, repeat from step 2 for 34 times; 6, 68°C for 5 minutes; 7, 15°C forever.

All PCR products were separated on an agarose gel, the fragments of 554bp were cut out and purified as per Section 3.2.2.2.

The amiRNA expression fragments were cloned into pCR8 as per Section 3.2.2.3 and transformed into Mach1 chemically competent cells as per Section 3.2.2.4. An *Eco*RV restriction endonuclease digest was performed as per Section 3.2.2.7 to screen the entry vectors. Sequence identity was confirmed using sequencing as per Section 2.2.3.8, with the primers G-4368 and G-4369. The entry vectors generated were; pCR8/Orphan19 amiRNA, pCR8/OsEREB67 amiRNA, pCR8/OsMYBR63 amiRNA, pCR8/OsbHLH17 amiRNA, pCR8/OsLUX amiRNA and pCR8/OsMYB54 amiRNA.

The amiRNA expression fragments were recombined into pMDC32 as per Section 3.2.3.1.1. Restriction endonuclease digests were performed using the following enzymes and buffers as per Section 3.2.2.7, to screen the expression clones;

pMDC32/Orphan19 amiRNA, *Sac*II, buffer 4; pMDC32/OsEREB67 amiRNA, *Hinc*II, buffer 3, BSA; pMDC32/OsMYBR63 amiRNA, *Bst*BI, *Pvu*II, buffer 4; pMDC32/OsbHLH17 amiRNA, *Sca*I, buffer 3; pMDC32/OsLUX amiRNA, *Acc*I, *Pac*I, buffer 4, BSA; pMDC32/OsMYB54 amiRNA, *Ssp*I, HindIII, buffer 2. The restriction digest products were separated by agarose gel electrophoresis and visualised in the gel using a transilluminator as per Section 2.2.3.6.

3.2.4. *Agrobacterium*-mediated transformation of japonica rice

3.2.4.1. Transformation of *Agrobacterium tumefaciens*

A. tumefaciens AGL1 was transformed with the vectors from Section 3.2.3 using a freeze-thaw method. The cells were thawed on the bench, 2µL of 1µg/µL vector was added, the cells were frozen in liquid nitrogen for one minute and then incubated in a 37°C water bath for five minutes. LB medium (200uL) was added and the cells were incubated at 28°C and 180rpm for two hours, then spread on LBA supplemented with rifampicin (50µg/mL) and kanamycin (50µg/mL) and incubated at 28°C for two to three days.

Restriction digests were used to screen for *A. tumefaciens* colonies that contained an expression vector. LB medium (2mL) supplemented with rifampicin (50µg/mL) and kanamycin (50µg/mL) was inoculated with an *A. tumefaciens* clone and incubated at 28°C overnight with shaking at 180rpm. The entire culture was added to 50mL LB medium supplemented with rifampicin (50µg/mL) and kanamycin (50µg/mL) and incubated at 28°C overnight with shaking at 180rpm. Plasmid DNA was extracted using a QiaPrep Spin Miniprep Kit, as per the manufacturer's instructions. Restriction endonuclease digests were performed as per Section 3.2.3.1.3 for 35S overexpression vectors except pMDC32/OsbHLH17, and as per Section 3.2.3.2.5 for salt inducible overexpression vectors. PCR was used to screen *A. tumefaciens* cultures containing the vector pMDC32/OsbHLH17 as per Section 3.2.2.5 with the following changes; primers were NOS2R and OsbHLH17.Code_F, the annealing temperature was 53°C, the extension time was 2.5 minutes and 40 cycles of amplification were performed. PCR was used to screen *A. tumefaciens* cultures containing amiRNA knockdown vectors as per Section 3.2.2.5 with the following changes; primers were the generic NOSR2 (Table 5.7) and primer I (Table 5.5), specific for each construct from Section 3.2.3.4, the annealing temperature was 53°C, the extension time was 1.5 minutes and 40 cycles of amplification were performed.

3.2.4.2. Rice transformation

O. sativa ssp. *japonica* cv. Nipponbare calli were transformed using *Agrobacterium*-mediated transformation (Sallaud *et al.*, 2003). Callus transformation and any derivations are outlined briefly, below. The calli were transformed with 20 different vectors: six 2x35S constitutive overexpression vectors and six salt inducible overexpression vectors driving expression of six transcription factor CDSs, six amiRNA

expression vectors to knockdown expression of the six transcription factors, one *salt inducible promoter:GFP reporter* construct and one *salt inducible promoter:uidA reporter* construct. All vectors are from Section 3.2.2.

Approximately 400 rice seeds were dehusked and placed in 70% ethanol for one minute with occasional agitation. The seeds were then immersed in 30% bleach and 20µL Tween20 for 30 minutes with occasional agitation, followed by five rinses in sterile MilliQ water. Ten seeds were placed on each plate of NB medium and sealed with Parafilm. The plates were incubated at 28ºC in the dark for four weeks to induce callus formation. Small yellow/white calli were transferred to fresh NB medium for a further two weeks of growth. The mature calli were coincubated with an *A. tumefaciens* suspension in liquid CCL medium for 15 minutes. This was prepared by culturing the *A. tumefaciens* on AB medium supplemented with rifampicin (50µg/mL) and kanamycin (50µg/mL) for three days, pelleting the cells and suspending in liquid CCL medium to an OD_{600} of 0.2-0.4 at 600nm. The calli were dried on sterile filter paper, transferred to CCS medium and incubated for three days at 25°C in the dark. The calli were then rinsed in MilliQ water supplemented with cefotaxime (400µg/mL) and vancomycin (100µg/mL) for ten minutes to eliminate the *A. tumefaciens*, before being dried on sterile filter paper and transferred to selective R2S medium. The calli were incubated at 28ºC in the dark for one week, rolled over to ensure that all surfaces had been in contact with the selective medium, then incubated for a further week. The calli were transferred to NBS medium and incubated at 28°C for one week, then rolled on the surface of the medium to dislodge the transformed regenerating calli. The calli were incubated at 28°C for a further two weeks, transferred to PRAG medium for seven days, then to RN medium for two days in the dark at 28°C. The plates of RN medium were then incubated at 28°C with a 12/12-hour (light/dark) photoperiod at an intensity of $100\mu mol.m^2.second^{-1}$ for four to six weeks. Individual regenerating shoots were selected from each callus and the surrounding callus excised. The regenerating shoots and roots were both trimmed to 20mm in length and the trimmed plantlets were inserted into tubs of P medium. These were incubated as above for three weeks. The plantlets were trimmed again and planted in Jiffy Peat Pots soaked in RO water. The plants were grown in a growth room at 28°C during the day and 22°C during the night, at 70-90% humidity with a 12/12-hour (light/dark) photoperiod at an intensity of $440\mu mol.m^2.second^{-1}$. After two weeks, the rice plants in their Jiffy Peat Pots were transferred to one litre pots filled with UC Davis soil mix, half submerged in RO water. The plants were fertilised with ACPFG nutrient solution (Table 5.8) weekly. After three

to four months when the plants had set seed, water was withheld until the plants were dry. The seed was harvested and dried in a 37°C drying oven for five days. The seed was stored at RT in the dark.

3.3. Results

3.3.1. Expression vector construction

3.3.1.1. Cloning transcription factor coding sequences

The transcription factor CDSs for the genes *OsOrphan19*, *OsEREB67*, *OsLUX* and *OsMYB54* were cloned from *O. sativa* ssp. *indica* cv. FL478 cDNA. The CDSs for the genes *OsMYBR63* and *OsbHLH17* were cloned from *O. sativa* ssp. *japonica* cv. Nipponbare genomic DNA.

The four CDSs cloned from *O. sativa* ssp. *indica* cv. FL478 cDNA, had 100% AA identity with the published sequence for *O. sativa* ssp. *japonica* cv. Nipponbare. The gene *OsMYBR63*, cloned from *O. sativa* ssp. *japonica* cv. Nipponbare, had 100% AA identity with the published sequence for that cultivar, but the gene *OsbHLH17* did not. It contained two AA substitutions, S45R and G68S, which are polar neutral to polar basic and nonpolar neutral to polar neutral changes, respectively. Both were outside of the bHLH DNA-binding domain and outside any other conserved regions.

3.3.1.2. 2x35S overexpression vectors

Six vectors were generated with the cauliflower mosaic virus dual 35S promoter driving expression of the six different transcription factor CDSs (e.g. *2x35S:OsOrphan19)*. The vector maps are present as Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8 and Figure 3.9.

Two vectors were generated with the salt inducible promoter, JRC0470, driving expression of the CDSs of *uidA* and *GFP* (e.g. *JRC0470:uidA*). The vector maps are present as Figure 3.10 and Figure 3.11, respectively.

Six vectors were generated with the salt inducible promoter, JRC0470, driving expression of the six different transcription factor CDSs (e.g. *JRC0470:OsOrphan19*). The vector maps are present as Figure 3.12, Figure 3.13, Figure 3.14, Figure 3.15, Figure 3.16 and Figure 3.17.

Six vectors were generated with the cauliflower mosaic virus dual 35S promoter driving expression of the six different amiRNA sequences, designed to knock down expression of the six different transcription factors (*2x35S:OsOrphan19 amiRNA*). The vector maps are present as Figure 3.18, Figure 3.19, Figure 3.21, Figure 3.22 and Figure 3.23.

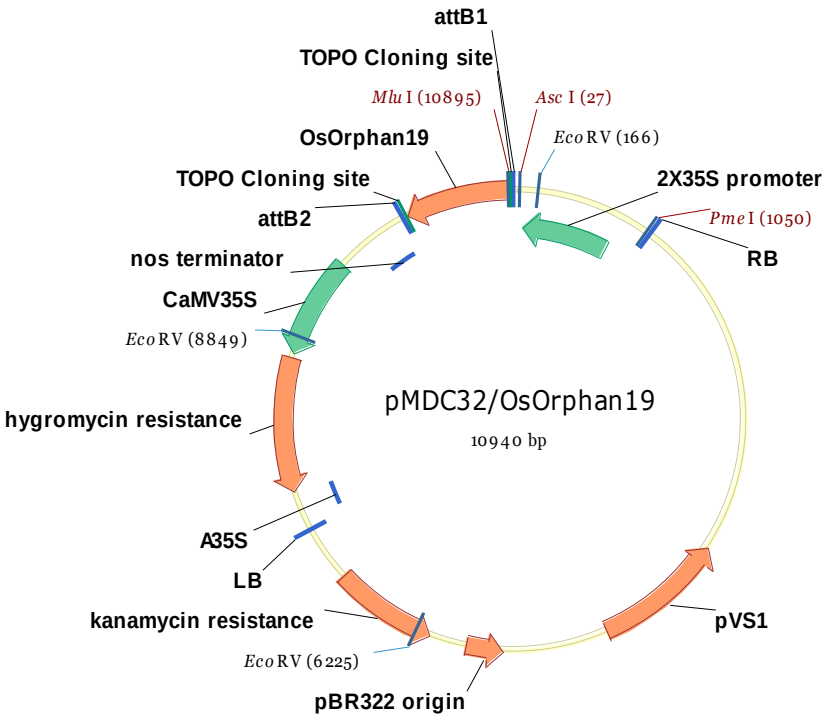


Figure 3.4 Schematic diagram representing the pMDC32/OsOrphan19 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; *OsOrphan19* transcription factor CDS (*2x35S:OsOrphan19*). For a detailed description of vector features, see the legend for Figure 3.2.

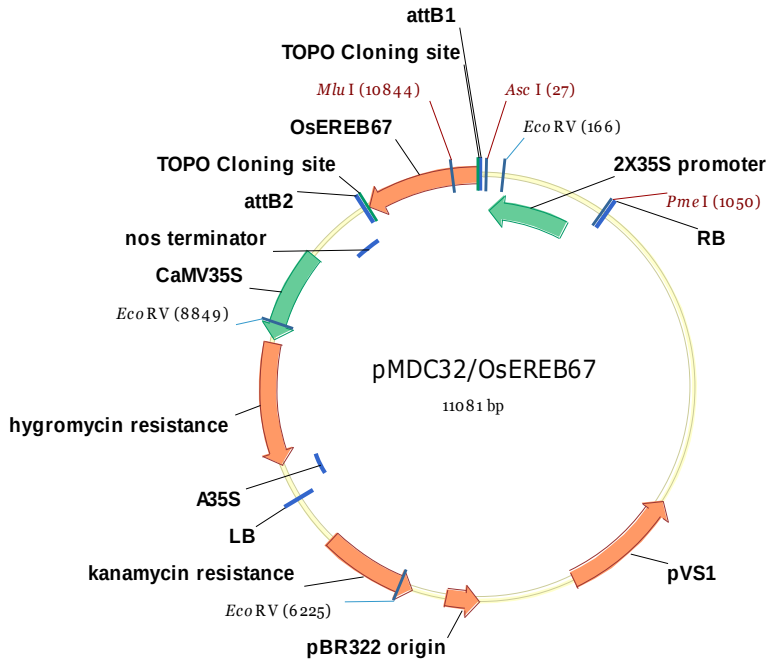


Figure 3.5 Schematic diagram representing the pMDC32/OsEREB67 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; *OsEREB67* transcription factor CDS (*2x35S:OsEREB67*). For a detailed description of vector features, see the legend for Figure 3.2.

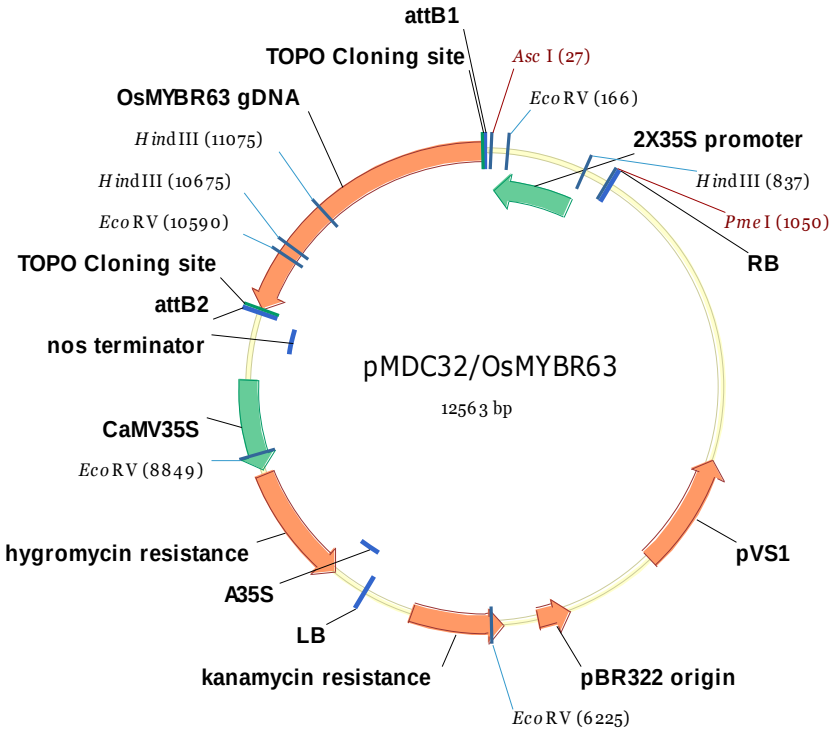


Figure 3.6 Schematic diagram representing the pMDC32/OsMYBR63 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; *OsMYBR63* transcription factor CDS (*2x35S:OsMYBR63*). For a detailed description of vector features, see the legend for Figure 3.2.

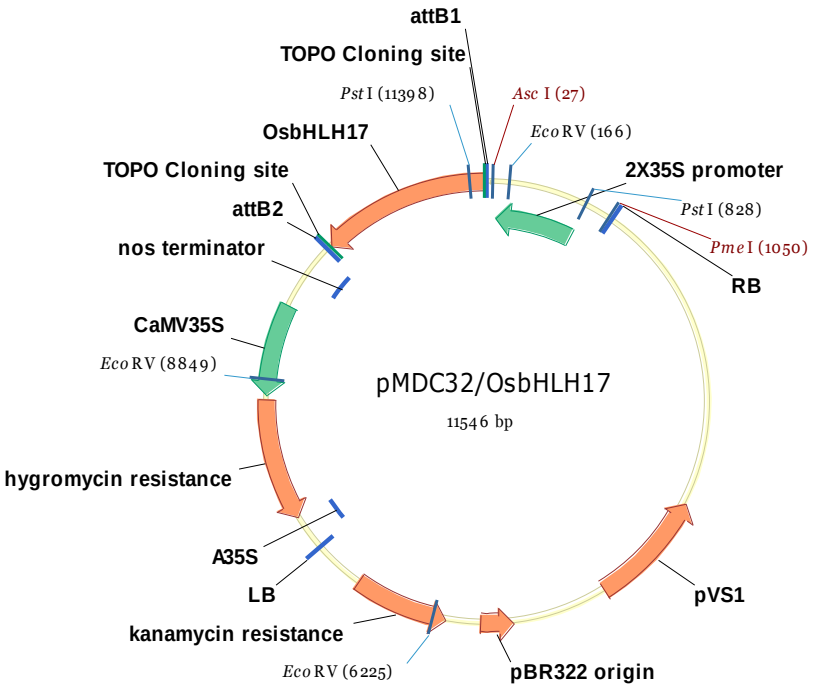


Figure 3.7 Schematic diagram representing the pMDC32/OsbHLH17 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; *OsbHLH17* transcription factor CDS (*2x35S:OsbHLH17*). For a detailed description of vector features, see the legend for Figure 3.2.

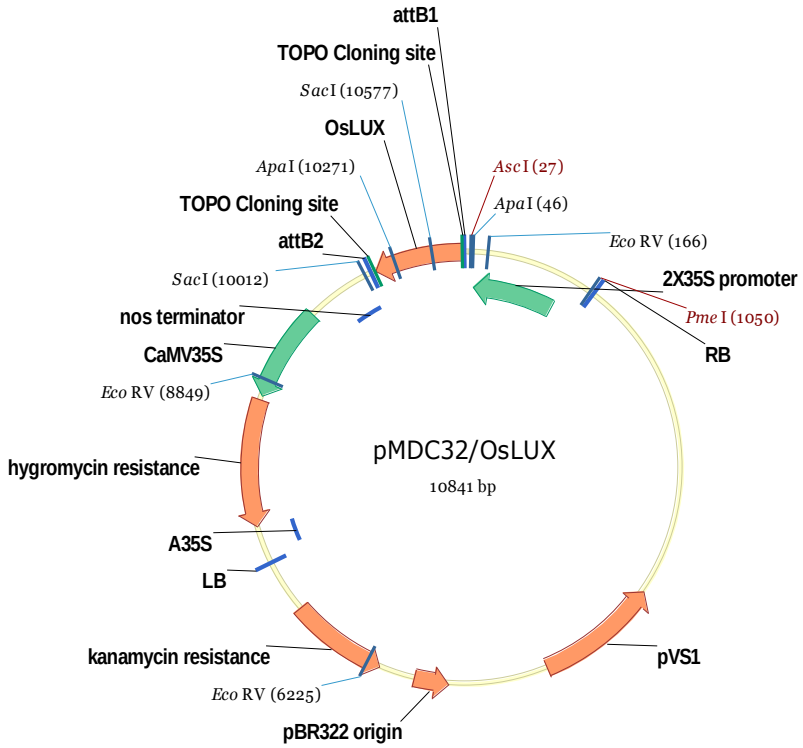


Figure 3.8 Schematic diagram representing the pMDC32/OsLUX expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; *OsLUX* transcription factor CDS (*2x35S:OsLUX*). For a detailed description of vector features, see the legend for Figure 3.2.

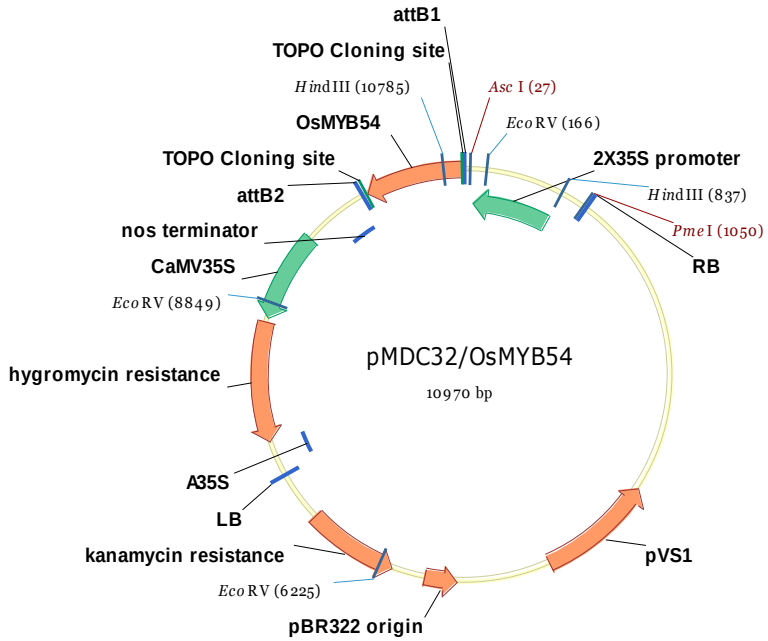


Figure 3.9 Schematic diagram representing the pMDC32/OsMYB54 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; *OsMYB54* transcription factor CDS (*2x35S:OsMYB54*). For a detailed description of vector features, see the legend for Figure 3.2.

3.3.1.3. Salt inducible expression vectors and reporter constructs

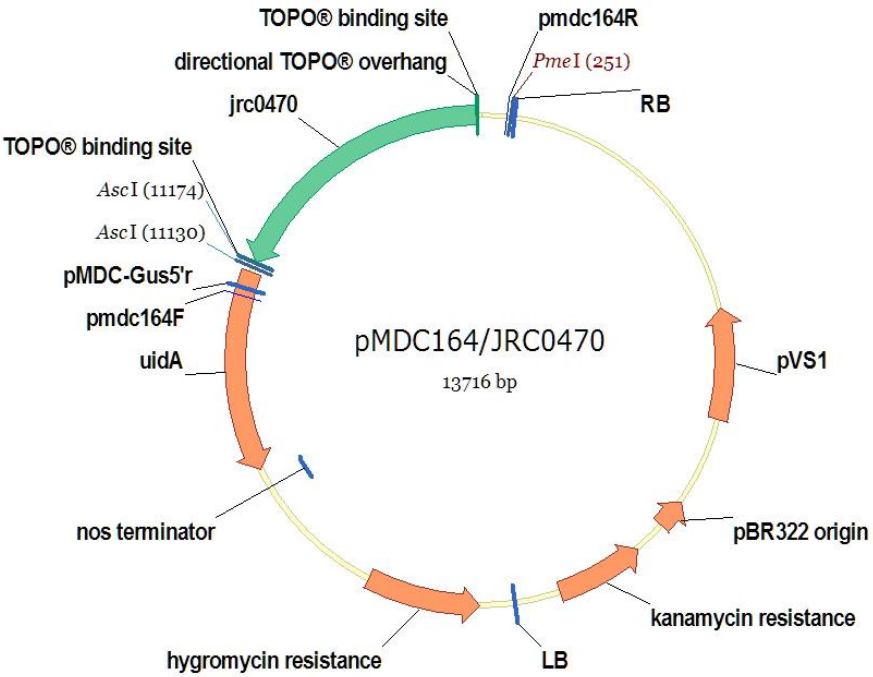


Figure 3.10 Schematic diagram representing the pMDC164/JRC0470 expression vector

The expression vector was generated for transformation into rice plants. The reporter construct was based on the pMDC32 backbone, with the T-DNA segment containing the salt inducible promoter, JRC0470, driving expression of the *uidA* reporter gene (*JRC0470:uidA*). The reporter construct gives spatial and temporal information about the JRC0470 promoter through detection of the β-glucuronidase gene product using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt staining. For a detailed description of vector features, see the legend for Figure 3.2.

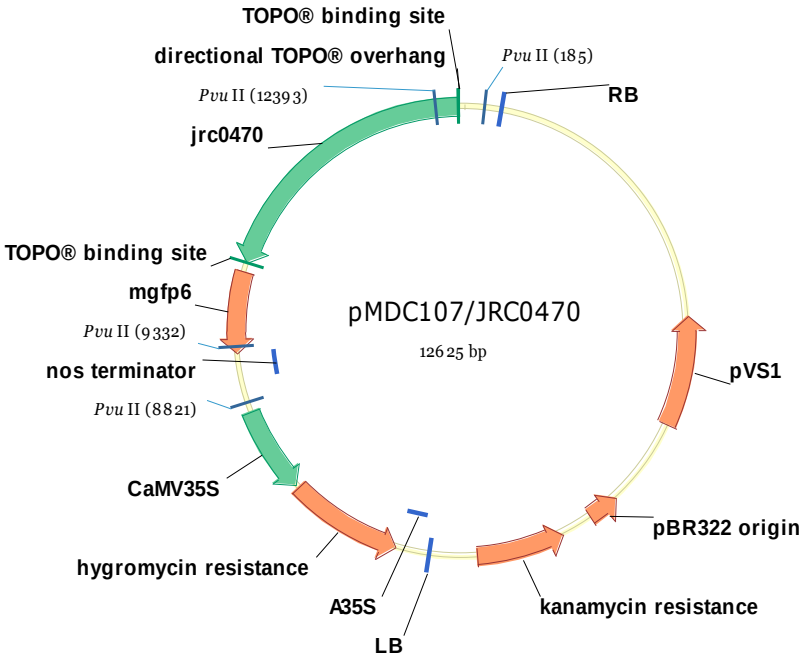


Figure 3.11 Schematic diagram representing the pMDC107/JRC0470 expression vector

The expression vector was generated for transformation into rice plants. The reporter construct was based on the pMDC32 backbone, with the T-DNA segment containing the salt inducible promoter, JRC0470, driving expression of the *mgfp6* reporter gene (*JRC0470:mgfp6*). The reporter construct gives spatial and temporal information about the JRC0470 promoter through detection of the GFP protein using fluorescence spectroscopy. For a detailed description of vector features, see the legend for Figure 3.2.

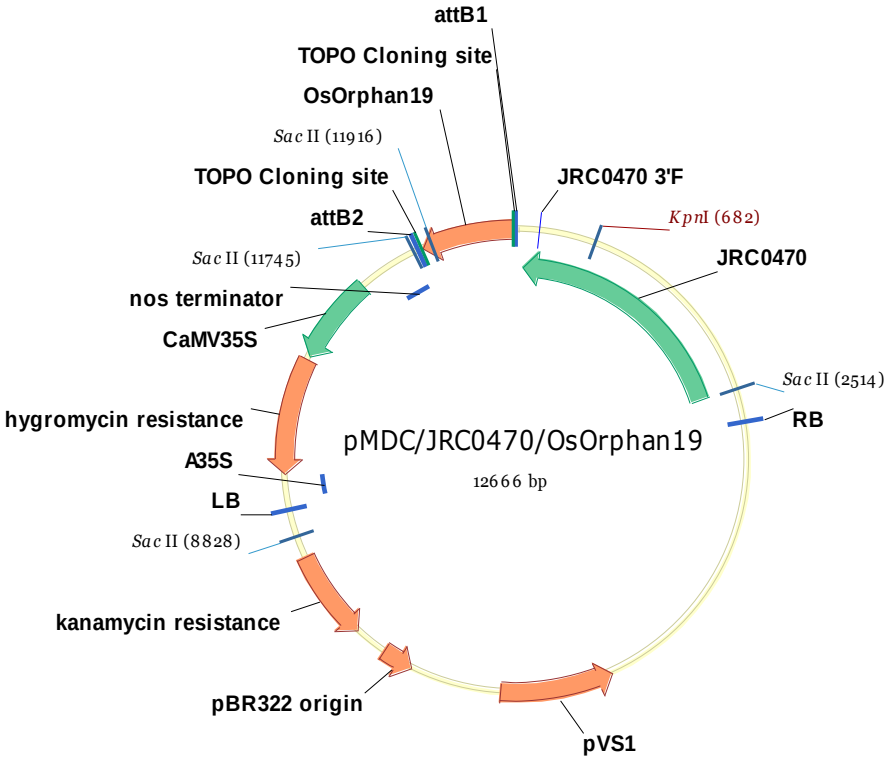


Figure 3.12 Schematic diagram representing the pMDC/JRC0470/OsOrphan19 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with the salt inducible promoter, JRC0470, driving expression of the insert; *OsOrphan19* transcription factor CDS (*JRC0470:OsOrphan19*). For a detailed description of vector features, see the legend for Figure 3.2.

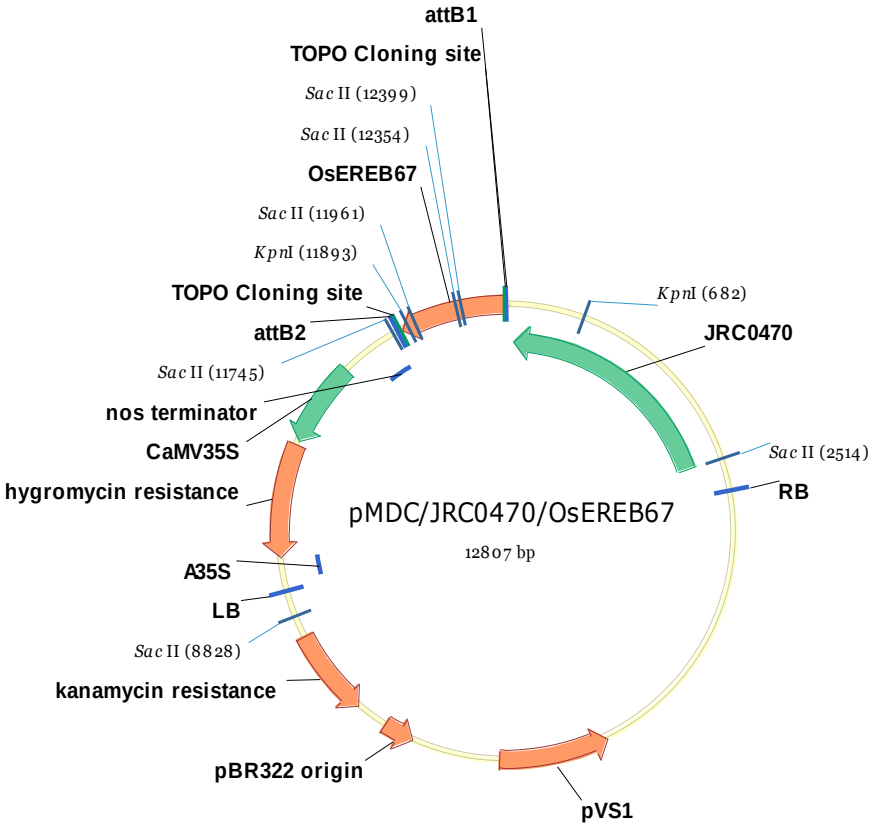


Figure 3.13 Schematic diagram representing the pMDC/JRC0470/OsEREB67 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with the salt inducible promoter, JRC0470, driving expression of the insert; *OsEREB67* transcription factor CDS (*JRC0470:OsEREB67*). For a detailed description of vector features, see the legend for Figure 3.2.

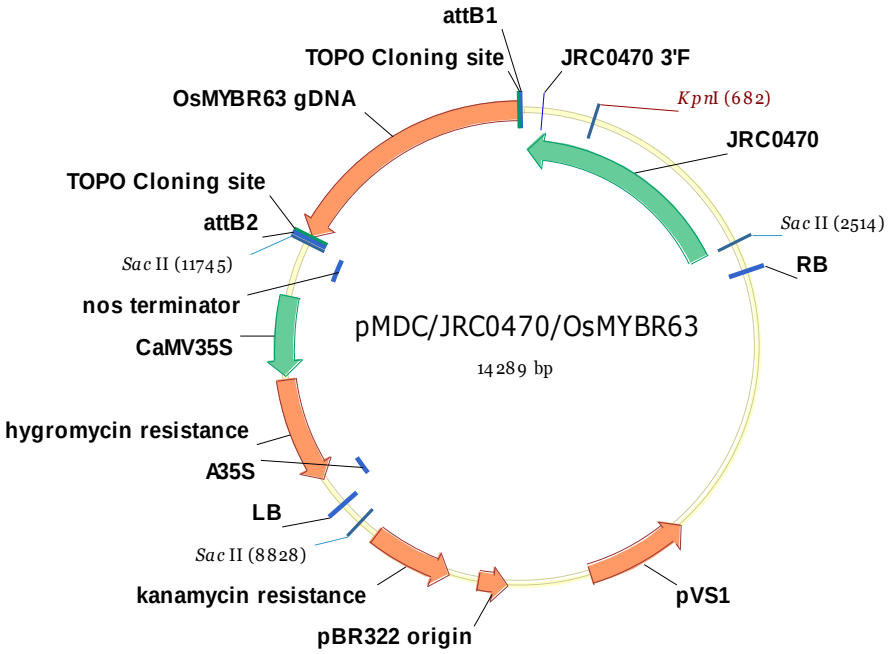


Figure 3.14 Schematic diagram representing the pMDC/JRC0470/OsMYBR63 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with the salt inducible promoter, JRC0470, driving expression of the insert; *OsMYB63* transcription factor CDS (*JRC0470:OsMYBR63*). For a detailed description of vector features, see the legend for Figure 3.2.

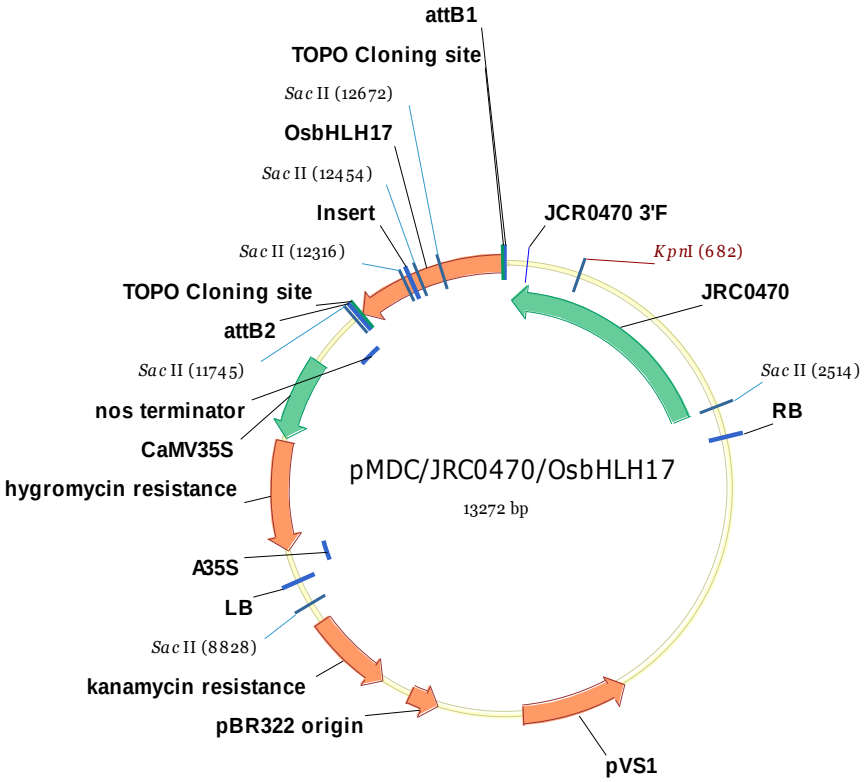


Figure 3.15 Schematic diagram representing the pMDC/JRC0470/OsbHLH17 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with the salt inducible promoter, JRC0470, driving expression of the insert; *OsbHLH17* transcription factor CDS (*JRC0470:OsbHLH17*). For a detailed description of vector features, see the legend for Figure 3.2.

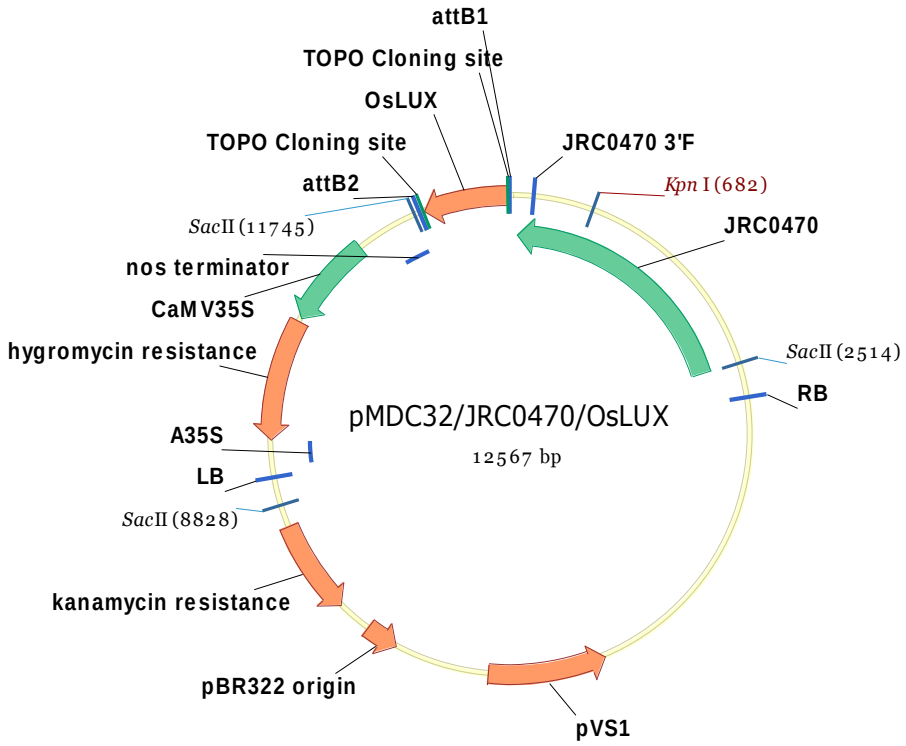


Figure 3.16 Schematic diagram representing the pMDC/JRC0470/OsLUX expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with the salt inducible promoter, JRC0470, driving expression of the insert; *OsLUX* transcription factor CDS (*JRC0470:OsLUX*). For a detailed description of vector features, see the legend for Figure 3.2.

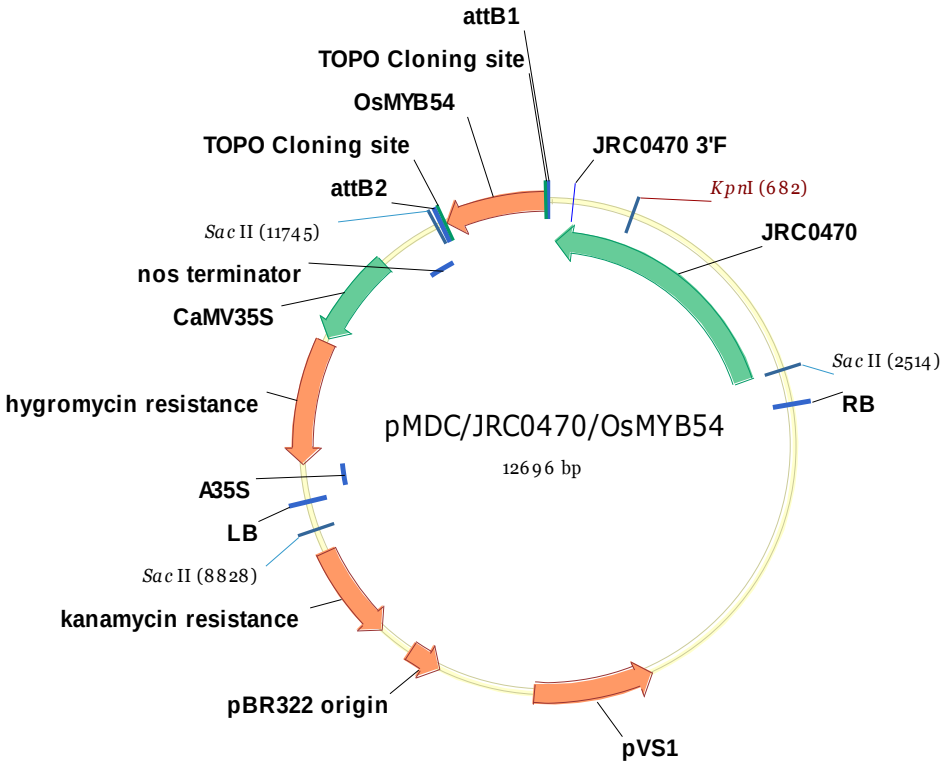


Figure 3.17 Schematic diagram representing the pMDC/JRC0470/OsMYB54 expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with the salt inducible promoter, JRC0470, driving expression of the insert; *OsMYB54* transcription factor CDS (*JRC0470:OsMYB54*). For a detailed description of vector features, see the legend for Figure 3.2.

3.3.1.4. Artificial microRNA knockdown expression vectors

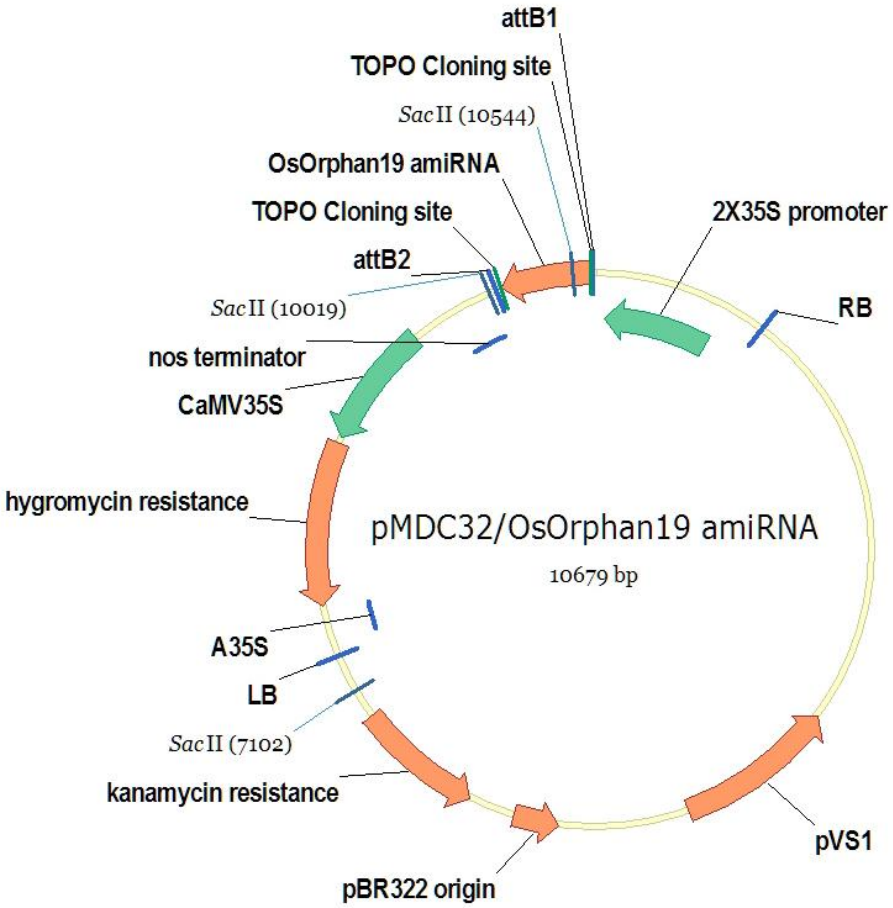


Figure 3.18 Schematic diagram representing the pMDC32/Orphan19 amiRNA expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; amiRNA targeting the *OsOrphan19* transcription factor CDS (*2x35S:OsOrphan19 amiRNA*). For a detailed description of vector features, see the legend for Figure 3.2.

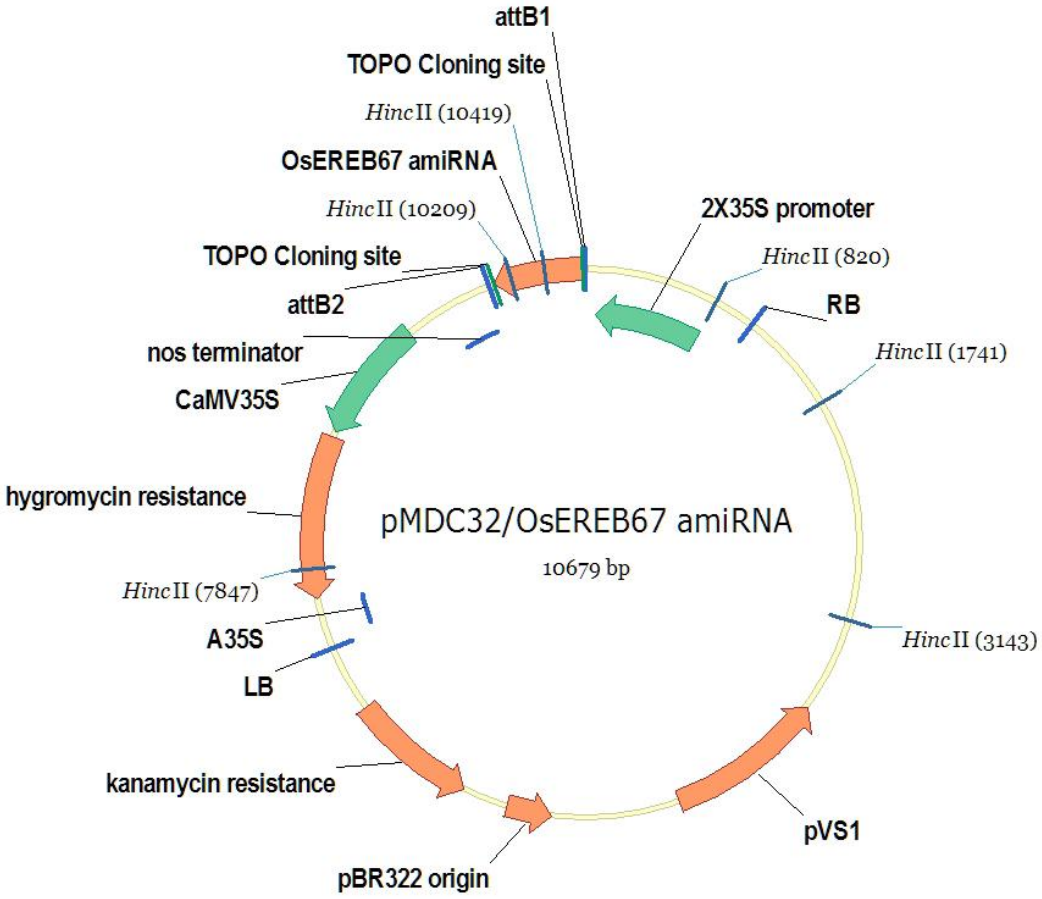


Figure 3.19 Schematic diagram representing the pMDC32/OsEREB67 amiRNA expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; amiRNA targeting the *OsEREB67* transcription factor CDS (*2x35S:OsEREB67 amiRNA*). For a detailed description of vector features, see the legend for Figure 3.2.

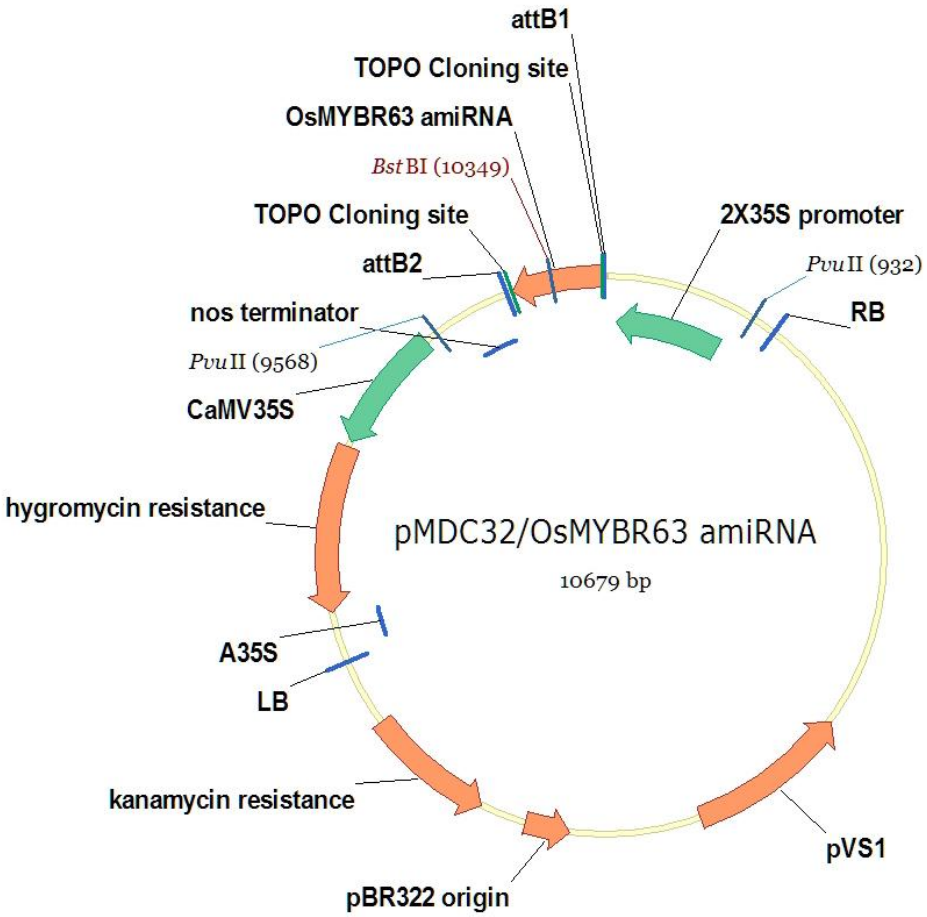


Figure 3.20 Schematic diagram representing the pMDC32/OsMYBR63 amiRNA expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; amiRNA targeting the *OsMYBR63* transcription factor CDS (*2x35S:OsMYBR63 amiRNA*). For a detailed description of vector features, see the legend for Figure 3.2.

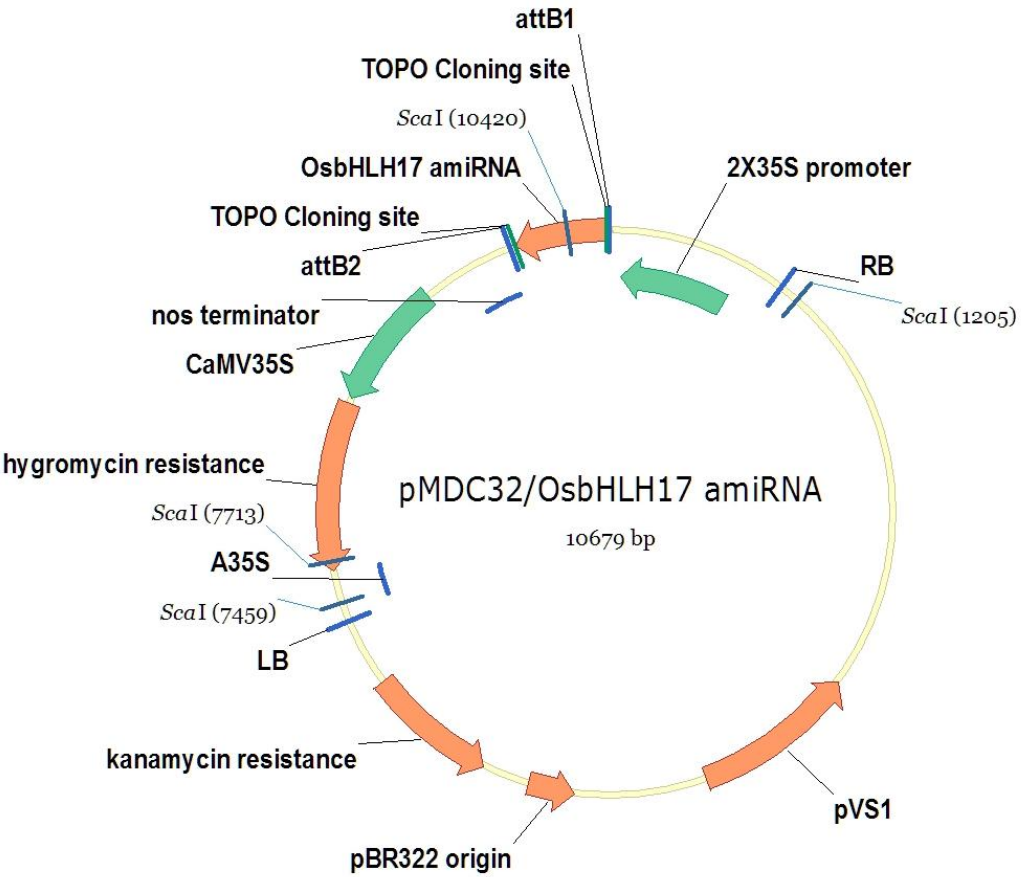


Figure 3.21 Schematic diagram representing the pMDC32/OsbHLH17 amiRNA expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; amiRNA targeting the *OsbHLH17* transcription factor CDS (*2x35S:OsbHLH17 amiRNA*). For a detailed description of vector features, see the legend for Figure 3.2.

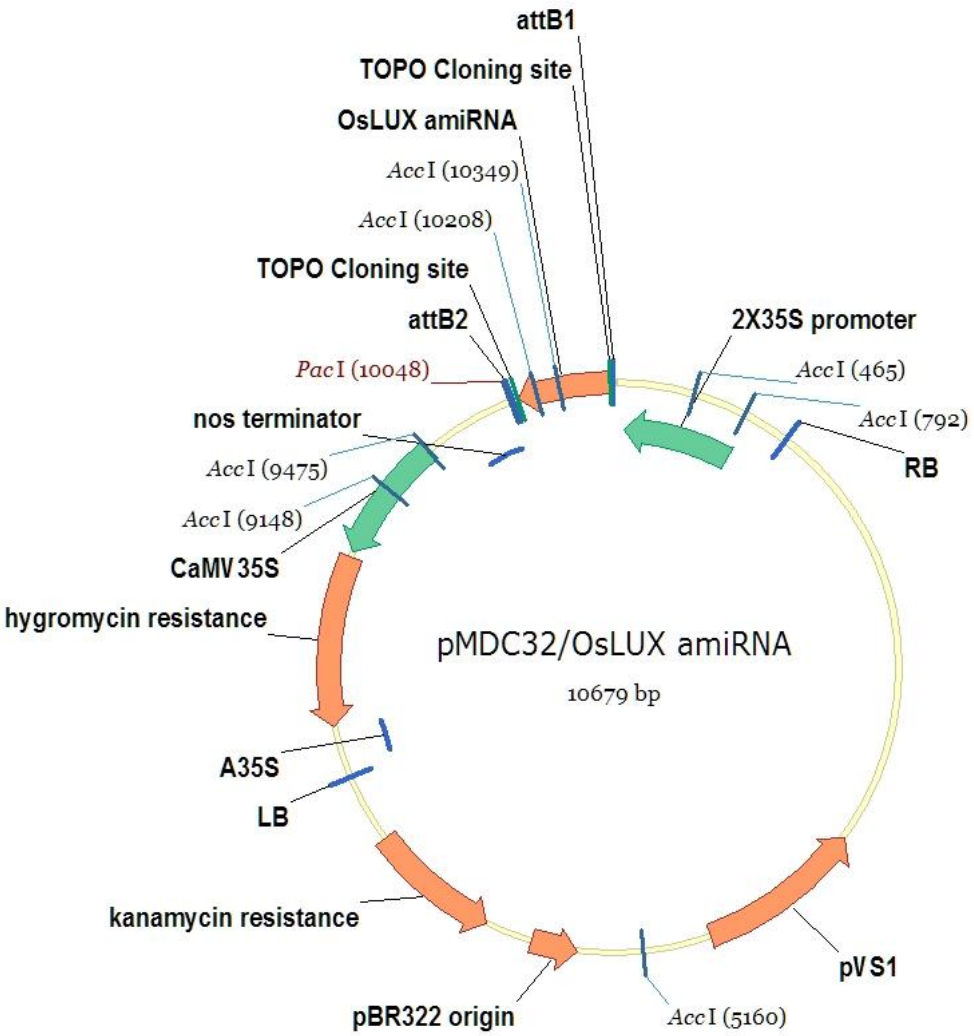


Figure 3.22 Schematic diagram representing the pMDC32/OsLUX amiRNA expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; amiRNA targeting the *OsLUX* transcription factor CDS (*2x35S:OsLUX amiRNA*). For a detailed description of vector features, see the legend for Figure 3.2.

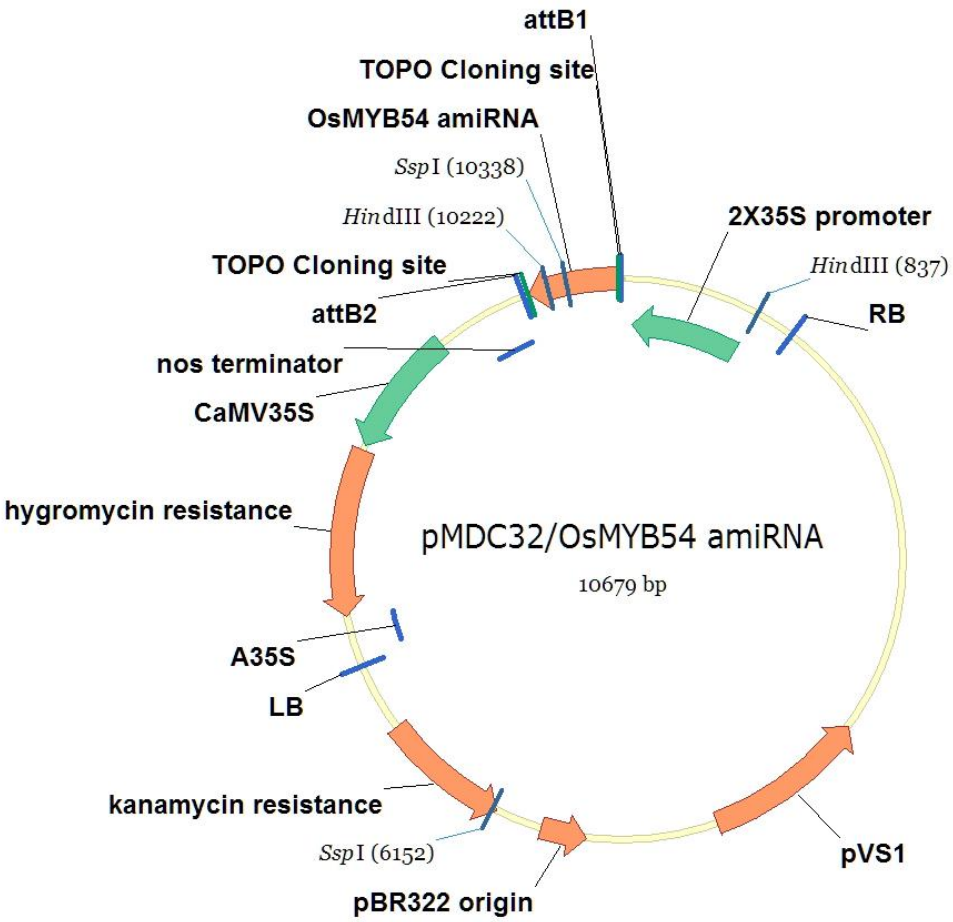


Figure 3.23 Schematic diagram representing the pMDC32/OsMYB54 amiRNA expression vector

The expression vector was generated for transformation into rice plants. The expression vector was based on the pMDC32 backbone, with a dual 35S promoter driving expression of the insert; amiRNA targeting the *OsMYB54* transcription factor CDS (*2x35S:OsMYB54 amiRNA*). For a detailed description of vector features, see the legend for Figure 3.2.

3.3.2. *Agrobacterium*-mediated transformation of japonica rice

A. tumefaciens strain AGL1 was used to introduce the T-DNA sequence from each of the vectors from Section 3.3.1 into callus from *O. sativa* ssp. *japonica* cv. Nipponbare. Transgenic shoots and roots were regenerated under selection to produce whole plants, from which seed was collected. Approximately 15 lines were generated per construct.

3.3.2.1. Rice plants with constitutive overexpression of transcription factor coding sequences displayed abnormal phenotypes

The transgenic rice lines with constitutive overexpression of transcription factor CDSs displayed abnormal phenotypes when regenerating from callus. The lines transformed with the construct *2x35S:OsEREB67*, were severely affected. Only 14% of calli produced shoots, compared to 40-87% for lines transformed with different constructs. Of all lines containing the *2x35S:OsEREB67* construct that regenerated from callus, only two gave seed and only one gave sufficient seed for further analysis. All others were sterile.

The lines transformed with the construct *2x35S:MYBR63* had poor root growth when regenerating from callus and the lines transformed with the construct *2x35S:OsMYB54* had abundant fine roots growing in all directions. The lines transformed with these two constructs, with *2x35S:OsOrphan19* and with *2x35S:OsLUX*, all had delayed seed set and poor grain filling, where some plants would only have a few florets fill per panicle.

3.3.2.2. Rice plants with salt inducible overexpression of transcription factor coding sequences displayed abnormal phenotypes

Rice callus transformed with some of the salt inducible constructs had poor shoot regeneration, resulting in a limited number of transgenic lines. These lines also had poor seed set. These are in the order of; construct-number of lines (lines that produced seed). *JRC0470:OsOrphan19*- 14(5); *JRC0470:OsEREB67* -3(2); *JCR0470:MYBR63* -5(1); *JRC0470:OsbHLH17* -8(4); *JRC0470:OsLUX* -13(7); *JRC0470:OsMYB54* -8(5). Most constructs yielded seed in more than half of the regenerating plants. *JCR0470:MYBR63* was the worst performing.

The salt inducible line transformed with the construct *JRC0470:OsMYB54*, had an abnormal phenotype relative to wild type plants. The tillers were thicker at the base and pushed against each other, forcing the tillers to spread out in a fan shape. As shown in Figure 3.24, the phenotype varied from mild to extreme amongst all lines containing the

construct. The plants were also drought stressed for 24 hours, accidentally, as they were on a three day schedule, but were not watered until the fourth day. The plants with the most extreme phenotype and that had delayed development turned brown and the leaves became crisp. See Figure 3.25. The plants with a less extreme phenotype only appeared dehydrated, however the florets with developing rice grains turned white and died. See Figure 3.26. This only occurred in plants containing the *JRC0470:OsMYB54* construct. There were no abnormal phenotypes in other lines that contained the salt inducible promoter, JCR0470, driving expression of transcription factor CDSs. No control plants at a similar developmental stage were available for comparison at the time when the photographs were taken.

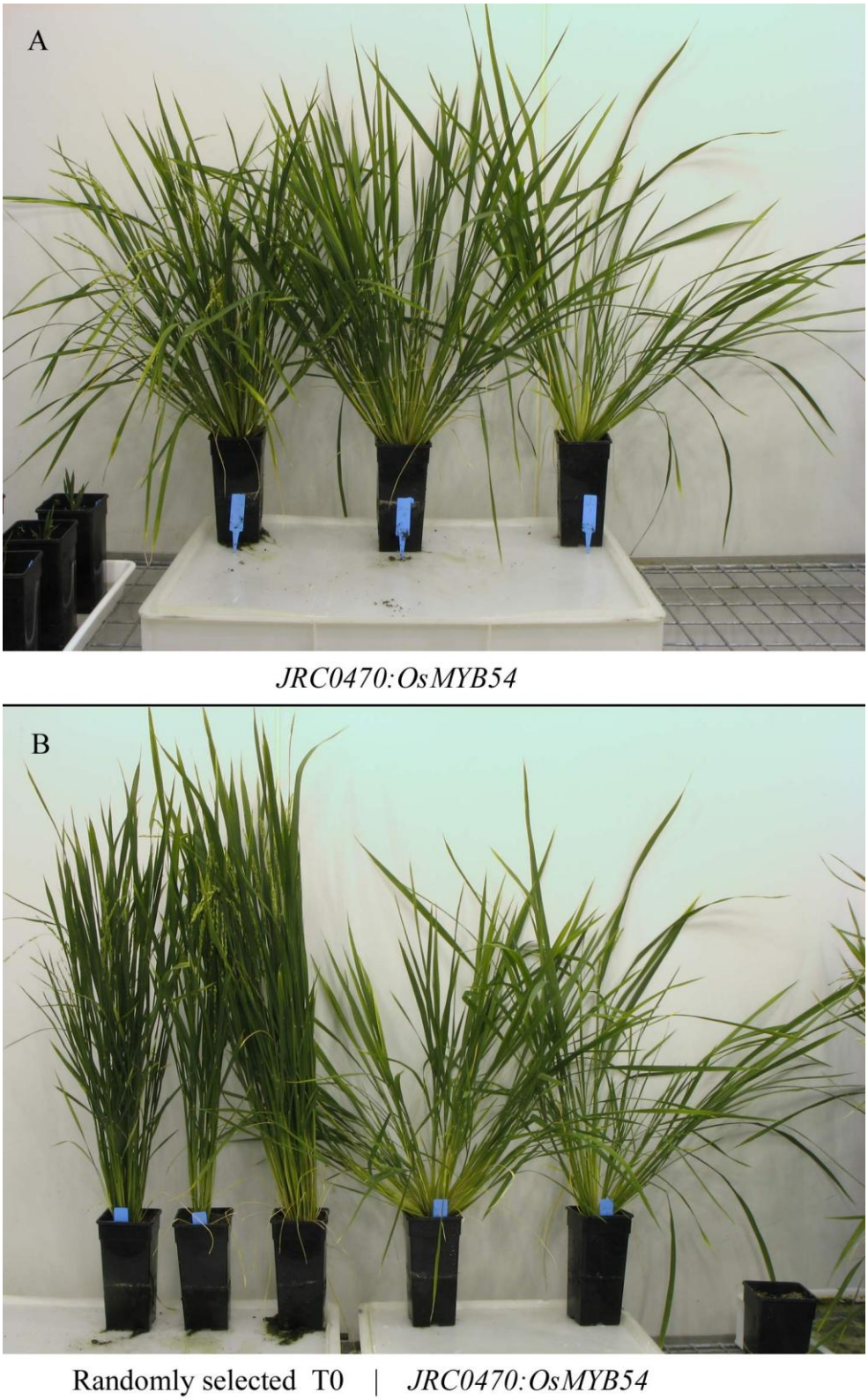


Figure 3.24 Photograph depicting the fan shaped phenotype of transgenic plants

The thicker tillers caused overcrowding and caused them to spread out in a fan shape, in transgenic plants containing the construct *JRC0470:OsMYB54*. Panel A shows three plants containing the construct *JRC0470:OsMYB54*. Panel B shows three randomly selected T0 transgenic lines containing different constructs on the left and two plants containing the construct *JRC0470:OsMYB54* on the right. Control plants were not available for comparison.

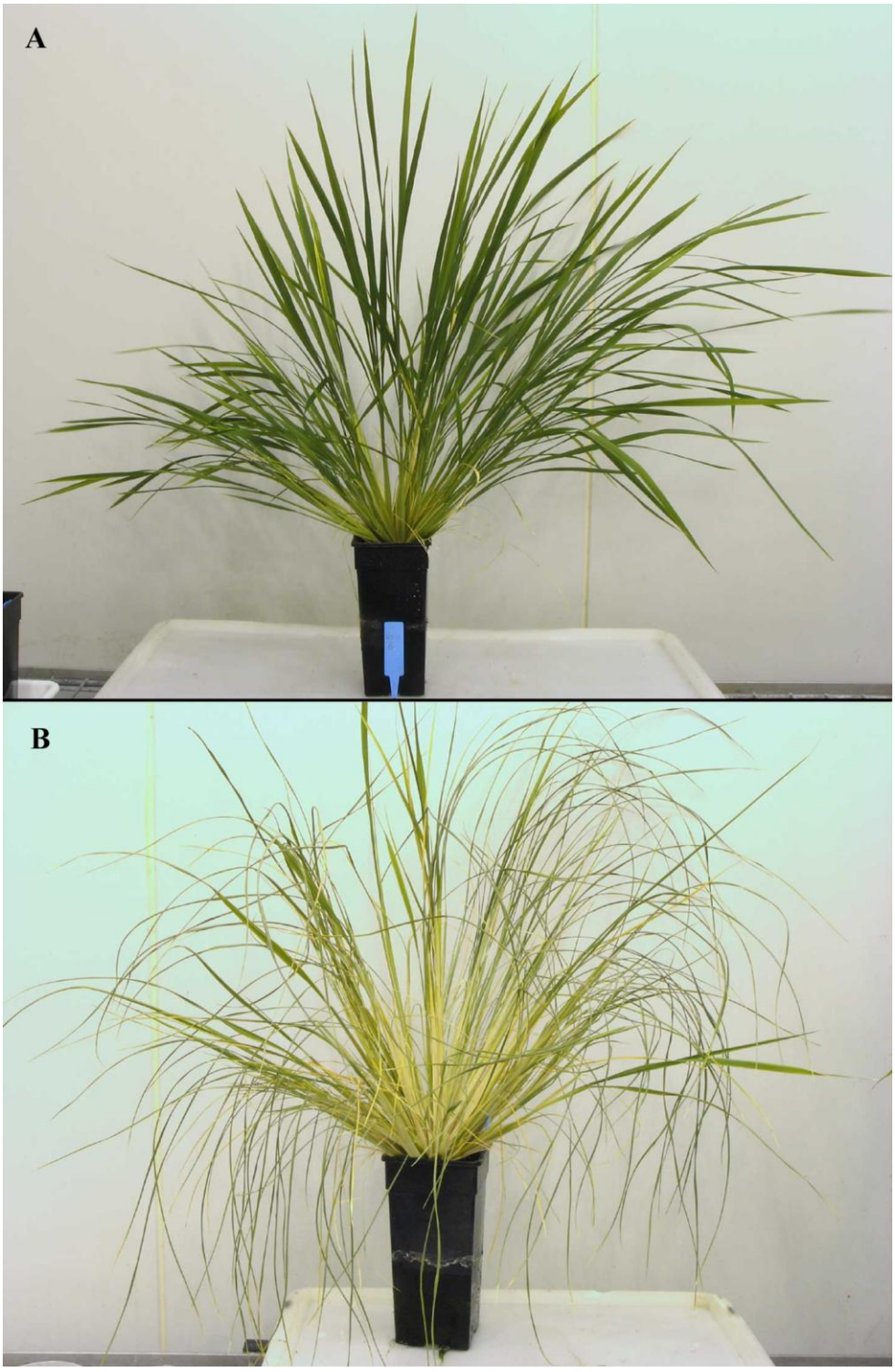


Figure 3.25 Photograph depicting the drought sensitive phenotype of *JRC0470:OsMYB54* rice

Regenerating transgenic rice plants were found to be extremely sensitive to water stress. The plants were on a three day watering cycle but were accidentally not watered until day four, so were water stressed for one day. The panels show a representative plant that had one of the most extreme bushy phenotypes. Panel A shows the plant several days before the drought event. Panel B shows the plant on the day following the drought event, where all the leaves are brown and crisp. Control plants were not available for comparison.

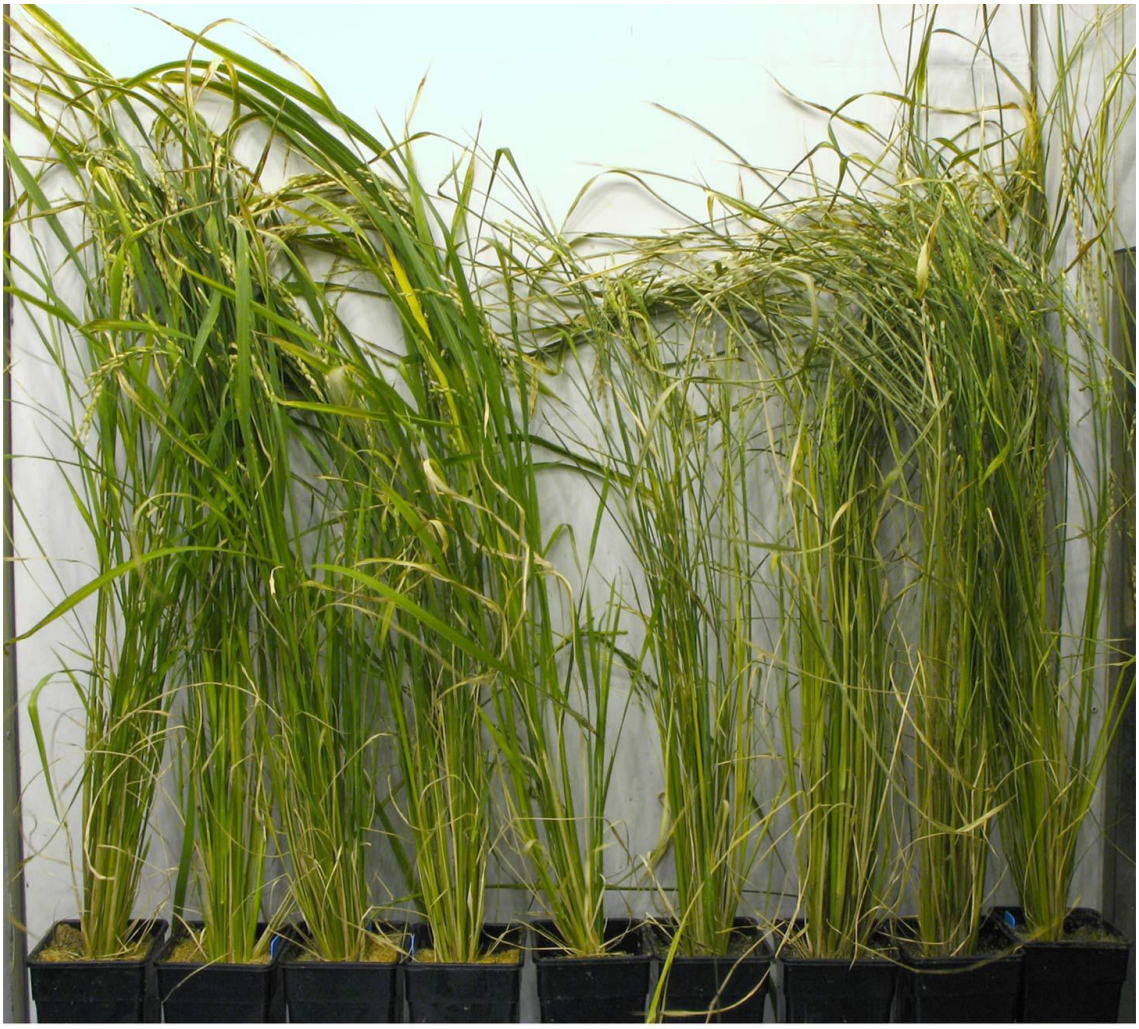
Figure 3.26 Photograph depicting the drought sensitive phenotype of *JRC0470:OsMYB54* rice

The photo shows panicles from water stress affected *JRC0470:OsMYB54* rice plants. The plants were on a three day watering cycle but were accidentally not watered until day four, thus were water stressed for one day. The panel shows panicles from several plants. The developing grains have been aborted and the tips of each floret have bleached white.

3.3.2.3. Rice plants with constitutive overexpression of artificial microRNA constructs displayed a stay-green phenotype

The amiRNA overexpressing lines regenerated well from callus and the regenerating transgenic plants had no abnormal phenotypes. Unlike the constitutive or salt inducible overexpression lines, there were no apparent negative influences on fertility.

The amiRNA overexpressing line with the construct *2x35S:OsbHLH17 amiRNA*, displayed a stay-green phenotype. When all plants had set seed, water was withheld to enhance final grain filling before harvest. The lines with the construct *2x35S:OsbHLH17 amiRNA* still had broad green leaves after a week of withholding water. All other transgenic lines with different constructs had dry shrivelled leaves. A comparison of *2x35S:OsbHLH17 amiRNA* plants and a random assortment of other transgenic lines can be seen in Figure 3.27.



2x35S:OsbHLH17 amiRNA | Randomly selected T0

Figure 3.27 Photograph depicting the stay-green phenotype of transgenic plants

Water was withheld from all transgenic plants after seed set. After one week, the transgenic plants containing the construct *2x35S:OsbHLH17 amiRNA* still had broad green leaves, whereas all other transgenic plants were dry. The figures shows the *2x35S:OsbHLH17 amiRNA* plants on the left and other randomly selected T0 transgenic plants on the right. Control plants were not available for comparison.

3.4. Discussion

The transgenic plants with constitutive overexpression of transcription factor CDSs displayed abnormal phenotypes and reduced fertility, likely due to expression in inappropriate tissues and/or at inappropriate developmental stages. Increasing transgene expression through the use of an inducible promoter may have overcome these deficiencies (Kasuga *et al.*, 1999), hence the use of the salt inducible promoter, JRC0470. However, the salt inducible promoter, JRC0470 appears to have been expressed during some developmental stages, evident in the bushy phenotype of some transgenic lines. The promoter is characterised in Chapter 4.

The gene *OsMYB54* displayed increased expression due to the imposition of a moderate salt stress (Chapter 2). *OsMYB54* may have a dual role in salt and water stress responses, as the transgenic plants with salt inducible expression of *OsMYB54* had increased sensitivity to water stress. The rice plants were grown in soil in one litre plastic pots which were in a tray half filled with water. The tray was allowed to become dry for 24 hours but the soil in the pots was still moist. Even so, the plants were severely affected and the leaves became crisp. Transgenic plants with salt inducible expression of different genes were unaffected. This suggests that appropriate regulation of *OsMYB54* is necessary for the maintenance of plant water relations.

The importance of *OsMYB54* in the plant responses to salt stress was confirmed through its inclusion in patent WO/2009/127443, entitled "Transcription factors involved in salt stress in plants". Also, homologs in rice and other species appear in patents claiming the development of plants with enhanced cold tolerance (US patent 7659446) and plants with improved tolerance to drought, shade and low nitrogen conditions (US patent 7663025).

OsbHLH17 had increased expression in the shoot of IR63731 due to the imposition of a moderate salt stress (Chapter 2). *OsbHLH17* may also have a dual role in salt and water stress responses, as transgenic plants with knocked down expression of *OsbHLH17* had a stay-green phenotype when water was withheld following grain filling. Water withholding encourages senescence and enhances grain filling (Yang *et al.*, 2003a). A stay-green phenotype is thought to be desirable; the idea being that senescence is delayed and photosynthetic capacity is maintained during and after grain filling, resulting in a yield increase. Unfortunately, yield data is not available for *OsbHLH17* amiRNA knockdown plants, as the T0 seed was quarantined at harvest following a fungal outbreak.

During senescence, nitrogen is remobilised as components of the photosynthetic apparatus are degraded (Hörtensteiner & Feller, 2002), including chlorophyll, carotenoids, the proteins of photosystem I and II and Rubisco (Humbeck *et al.*, 1996). Senescence is promoted by ethylene (Khan, 2006; Pratt & Goeschl, 1969), methyl-jasmonate (Ananieva *et al.*, 2004; Ueda *et al.*, 1981) and exogenous ABA (Yang *et al.*, 2003b). However, the ABA deficient mutants, *aba1-1* and *aba2-1*, underwent sugar-dependent senescence more rapidly than wild type plants, while the ABA insensitive mutants, *abi1-1*, *abi2-1* and *abi4-1* but not *abi5-1*, underwent sugar-dependent senescence at the same rate as wild type plants, which makes the role of ABA in senescence unclear (Pourtau *et al.*, 2004). Senescence can be delayed by increasing cytokinin levels at appropriate developmental stages. Increased cytokinin levels delay senescence through preventing chlorophyll degradation and by increasing transpiration (Badenoch-Jones *et al.*, 1996; Gan & Amasino, 1995; Yang *et al.*, 2003b). Senescence promoting and inhibiting compounds also repress the effects of each other. Methyl-jasmonate inhibits the effects of cytokinin retarding senescence on oak leaf segments and methyl-jasmonate is also able to reduce endogenous cytokinin levels in *Cucurbita pepo* (zucchini) seedlings (Ananieva *et al.*, 2004; Ueda *et al.*, 1981).

The stay-green phenotype of *OsbHLH17* knock down plants could be the result of increased cytokinin levels, which would reduce chlorophyll degradation. Methyl-jasmonate reduces endogenous cytokinin levels and results in the rapid chloroplast destruction (Ananieva *et al.*, 2004). Methyl-jasmonate also inhibits the cytokinin induced retardation of senescence in oak leaf segments (Ueda *et al.*, 1981). So, methyl-jasmonate and cytokinin work antagonistically to promote and inhibit senescence, respectively. Since the OsbHLH17 homolog AtMYC2 is involved in jasmonate signalling, is it plausible that OsbHLH17 is also. Therefore it is possible that OsbHLH17 positively regulates JA-mediated gene expression, so reduced expression of *OsbHLH17* may reduce JA-mediated gene expression and JA levels in the leaf, raising the effective cytokinin level to slow senescence.

No other unusual phenotypes were observed in rice plants with altered expression of other transcription factors during tissue culture, regeneration or later growth stages. In the T1 generation, transgenic plants were analysed for salinity tolerance.

Chapter 4. Genotypic and phenotypic analysis of transgenic rice

4.1. Introduction

This chapter outlines the analysis performed on the transgenic lines that were generated in Chapter 3. The lines were first selected based on the number of T-DNA inserts. Lines with low copy numbers were further selected based on performance in salinity tolerance assays. Transgene expression analysis was then performed on the selected lines.

4.2. Materials and methods

4.2.1. Materials

The Klenow enzyme and DNA marker of bacteriophage Lambda DNA digested with *Hin*dIII were purchased from New England Biolabs, Ipswich, Massachusetts, USA. The Whatmann® 3MM filter paper was purchased from Whatmann®, Kent, United Kingdom. The Cling Wrap was purchased from Glad®, Oakland, California, USA. The Platinum® Taq was purchased from Invitrogen, San Diego, California, USA.EasyTides® [α-^{32}P]dCTP was purchased from PerkinElmer, Waltham, Massachusetts, USA. Sephadex G-100, Hybond N^+ membranes, and agarose NA were purchased from GE Life Sciences, NSW, Australia. The HR-T X-ray film and autoradiography cassette was purchased from Fujifilm, Stamford, Connecticut, USA. The SYBR® safe was purchased from Invitrogen, San Diego, California, USA. Ammonium acetate, 4mm steel ball bearings, tri-sodium citrate, sodium chloride and calcium chloride were supplied by BDH Laboratory Supplies, Victoria, Australia. The Pasteur pipettes, dextran sulphate, salmon sperm DNA, BSA, Ficoll 400, polyvinyl-pyrrolidone 360, bromphenol blue, xylene cyanol FF, Pipes, Triton X-100, potassium ferrocyanide, potassium ferricyanide, 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt (X-gluc), disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate monohydrate, dimethylformamide and chemicals for ACPFG growth solution were purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

The LEICA MZFLIII fluorescence stereomicroscope was purchased from Leica Microsystems, VIC, Australia. CP1000 automatic film processer was manufactured by AGFA, Belgium. Canon Powershot S3 was manufactured by Canon, Tokyo, Japan. The flame photometer model 420 was manufactured by Sherwood Scientific limited, Cambridge, UK.

4.2.2. Southern analysis of T0 rice plants

The number of T-DNA inserts in the transgenic lines was determined using Southern blots (Southern, 1975). This method and any derivations are described below.

4.2.2.1. Isolation of rice genomic DNA

Rice plant leaf tissue was harvested from three week old plants and frozen in liquid nitrogen. Tissue was ground and 100mg aliquots were made in 1.5mL microfuge tubes. The tissue was allowed to thaw and reach RT, 600µL of DNA Extraction Buffer (Section 5.2) was added, tubes were shaken vigorously and incubated at 65°C for 30 minutes. Samples were allowed to cool to RT at –20°C, 300µL 6M ammonium acetate was added, the tubes were shaken vigorously and incubated at 4°C for 15 minutes, followed by centrifugation at 7000*g* for five minutes. The supernatant (600µL) was transferred to a fresh tube containing 360µL of 100% isopropanol, tubes were shaken, incubated for five minutes at RT then centrifuged at 7000*g* for five minutes. The tubes were inverted over paper towels to remove the isopropanol, the pellets were washed with 300µL of 70% ethanol, centrifuged for five minutes at 7000*g* and the ethanol was removed. The pellets were dried in a 37°C oven and resuspended in 30µL R40 (Section 5.2) overnight at 4°C.

4.2.2.2. Restriction digests of rice genomic DNA

Rice genomic DNA was digested with *Hind*III restriction endonuclease which cuts 100bp inside the Right Border of the T-DNA. Each restriction digest contained; 6µL genomic DNA, 1µL Buffer B, 0.5µL *Hind*III, 2.5µL MilliQ water. The digests were incubated at 37°C overnight, then 2µL 6X ficoll dye (Section 5.2) was added prior to gel loading.

4.2.2.3. Gel electrophoresis of digested rice genomic DNA

The digested DNA was separated on a 1% agarose NA 1X TAE gel at 20 volts for 24 hours. The gel was stained in ethidium bromide (1µg/mL) and DNA was visualised in the gel using a transilluminator.

4.2.2.4. DNA transfer to nylon membranes

The DNA was transferred to a Hybond N^+ nylon membrane using the capillary transfer method (Southern, 1975), with 0.4M sodium hydroxide as the liquid carrier. The DNA was allowed to transfer for 18 hours. Following transfer, the membrane was soaked in

2X SSC buffer (Section 5.2) for 1 minute, then blotted dry on Whatmann filter paper. The gel and membrane were checked using a transilluminator to ensure that DNA transfer was complete and to cross-link the DNA to the membrane. The membrane was wrapped in Cling Wrap and stored at –20°C until use.

4.2.2.5. Oligo labelling of DNA probes

Probes were designed to target the hygromycin resistance gene. The hygromycin resistance gene CDS was amplified using PCR. The reaction contained the following; 1µL HYG1 primer (Table 5.7), 1µL HYG2 primer (Table 5.7), 1µL template DNA (200 ng/µL), 2.5µL 10X buffer, 0.75µL $MgCl_2$, 0.1µL Platinum Taq and 17.65µL MilliQ water. The thermocycler was programmed as follows; 1, 94ºC for 2 minutes; 2, 94ºC for 30 seconds; 3, 56ºC for 30 seconds; 4, 72ºC for 1 minute; 5, repeat from step 2 for 39 times; 6, 15ºC forever. The 1026bp PCR product of the hygromycin resistance gene CDS was gel purified and cleaned up as per Section 3.2.2.2. The purified DNA was diluted with MilliQ water to a concentration of approximately 20ng/µL.

The probe was synthesised by adding 3µL of hygromycin resistance gene DNA (approximately 50 ng), 3µL of 9 mer random primer mix (0.1µg/mL) and 1.5µL of MilliQ water into a 1.5mL microfuge tube. The tube was placed in a boiling rack and boiled for 5 minutes. The tube was then incubated on ice for 5 minutes. Oligo buffer (12.5µL) (Section 5.2) was added to the sample, mixed, and centrifuged for 5 seconds in a micro-centrifuge. Klenow (1µL) and 4µL [α-^{32}P]dCTP were added, mixed with the pipette tip, and incubated at 37°C for 45 minutes. The probe was stored on ice until use.

The probe was purified using a Sephadex® G-100 (Section 5.2) mini-column. Drops leaving the column with a radioactivity greater than 100 cps were collected until the radioactivity peaked at 1kcps and dropped to 500 counts per second.

4.2.2.6. Hybridisation

The membranes were soaked in 5x SSC for 1 minute, rolled into a cylinder 20mm in diameter and placed into a hybridisation bottle. Pre-hybridisation solution (Section 5.2) (30mL) was added to each bottle and incubated at 65°C overnight in a rotisserie oven. The solution in the bottle was discarded and replaced with 8mL of hybridisation solution (Section 5.2) then the bottle was returned to the hybridisation oven for 30 minutes. To the labelled Hyg probe, 500µL of carrier DNA (5mg/ml) was added. The probe was boiled for 5 minutes, chilled on ice for 5 minutes and added to the hybridisation solution in the bottom of the bottle. The bottle was returned to the

hybridisation oven overnight. The hybridisation mix was discarded, leaving the membrane in the bottle. The membrane was washed in the bottle with wash #1, 40mL 2x SSC, 0.1% SDS and returned to the oven for 20 minutes. The membrane was transferred to a plastic box and washed successively with 200mL of the following for 20 minutes until radioactivity reached just above background levels; #2, 1x SSC, 0.1% SDS; #3, 0.5x SSC, 0.1% SDS and #4, 0.2x SSC, 0.1% SDS.

4.2.2.7. Autoradiography

The membrane was blotted dry on paper towel and the radioactivity measured. If the radioactivity was approximately 1 cps, the membrane was taped in an autoradiography cassette with a sheet of HR-T X-ray film, then incubated at -80°C for two to eight days. The film was developed in a CP1000 automatic film processor. The membranes were stripped prior to storage at -20°C by placing them in 200mL of boiling stripping solution (Section 5.2) for 30 minutes on a rocking platform.

4.2.3. Analysis of reporter plants

Induction of the salt inducible promoter JRC0470 was analysed in reporter plants generated in Section 3.2.3.3. For the construct *JRC0470:uidA*, the lines used were 1, 5 and 10. For the construct *JRC0470:mgfp*, the lines used were 2-5, 2-7, 2-9, 3-2, 3-8 and 3-9. The rice seed was sterilised as per Section 3.2.4.2, germinated on plates and transferred to two tanks of supported hydroponics as per Section 4.2.5. The plants were grown for 24 hours and sampled (time zero hours). To one tank, a 25mM NaCl stress was applied for 24 hours and the plants were sampled (time 24 hours). A 50mM NaCl stress was applied and the plants were sampled 24 and 48 hours later (time 48 and 72 hours). Plants were sampled from the control and salt treatment tanks at all time points. To keep the available level of calcium constant, $CaCl_2$ was added to a final concentration of 0.825mM for each addition of NaCl.

4.2.3.1. GUS staining and imaging of reporter plants

Plants containing the construct *JRC0470:uidA* were harvested and immediately submerged in GUS staining solution (Section 5.2). Samples were vacuum infiltrated at 26inHg for 22 minutes and incubated at 37°C in the dark overnight. The staining solution was discarded and samples received two 50% ethanol washes for three hours

each at 30rpm and repeated washes in 75% ethanol until all chlorophyll had been removed.

Images were taken of all *JRC0470:uidA* plants from each time point using a Canon Powershot S3. Close images of *JRC0470:uidA* and *JRC0470:mgfp6* plants were taken using a Leica MZ FLIII Fluorescence Stereo Microscope.

4.2.4. Salinity tolerance assays of transgenic plants with altered transcription factor expression

Transgenic rice lines with one or few T-DNA insertions were screened for salinity tolerance in a supported hydroponics system. The lines selected were: pMDC32 empty vector, 1D, 3B, 4D; *2x35S:OsOrphan19*, 8A, 8E, 9B, 9D; *2x35S:OsEREB67*, 10B; *2x35S:OsMYBR63*, 11A, 11C, 13A, 14A, 15E; *2x35S:OsLUX*, 16E, 17B, 17C, 18E, 19C; *2x35S:OsMYB54*, 22A, 22C, 22E, 23C, 23E, 25A. Nipponbare rice was used as a wild-type control. The rice seed was sterilised as per Section 3.2.4.2 and placed on filter paper soaked in sterile MilliQ water in a deep Petri dish, with ten seeds per plate. The plates were incubated for six days in the Australian Genome Research Facility (AGRF) growth room Controlled Environment 1 (CE1) at 28°C day, 25ºC night, 60-95% humidity, with a 12 hour light cycle at an intensity of 400µmol.m^{-2}.s^{-1}. The seedlings were then transferred to supported hydroponics tanks filled with ACPFG nutrient solution (Table 5.8). The nutrient solution was changed weekly for the remainder of the experiment. The tanks measured 400mm x 300mm x 140mm and held 100 plants per tank, see Figure 4.1. At 14 days after germination, NaCl was added incrementally over time to make the final concentration in the growth solution as follows; 25mM for 1 day, 50mM for 2 days, 75mM for 2 days, 100mM for 3 days, 125mM for 5 days, 150mM for 5 days, 175mM for 14 days. To keep the available level of calcium constant, $CaCl_2$ was added to a final concentration of 0.825mM for each addition of NaCl. Plant heights were recorded daily. Plants were scored daily for health on a scale of one to nine as follows; 1, healthy; 2, 1-15% chlorotic; 3, 16-30% chlorotic; 4, 31-45% chlorotic; 5, 46-60% chlorotic; 6, 61-75% chlorotic; 7, 76-90% chlorotic; 8, 91-99% chlorotic; 9, dead. A leaf sample was taken on day 18 to genotype the plants. Genomic DNA was extracted as per Section 4.2.2.1 and PCR was performed as per Section 2.2.1.5. Changes include using a one minute extension time and the primers HYG1 and HYG2 (Table 5.7) to amplify the hygromycin resistance gene. The PCR products were separated using agarose gel electrophoresis and visualised in the gel using a transilluminator as per

Section 2.2.1.6. Nipponbare health scores were plotted over time and a regression line fitted. The point on the regression line that intersected a health score of 5 (when half of the plants were dead), fell on day 22. Data from all lines were compared to Nipponbare, empty vector transformants and null segregants on day 22.

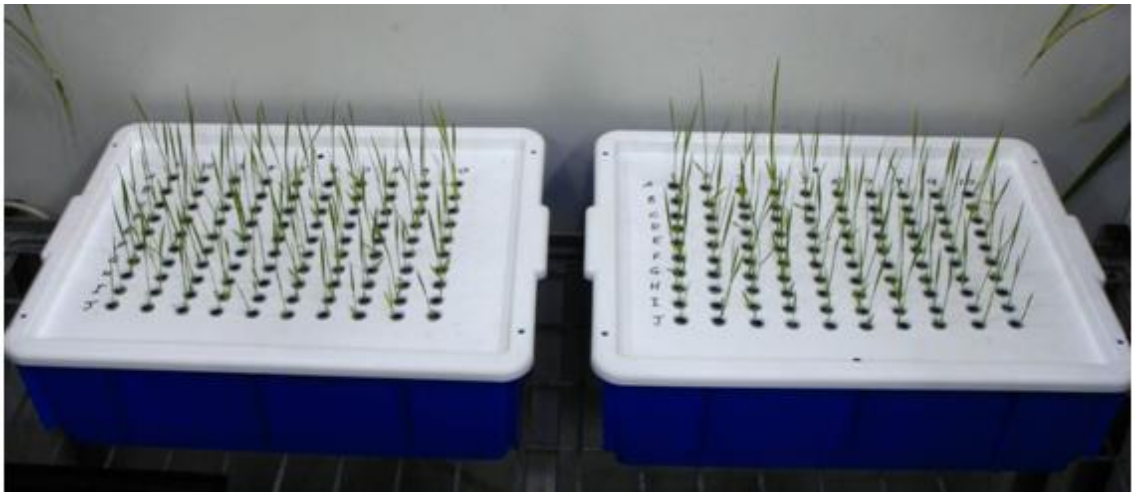


Figure 4.1 Photograph of hydroponics tanks used for salinity tolerance assays

Salinity tolerance assays were conducted in 10L hydroponics tanks filled with ACPFG nutrient solution. There were 96-100 plants per tank. Rice seedlings were placed in Eppendorf tubes with the bottom cut off, so the roots would be immersed in nutrient solution.

4.2.5. Analysis of sodium and potassium accumulation in transgenic plants

Sodium and potassium accumulation under salt stress was examined in transgenic lines with the highest health scores and greatest plant heights, from the salinity tolerance assay. Three replicates of the experiment were performed. Two were under the same conditions as Section 4.2.4. The third was performed in The Plant Accelerator® at the Australian Plant Phenomics Facility. The greenhouse conditions were; 28°C day, 25ºC night, 5-50% humidity and a light intensity that varied depending on the weather. Light intensity peaked at 200-500µmol.m^{-2}.s^{-1} and only ever reached or exceeded 200µmol.m^{-2}.s^{-1} for ≤ six hours of the day. The total day length was approximately 9h 57min. The lines used in the first replicate were: *2x35S:OsOrphan19*, 8A, 9D; *2x35S:OsEREB67*, 10B; *2x35S:OsMYBR63*, 11A, 11C; *2x35S:OsLUX*, 17C, 19C; *2x35S:OsMYB54*, 22C, 22E. The following lines were added to the second and third replicates:

2x35S:OsOrphan19, 8C, 9B; *2x35S:OsMYB54*, 23E, 25A. The rice seed was sterilised as per Section 3.2.4.2, germinated on plates and transferred to supported hydroponics as per Section 4.2.5. The ACPFG nutrient solution was changed weekly and maintained at approximately pH 5.5. Sodium chloride stress was imposed on the day the fourth leaf emerged on the first plant. NaCl was added to a final concentration of 25mM and maintained for 24 hours, then increased to 50mM and maintained for the remainder of the experiment. To keep the available level of calcium constant, $CaCl_2$ was added to a final concentration of 0.825mM for each addition of NaCl. The day of fourth leaf emergence was recorded for every plant. Thirteen days post fourth leaf emergence, the plants were harvested and the root material was blotted dry with paper towels. Fresh weights were recorded for the shoot, root, fourth leaf and a small piece of leaf for genotyping. In an alternative method, total plant fresh weights were recorded and the plants were transferred to soil and grown until seed set. The plants were genotyped as per Section 4.2.5. The plant material was dried in a 65°C drying oven for a minimum of 72 hours and dry weights were recorded. The fourth leaf of each plant was digested in 1% nitric acid at 85°C for four hours and the sodium and potassium concentration determined using a flame photometer.

The method above was also used to analyse sodium and potassium accumulation in transgenic rice plants expressing transcription factor CDSs under the control of a salt inducible promoter and in plants constitutively overexpressing amiRNAs targeting those transcription factors. The salt inducible lines were: *JRC0470:OsOrphan19 4*, *6*, *11*; *JRC0470:OsEREB67 1*, *3*; *JRC0470:OsMYBR63 4*; *JRC0470:OsbHLH17 2*, *3*, *5*; *JRC0470:OsLUX 1*, *2*, *12* and *JRC0470:OsMYB54 3*, *5*, *7*. All these lines were included in a growth experiment that was halted due to a fungal outbreak in the growth room. Lines containing the constructs *JRC0470:OsOrphan19* and *JRC0470:OsLUX* were selected for a repeat growth experiment based on their greater biomass, relative to lines containing the other constructs.

The amiRNA knockdown lines were: *2x35S:OsOrphan19 amiRNA 2, 5, 9*: *2x35S:OsEREB67 amiRNA 1, 8, 10*; *2x35S:OsMYBR63 amiRNA 1, 2, 15*; *2x35S:OsbHLH17 amiRNA 1, 8, 14*; *2x35S:OsLUX amiRNA 1, 3, 9* and *2x35S:OsMYB54 amiRNA 2, 3, 4*.

4.2.6. Semi-quantitative RT-PCR analysis of transgenic rice plants

Transgene transcript levels were analysed in transgenic rice plants constitutively and salt-inducibly overexpressing transcription factor CDSs using semi-quantitative RT-PCR. Endogenous transcription factor transcript levels were analysed in transgenic rice plants constitutively overexpressing amiRNAs.

The rice plant leaf tissue was harvested and snap frozen in liquid nitrogen. The frozen tissue was ground with 3 x 4mm steel ball bearings and total RNA was extracted as per Section 2.2.3.2. Contaminating DNA was removed as per Section 2.2.3.3. cDNA was synthesised as per Section 2.2.3.4. PCR products from endogenous transcripts were amplified as per Section 2.2.3.5 with the following modifications; the annealing temperature was 54°C, the cDNA template was diluted 1:15 with MilliQ water the number of cycles of amplification were 29 for *OsOrphan19,* 30 for *OsEREB67,* 25 for *OsMYBR63,* 40 for *OsbHLH17,* 35 for *OsLUX,* 35-40 for *OsMYB54* and 29 cycles for the α-tubulin control, using primers from Table 5.1. PCR products from transgene transcripts were amplified as per Section 2.2.3.5 with the following modifications; the cDNA template was diluted 1:15 in MilliQ water, the annealing temperature was 54°C, the number of cycles of amplification were 25 for *OsMYBR63*, 29 for *OsOrphan19*, *OsLUX*, *OsMYB54*, and 35 *OsEREB67*, the forward primers were from Table 5.6 and the reverse primer, NOSR2, was from Table 5.7. PCR products were separated on 1X TAE 1% agarose gel containing 1X SYBR® safe, at 80 volts for 40 minutes and DNA was visualised in the gel using a UV transilluminator and photographed.

4.2.7. Yield

Yield attributes were recorded, including; the number of seeds harvested and 100-grain weight. The rice seeds were photographed with husks and dehusked from a distance of 100mm, on a 90mm disc of Whatman™ grade 1 filter paper, using a Canon Powershot S3 camera.

4.3. Results

4.3.1. Southern analysis of T0 rice plants

Southern analysis was performed to select transgenic lines with one or two T-DNA inserts. The probe targeted the hygromycin resistance gene present once in each T-DNA inserted in the rice genomic DNA, assuming no tandem repeats or truncations. Southern analysis was performed for the transgenic lines. The number of T-DNA insertions are in brackets after each line name; pMDC32 empty vector and *2x35S:OsOrphan19* 8A(1), 8C(1), 9B(1), 9D(1) (Figure 4.2), *2x35S:OsEREB67* 10B(3) and *2x35S:OsMYBR63* 11A(2), 11C(2) (Figure 4.3), *2x35S:OsLUX* 17C(2), 19C(2) (Figure 4.4), *2x35S:OsMYB54* 22C(2), 22E(3), 23E(2), 25A(1) (Figure 4.5), *JRC0470:OsOrphan19* 4(1), 6(1), 11(1), *JRC0470:OsEREB67* 1(1), 3(1) and *JRC0470:OsMYBR63* 4(1) (Figure 4.6), *JRC0470:OsbHLH17* 2(1), 3(1), 5(5), *JRC0470:OsLUX* 1(1), 2(1), 12(1) and *JRC0470:OsMYB54* 3(1), 5(1), 7(1) (Figure 4.7). Transgenic rice overexpressing amiRNA constructs were selected for salinity tolerance assays based on transcript level, not on T-DNA insert number. See subsections of Section 4.3.4 below.

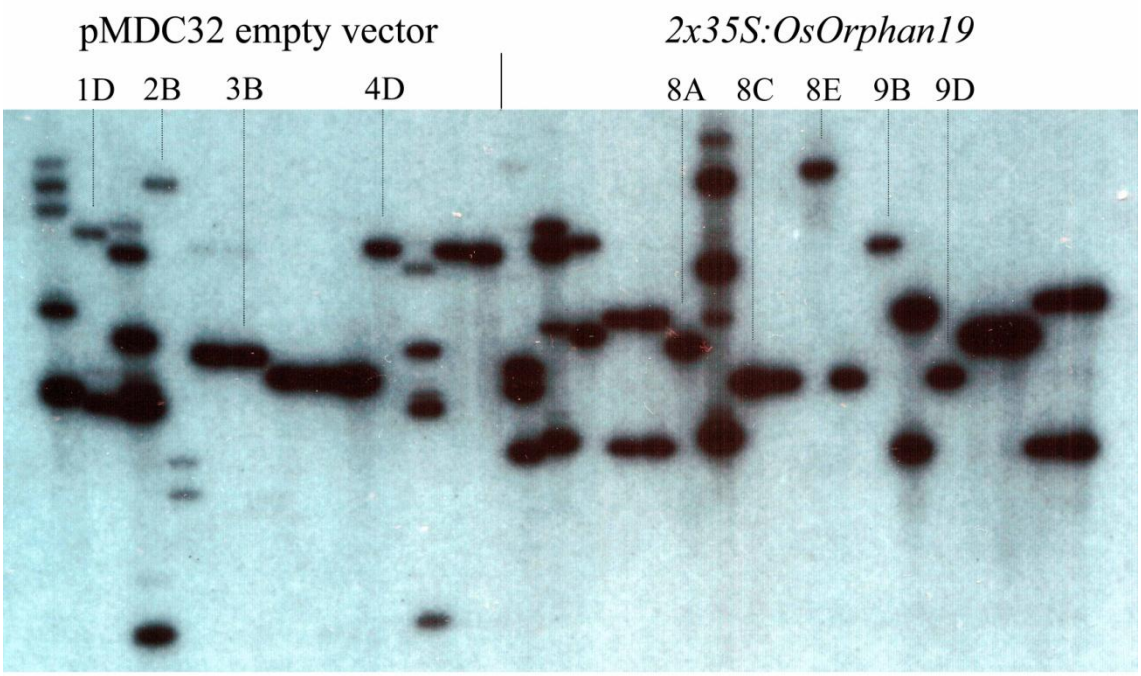


Figure 4.2 Image of an autoradiograph of *Hind*III digested T0 rice genomic DNA for the transgenic lines containing the pMDC32 empty vector and *2x35S:OsOrphan19*

DNA from transgenic rice was digested with *Hind*III, separated using gel electrophoresis, blotted onto a nitrocellulose membrane and hybridised to a [α-^{32}P]dCTP labelled probe targeting the hygromycin resistance gene present in the T-DNA. The membrane was successively washed in 2x, 1x, 0.5x and 0.2x SSC with 0.1% SDS until radioactivity was at background levels, laid against a sheet of HR-T X-ray film at -80°C for two to eight days, developed and photographed. The left panel is from rice transformed with the pMDC32 empty vector and includes lines 1D, 2B, 3B and 4D. The right panel is from rice transformed with *2x35S:OsOrphan19* and includes lines 8A, 8C, 8E, 9B and 9D. Lines not used in subsequent experiments are not labelled.

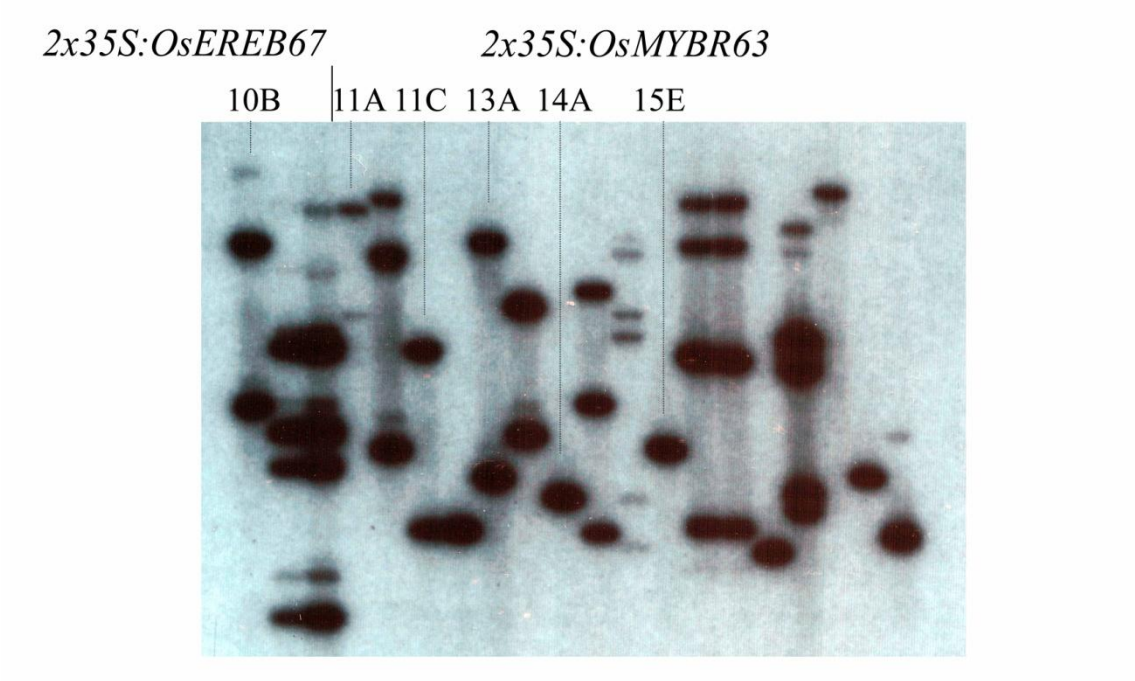


Figure 4.3 Image of an autoradiograph of *Hind*III digested T0 rice genomic DNA for the transgenic lines containing the constructs *2x35S:OsEREB67* and *2x35S:OsMYBR63*

The autoradiograph was produced as described in the legend for Figure 4.2. The left panel is from rice transformed with *2x35S:OsEREB67* and includes line 10B. The right panel is from rice transformed with *2x35S:OsMYBR63* and includes lines 11A, 11C, 13A, 14A and 15E. Lines not used in subsequent experiments are not labelled.

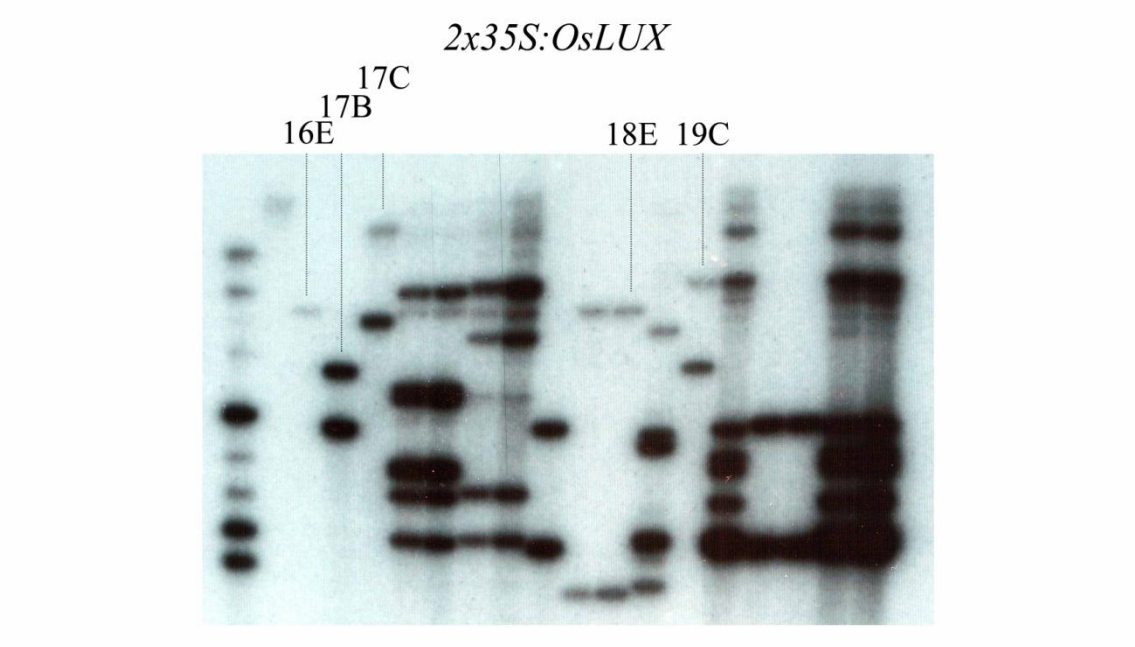


Figure 4.4 Image of an autoradiograph of *Hind*III digested T0 rice genomic DNA for the transgenic line containing the construct *2x35S:OsLUX*

The autoradiograph was produced as described in the legend for Figure 4.2. The autoradiograph is from rice transformed with *2x35S:OsLUX* and includes lines 16E, 17B, 17C, 18E and 19C. Lines not used in subsequent experiments are not labelled.

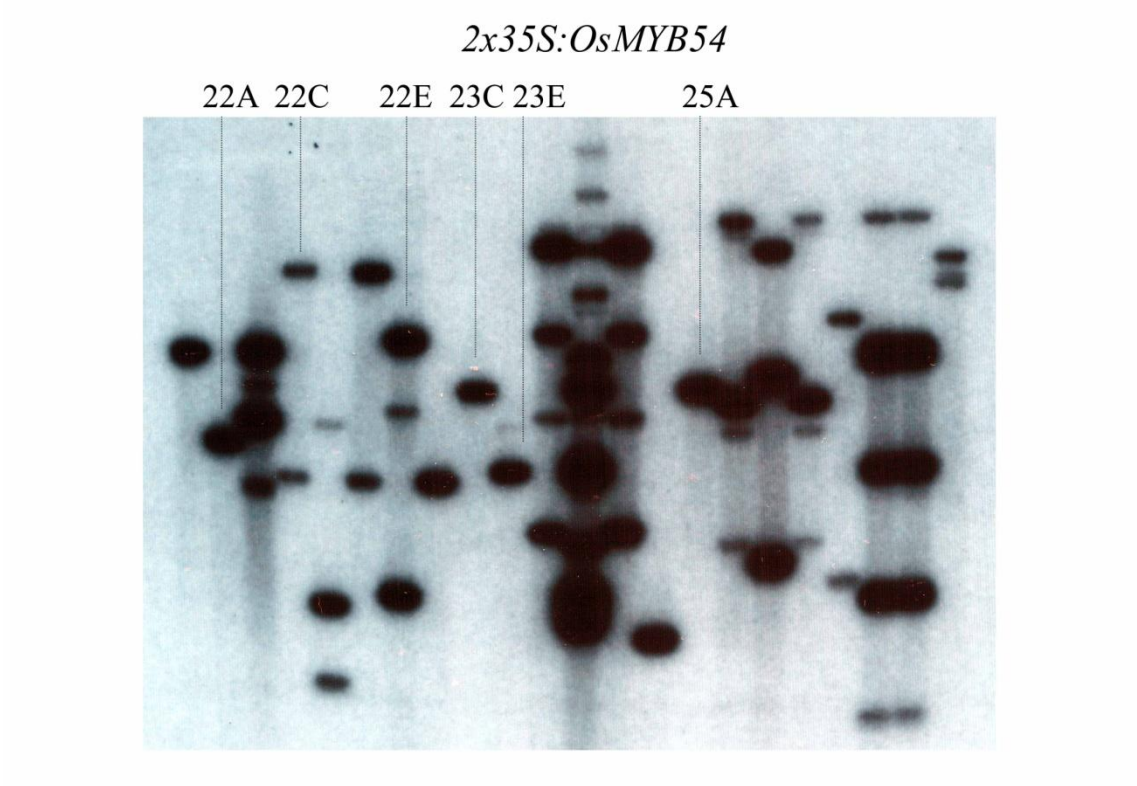


Figure 4.5 Image of an autoradiograph of *Hind*III digested T0 rice genomic DNA for the transgenic line containing the construct *2x35S:OsMYB54*

The autoradiograph was produced as described in the legend for Figure 4.2. The autoradiograph is from rice transformed with *2x35S:OsMYB54* and includes lines 22A, 22C, 22E, 23C, 23E and 25A. Lines not used in subsequent experiments are not labelled.

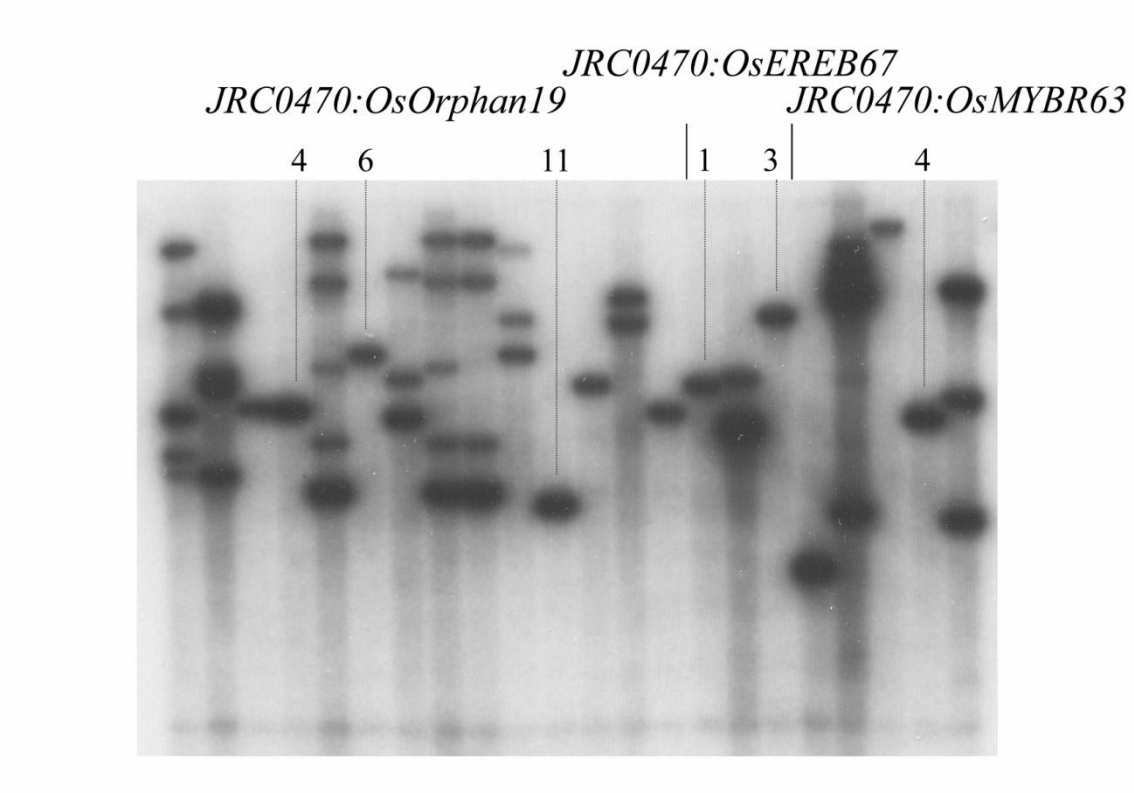


Figure 4.6 Image of an autoradiograph of *Hind*III digested T0 rice genomic DNA for the transgenic line containing the constructs *JRC0470:OsOrphan19*, *JRC0470:OsEREB67* and *JRC0470:OsMYBR63*.

The autoradiograph was produced as described in the legend for Figure 4.2. The left panel is from rice transformed with *JRC0470:OsOrphan19* and includes lines 4, 6 and 11. The middle panel is from rice transformed with *JRC0470:OsEREB67* and includes lines 1 and 3. The right panel is from rice transformed with *JRC0470:OsMYBR63* and includes line 4. Lines not used in subsequent experiments are not labelled.

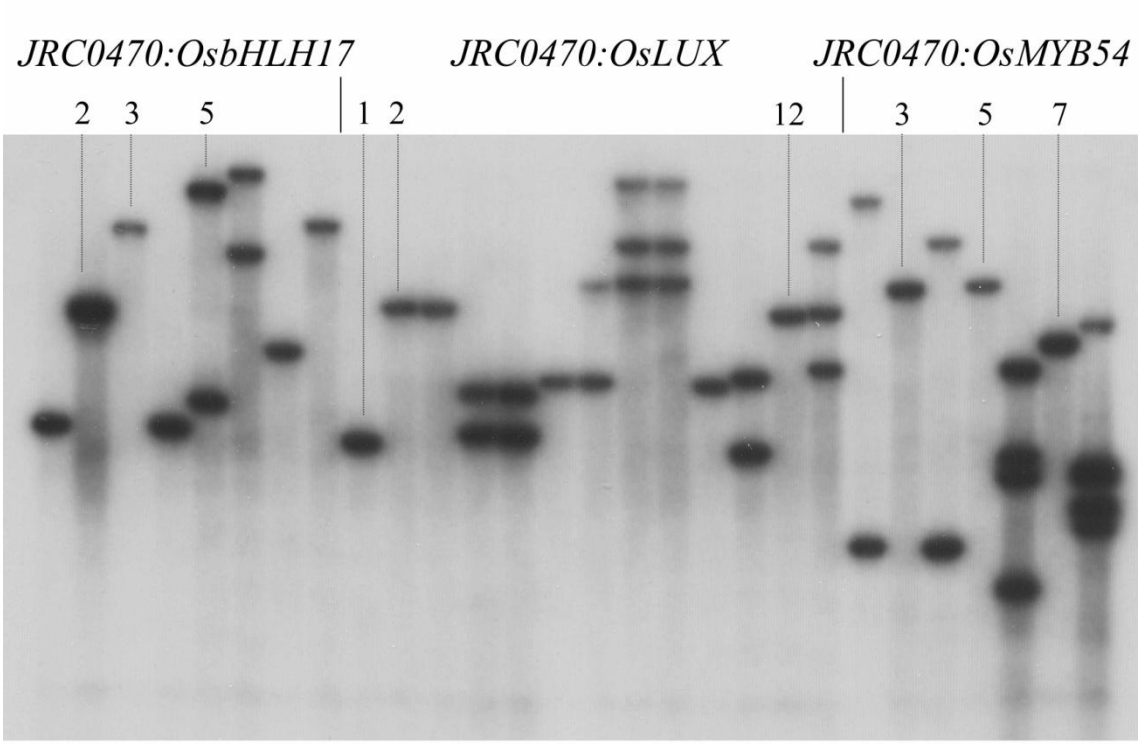


Figure 4.7 Image of an autoradiograph of *Hind*III digested T0 rice genomic DNA for the transgenic line containing the constructs *JRC0470:OsbHLH17*, *JRC0470:OsLUX* and *JRC0470:OsMYB54*.

The autoradiograph was produced as described in the legend for Figure 4.2. The left panel is from rice transformed with *JRC0470:OsbHLH17* and includes lines 2, 3 and 5. The middle panel is from rice transformed with *JRC0470:OsLUX* and includes lines 1, 2 and 12. The right panel is from rice transformed with *JRC0470:OsMYB54* and includes lines 3, 5 and 7. Lines not used in subsequent experiments are not labelled.

4.3.2. Analysis of the JRC0470 salt inducible promoter

Reporter plants containing the *JRC0470:uidA* construct were subjected to salt stress and the effect on β-D-glucuronoside glucuronosohydrolase (GUS) levels was analysed with histochemical straining using X-gluc. Reporter plants containing the *JRC0470:mgfp6* construct were used in the same experiment and the effect on GFP fluorescence was analysed. The plants were photographed at zero hours (Figure 4.8), 24 hours (Figure 4.9), 48 hours (Figure 4.10) and 72 (Figure 4.11) hours after the application of salt stress.

The results show that the transgenic plants had GUS staining in the root vasculature, first, second and third leaves without salt stress. With the application of salt stress, GUS staining was slightly greater in the roots, second and third leaves. Minimal GUS staining was present in the wild type plants, with or without salt stress. These results show that the JRC0470 promoter is induced at a low constitutive level in the root vasculature and in the leaves and that the application of salt stress induces the promoter further in the same tissues.

No GFP fluorescence was visible in the transgenic lines at any time point (data not shown).

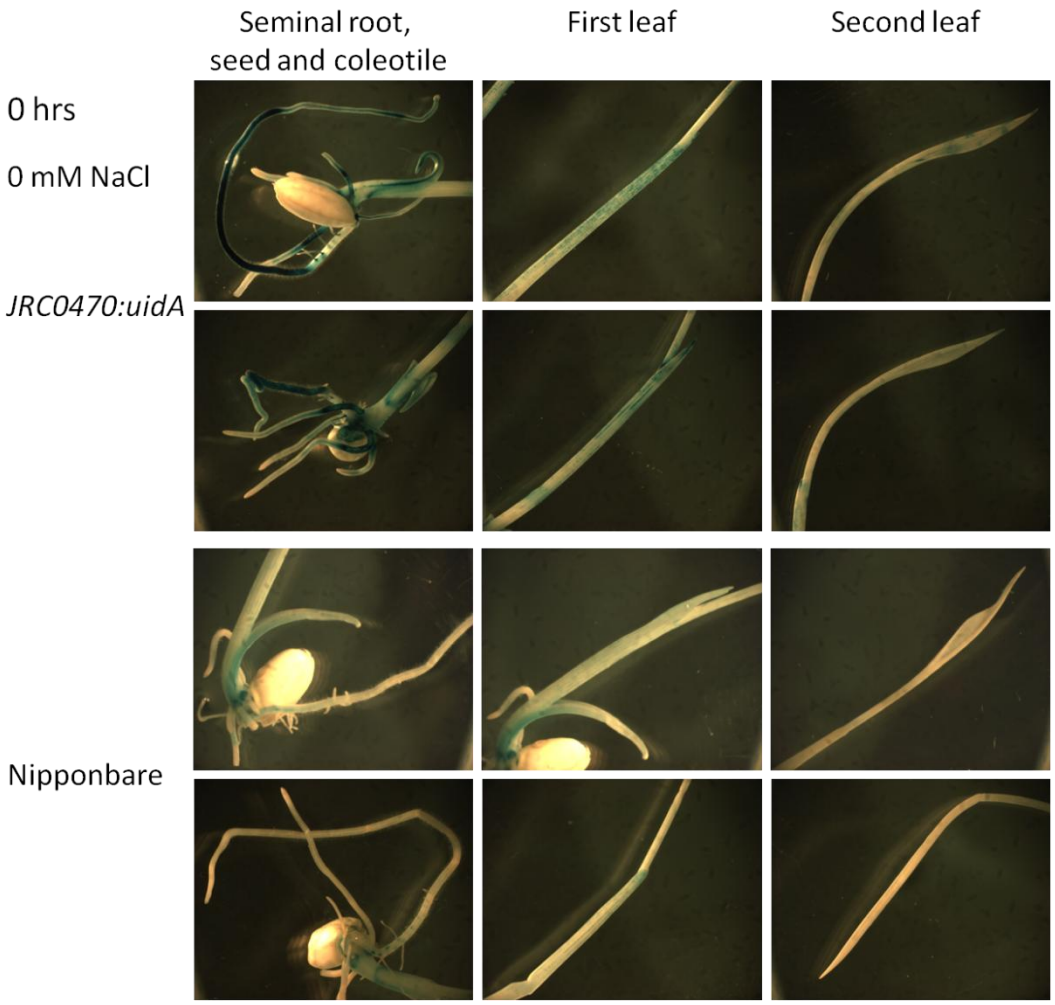


Figure 4.8 GUS staining in JRC0470:uidA reporter lines – 0 hours, 0mM NaCl

The figure shows photos of X-gluc treated transgenic rice plants containing the reporter construct *JRC0470:uidA* (top panels) and Nipponbare control plants (bottom panels) at 0 hours with a 0mM NaCl stress. In this experiment the plants were treated with 25mM NaCl for 24 hours, then 50mM NaCl for 48 hours. Plants were sampled at four time points; prior to salt stress imposition and every 24 hours thereafter, up to 72 hours.

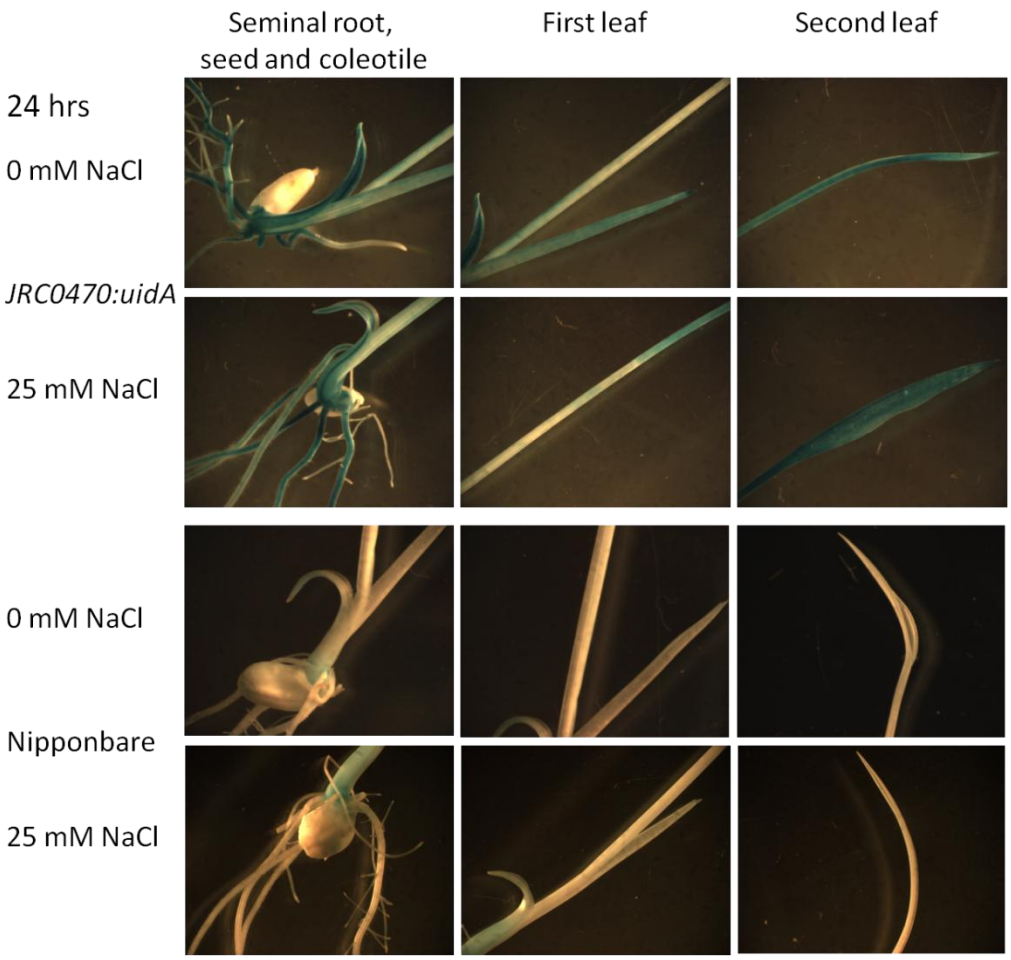


Figure 4.9 GUS staining in JRC0470:uidA reporter lines – 24 hours, 25mM NaCl

The figure shows photos of X-gluc treated transgenic rice plants containing the reporter construct *JRC0470:uidA* and Nipponbare control plants at 24 hours with a 25mM NaCl stress. The top panels show *JRC0470:uidA* rice plants with a 0mM and 25mM NaCl stress. The bottom panels show Nipponbare control rice plants with a 0mm and 25mM NaCl stress.

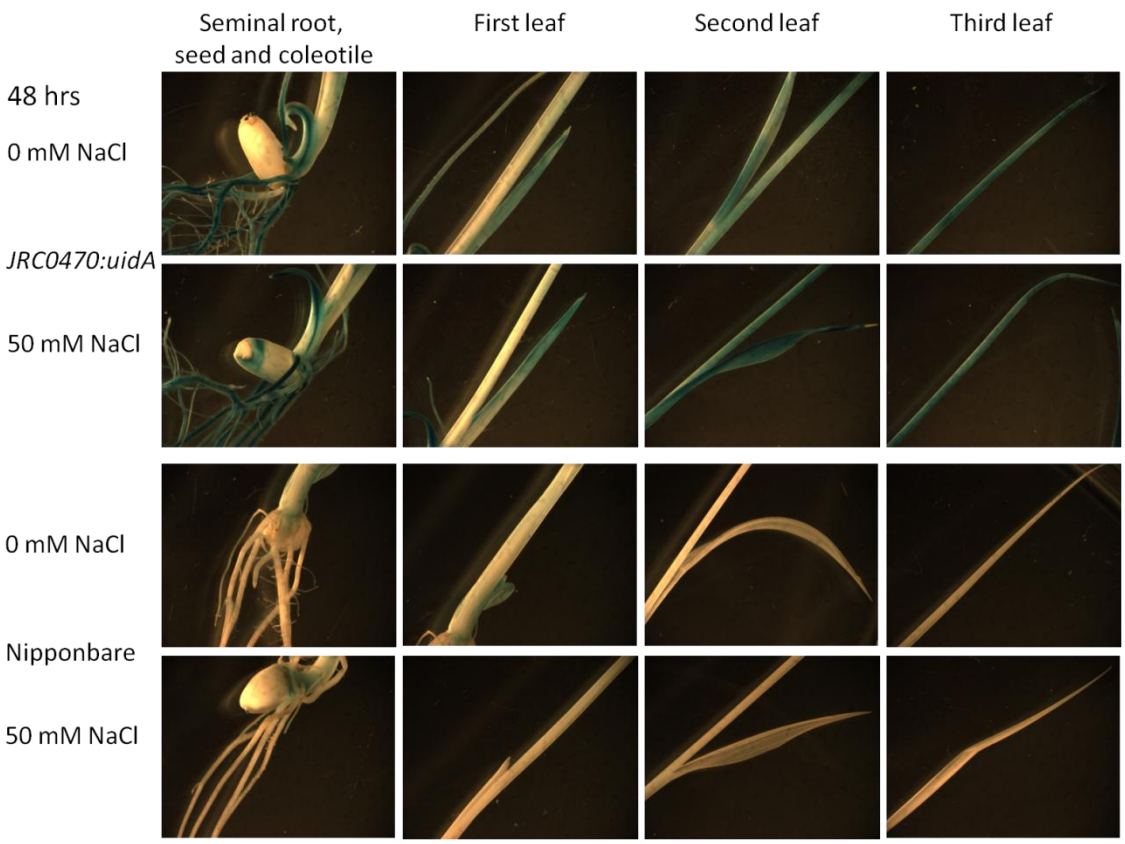


Figure 4.10 GUS staining in JRC0470:uidA reporter lines – 48 hours, 50mM NaCl

The figure shows photos of X-gluc treated transgenic rice plants containing the reporter construct *JRC0470:uidA* and Nipponbare control plants at 48 hours with a 50mM NaCl stress. The top panels show *JRC0470:uidA* rice plants with a 0mM and 50mM NaCl stress. The bottom panels show Nipponbare control rice plants with a 0mm and 50mM NaCl stress.

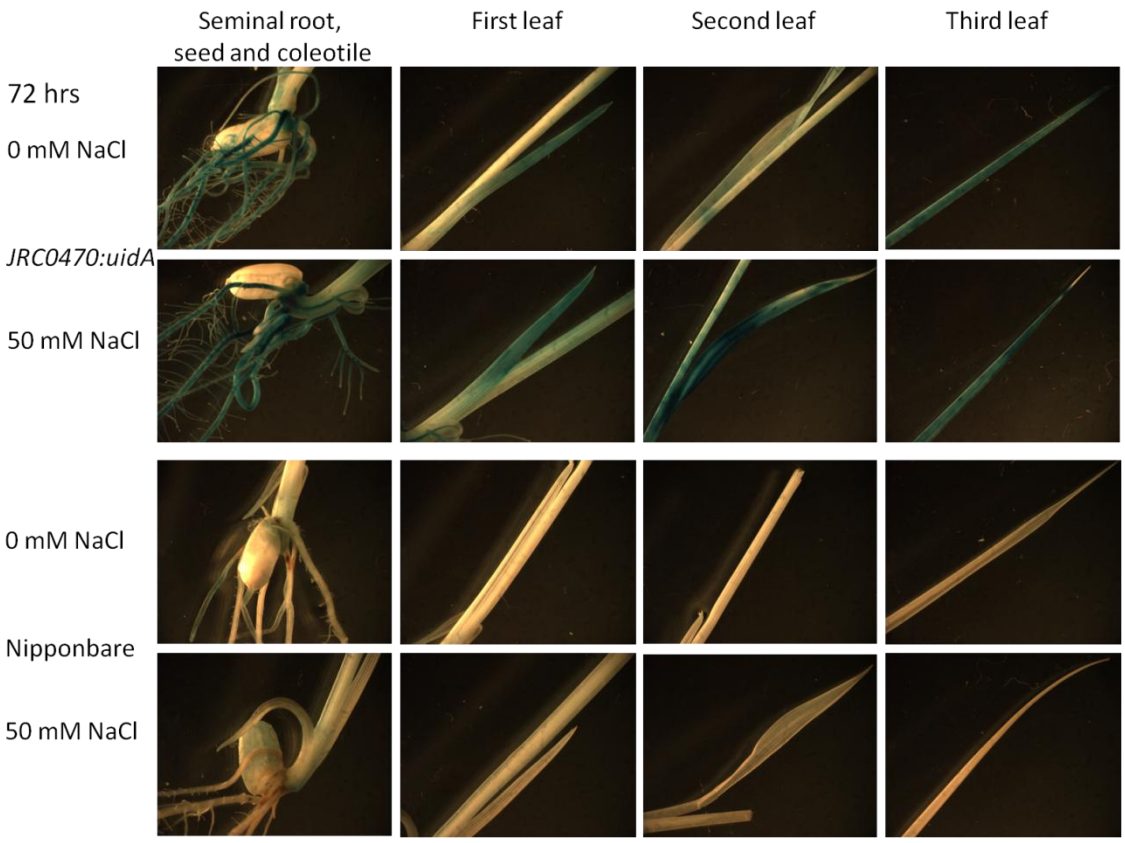


Figure 4.11 GUS staining in JRC0470:uidA reporter lines – 72 hours, 50mM NaCl

The figure shows photos of X-gluc treated transgenic rice plants containing the reporter construct *JRC0470:uidA* and Nipponbare control plants at 72 hours with a 50mM NaCl stress. The top panels show *JRC0470:uidA* rice plants with a 0mM and 50mM NaCl stress. The bottom panels show Nipponbare control rice plants with a 0mm and 50mM NaCl stress.

4.3.3. Salinity tolerance assay of T1 plants

The salinity tolerance of transgenic rice plants constitutively overexpressing transcription factor CDSs was analysed in a salinity tolerance assay examining plant health scores and heights. NaCl was added to the growth medium so as to slowly increase the stress from 0mM to 175mM, over a 19 day period and then maintain the stress for a further 13 days. The health scores and heights were compared between the transgenic lines and the control line on day 22 when half of the control plants were dead. This was determined by plotting the control line health scores against time and fitting a regression line to determine on which day the control line had an average health score of 5; equating to the plants being 46-60% chlorotic (Figure 4.12).

Graphs containing the plant heights and health scores that compare each transgenic line with pooled data for the wild type cultivar Nipponbare, empty vector transformants and null segregants, are present in the following pages. The graphs shown only represent lines used in subsequent experiments.

The transgenic lines *2x35S:OsOrphan19*, 8A (Figure 4.13) and 9D (Figure 4.16) both had health scores of mostly 4 and 4-5, respectively. Line 8A was shorter than most wild type plants, whereas line 9D consisted of mostly taller plants. For both lines, the taller plants generally had better health scores. The other lines 8C (Figure 4.14) and 9B (Figure 4.15) had better performing taller plants and poor performing shorter plants. For the transgenic line *2x35S:OsEREB67*, 10B (Figure 4.17) all plants had a health score of 3 or 4 and were of average height, relative to wild type plants. The transgenic lines *2x35S:OsMYBR63*, 11A (Figure 4.18) and 11C (Figure 4.19) were quite different. Line 11A had an even spread of better performing taller plants and poor performing shorter plants. Line 11C had mostly well performing plants of an average height, relative to wild type plants. The transgenic lines *2x35S:OsLUX*, 17C (Figure 4.20) and 19C (Figure 4.21) had mostly average performing shorter plants and better performing average height plants, respectively. The transgenic lines *2x35S:OsMYB54*, 22C (Figure 4.22) and 22E (Figure 4.25) were quite different. Line 22C had a spread of plant performance, with all plants of average height. Line 22E had mostly well performing taller plants. Both lines 23E (Figure 4.24) and 25A (Figure 4.25) had mostly well performing shorter plants.

Transgenic lines were analysed for Na^+ and K^+ accumulation under salt stressed conditions.

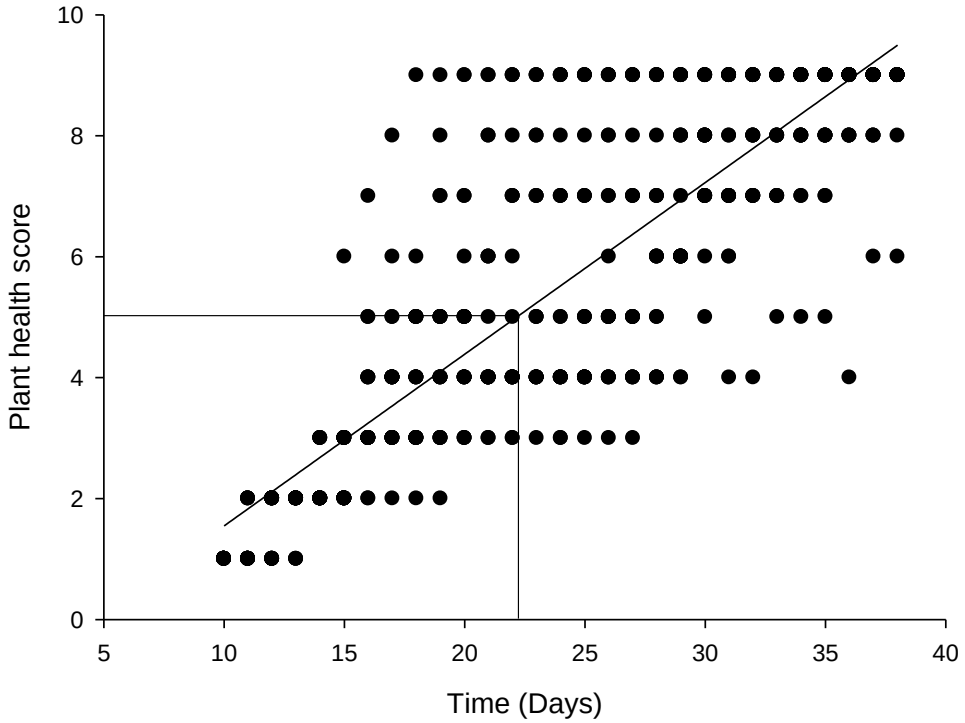


Figure 4.12 Graph depicting plant health scores over time in salt stressed *O. sativa* cv. Nipponbare plants to determine on which day the average health score was 5.

The plants were grown in supported hydroponics in ACPFG nutrient solution. At 14 days after germination NaCl was added to the following concentrations; 25mM for 1 day, 50mM for 2 days, 75mM for 2 days, 100mM for 3 days, 125mM for 5 days, 150mM for 5 days, 175mM for 14 days. To keep the available calcium constant, $CaCl_2$ was added to a final concentration of 0.825mM for every 50mM NaCl. Plant heights and health scores were recorded daily. Health scores were; 1, healthy; 2, 1-15% chlorotic; 3, 16-30% chlorotic; 4, 31-45% chlorotic; 5, 46-60% chlorotic; 6, 61-75% chlorotic; 7, 76-90% chlorotic; 8, 91-99% chlorotic; 9, dead. The graph shows health scores over time for the control line, Nipponbare. Each dot represents one or more plants. The day on which the average health score was 5, day 22, was determined by fitting a regression line and drop lines to the axes. The NaCl stress was 150mM on day 22. Data from day 22 for Nipponbare was then compared to the transgenic lines (Figure 4.13 to Figure 4.25).

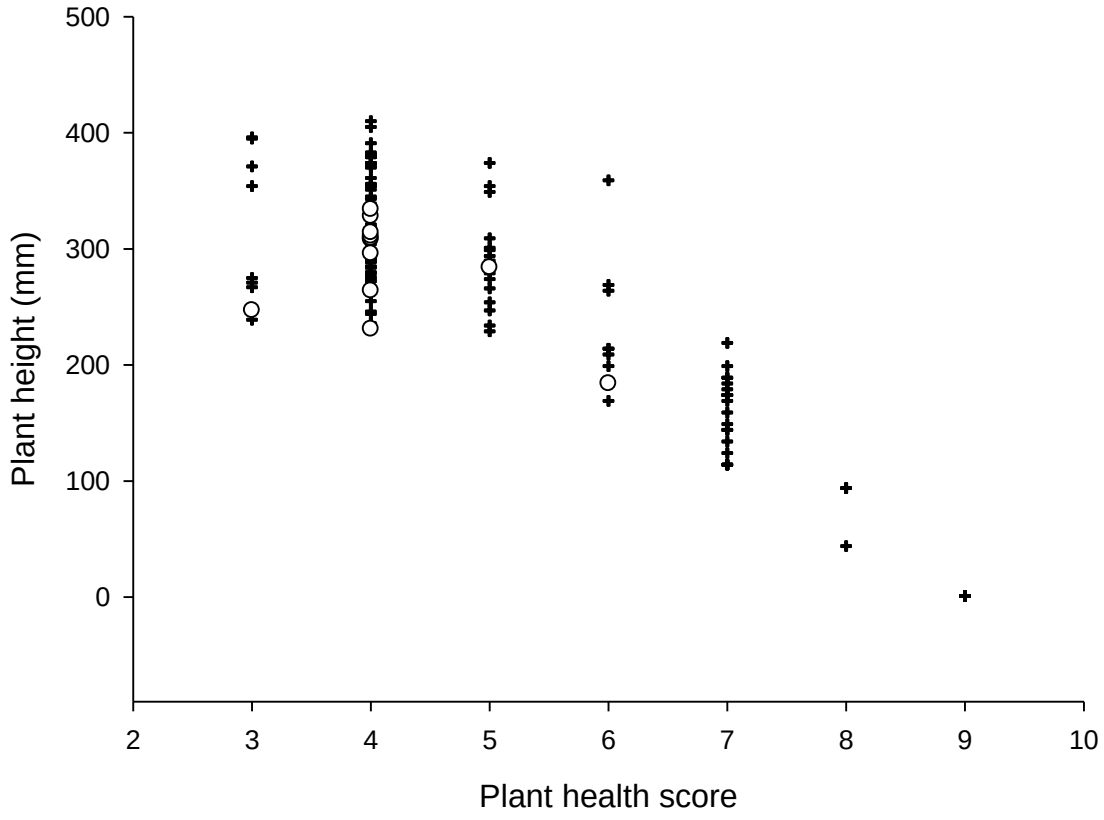


Figure 4.13 Graph of plant heights and health scores comparing the rice line *2x35S:OsOrphan19* 8A and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsOrphan19* 8A (white dots). Each data point represents one plant.

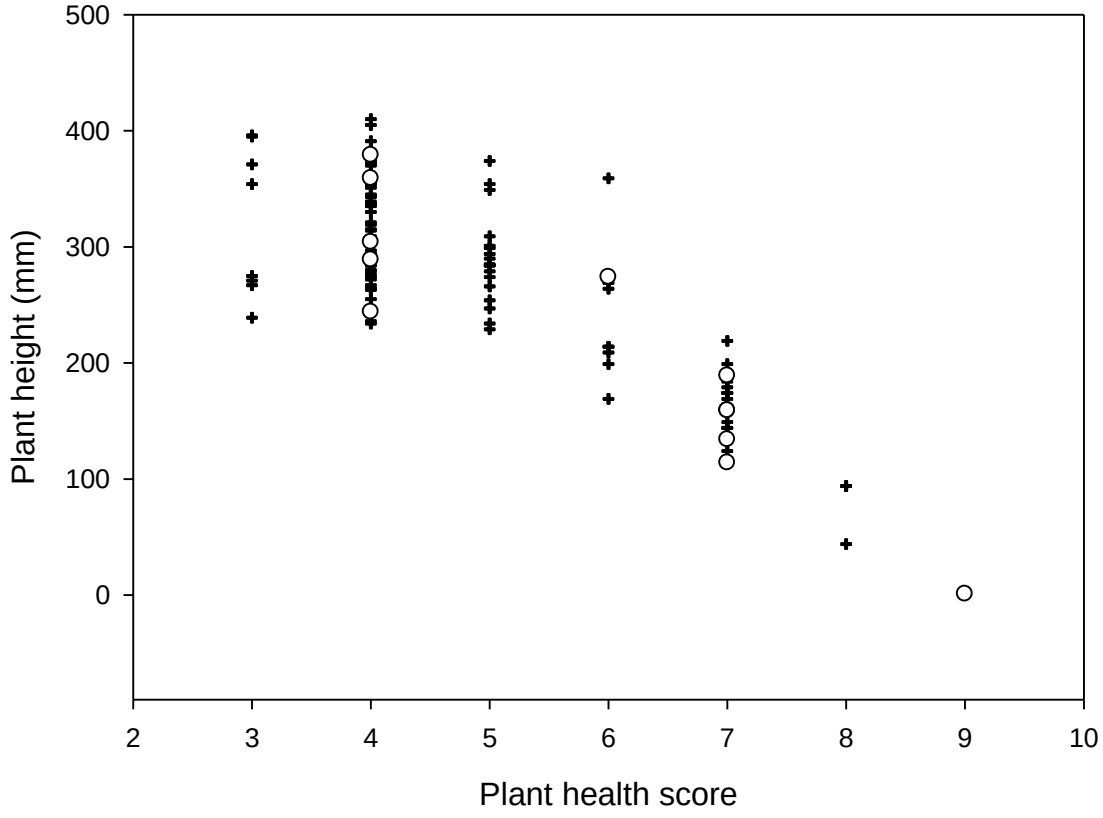


Figure 4.14 Graph of plant heights and health scores comparing the rice line *2x35S:OsOrphan19* 8C and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsOrphan19* 8C (white dots). Each data point represents one plant.

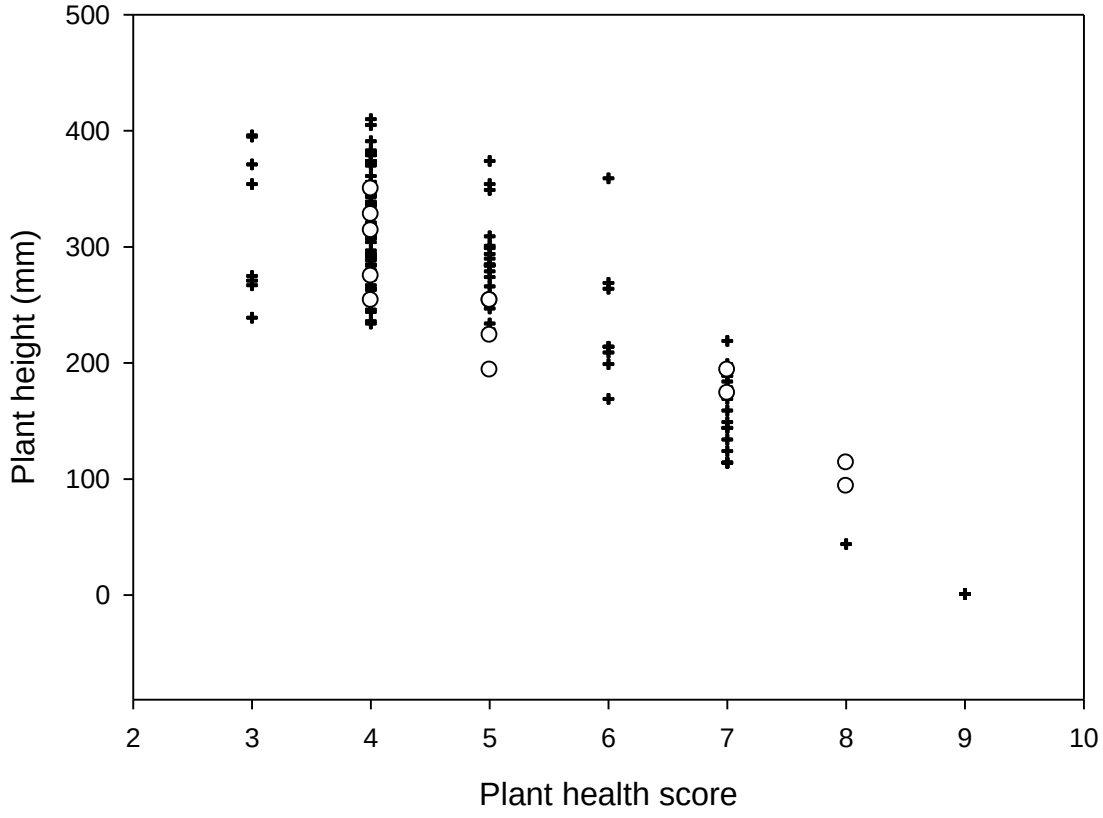


Figure 4.15 Graph of plant heights and health scores comparing the rice line *2x35S:OsOrphan19* 9B and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsOrphan19* 9B (white dots). Each data point represents one plant.

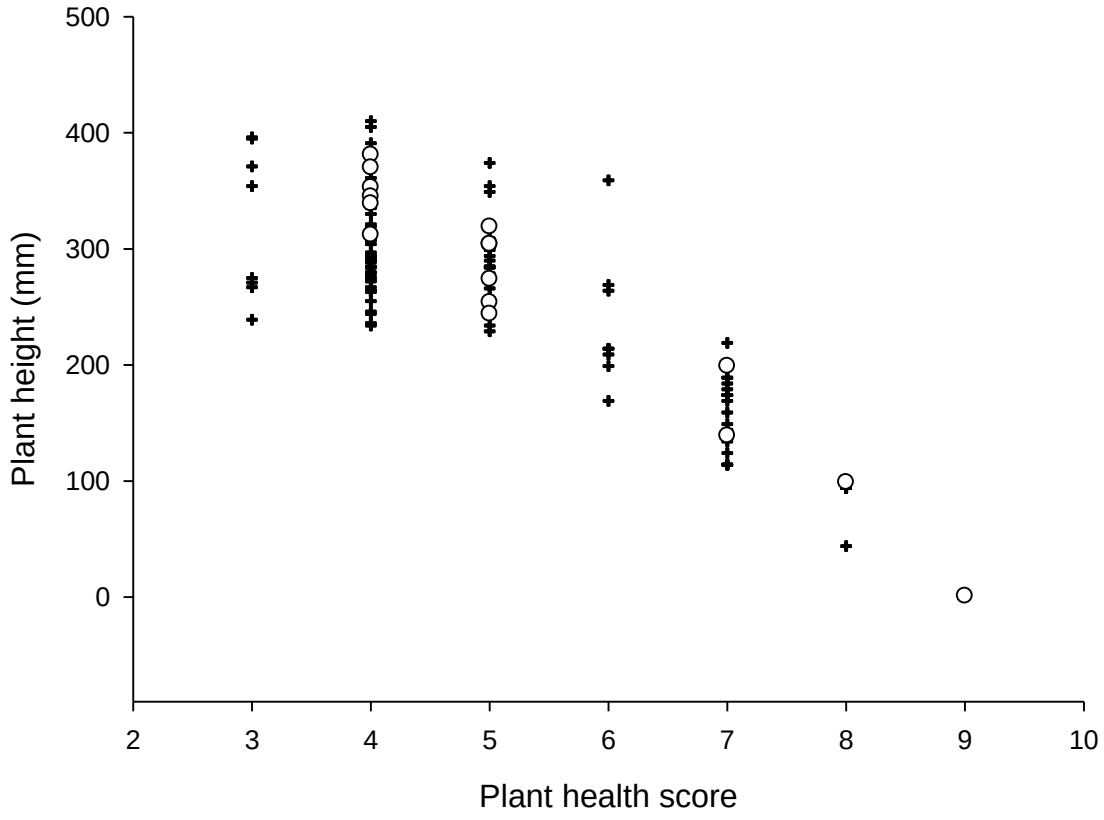


Figure 4.16 Graph of plant heights and health scores comparing the rice line *2x35S:OsOrphan19* 9D and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsOrphan19* 9D (white dots). Each data point represents one plant.

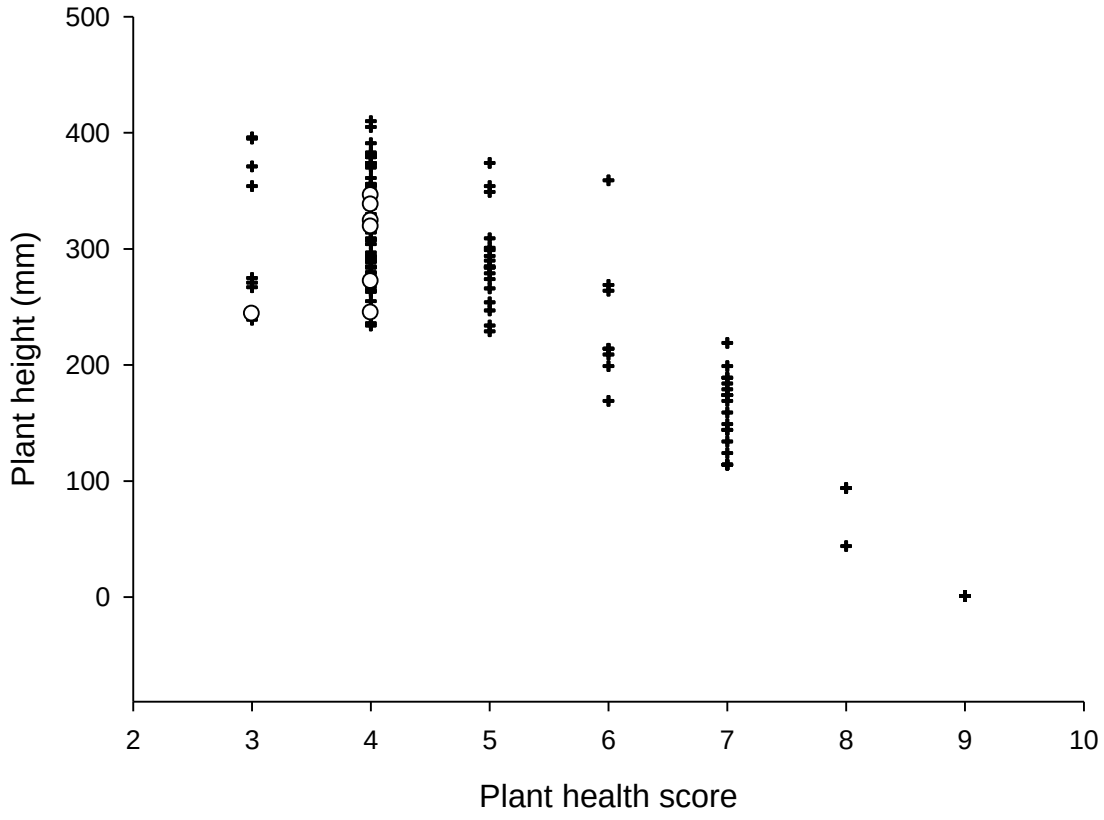


Figure 4.17 Graph of plant heights and health scores comparing the rice line *2x35S:OsEREB67* 10B and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsEREB67* 10B (white dots). Each data point represents one plant.

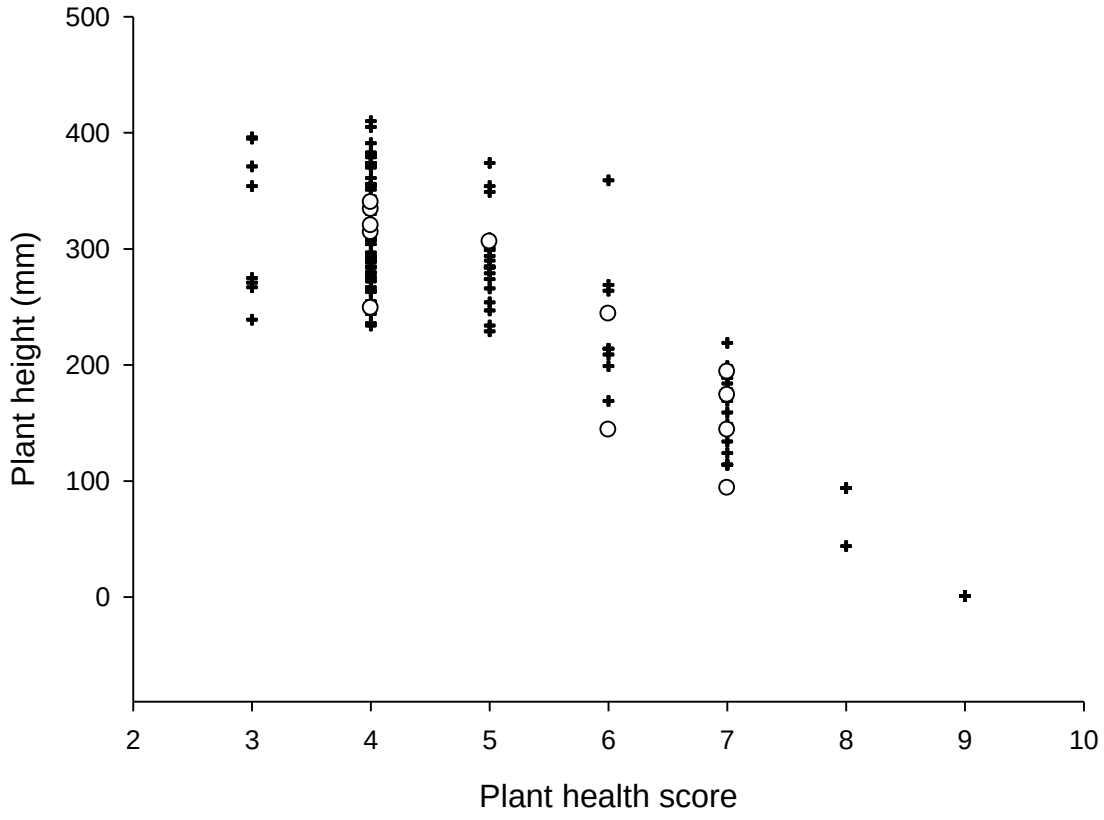


Figure 4.18 Graph of plant heights and health scores comparing the rice line *2x35S:OsMYBR63* 11A and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsMYBR63* 11A (white dots). Each data point represents one plant.

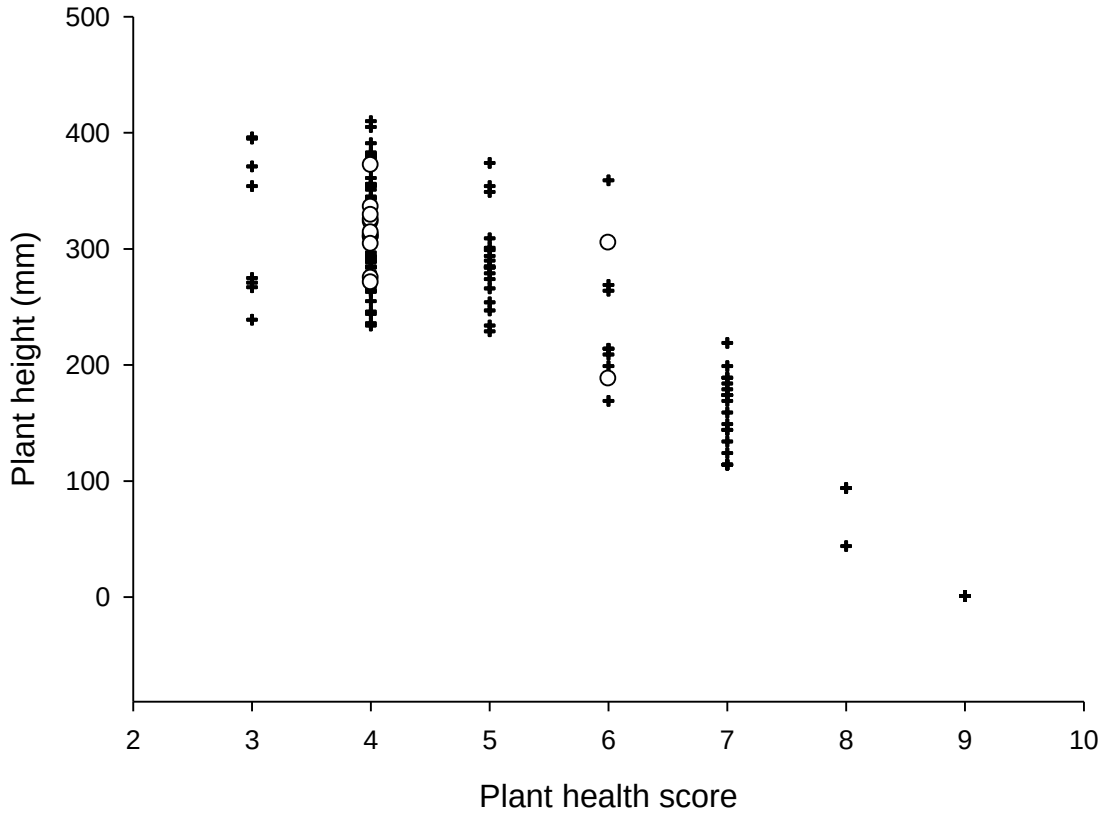


Figure 4.19 Graph of plant heights and health scores comparing the rice line *2x35S:OsMYBR63* 11C and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsMYBR63* 11C (white dots). Each data point represents one plant.

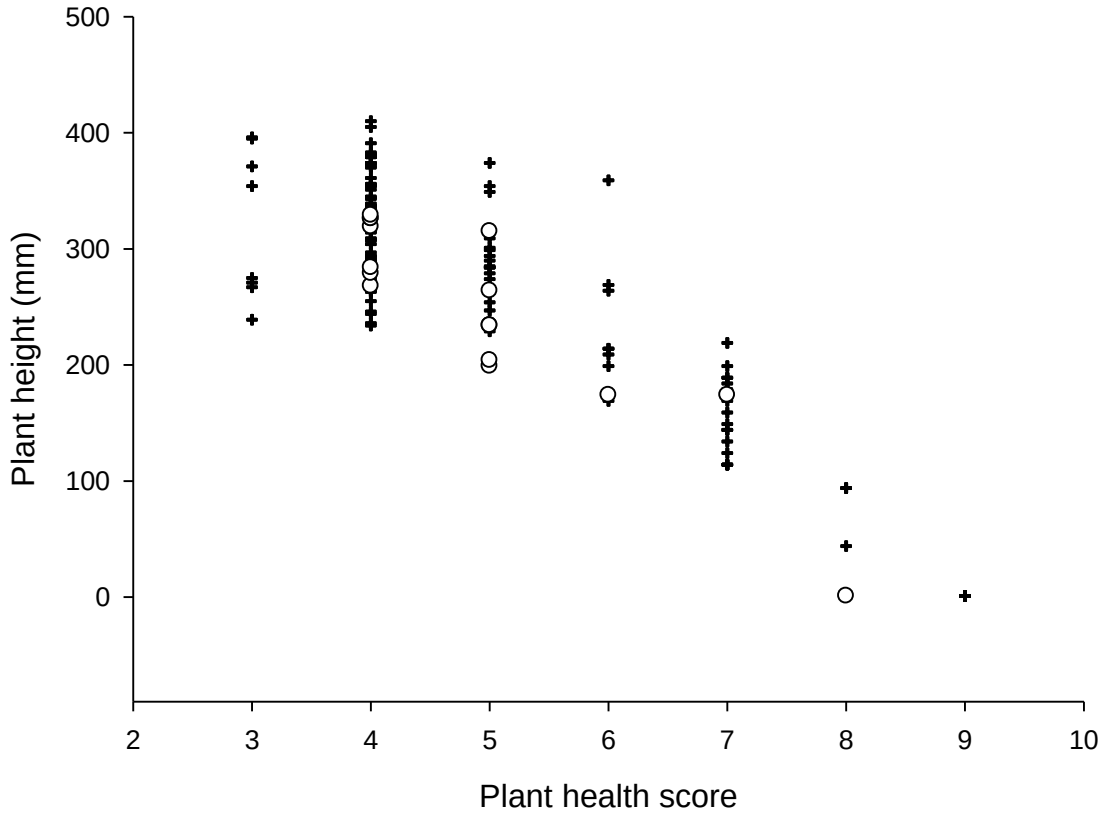


Figure 4.20 Graph of plant heights and health scores comparing the rice line *2x35S:OsLUX* 17C and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsLUX* 17C (white dots). Each data point represents one plant.

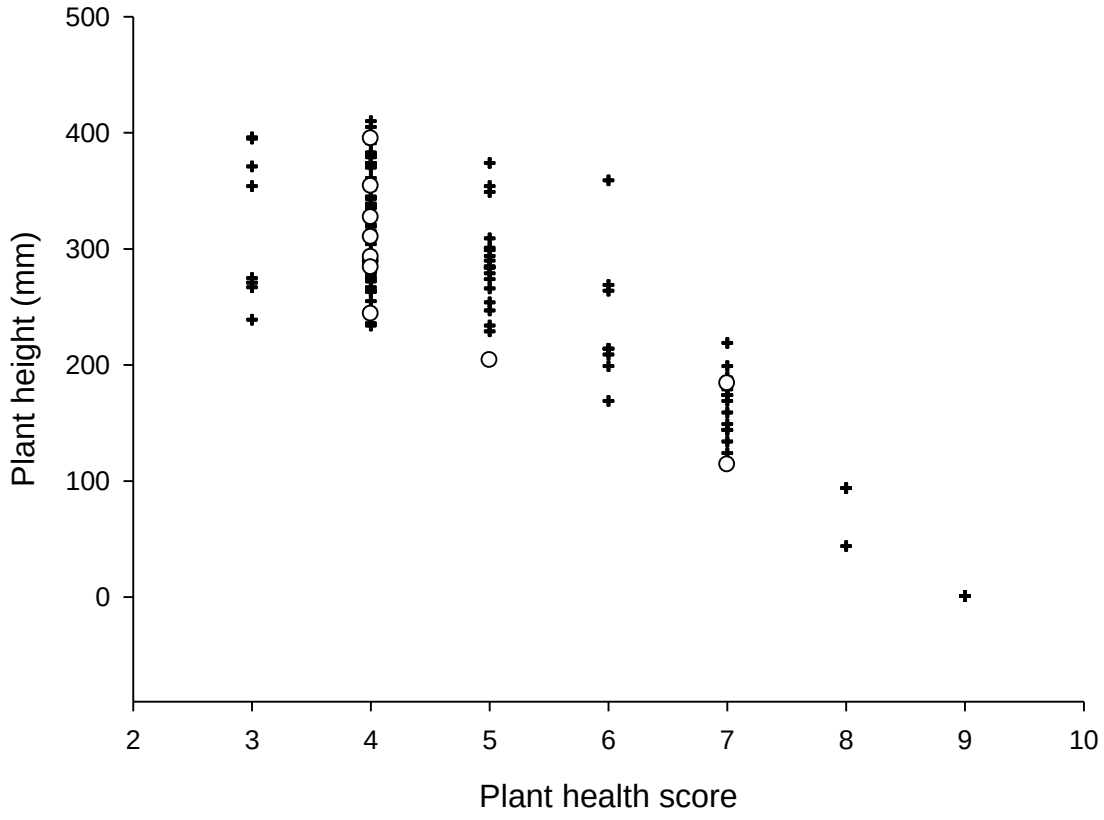


Figure 4.21 Graph of plant heights and health scores comparing the rice line *2x35S:OsLUX* 19C and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsLUX* 19C (white dots). Each data point represents one plant.

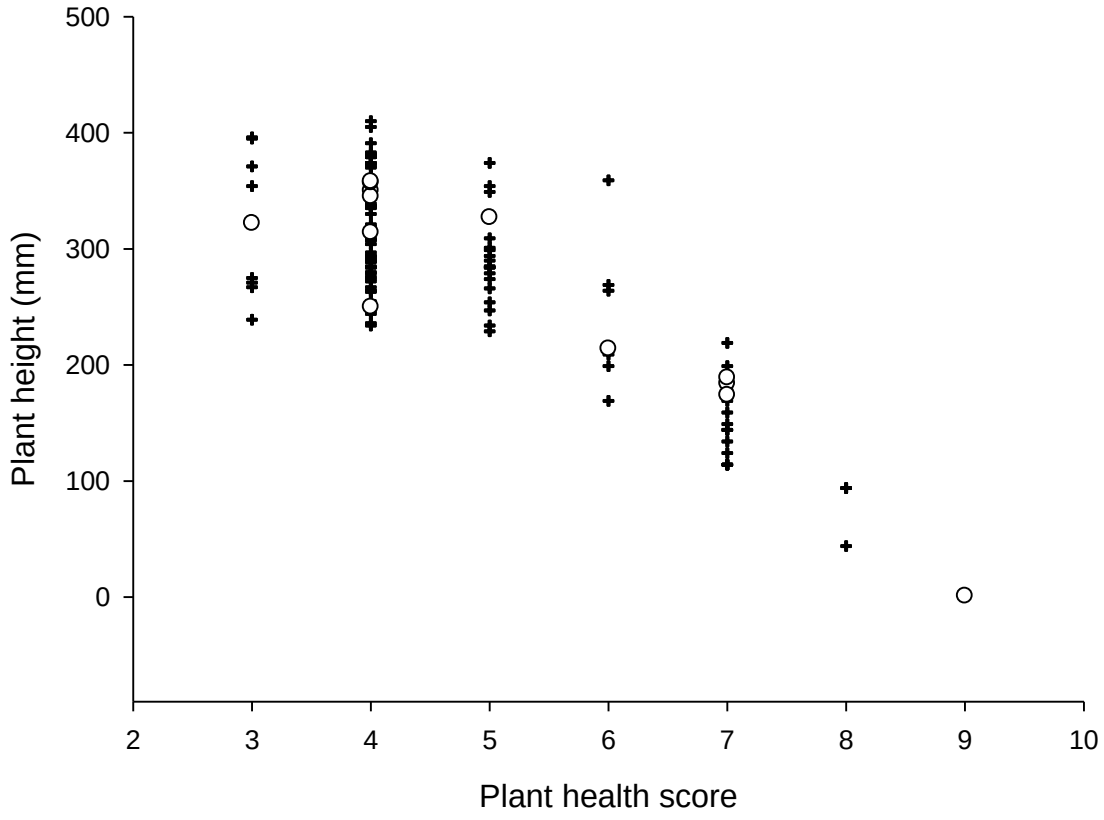


Figure 4.22 Graph of plant heights and health scores comparing the rice line *2x35S:OsMYB54* 22C and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsMYB54* 22C (white dots). Each data point represents one plant.

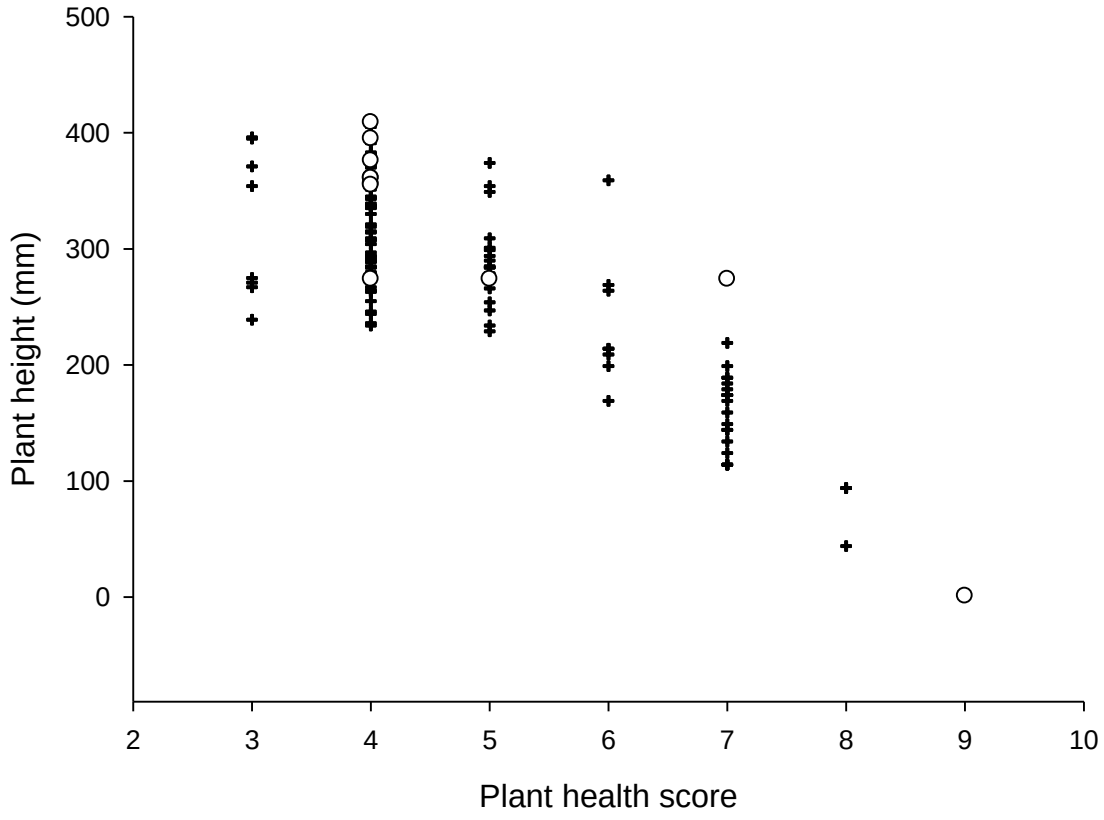


Figure 4.23 Graph of plant heights and health scores comparing the rice line *2x35S:OsMYB54* 22E and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsMYB54* 22E (white dots). Each data point represents one plant.

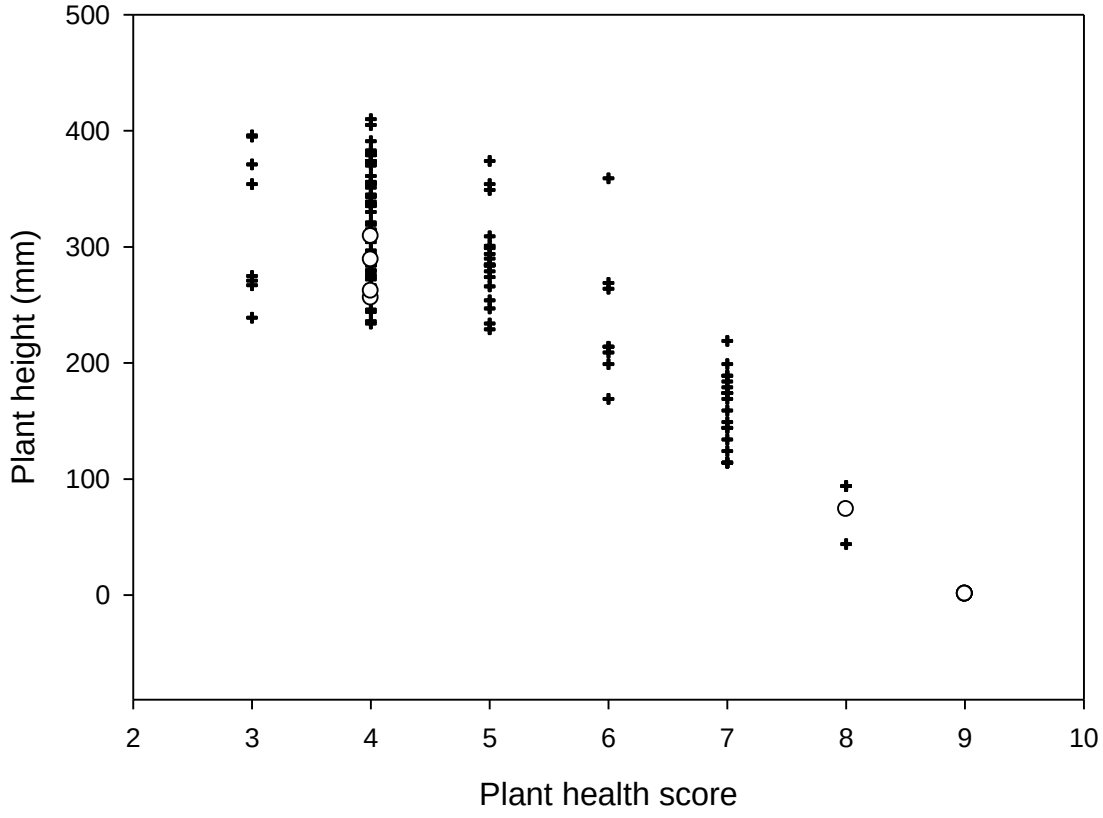


Figure 4.24 Graph of plant heights and health scores comparing the rice line *2x35S:OsMYB54* 23E and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsMYB54* 23E (white dots). Each data point represents one plant.

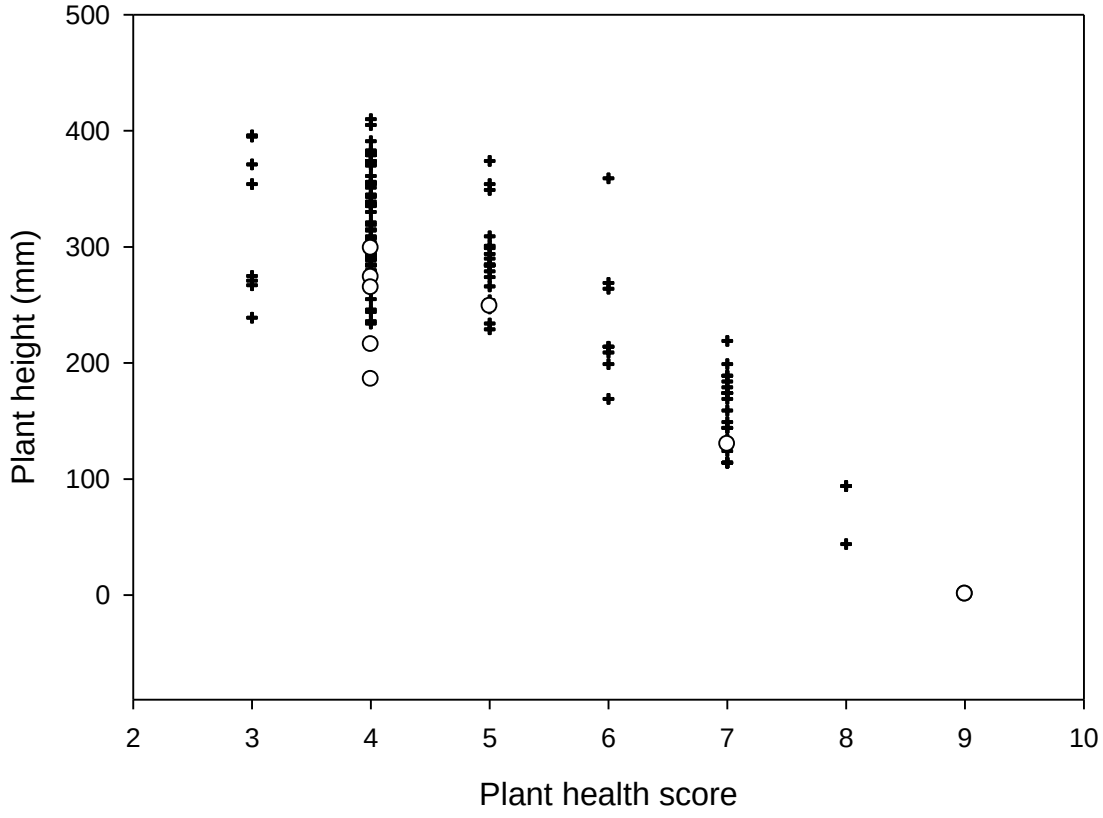


Figure 4.25 Graph of plant heights and health scores comparing the rice line *2x35S:OsMYB54* 25A and wild type rice plants

The data show plant heights against plant health scores on day 22 with a 150mM NaCl stress. Data from Nipponbare, empty vector transformants and null segregants (black crosses) are compared with data from *2x35S:OsMYB54* 25A (white dots). Each data point represents one plant.

4.3.4. Analysis of sodium and potassium accumulation in T1 plants

Sodium and potassium accumulation was examined in transgenic plants with constitutive and salt-inducible overexpression of transcription factor CDSs and with constitutive expression of amiRNA constructs, designed to knock down transcription factor transcript levels. Three replicates were conducted with plants with constitutive overexpression of transcription factor CDSs, albeit in different locations and with slightly different growth conditions. A single replicate was conducted for plants with salt-inducible overexpression of transcription factor CDSs and with constitutive expression of amiRNA constructs.

There were two factors that affected plant growth and sodium accumulation, which distort the results and make comparisons more difficult between the different sodium and potassium accumulation experiments. These were; changes to the growth solution and a change of growth room.

The growth solution was modified two times to improve plant health (Table 4.1). The growth solution initially contained 0.5mM sodium silicate, which gave the rice plants symptoms of nitrogen and iron deficiency. The sodium silicate was then removed from the growth solution, which resulted in the plants accumulating two-fold or greater sodium and increased mortality. Finally 0.05mM sodium silicate was used, resulting in healthy plants and reduced sodium accumulation.

Due to a fungal outbreak in CE1, plant growth experiments were moved to a greenhouse in The Plant Accelerator®. This occurred during the first experiment involving transgenic rice with the salt inducible promoter driving expression of transcription factor CDSs (*JRC0470:OsOrphan19* etc). The plants from this experiment were subsequently destroyed. The differences between the two locations are outlined in Table 4.1. The notable differences between the two locations are light intensity, day length and humidity. Light intensity was sustained for the entire day length of 12 hours in CE1, where plants were grown under artificial light. Whereas the light intensity was high for approximately six hours in The Plant Accelerator®, as the light source was natural sunlight. Accordingly, the rice plants grown in The Plant Accelerator® had reduced fresh weights, relative to those grown in the growth room, CE1. The lower humidity in The Plant Accelerator would have affected pollen vitality and stigma receptivity, had the plants been grown until flowering (Xu & Li, 1986).

The fungal contamination was suspected to have gone undetected for a significant period of time prior to the official outbreak. A symptom of contamination included black marks on harvested seed. These were seen on seed harvested a year prior to the outbreak from plants with constitutive overexpression of transcription factor coding sequences.

The results of the sodium and potassium accumulation experiments have been divided into sections based on which transcription factor had altered expression.

Table 4.1 Experimental conditions for sodium and potassium accumulation experiments

The table outlines the differences in plant growth conditions between the different sodium and potassium accumulation experiments. This includes the constructs involved, the two locations at which the plants were grown, CE1 at the AGRF and The Plant Accelerator® (TPA) at the Australian Plant Phenomics Facility, the differing conditions between the facilities and the changes in growth solution.

Transgenics	Location	Conditions	Medium
Rep 1-2x35S:OsTFCDS	CE1	12h light 400 µmol.m^{-2}.s^{-1} 70-90% humidity 28°C day 25°C night	0.5mM silicate
Rep 2-2x35S:OsTFCDS	CE1	12h light 400 µmol.m^{-2}.s^{-1} 70-90% humidity 28°C day 25°C night	0.5mM silicate
Rep 1-JRC0470:OsTFCDS	CE1	12h light 400 µmol.m^{-2}.s^{-1} 70-90% humidity 28°C day 25°C night	0mM silicate
Rep 3-2x35S:OsTFCDS	TPA	9h 57min light, only ≤6h light at 200-500µmol.m^{-2}.s^{-1} 5-50% humidity 28°C day 25°C night	0mM silicate
Rep 2-JRC0470:OsTFCDS	TPA	9h 57min light, only ≤6h light at 200-500µmol.m^{-2}.s^{-1} 5-50% humidity 28°C day 25°C night	0mM silicate
Rep 1-2x35S:amiRNA	CE1	12h light 400 µmol.m^{-2}.s^{-1} 70-90% humidity 28°C day 25°C night	0.05mM silicate

4.3.4.1. OsOrphan19

Expression of the transcription factor *OsOrphan19* was altered in rice plants and indicators of salt tolerance assessed. Semi-quantitative PCR was used to analyse transgene transcript levels in *2x35S:OsOrphan19* (Figure 4.26) and *JRC0470:OsOrphan19* expressing rice plants (Figure 4.27) and endogenous gene transcript levels in *2x35S:OsOrphan19 amiRNA* expressing rice plants (Figure 4.28). The *2x35S:OsOrphan19* and *JRC0470:OsOrphan19* lines were found to have high transgene transcript levels. The amiRNA expressing lines had strongly reduced *OsOrphan19* transcript levels in the T0 generation and variable levels comparable to wild type plants in the T1 generation.

Increased expression of *OsOrphan19* reduced sodium accumulation and reduced expression increased sodium accumulation. This can be seen in Figure 4.29, where the *2x35S:OsOrphan19* lines 8A, 8C and 9D, and the *JRC0470:OsOrphan19* lines have reduced sodium accumulation relative to wild type and null plants. The large error bars for 9B are due to two plants having leaf sodium concentrations of 27mM and 39mM, and two having 217mM and 273mM. The amiRNA expressing lines have increased sodium relative to wild type plants and nulls. This suggests increased expression reduces sodium accumulation and reduced expression increases sodium accumulation.

Altered expression of *OsOrphan19* greatly affected the leaf K^+:Na^+ ratios. This can be seen in Figure 4.30. The *2x35S:OsOrphan19* and salt inducible lines had comparable or improved K^+:Na^+ ratios, whereas the amiRNA expressing lines had poor K^+:Na^+ ratios, relative to wild type and null plants.

Increased expression of *OsOrphan19* either did not affect or increased fresh weight, whereas reduced expression decreased fresh weight. This can be seen in Figure 4.31. The only statistically significant differences were between the two transgenic lines, 8A and 9D (P=<0.001) and between 9D and the wild type, Nipponbare (P=0.001) (See Section 5.3.1.1 for ANOVA). No other fresh weight differences were statistically significant. The low fresh weights for the *2x35S:OsOrphan19* lines in experiment three (green dots) were due to low light, variable temperatures and a short cold treatment in the second week of growth due to a malfunction in the growth room temperature control system. The variability seen in fresh weights between the *JRC0470:OsOrphan19* lines in Exp2 (pink) and all other lines was due to different growth conditions. The terminated experiment for the *JRC0470:OsOrphan19* lines was in CE1 (orange dots), where the fresh weights were higher due to the different growth conditions. The fresh

weights for the *JRC0470:OsOrphan19* plants were comparable to wild type and nulls under salt stress in both experiments/growth environments. AmiRNA expressing lines consistently had reduced fresh weights relative to wild type and nulls plants.

Plants with constitutive overexpression of *OsOrphan19* had reduced germination (Table 5.10), relative to plants with salt inducible overexpression (Table 5.11) or reduced expression of *OsOrphan19* (Table 5.9) and wild type plants (Table 5.9, Table 5.10, Table 5.11). Plants with constitutive overexpression of *OsOrphan19* also had reduced 100 grain weight ((Table 5.10), relative to plants with salt inducible overexpression of *OsOrphan19* (Table 5.11) and wild type plants (Table 5.9, Table 5.10, Table 5.11). Six days following germination, the plants with constitutive overexpression of *OsOrphan19* had short coleoptiles and long seminal roots, whereas the two of three amiRNA knockdown lines had large plants with vigorous shoot growth (Table 5.9, Table 5.10).

The data show that increased expression of *OsOrphan19* can increase fresh weight, improve the K^+:Na^+ ratio and reduce sodium accumulation, and that reduced expression of *OsOrphan19* reduces fresh weight, has a negative impact upon the K^+:Na^+ ratio and increases sodium accumulation.

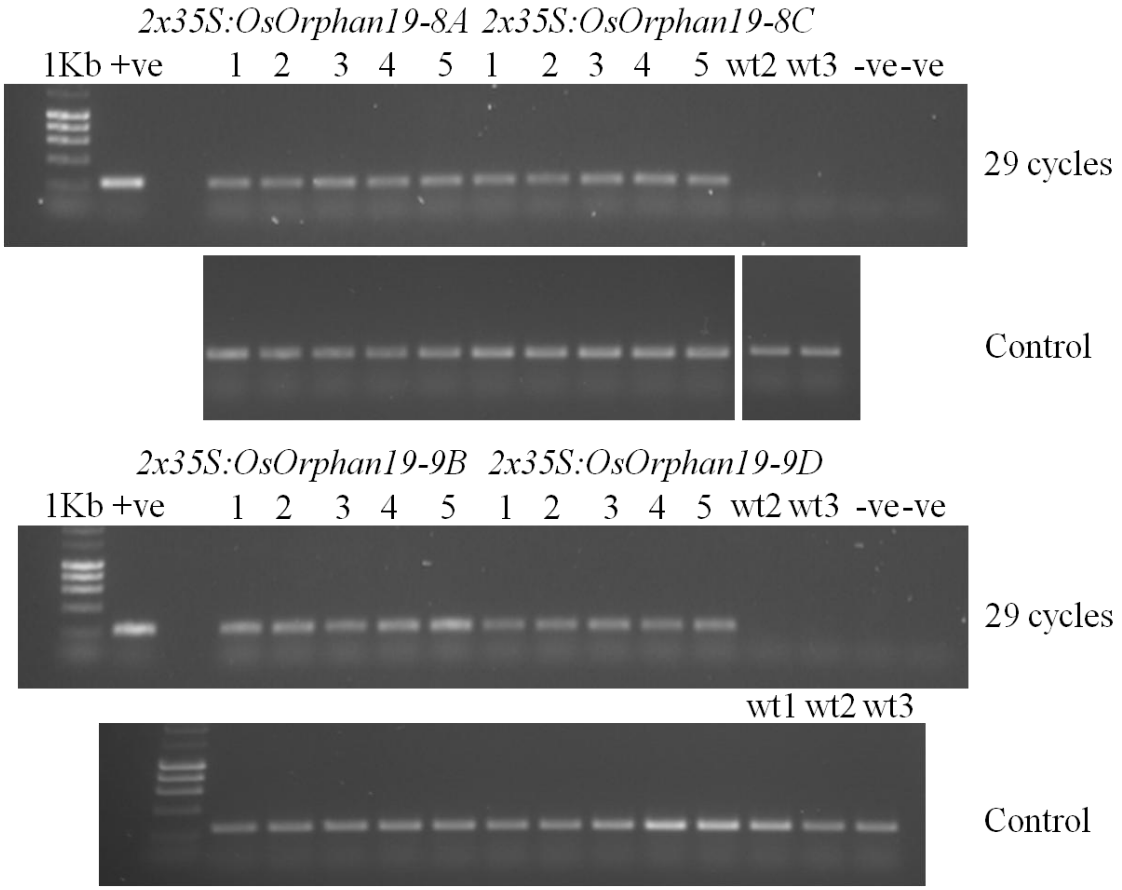


Figure 4.26 Semi quantitative RT-PCR analysis of transgene transcript levels in *2x35S:OsOrphan19* rice plants

Transgene transcript levels were analysed in rice plants with constitutive overexpression of the *OsOrphan19* CDS. Tissue was harvested from T0 primary transformant leaf tissue at the vegetative stage, growing in soil and immersed in ACPFG nutrient solution. The tissue was snap frozen in liquid nitrogen, total RNA was extracted using TRIzol, DNase treated and cDNA synthesised using oligo dT primers. Transgene transcript levels were analysed using primers specific for *OsOrphan19* and the nopaline synthase terminator; expected size 193bp, 29 cycles. The control gene amplified was α-tubulin, as this had the most consistent expression in previous qRT-PCR experiments using Nipponbare rice plants; expected size 234bp, 29 cycles. Positive control template was a sequenced plasmid. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

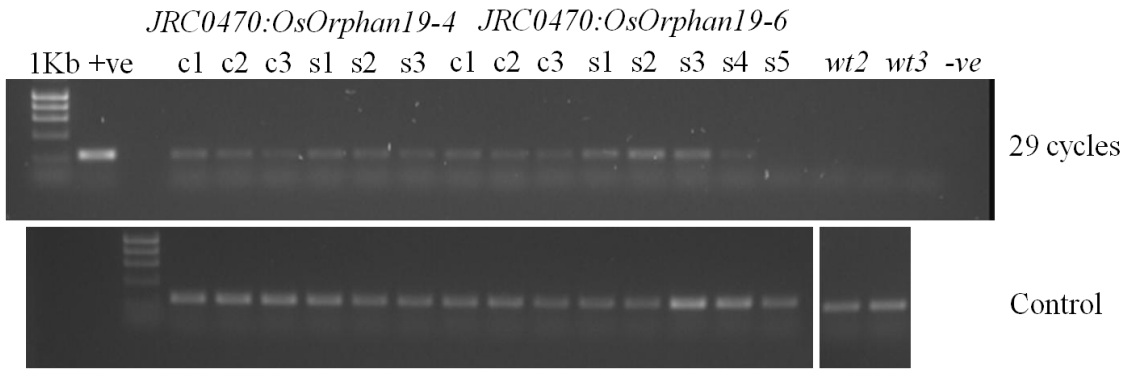


Figure 4.27 Semi quantitative RT-PCR analysis of transgene transcript levels in *JRC0470:OsOrphan19* rice plants

Transgene transcript levels were analysed in plants with salt-inducible overexpression of the *OsOrphan19* CDS. Transgene transcript levels were analysed using primers specific for *OsOrphan19* and the nopaline synthase terminator; expected size 193bp, 29 cycles. α-tubulin was used as the control gene; expected size 234bp, 29 cycles. Control treatment c1-c3. Salt stress treatment s1-s5. Positive control template was a sequenced plasmid. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

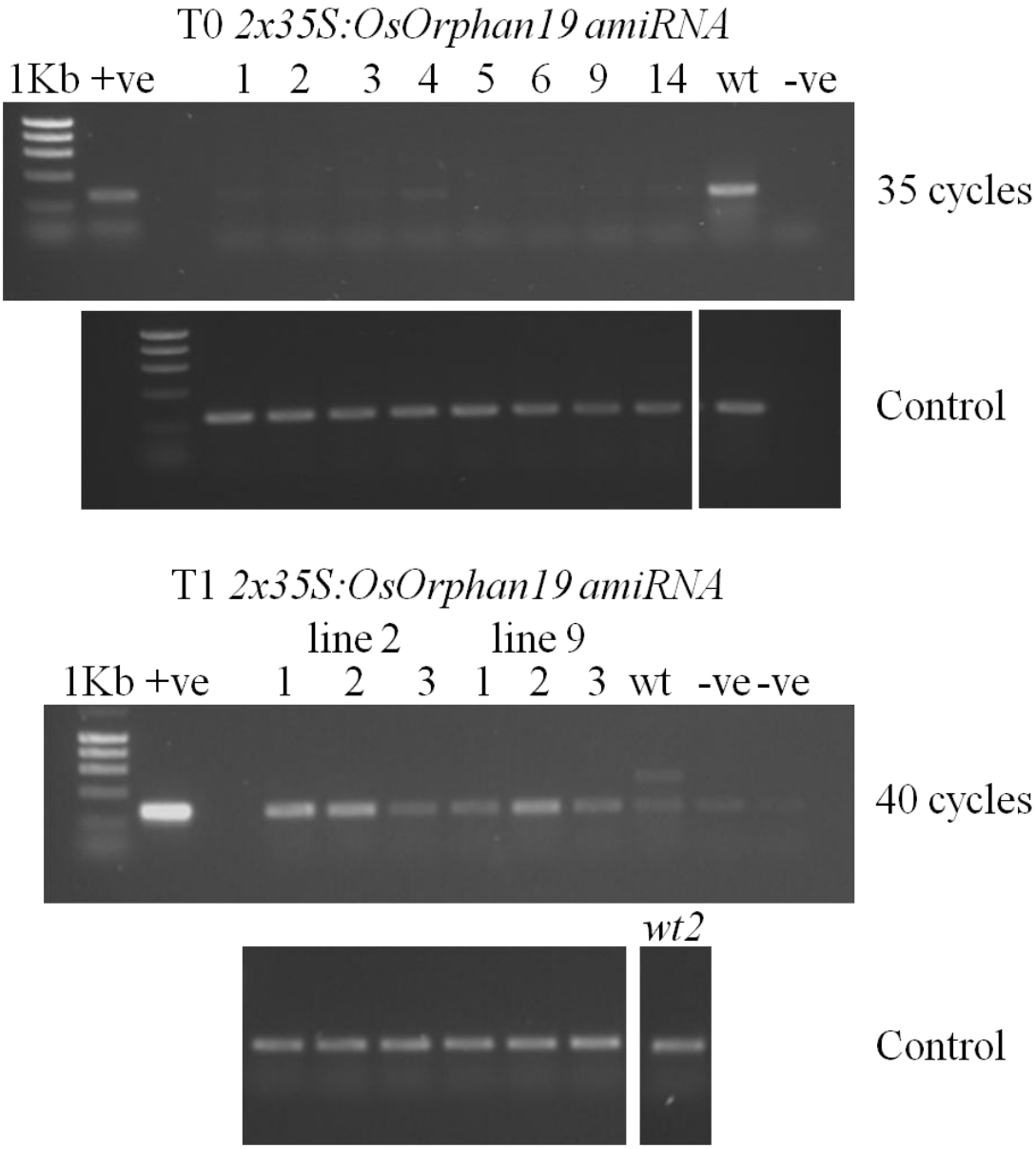


Figure 4.28 Semi quantitative RT-PCR analysis of endogenous transcript levels in *2x35S:OsOrphan19 amiRNA* rice plants

Endogenous transcript levels of *OsOrphan19* were analysed in plants with constitutive overexpression of an amiRNA construct targeting *OsOrphan19*. Transcript levels were analysed using primers specific for *OsOrphan19*; expected size 193bp, 29 cycles. α-tubulin was used as the control gene; expected size 234bp, 29 cycles. Positive control template was a sequenced PCR product. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

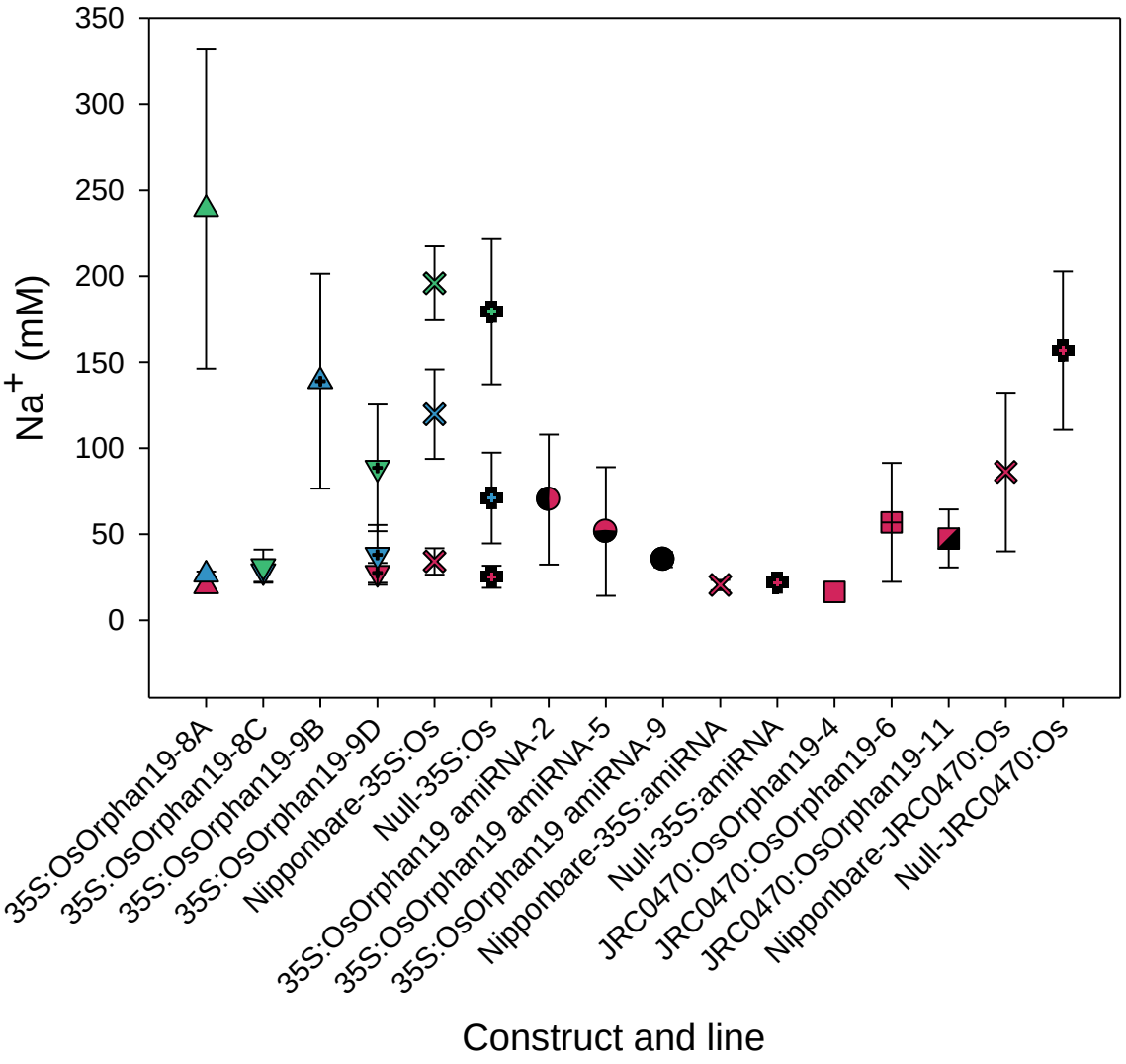


Figure 4.29 Graph of sodium accumulation in rice plants with altered expression of *OsOrphan19* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry. The graph contains data from *2x35S:OsOrphan19* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsOrphan19 amiRNA* (centre) and *JRC0470:OsOrphan19* (right) rice. Error bars represent SEM. n≥3.

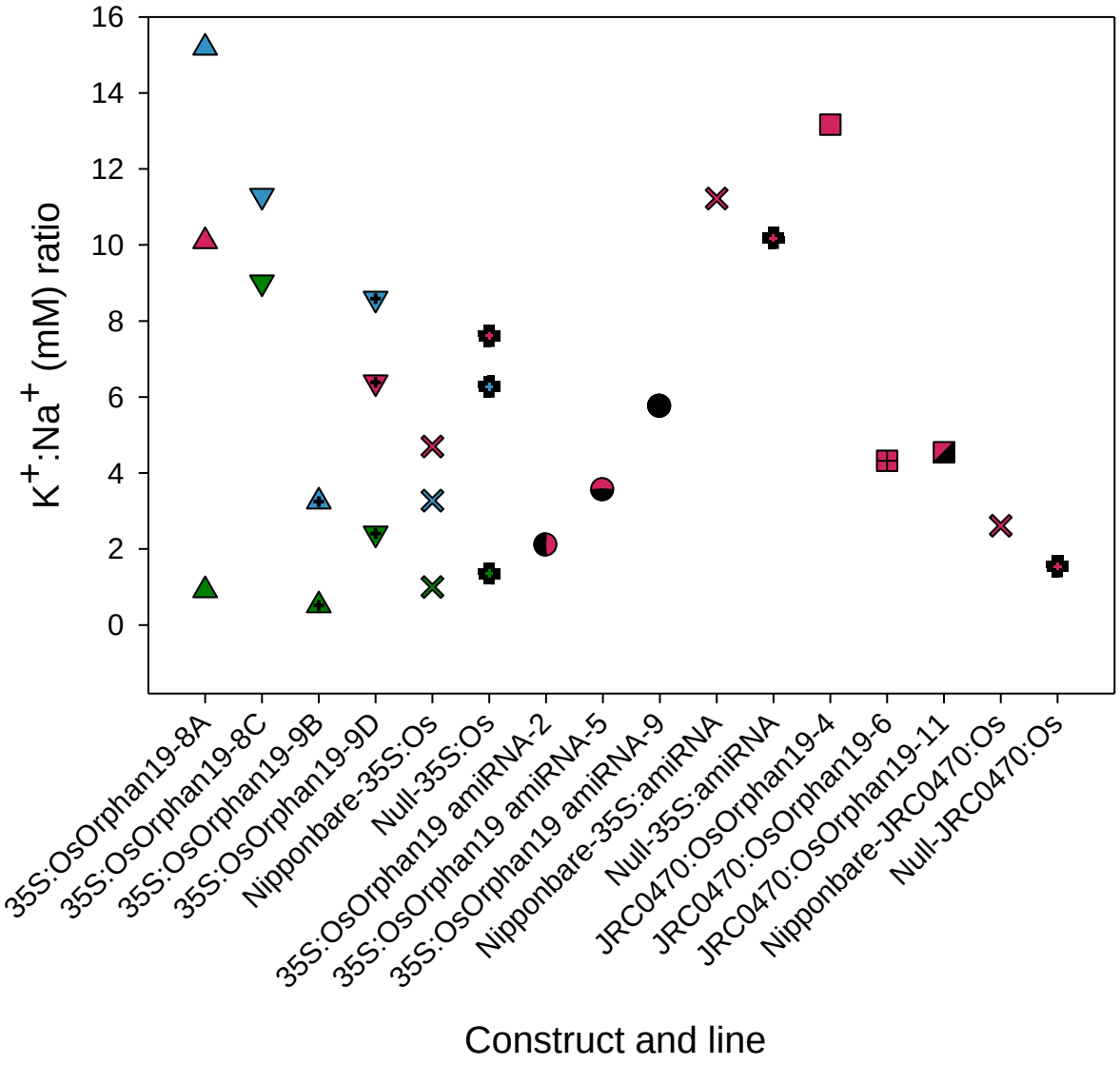


Figure 4.30 Graph of K^+:Na^+ ratio in rice plants with altered expression of *OsOrphan19* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry and the K^+:Na^+ ratio calculated. The graph contains data from *2x35S:OsOrphan19* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsOrphan19 amiRNA* (centre) and *JRC0470:OsOrphan19* (right) rice.

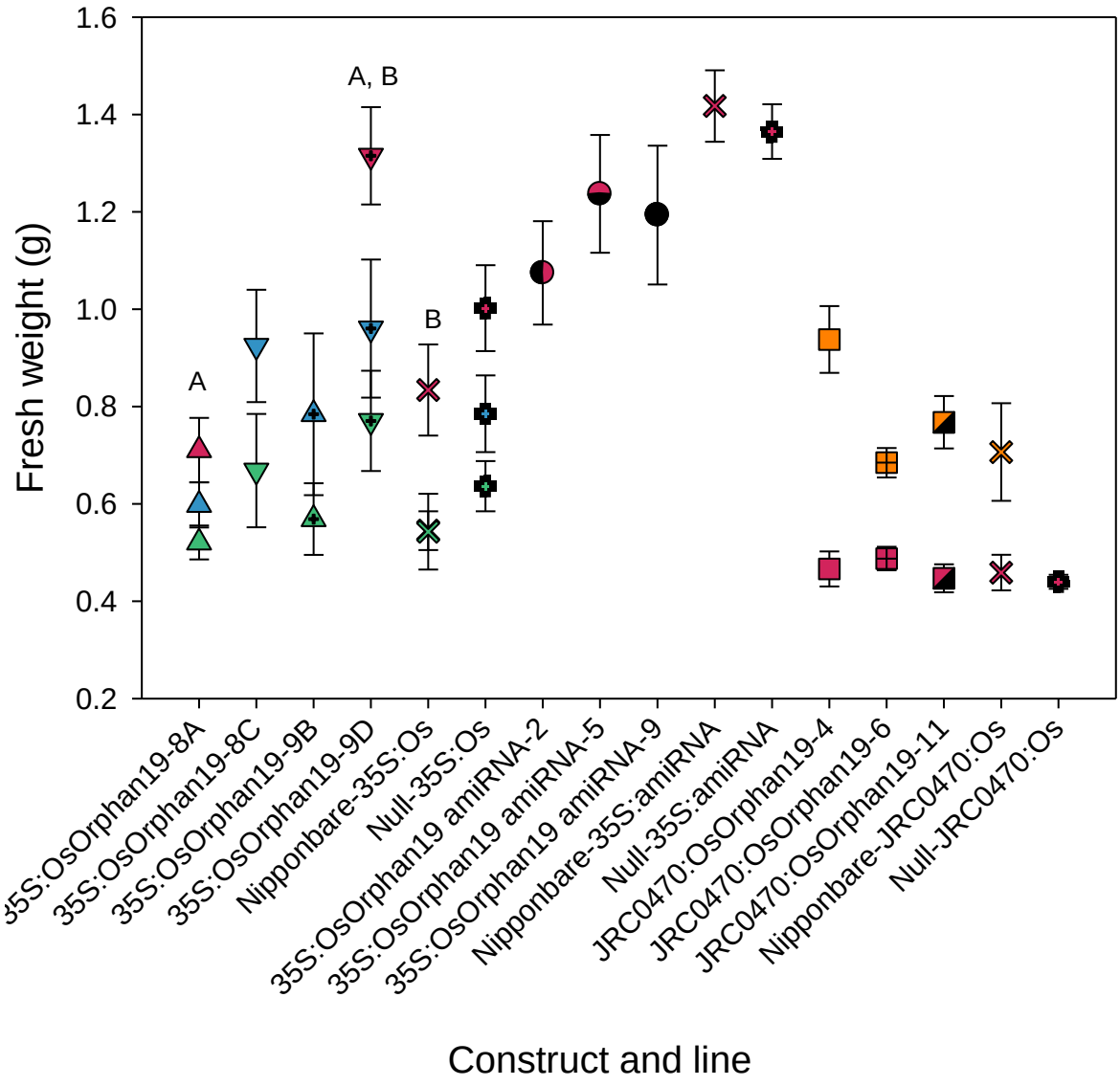


Figure 4.31 Graph of fresh weight in rice plants with altered expression of *OsOrphan19* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The fresh weight was then measured. The graph contains data from *2x35S:OsOrphan19* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsOrphan19 amiRNA* (centre) and *JRC0470:OsOrphan19* (right) rice. Error bars represent SEM. n≥3.

4.3.4.2. OsEREB67

Expression of the transcription factor *OsEREB67* was altered in rice plants and indicators of salt tolerance assessed. Semi-quantitative PCR was used to analyse transgene transcript levels in *2x35S:OsEREB67* expressing rice plants (Figure 4.32) and endogenous gene transcript levels in *2x35S:OsEREB67 amiRNA* expressing rice plants (Figure 4.33). The *2x35S:OsEREB67* line had very low transcript levels of the transgene and the amiRNA expressing lines had variably reduced endogenous gene transcript in the T0 and T1 generations.

Increased expression of *OsEREB67* reduced sodium accumulation and decreased expression increased sodium accumulation. This can be seen in Figure 4.34. The *2x35S:OsEREB67* line 10B had reduced sodium accumulation in all experiments. The amiRNA expressing lines had increased sodium accumulation relative to null and wild type plants. The variation in sodium accumulation in the wild type and null plants between experiments can be attributed to the use of silicate.

Altered expression of *OsEREB67* also altered the K^+:Na^+ ratio of the rice plants, which can be seen in Figure 4.35. The *2x35S:OsEREB67* line 10B had an improved K^+:Na^+ ratio relative to wild type and null plants, whereas all amiRNA expressing lines had poor K^+:Na^+ ratios relative to wild type and nulls plants; approximately 3-fold worse.

Altered expression of *OsEREB67* had a minimal effect upon plant fresh weight. This can be seen in Figure 4.36. The *2x35S:OsEREB67* line 10B had reduced fresh weight in experiments one and three, but increased fresh weight in experiment two and the amiRNA expressing lines had reduced fresh weight under salt stress.

Plants with constitutive overexpression of *OsEREB67* had reduced germination (Table 5.10), relative to plants with reduced expression of *OsEREB67* (Table 5.9) and wild type plants (Table 5.9, Table 5.10).

The data show that increased expression of *OsEREB67* decreases sodium accumulation and improves the K^+:Na^+ ratio and may reduce fresh weight, and that decreased expression of *OsEREB67* increases sodium accumulation, negatively affects the K^+:Na^+ ratio and may also reduce fresh weight.

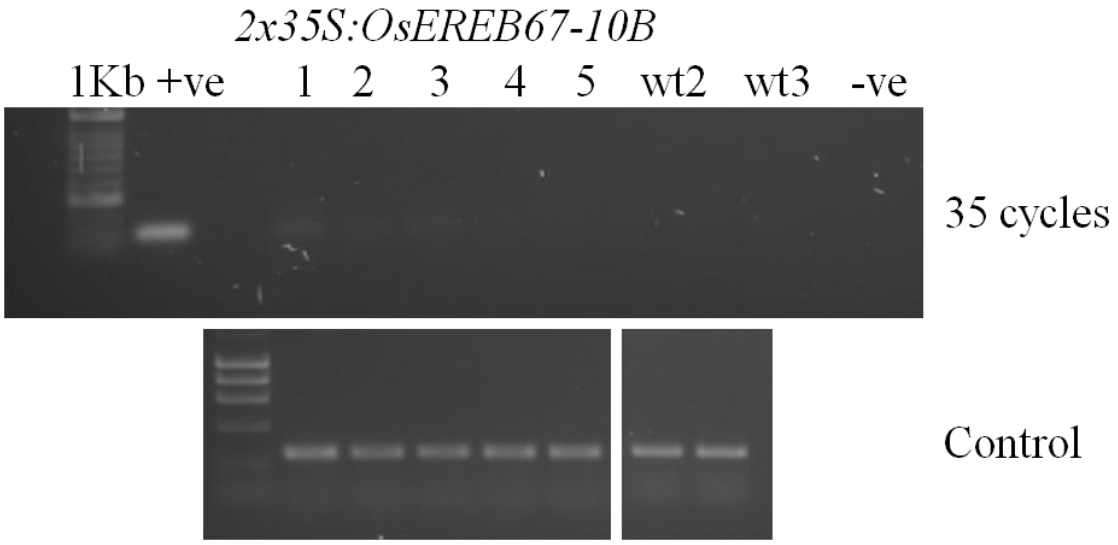


Figure 4.32 Semi quantitative RT-PCR analysis of transgene transcript levels in 2x35S:OsEREB67 rice plants

Transgene transcript levels were analysed in rice plants with constitutive overexpression of the *OsEREB67* CDS. Tissue was harvested from T0 primary transformant leaf tissue at the vegetative stage, growing in soil and immersed in ACPFG nutrient solution. The tissue was snap frozen in liquid nitrogen, total RNA was extracted using TRIzol, DNase treated and cDNA synthesised using oligo dT primers. Transgene transcript levels were analysed using primers specific for *OsEREB67* and the nopaline synthase terminator; expected size 131bp, 35 cycles. The control gene amplified was α-tubulin, as this had the most consistent expression in previous qRT-PCR experiments using Nipponbare rice plants; expected size 234bp, 29 cycles. Positive control template was a sequenced plasmid. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

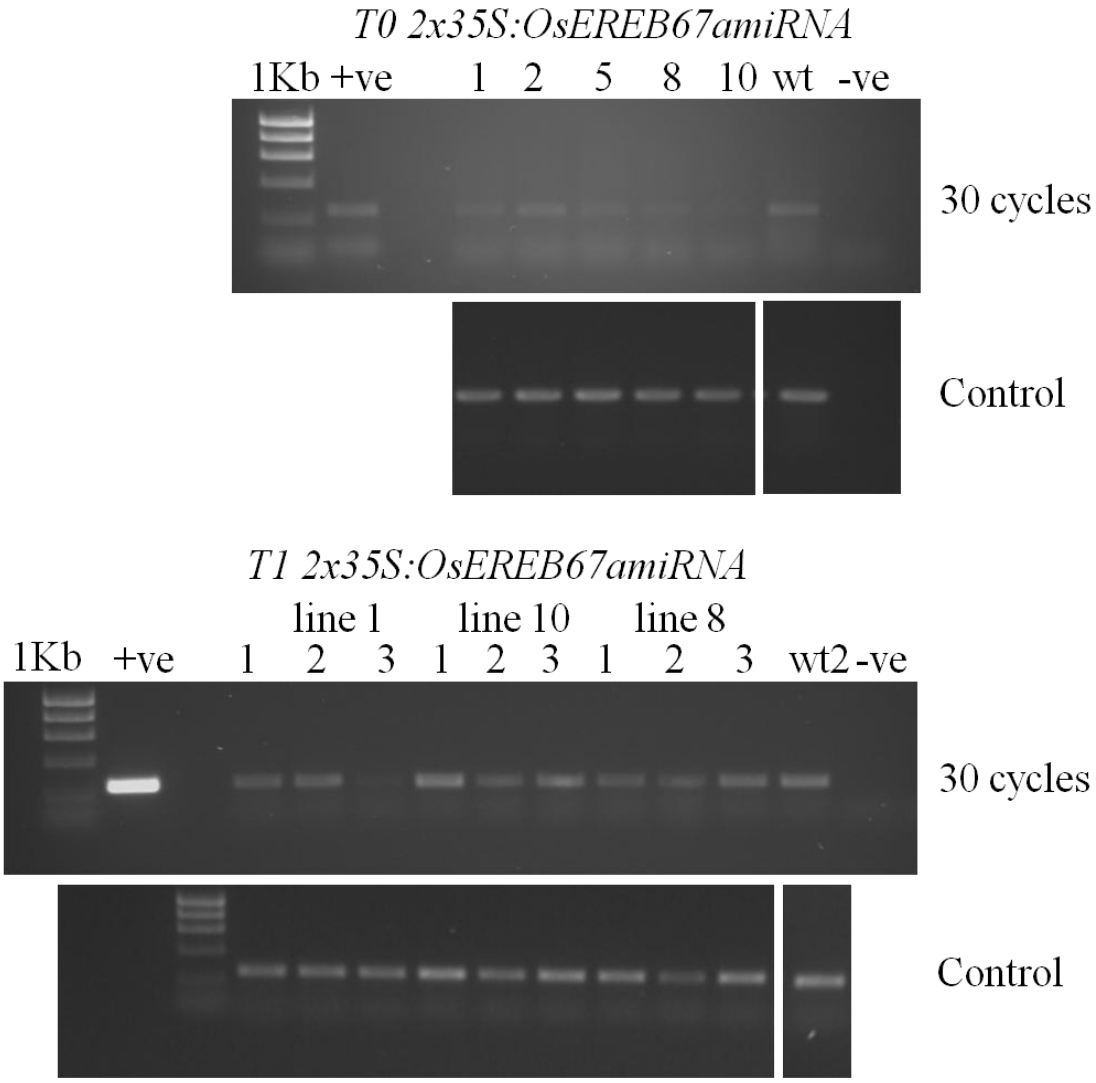


Figure 4.33 Semi quantitative RT-PCR analysis of endogenous transcript levels in 2x35S:OsEREB67 amiRNA rice plants

Endogenous transcript levels of *OsEREB67* were analysed in plants with constitutive overexpression of an amiRNA construct targeting *OsEREB67*. Transcript levels were analysed using primers specific for *OsEREB67*; expected size 236bp, 30 cycles. α-tubulin was used as the control gene; expected size 234bp, 29 cycles. Positive control template was a sequenced PCR product. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

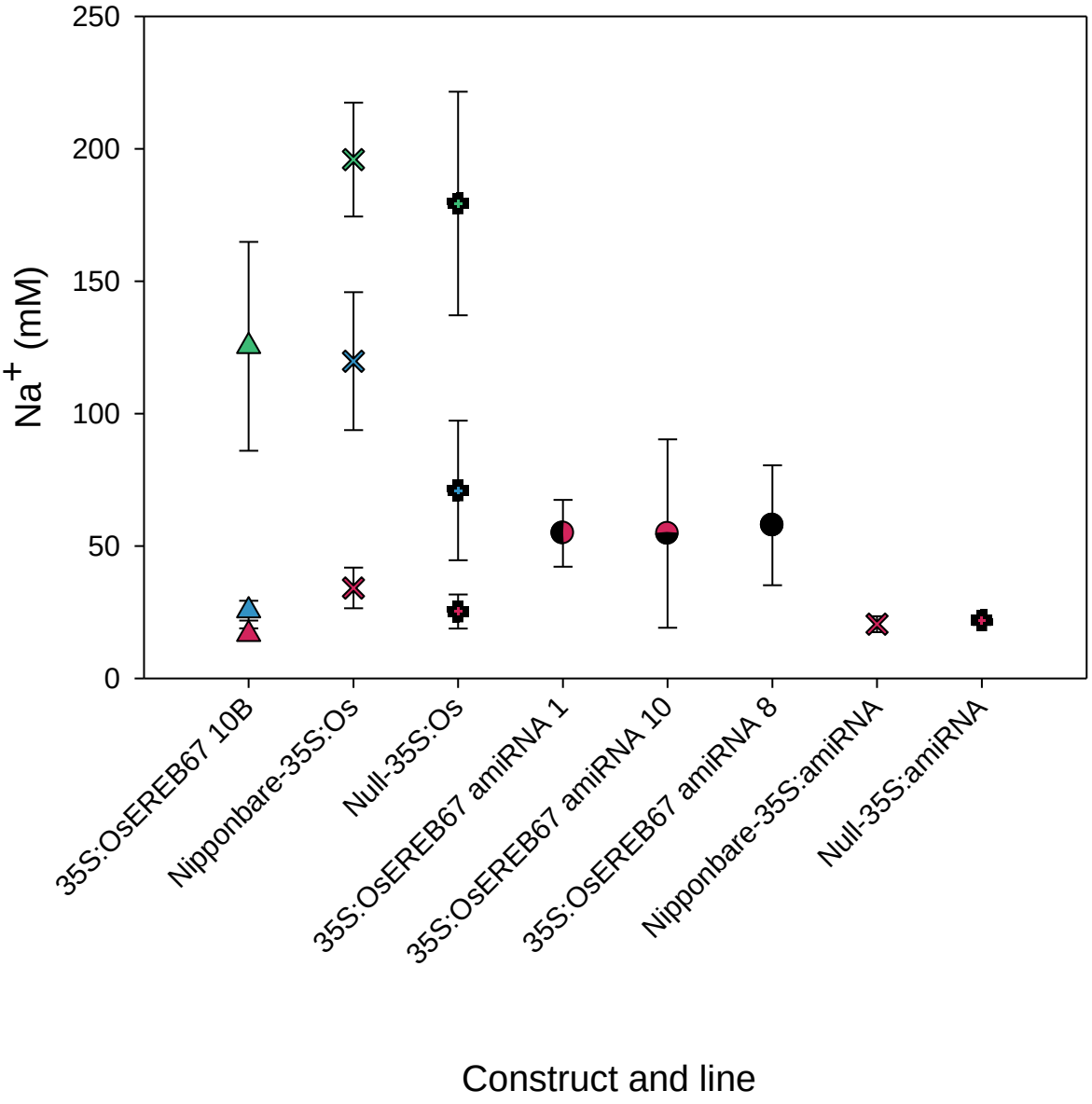


Figure 4.34 Graph of sodium accumulation in rice plants with altered expression of *OsEREB67* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry. The graph contains data from *2x35S:OsEREB67* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsEREB67 amiRNA* (centre) and *JRC0470:OsEREB67* (right) rice. Error bars represent SEM. n≥3.

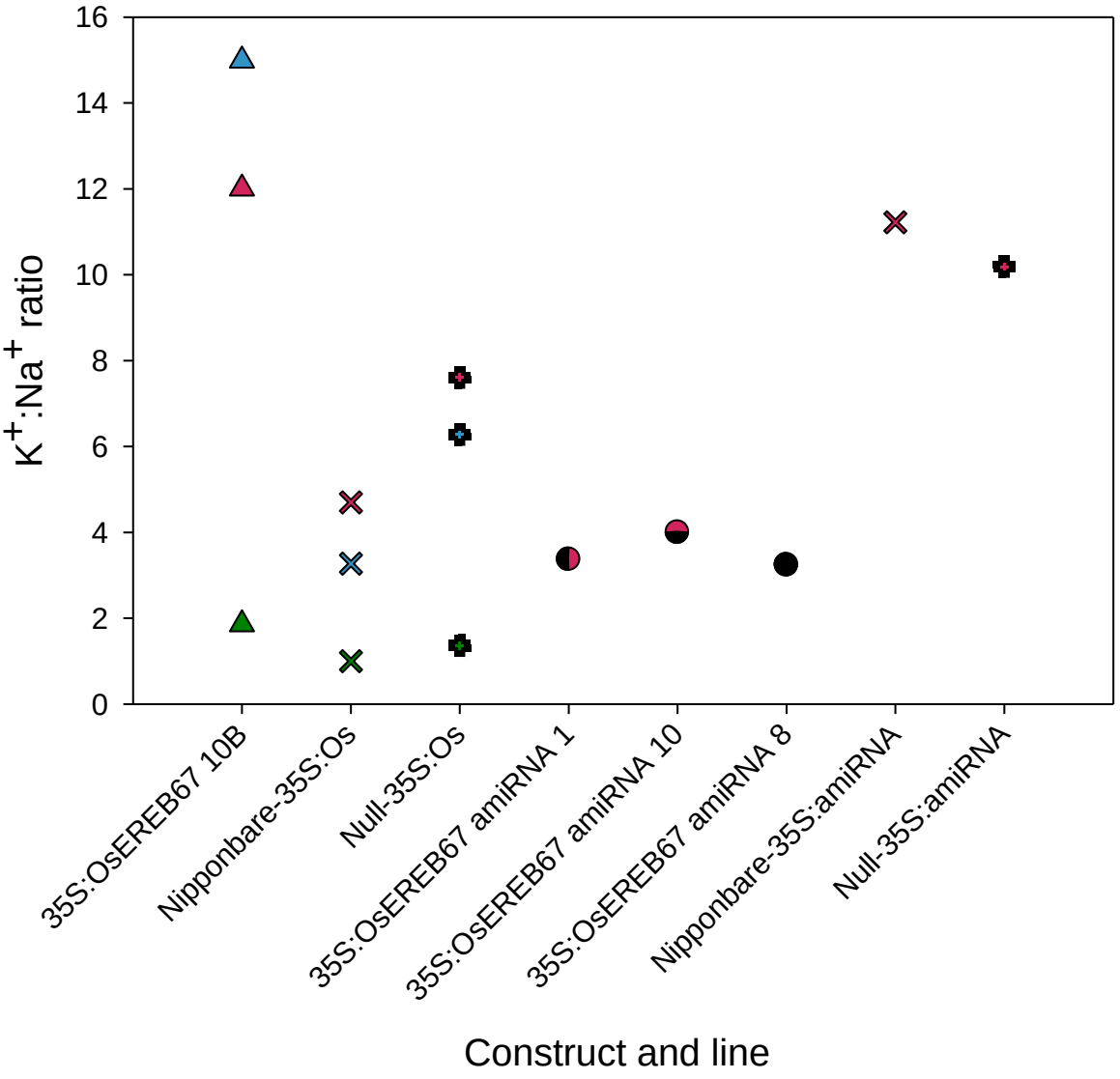


Figure 4.35 Graph of K^+:Na^+ ratio in rice plants with altered expression of *OsEREB67* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry and the K^+:Na^+ ratio calculated. The graph contains data from *2x35S:OsEREB67* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsEREB67 amiRNA* (centre) and *JRC0470:OsEREB67* (right) rice.

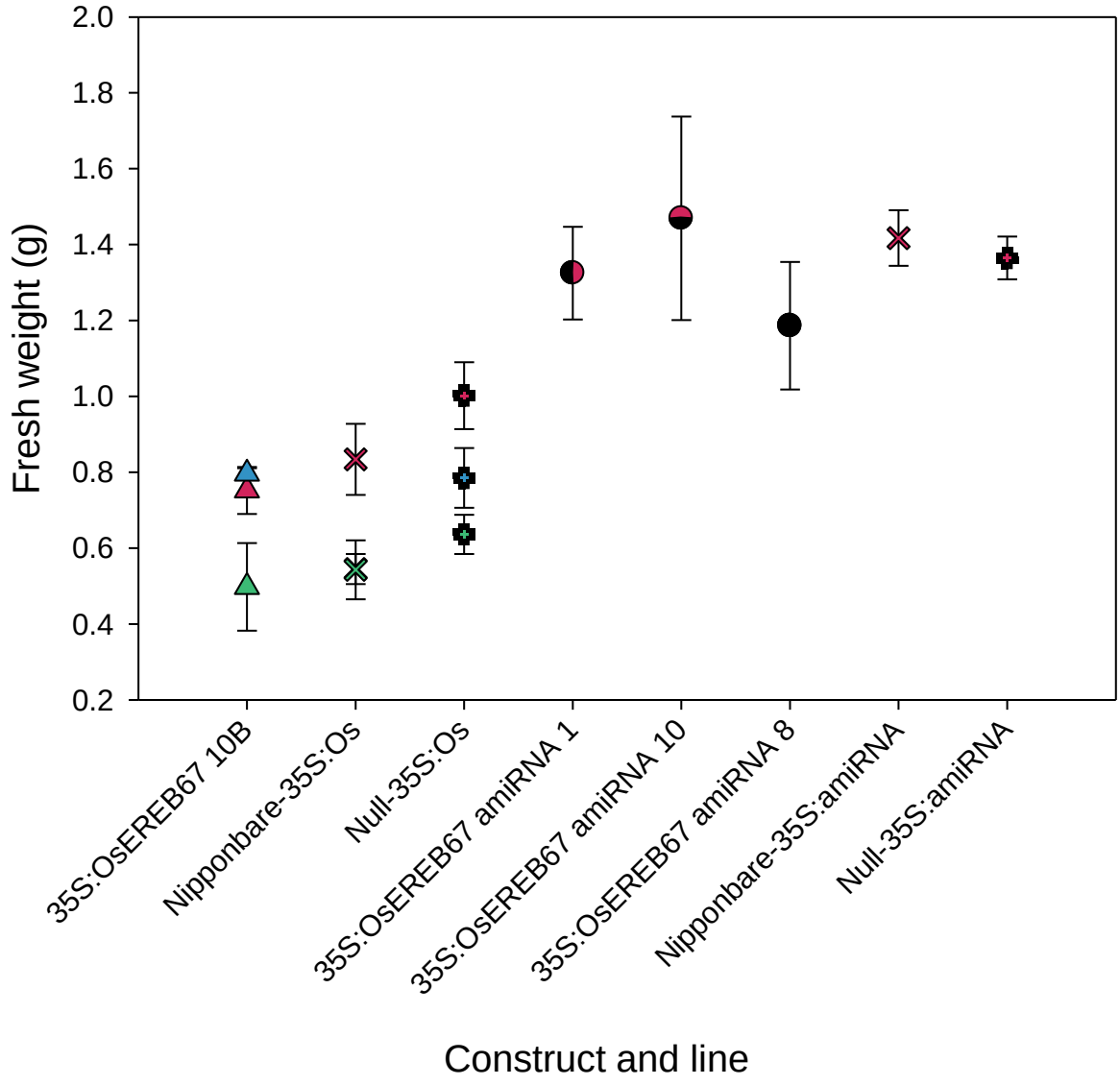


Figure 4.36 Graph of fresh weight in rice plants with altered expression of *OsEREB67* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The fresh weight was then measured. The graph contains data from *2x35S:OsEREB67* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsEREB67 amiRNA* (centre) and *JRC0470:OsEREB67* (right) rice. Error bars represent SEM. n≥3.

4.3.4.3. OsMYBR63

Expression of the transcription factor *OsMYBR63* was altered in rice plants and indicators of salt tolerance assessed. Semi-quantitative PCR was used to analyse transgene transcript levels in *2x35S:OsMYBR63* expressing rice plants (Figure 4.37) and endogenous gene expression in *2x35S:OsMYBR63 amiRNA* expressing rice plants (Figure 4.38). The expression data shows a high level of transgene transcript levels and that the amiRNA targeting *OsMYBR63* had a minimal effect upon transcript levels.

Altered expression of *OsMYBR63* had a minimal effect upon sodium and potassium accumulation. This can be seen in Figure 4.39. The *2x35S:OsMYBR63* lines had comparable levels or less sodium in experiment one, less sodium in experiment two and comparable levels or more sodium in experiment three, relative to wild type and null plants. The amiRNA expressing lines -1 and -2 had comparable levels of sodium accumulation and line -15 had higher sodium accumulation, relative to wild type and null plants.

Altered expression of *OsMYBR63* had a minimal effect upon the K^+:Na^+ ratio. This can be seen in Figure 4.40. In experiments one and two, the *2x35S:OsMYBR63* lines had improved K^+:Na^+ ratios and in experiment three, had K^+:Na^+ ratios comparable to wild type and null plants. The amiRNA expressing lines had variable K^+:Na^+ ratios. Lines amiRNA-1 and amiRNA-2 had improved K^+:Na^+ ratios relative to wild type and null plants and line amiRNA-15 had a poor K^+:Na^+ ratio.

Altered expression of *OsMYBR63* had a minimal but variable effect upon plant fresh weight. This can be seen in Figure 4.41. The *2x35S:OsMYBR63* lines all had greater fresh weights than wild type plants but were comparable to null plants. The amiRNA expressing line -1 had reduced fresh weight, line -15 was comparable and line -2 had a greater fresh weight, relative to wild type and null plants.

Plants with constitutive overexpression of *OsMYBR63* had reduced germination (Table 5.10), relative to plants with reduced expression of *OsMYBR63* (Table 5.9) and wild type plants (Table 5.9, Table 5.10).

The data show that altered expression of *OsMYBR63* may alter sodium accumulation and plant fresh weight, but a trend is not clear.

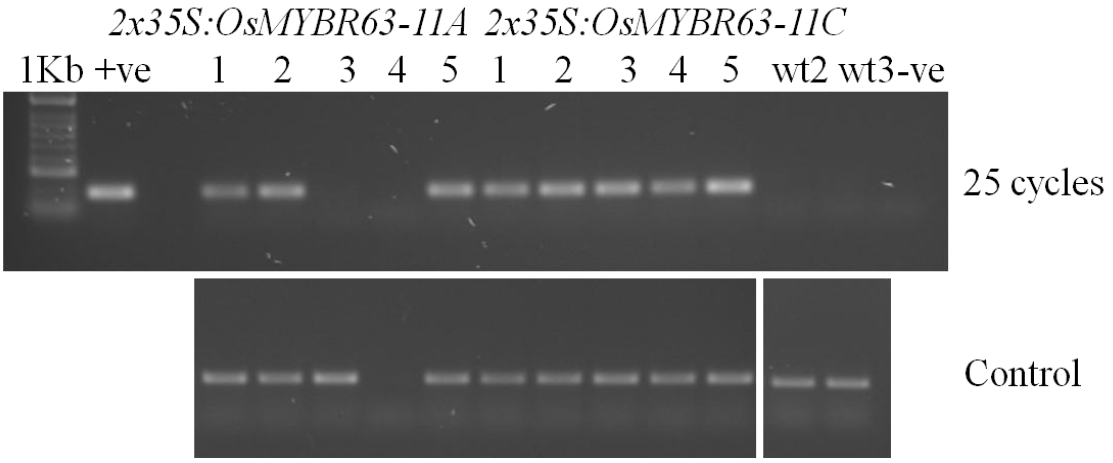


Figure 4.37 Semi quantitative RT-PCR analysis of transgene transcript levels in 2x35S:OsMYBR63 rice plants

Transgene transcript levels were analysed in rice plants with constitutive overexpression of the *OsMYBR63* CDS. Tissue was harvested from T0 primary transformant leaf tissue at the vegetative stage, growing in soil and immersed in ACPFG nutrient solution. The tissue was snap frozen in liquid nitrogen, total RNA was extracted using TRIzol, DNase treated and cDNA synthesised using oligo dT primers. Transgene transcript levels were analysed using primers specific for *OsMYBR63* and the nopaline synthase terminator; expected size 181bp, 25 cycles. The control gene amplified was α-tubulin, as this had the most consistent expression in previous qRT-PCR experiments using Nipponbare rice plants; expected size 234bp, 29 cycles. Positive control template was a sequenced plasmid. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

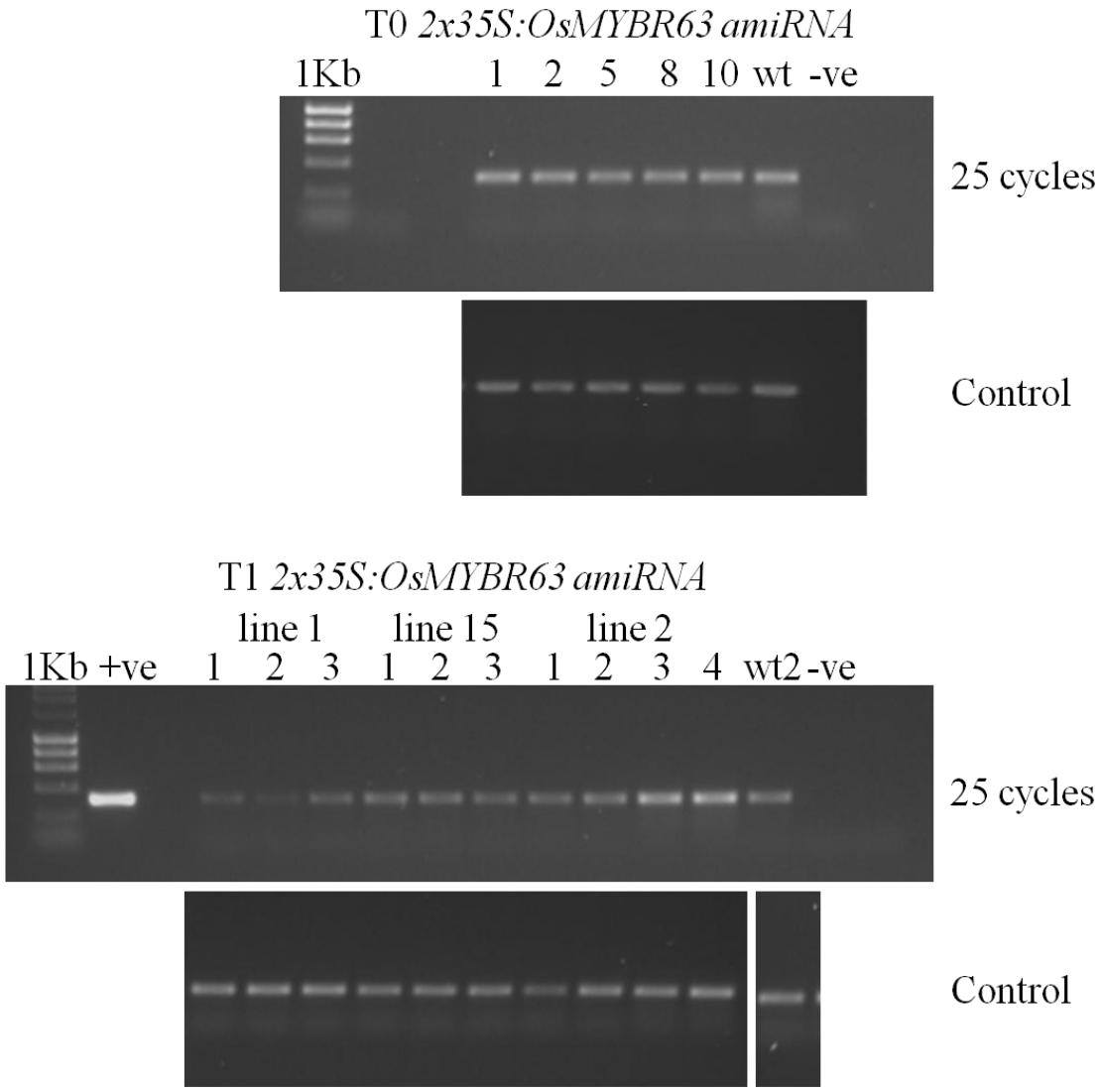


Figure 4.38 Semi quantitative RT-PCR analysis of endogenous transcript levels in 2x35S:OsMYBR63 amiRNA rice plants

Endogenous transcript levels of *OsMYBR63* were analysed in plants with constitutive overexpression of an amiRNA construct targeting *OsMYBR63*. Transcript levels were analysed using primers specific for *OsMYBR63*; expected size 300bp, 25 cycles. α-tubulin was used as the control gene; expected size 234bp, 29 cycles. Positive control template was a sequenced PCR product. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

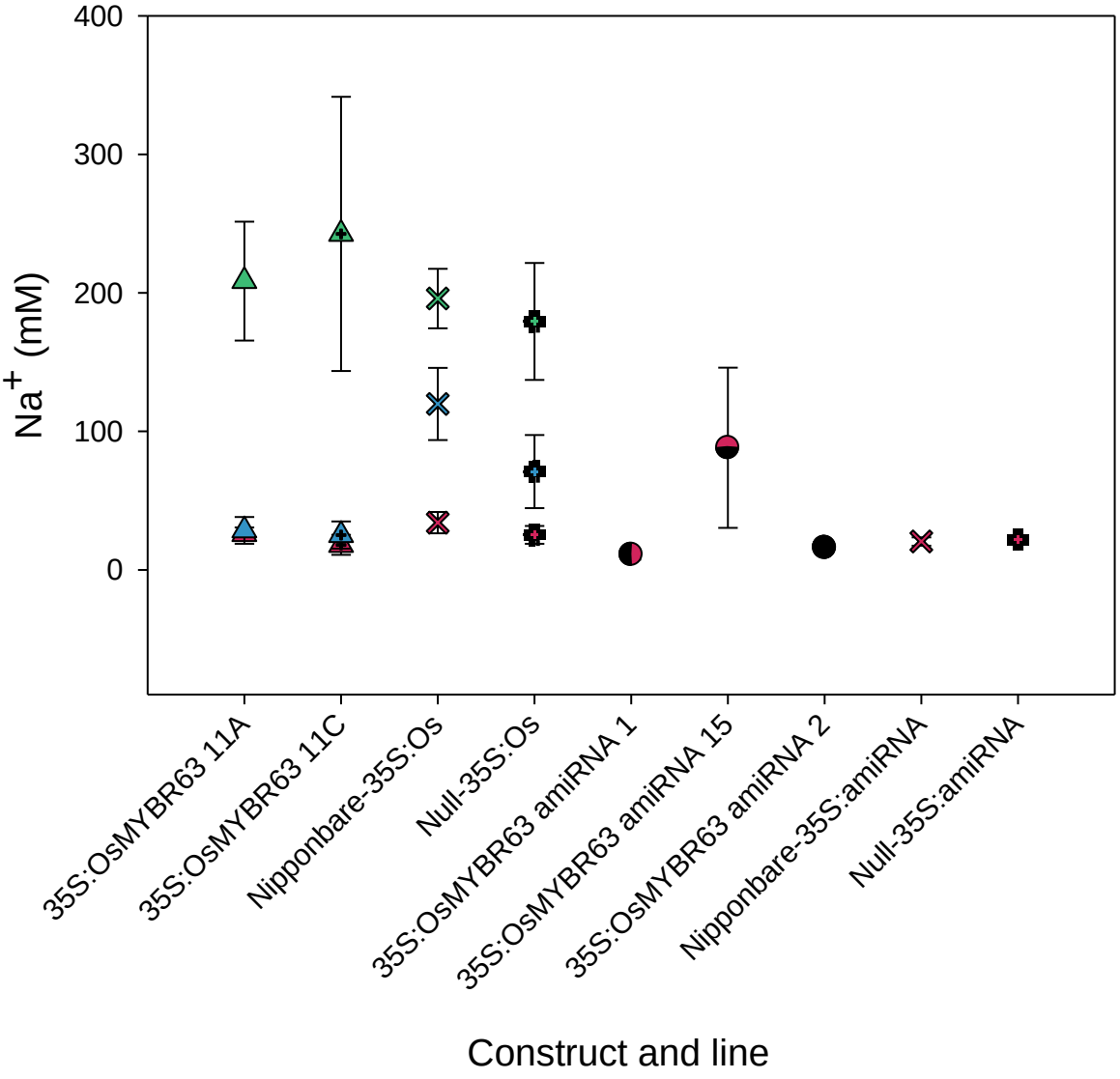


Figure 4.39 Graph of sodium accumulation in rice plants with altered expression of *OsMYBR63* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry. The graph contains data from *2x35S:OsMYBR63* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsMYBR63 amiRNA* (centre) and JRC0470:*OsMYBR63* (right) rice. Error bars represent SEM. n≥3.

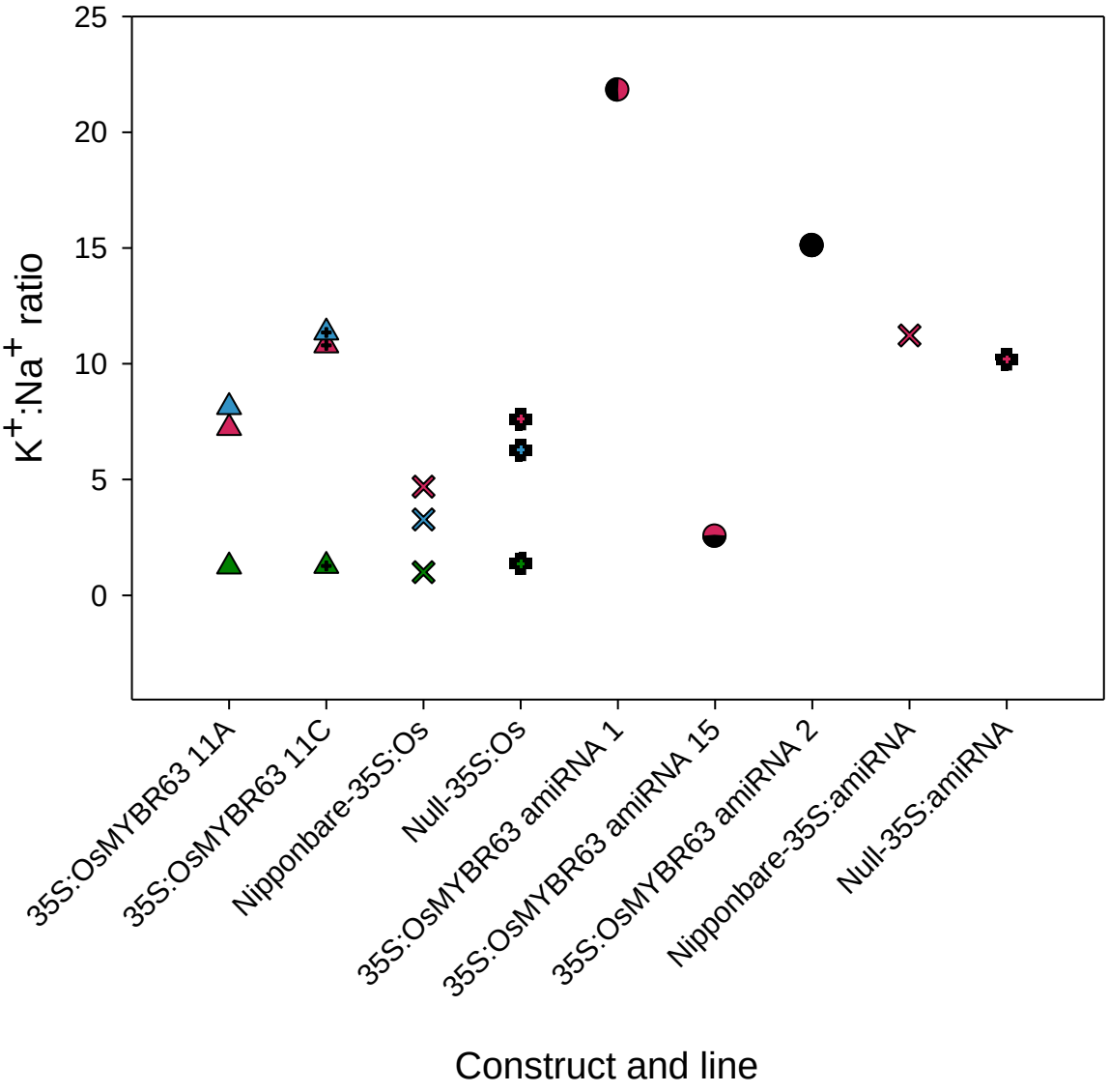


Figure 4.40 Graph of K^+:Na^+ ratio in rice plants with altered expression of *OsMYBR63* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry and the K^+:Na^+ ratio calculated. The graph contains data from *2x35S:OsMYBR63* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsMYBR63 amiRNA* (centre) and JRC0470:*OsMYBR63* (right) rice.

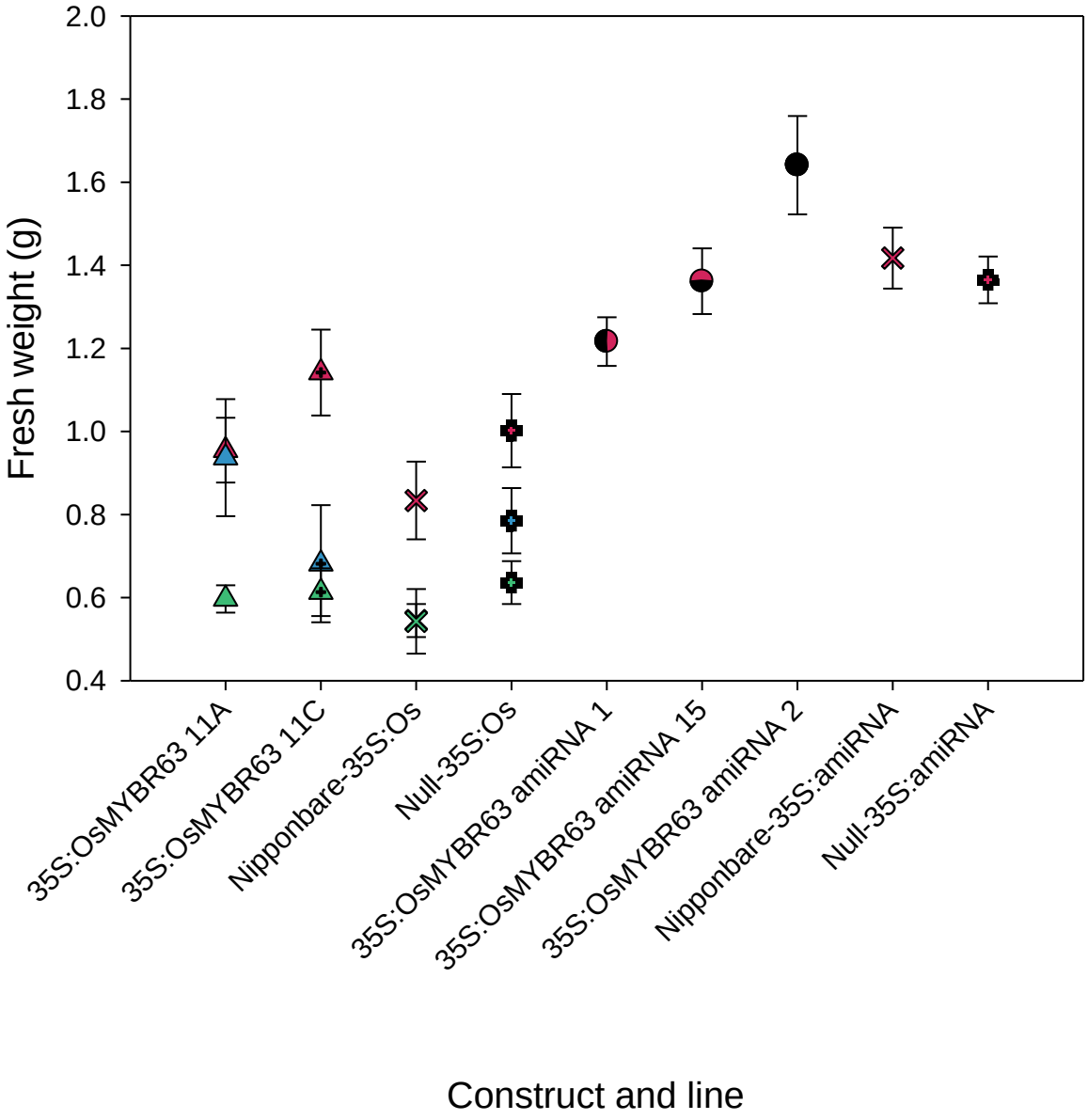


Figure 4.41 Graph of fresh weight in rice plants with altered expression of *OsMYBR63* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The fresh weight was then measured. The graph contains data from *2x35S:OsMYBR63* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsMYBR63 amiRNA* (centre) and JRC0470:*OsMYBR63* (right) rice. Error bars represent SEM. n≥3.

4.3.4.4. OsbHLH17

Expression of the transcription factor *OsbHLH17* was altered in rice plants and indicators of salinity tolerance assessed. Semi-quantitative PCR was used to analyse endogenous gene transcript levels in *2x35S:OsbHLH17 amiRNA* expressing rice plants (Figure 4.42). The expression data shows that the amiRNA targeting *OsbHLH17* had a minimal effect upon transcript levels in the T1 generation.

For *OsbHLH17*, only the lines expressing amiRNAs were analysed in sodium and potassium accumulation experiments. The gene CDS was difficult to clone, so the *2x35S:OsbHLH17* lines were not analysed. Altered expression of *OsbHLH17* increased sodium accumulation but the effect was variable. This can be seen in Figure 4.43. Lines amiRNA-1 and -8 were comparable to wild type and null plants, whereas amiRNA-14 accumulated approximately 4-fold more sodium.

Altered expression of *OsbHLH17* had a negative effect upon plant K^+:Na^+ ratios. This can be seen in Figure 4.45. Under salt stress, amiRNA-8 had a K^+:Na^+ ratio comparable to wild type and null plants, whereas amiRNA-1 had a slightly worse ratio and amiRNA-14 had a significantly worse ratio.

Altered expression of *OsbHLH17* reduced plant fresh weight. This can be seen in Figure 4.44. Under salt stress, amiRNA-1 and -14 had comparable fresh weights which were slightly less than wild type and null plants. amiRNA-8 had an average fresh weight 30% less than wild type and null plants.

The results show that reduced expression of *OsbHLH17* had a negative effect upon the plant K^+:Na^+ ratio and reduced plant fresh weight.

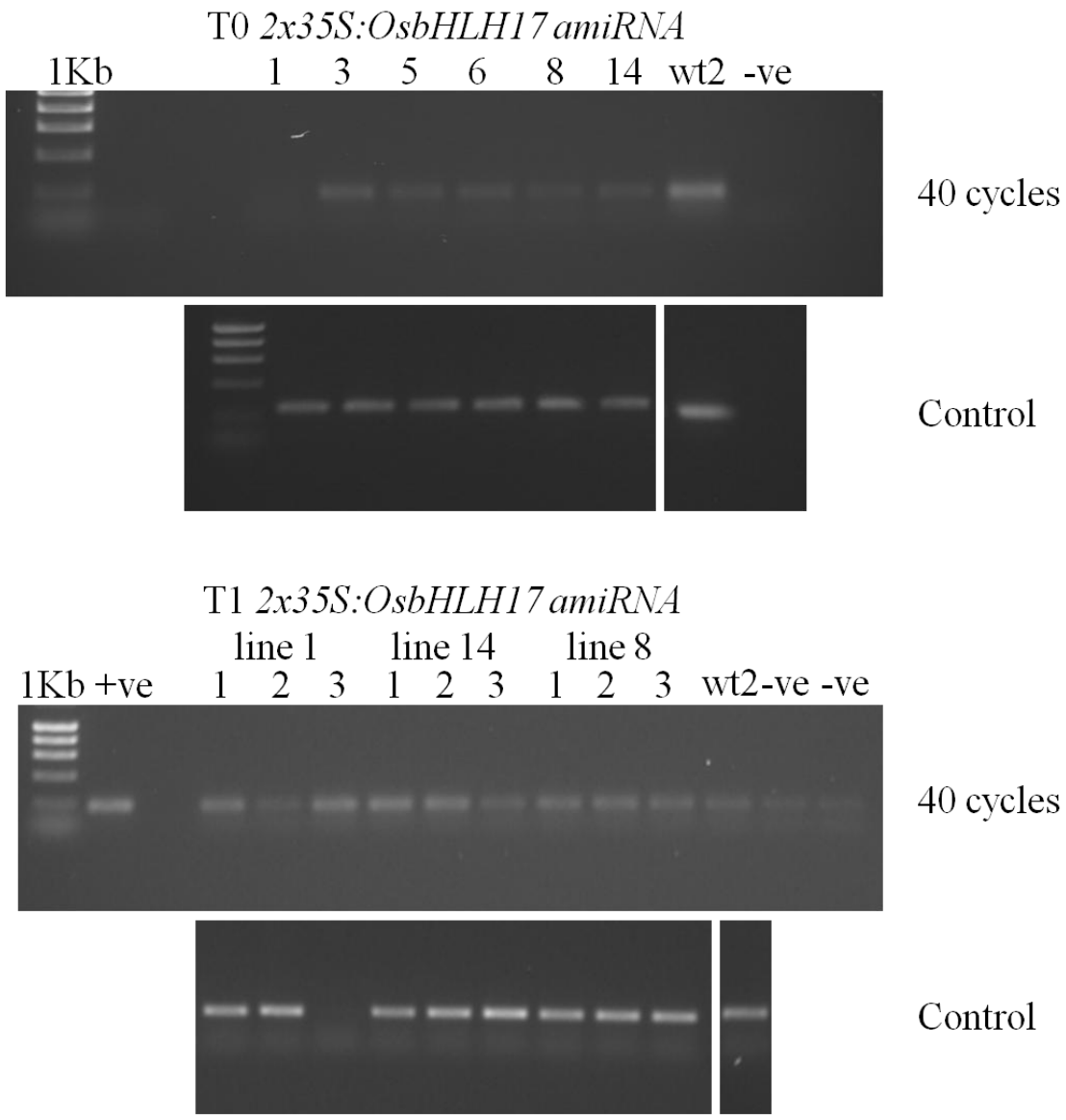


Figure 4.42 Semi-quantitative RT-PCR analysis of endogenous transcript levels in 2x35S:OsbHLH17 amiRNA rice plants

Endogenous transcript levels of *OsbHLH17* were analysed in plants with constitutive overexpression of an amiRNA construct targeting *OsbHLH17*. Transcript levels were analysed using primers specific for *OsbHLH17*; expected size 180bp, 40 cycles. α-tubulin was used as the control gene; expected size 234bp, 29 cycles. Positive control template was a sequenced PCR product. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

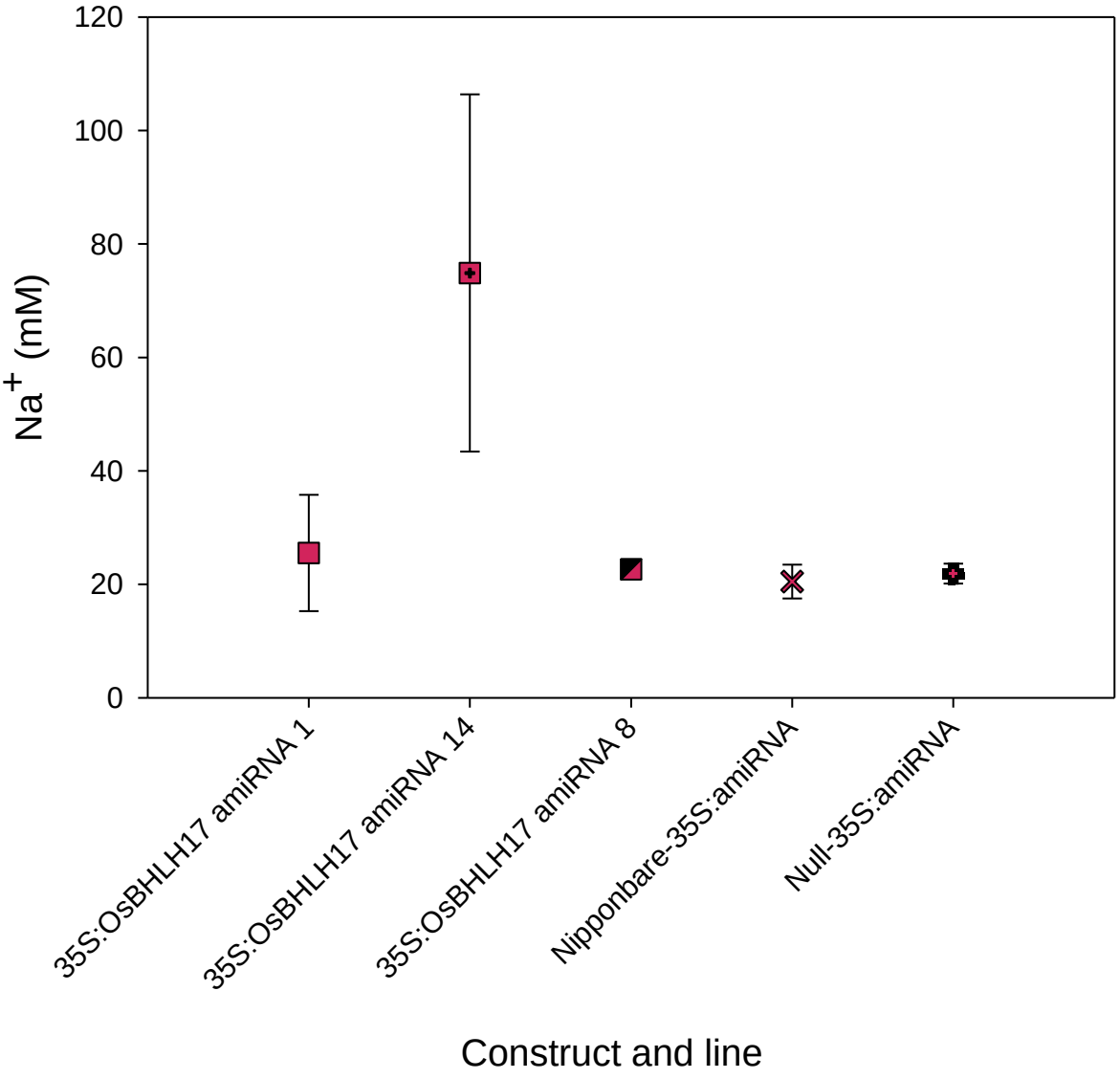


Figure 4.43 Graph of sodium accumulation in rice plants with altered expression of *OsbHLH17* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry. The graph contains data from *2x35S:OsbHLH17 amiRNA* rice. Error bars represent SEM. n≥3.

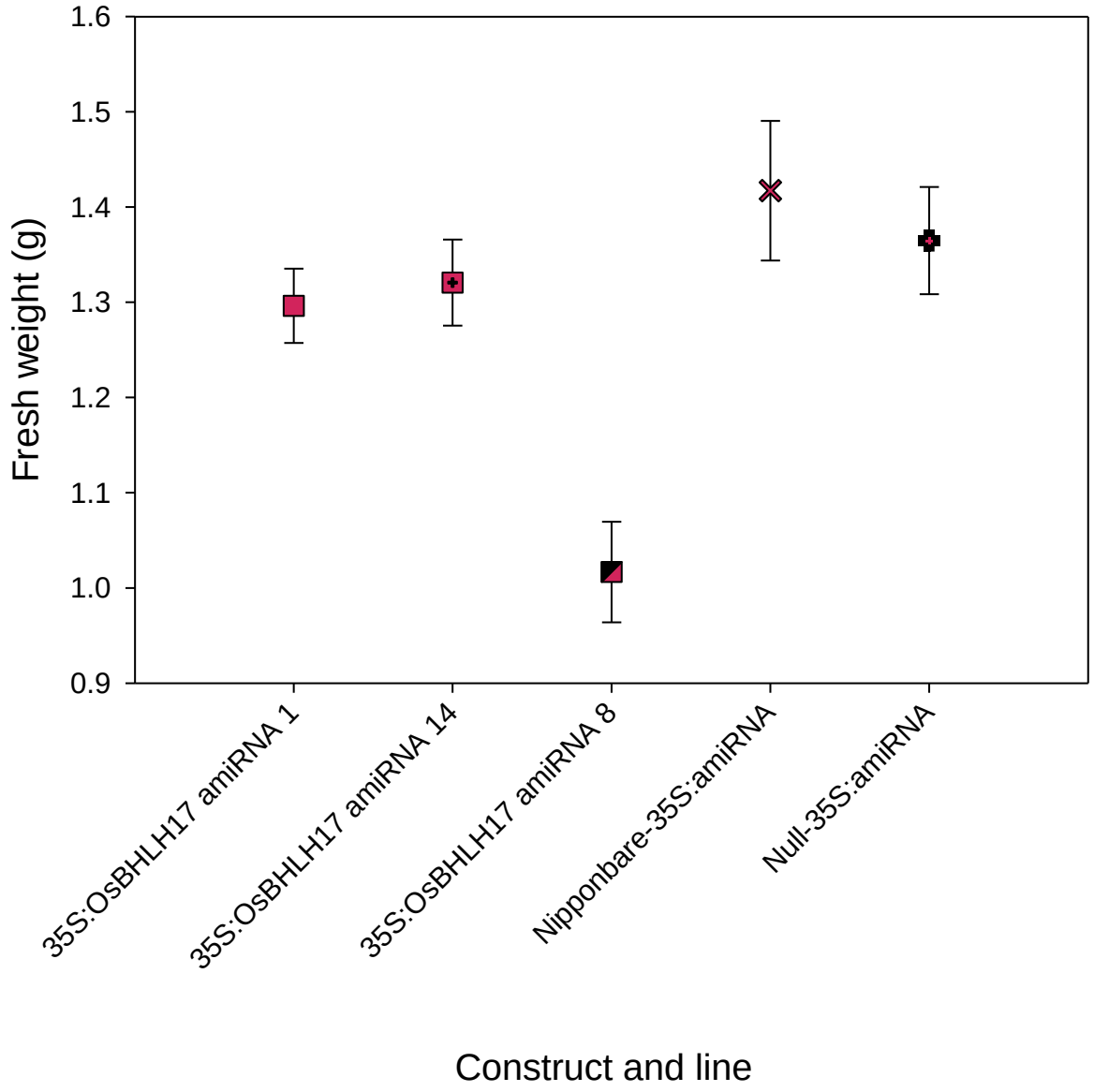


Figure 4.44 Graph of fresh weight in rice plants with altered expression of *OsbHLH17* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The fresh weight was then measured. The graph contains data from *2x35S:OsbHLH17 amiRNA* rice. Error bars represent SEM. n≥3.

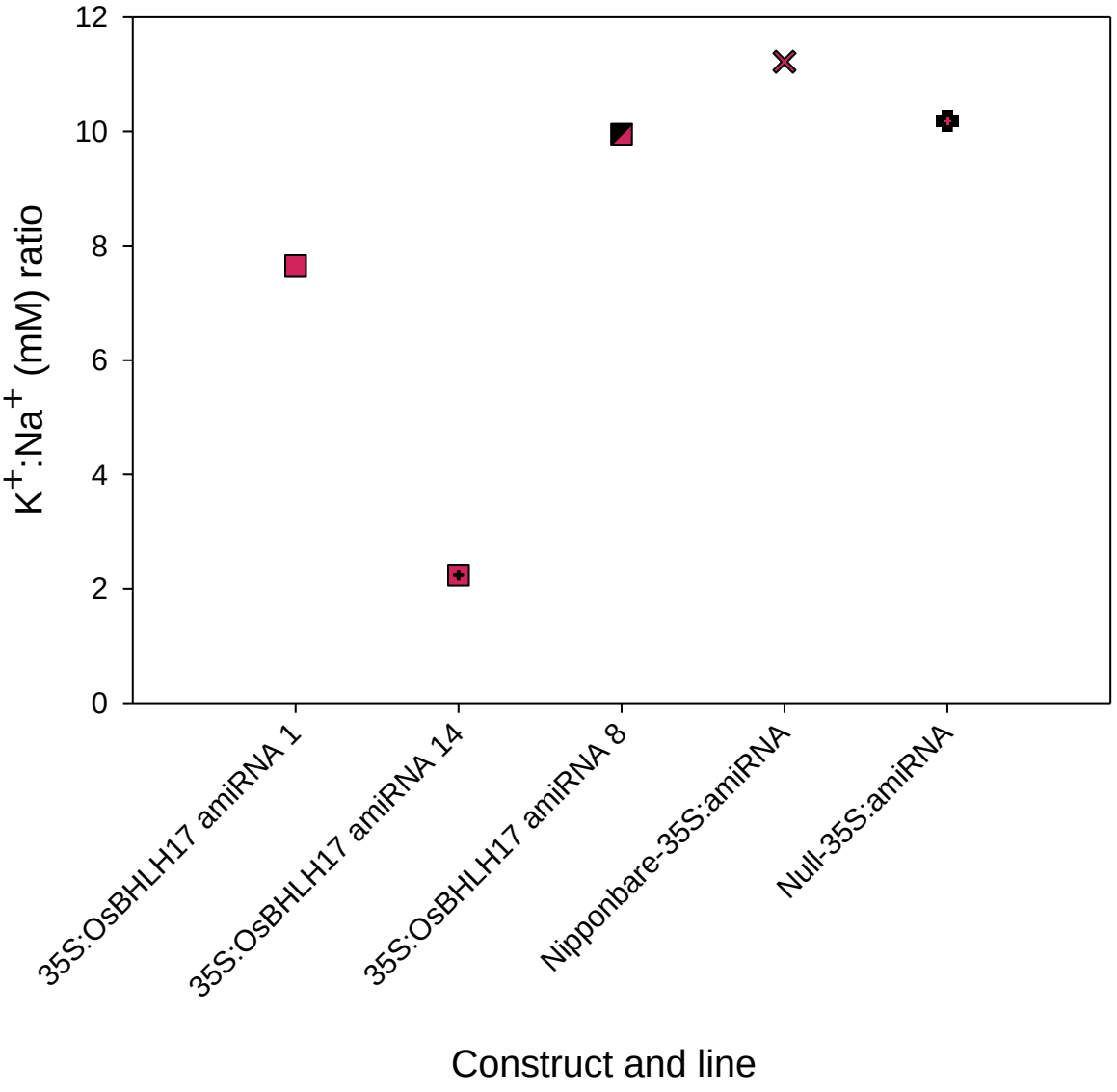


Figure 4.45 Graph of K^+:Na^+ ratio in rice plants with altered expression of *OsbHLH17* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry and the K^+:Na^+ ratio calculated. The graph contains data from *2x35S:OsbHLH17 amiRNA* rice.

4.3.4.5. OsLUX

Expression of *OsLUX* was altered in rice plants and indicators of salinity tolerance assessed. Semi-quantitative RT-PCR was used to analyse transgene transcript levels in *2x35S:OsLUX* (Figure 4.46) and *JRC0470:OsLUX* expressing rice plants (Figure 4.47) and endogenous gene expression in *2x35S:OsLUX amiRNA* expressing rice plants (Figure 4.48). The *2x35S:OsLUX* lines had high constitutive transcript levels and the *JRC0470:OsLUX* lines had moderate constitutive and mild salt inducible transcript levels. The *2x35S:OsLUX amiRNA* lines had reduced *OsLUX* transcript levels in the T0 and T1 generations.

Altered expression of *OsLUX* affected sodium and potassium accumulation. This can be seen in Figure 4.49. The *2x35S:OsLUX* line 17C had comparable sodium accumulation in experiment one and reduced sodium accumulation in experiments two and three, relative to wild type and control plants. *2x35S:OsLUX* line 19C had comparable sodium accumulation in experiment one and increased sodium accumulation in experiment two, relative to wild type and control plants. Line 19C, grown with the salt inducible lines (far right of figure), had reduced sodium accumulation relative to wild type and null plants. All salt inducible lines had reduced sodium accumulation relative to wild type and null plants. The amiRNA expressing lines had comparable or increased sodium accumulation relative to wild type and null plants.

The K^+:Na^+ ratios were also affected by altered *OsLUX* expression. This can be seen in Figure 4.50. The *2x35S:OsLUX* lines 17C and 19C, and the salt inducible overexpression lines -1, -2 and -12 had improved K^+:Na^+ ratios relative to wild type and null plants. The amiRNA expressing lines -1 and -9 had worse K^+:Na^+ ratios, relative to wild type and null plants, whereas the amiRNA expressing line -3 had an improved K^+:Na^+ ratio. The differences in K^+:Na^+ ratio between the amiRNA expressing lines are consistent with *OsLUX* transcript levels.

Altered expression of *OsLUX* affected plant fresh weight. This can be seen in Figure 4.51. In experiments one and three, the *2x35S:OsLUX* lines had comparable fresh weights to wild type and null plants. In experiment two, 17C had a slightly higher average fresh weight, relative to wild type and null plants. Under salt stress conditions, the amiRNA expressing lines had reduced fresh weights relative to wild type and null plants. The salt inducible lines had comparable fresh weights, relative to wild type and null plants.

Plants with constitutive overexpression of *OsLUX* had reduced germination (Table 5.10), relative to plants with salt inducible overexpression (Table 5.11) or reduced expression of *OsLUX* (Table 5.9) and wild type plants (Table 5.9, Table 5.10, Table 5.11). Plants with constitutive overexpression of *OsLUX* also had reduced 100 grain weight (Table 5.10), relative to plants with salt inducible overexpression of *OsLUX* (Table 5.11) and wild type plants (Table 5.9, Table 5.10, Table 5.11).

The data show that constitutive overexpression of *OsLUX* did not have a clear effect upon sodium accumulation, but did improve the K^+:Na^+ ratio. However, salt inducible overexpression of *OsLUX* appeared to reduce sodium accumulation and improve the K^+:Na^+ ratio, whereas reduced expression of *OsLUX* had the opposite effect. Reduced expression of *OsLUX* also reduced fresh weight.

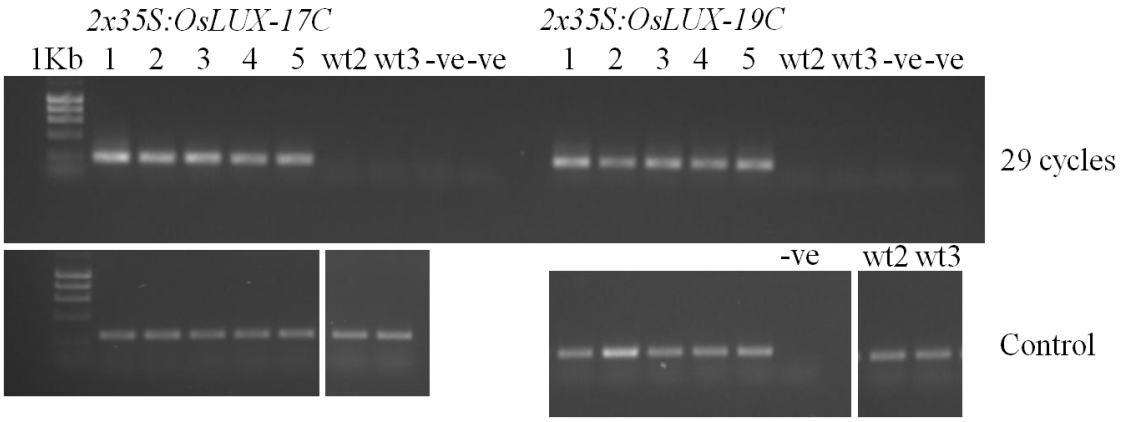


Figure 4.46 Semi quantitative RT-PCR analysis of transgene transcript levels in 2x35S:OsLUX rice plants

Transgene transcript levels were analysed in rice plants with constitutive overexpression of the *OsLUX* CDS. Tissue was harvested from T0 primary transformant leaf tissue at the vegetative stage, growing in soil and immersed in ACPFG nutrient solution. The tissue was snap frozen in liquid nitrogen, total RNA was extracted using TRIzol, DNase treated and cDNA synthesised using oligo dT primers. Transgene transcript levels were analysed using primers specific for *OsLUX* and the nopaline synthase terminator; expected size 146bp, 29 cycles. The control gene amplified was α-tubulin, as this had the most consistent expression in previous qRT-PCR experiments using Nipponbare rice plants; expected size 234bp, 29 cycles. Positive control template was a sequenced plasmid. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

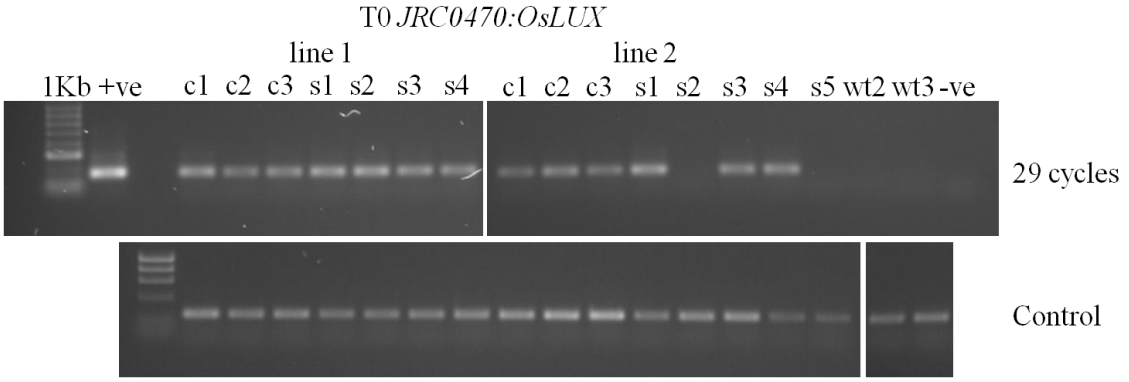


Figure 4.47 Semi quantitative RT-PCR analysis of transgene transcript levels in JRC0470:OsLUX rice plants

Transgene transcript levels were analysed in plants with salt-inducible overexpression of the *OsLUX* CDS. Transgene transcript levels were analysed using primers specific for *OsLUX* and the nopaline synthase terminator; expected size 193bp, 29 cycles. α-tubulin was used as the control gene; expected size 146bp, 29 cycles. Control treatment c1-c3. Salt stress treatment s1-s5. Positive control template was a sequenced plasmid. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

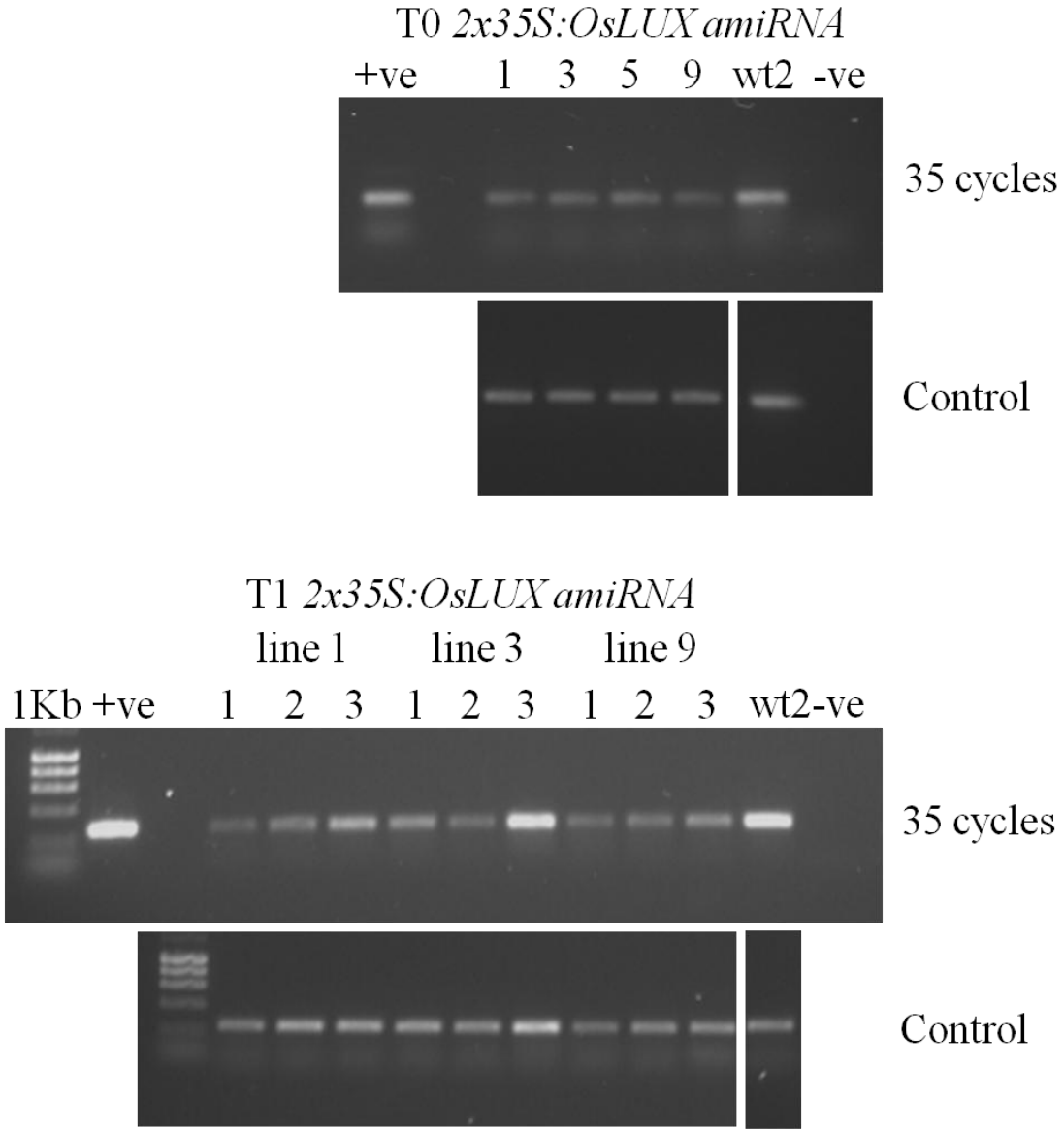


Figure 4.48 Semi quantitative RT-PCR analysis of endogenous transcript levels in 2x35S:OsLUX amiRNA rice plants

Endogenous transcript levels of *OsLUX* were analysed in plants with constitutive overexpression of an amiRNA construct targeting *OsLUX*. Transcript levels were analysed using primers specific for *OsLUX*; expected size 254bp, 35 cycles. α-tubulin was used as the control gene; expected size 234bp, 29 cycles. Positive control template was a sequenced PCR product. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

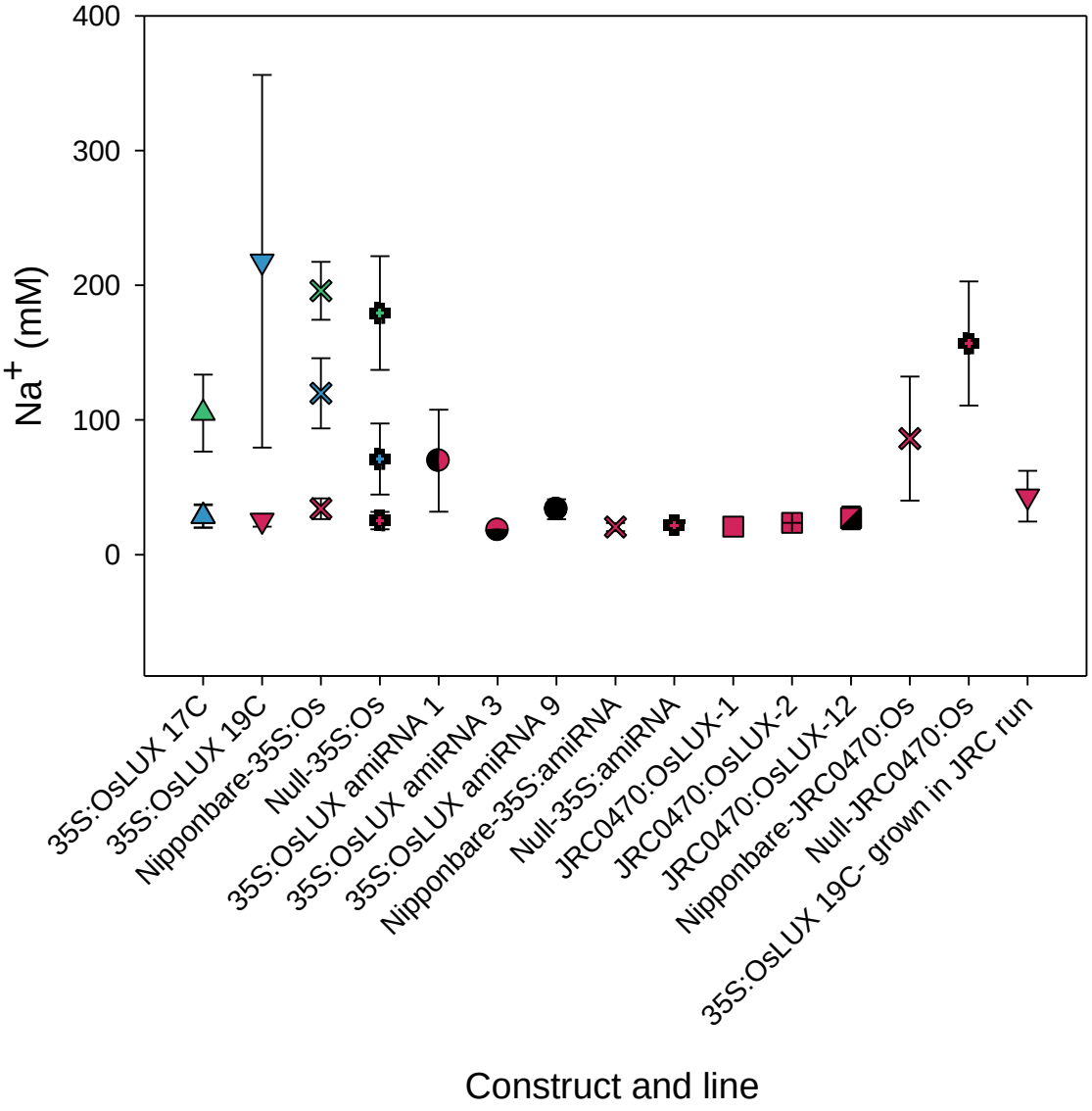


Figure 4.49 Graph of sodium accumulation in rice plants with altered expression of *OsLUX* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry. The graph contains data from *2x35S:OsLUX* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsLUX amiRNA* (centre) and *JRC0470:OsLUX* (right) rice. Error bars represent SEM. n≥3.

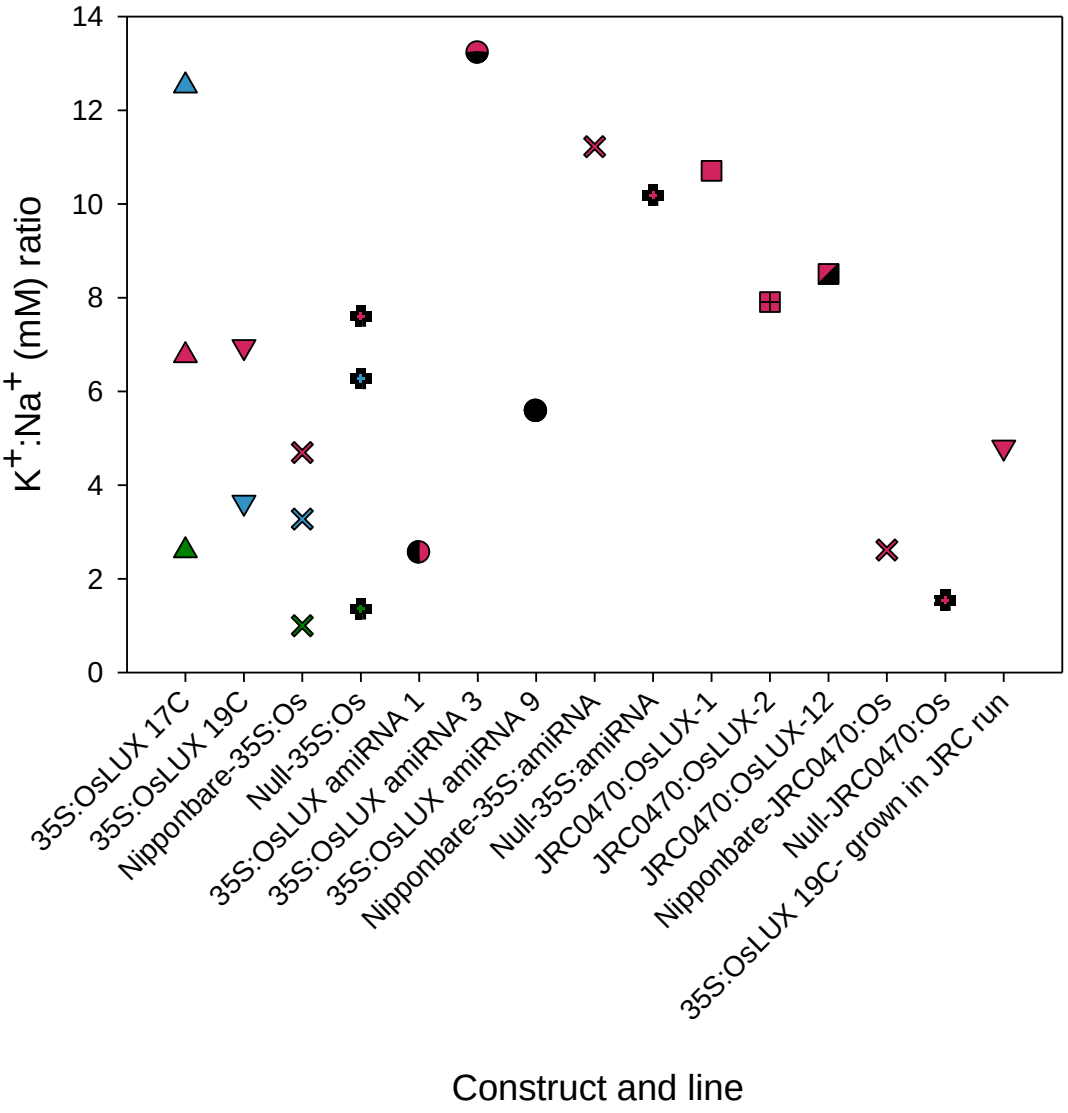


Figure 4.50 Graph of K^+:Na^+ ratio in rice plants with altered expression of *OsLUX* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry and the K^+:Na^+ ratio calculated. The graph contains data from *2x35S:OsLUX* (left) rice from the first ( ), second ( ) and third ( ) repetition and from *2x35S:OsLUX amiRNA* (centre) and *JRC0470:OsLUX* (right) rice. Error bars represent SEM. n≥3.

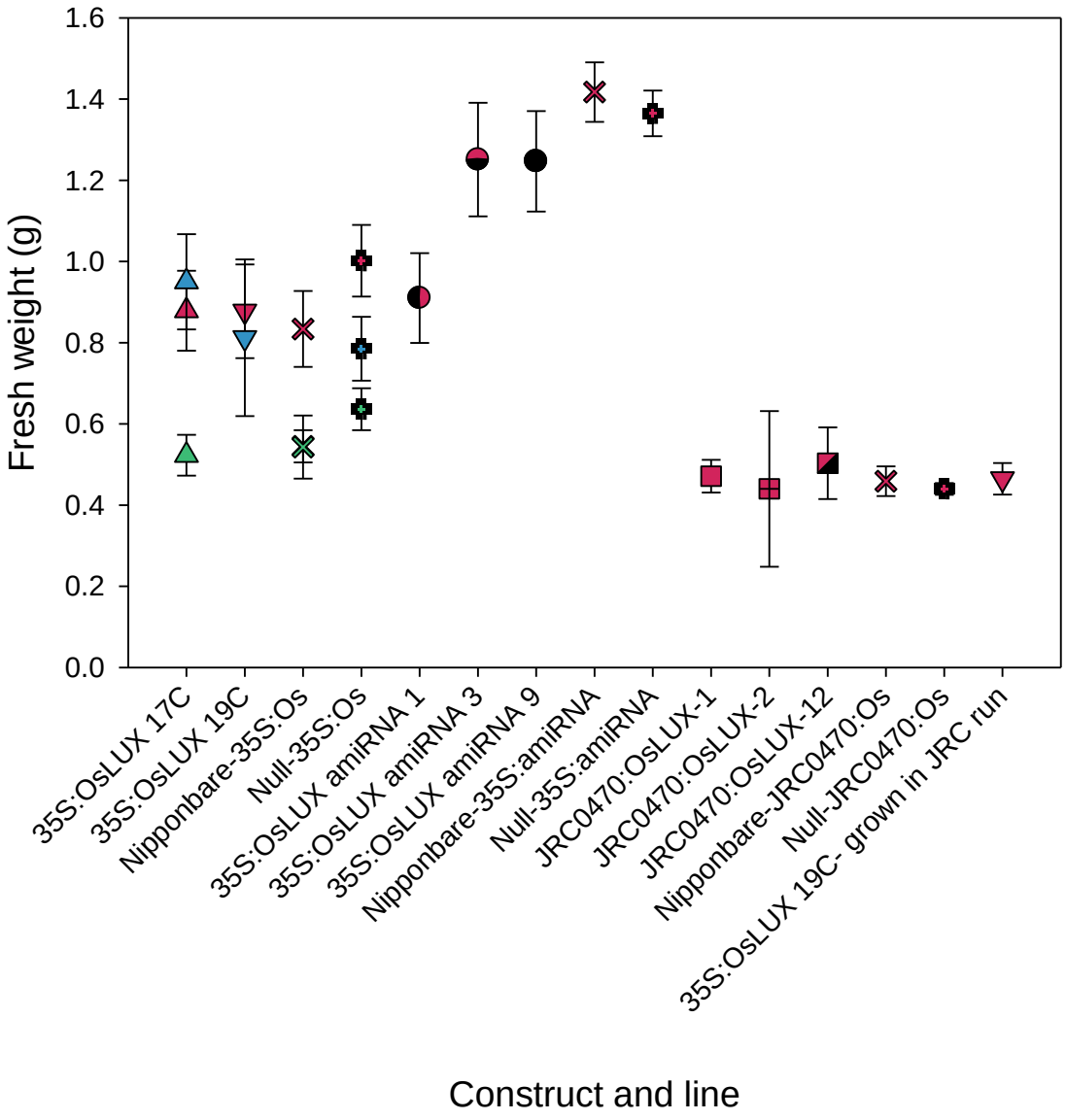


Figure 4.51 Graph of fresh weight in rice plants with altered expression of *OsLUX* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The fresh weight was then recorded. The graph contains data from *2x35S:OsLUX* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsLUX amiRNA* (centre) and *JRC0470:OsLUX* (right) rice. Error bars represent SEM. n≥3.

4.3.4.6. OsMYB54

Expression of *OsMYB54* was altered in rice plants and indicators of salinity tolerance assessed. Semi-quantitative RT-PCR was used to analyse transgene transcript levels in *2x35S:OsMYB54* expressing rice plants (Figure 4.52) and endogenous gene transcript levels in *2x35S:OsMYB54 amiRNA* (Figure 4.53) expressing rice plants. The *2x35S:OsMYB54* line 22C had very low transgene transcript levels, whereas lines 23E and 25A had high transcript levels. The *2x35S:OsMYB54* amiRNA lines had reduced *OsMYB54* transcripts in the T0 generation, but transcripts levels were variable and comparable to wild type in the T1 generation.

Altered expression of *OsMYB54* had an effect upon sodium accumulation. This can be seen in Figure 4.54. The *2x35S:OsMYB54* lines have variable sodium accumulation. Line 23E had very high sodium accumulation in experiments two and three, relative to wild type and null plants, so the 23E data points were removed to facilitate interpretation of the remaining data (Figure 4.55). In experiments one and two, 22C had comparable accumulation and 22E had reduced accumulation, relative to wild type plants. 25A had reduced accumulation in experiment two and increased accumulation in experiment three, relative to wild type and null plants. The amiRNA overexpression lines had increased sodium accumulation relative to wild type and null plants.

K^+:Na^+ ratios were also affected by altered *OsMYB54* expression. This can be seen in Figure 4.56. The amiRNA expressing lines had poor K^+:Na^+ ratios, relative to wild type and null plants. In experiments one and three, the constitutive overexpression lines had comparable or worse K^+:Na^+ ratios relative to wild type and null plants. In experiment two, constitutive overexpression lines 22C, 22E and 25A had improved K^+:Na^+ ratios and line 23E had a worse K^+:Na^+ ratio, relative to wild type and null plants.

Plant fresh weights were affected by altered expression of *OsMYB54*. This can be seen in Figure 4.57. Plants with constitutive overexpression of *OsMYB54* had fresh weights either comparable to or higher than wild type plants. However, line 22C with the lowest overexpression consistently had a higher fresh weight, relative to lines 23E and 25A with higher overexpression that consistently had the lowest fresh weights. Similarly, the K^+:Na^+ ratio was higher for line 22C and lower for lines 23E and 25A. Unfortunately, expression data was not obtained for line 22E, which had a high fresh weight and high K^+:Na^+ ratio. Two of the amiRNA expressing lines had reduced fresh weight relative to wild type plants and one had comparable fresh weight. The variation in fresh weight was not consistent with *OsMYB54* expression.

Plants with constitutive overexpression of *OsMYB54* had reduced germination (Table 5.10), relative to plants with reduced expression of *OsMYB54* (Table 5.9) and wild type plants (Table 5.9, Table 5.10).

Increased expression of *OsMYB54* may reduce sodium accumulation, improve the K^+:Na^+ ratio and increase fresh weight, but the results are dependent on the level of *OsMYB54* expression and vary between experiments. Decreased expression of *OsMYB54* increased sodium accumulation and had a negative effect upon the K^+:Na^+ ratio, however the effect on decreasing fresh weight was variable between events.

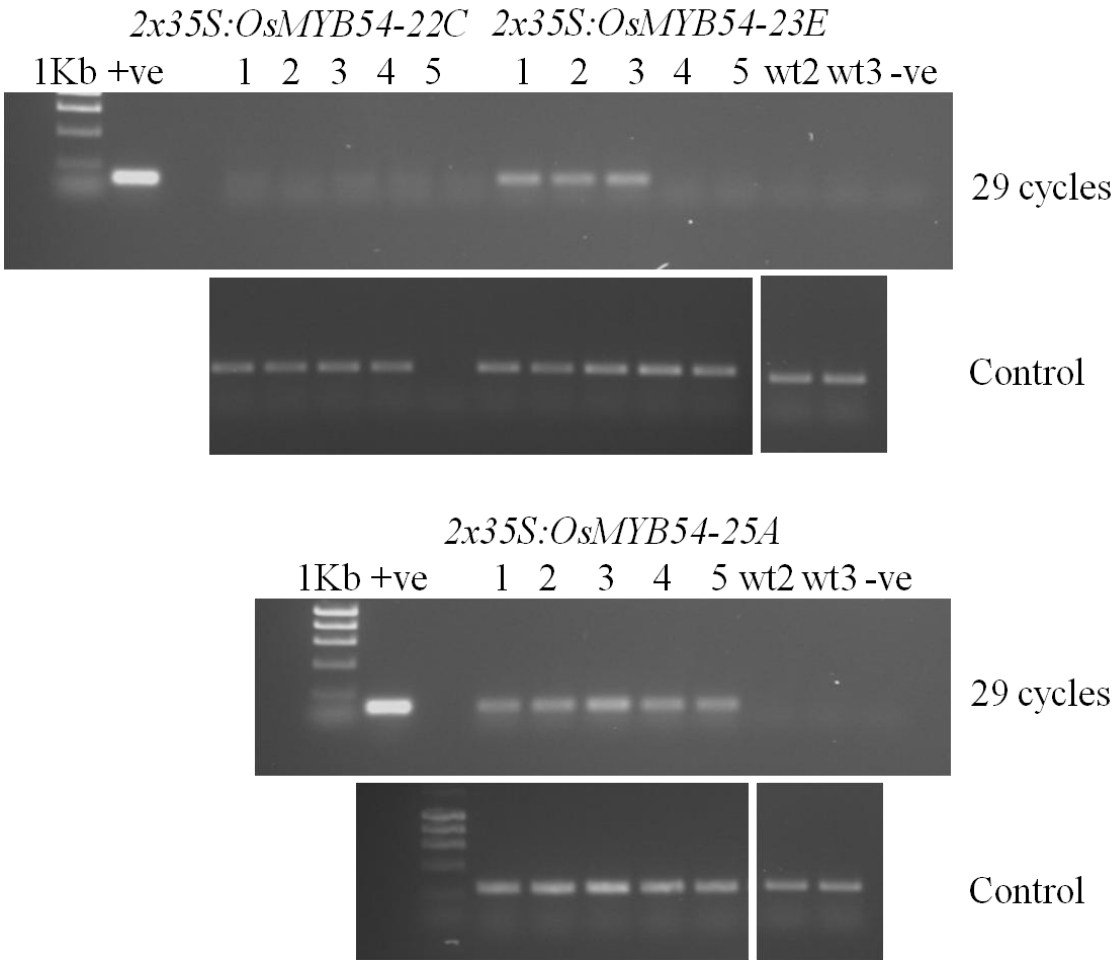


Figure 4.52 Semi quantitative RT-PCR analysis of transgene expression in 2x35S:OsMYB54 rice plants

Transgene transcript levels were analysed in rice plants with constitutive overexpression of the *OsMYB54* CDS. Tissue was harvested from T0 primary transformant leaf tissue at the vegetative stage, growing in soil and immersed in ACPFG nutrient solution. The tissue was snap frozen in liquid nitrogen, total RNA was extracted using TRIzol, DNase treated and cDNA synthesised using oligo dT primers. Transgene transcript levels were analysed using primers specific for *OsMYB54*and the nopaline synthase terminator; expected size 129bp, 29 cycles. The control gene amplified was α-tubulin, as this had the most consistent expression in previous qRT-PCR experiments using Nipponbare rice plants; expected size 234bp, 29 cycles. Positive control template was a sequenced plasmid. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

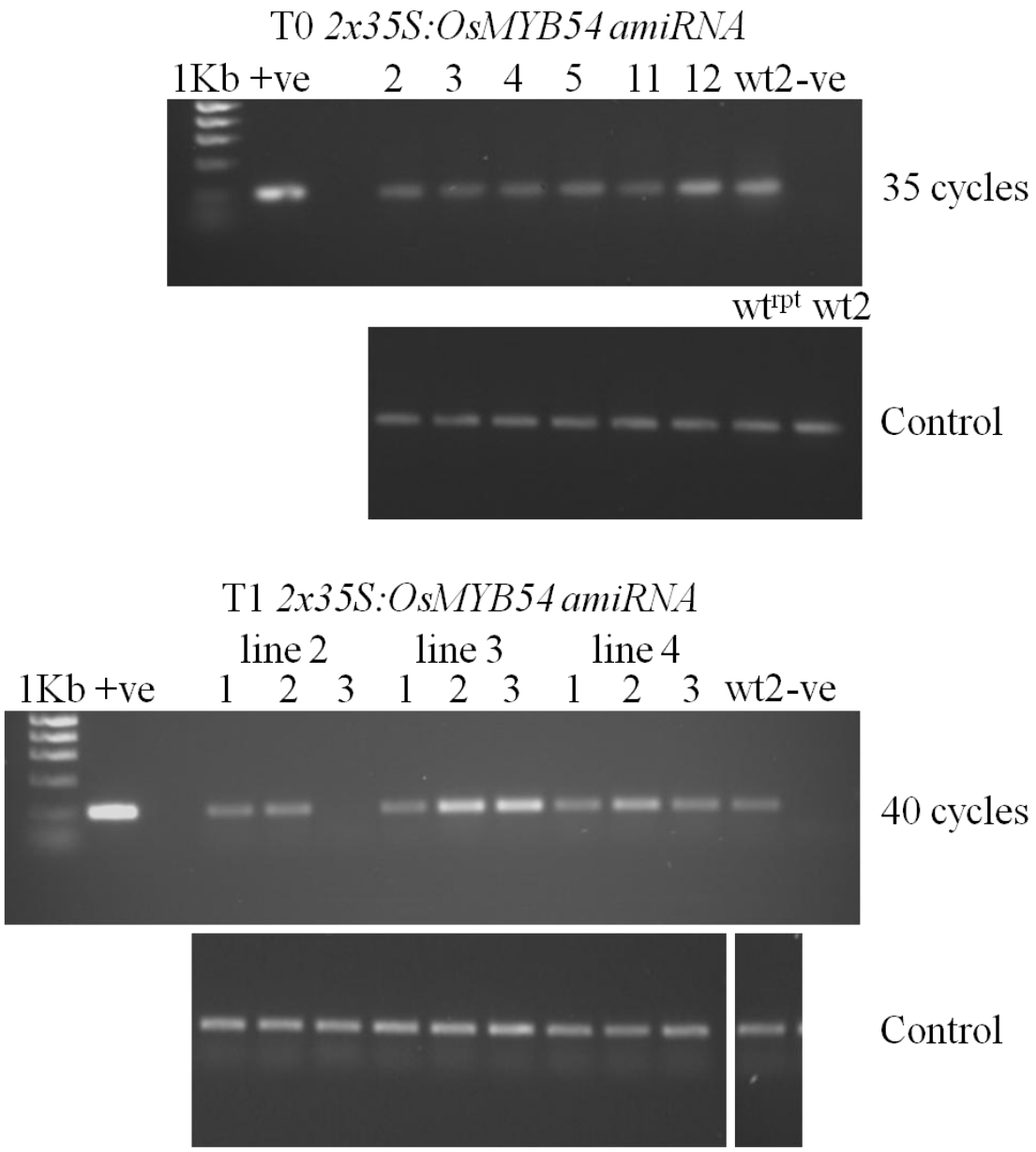


Figure 4.53 Semi quantitative RT-PCR analysis of endogenous gene expression in JRC0470:OsMYB54 rice plants

Endogenous transcript levels of *OsMYB54*were analysed in plants with constitutive overexpression of an amiRNA construct targeting *OsMYB54*. Transcript levels were analysed using primers specific for *OsMYB54*; expected size 198bp, 35-40 cycles. α-tubulin was used as the control gene; expected size 234bp, 29 cycles. Positive control template was a sequenced PCR product. Wild type sample (wt) was *O. sativa* cv. Nipponbare. –ve was no template control.

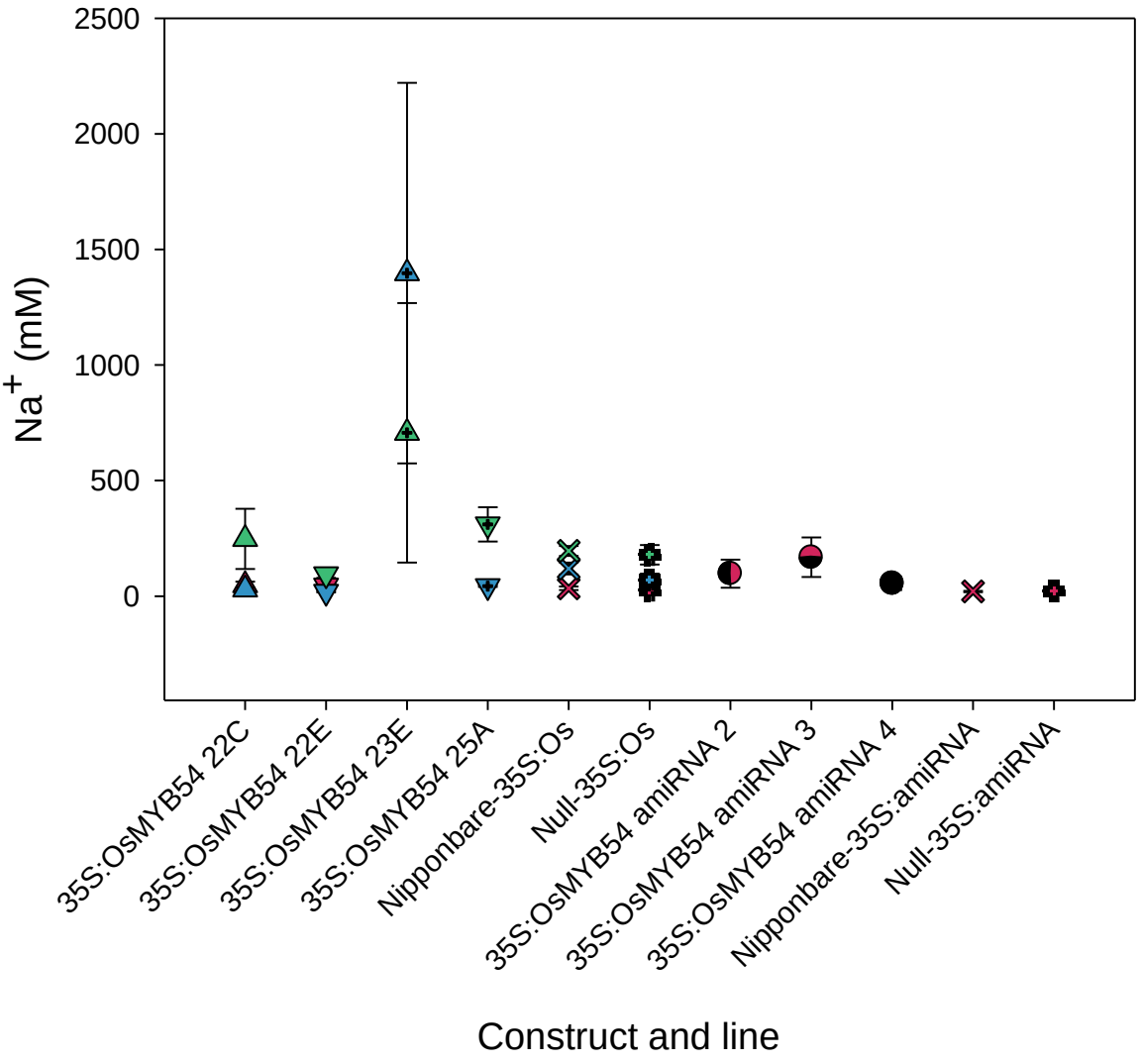


Figure 4.54 Graph of sodium accumulation in rice plants with altered expression of *OsMYB54* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry. The graph contains data from *2x35S:OsMYB54* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsMYB54 amiRNA* (centre) and *JRC0470:OsMYB54* (right) rice. Error bars represent SEM. n≥3.

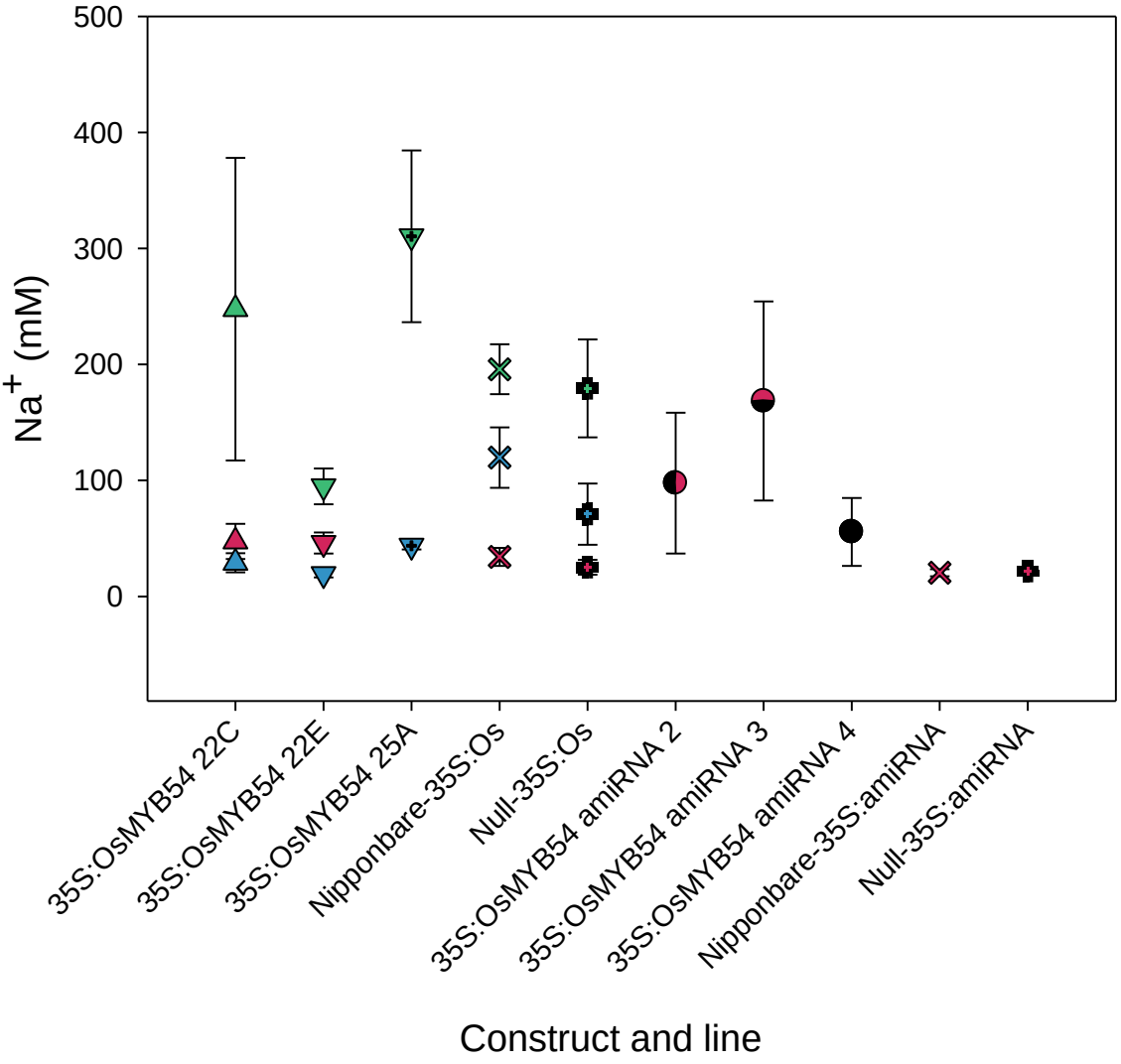


Figure 4.55 Graph of sodium accumulation in rice plants with altered expression of *OsMYB54* stressed with 50mM NaCl (line 23E removed)

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry. The graph contains data from *2x35S:OsMYB54* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsMYB54 amiRNA* (centre) and *JRC0470:OsMYB54* (right) rice. Error bars represent SEM. n≥3.

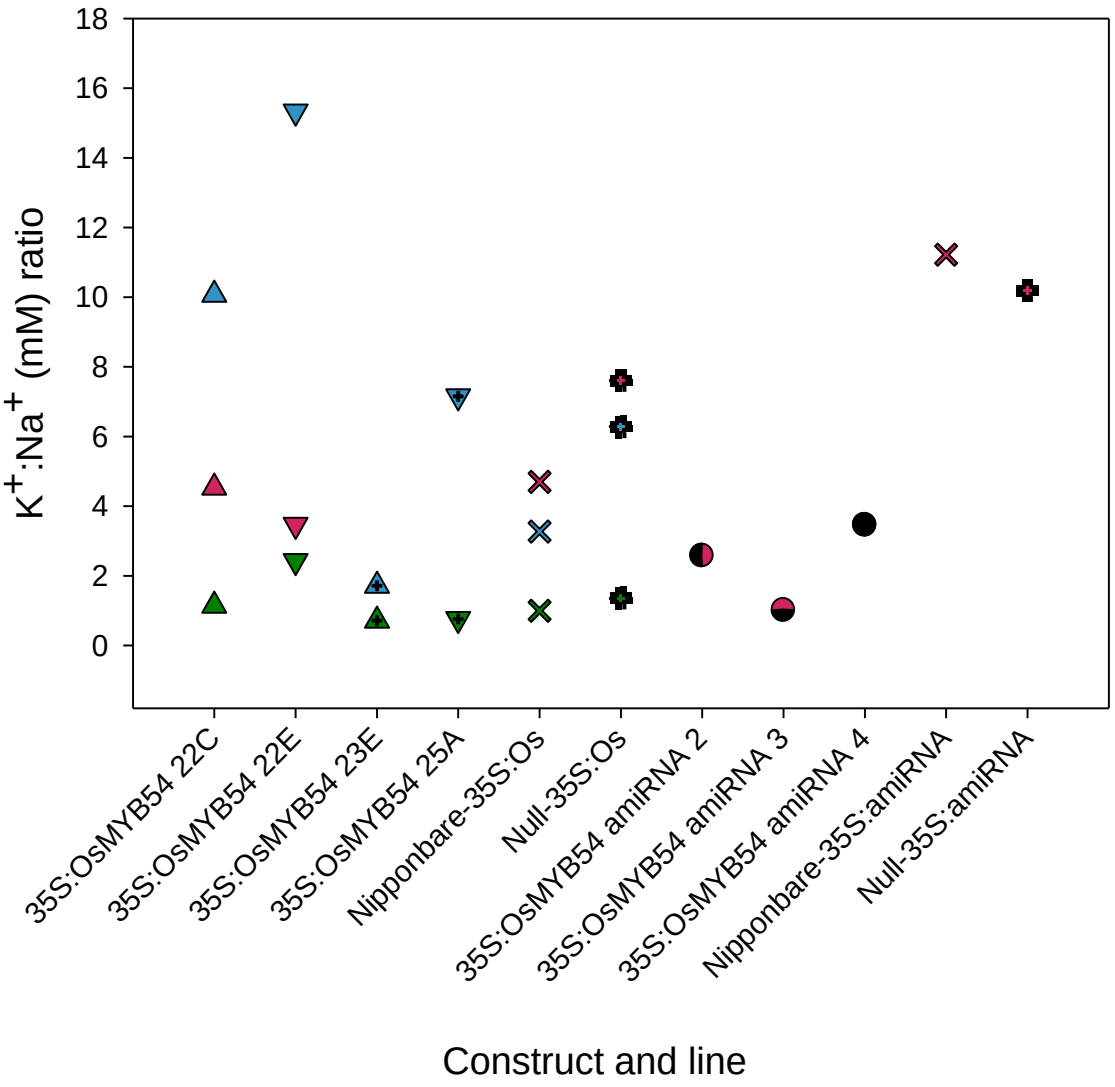


Figure 4.56 Graph of K^+:Na^+ ratio in rice plants with altered expression of *OsLUX* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The sodium and potassium concentration of the fourth leaf was determined using flame photometry and the K^+:Na^+ ratio calculated. The graph contains data from *2x35S:OsMYB54* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsMYB54 amiRNA* (centre) and *JRC0470:OsMYB54* (right) rice.

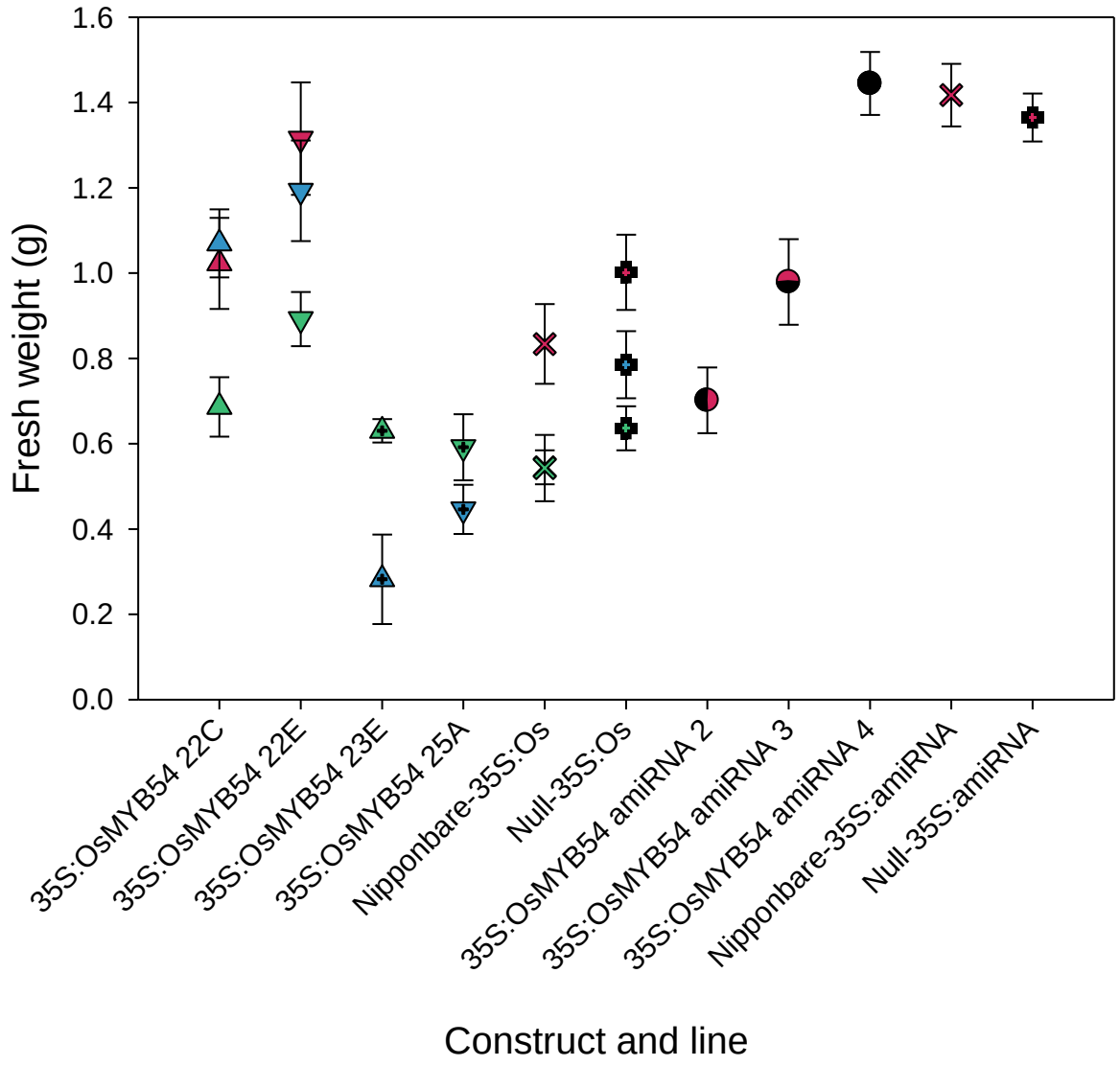


Figure 4.57 Graph of fresh weight in rice plants with altered expression of *OsMYB54* stressed with 50mM NaCl

The rice seeds were surface sterilised, germinated on filter paper, transferred to supported hydroponics in ACPFG growth solution and grown until fourth leaf emergence. A 25mM, then 50mM NaCl stress was applied over 24 hours and the plants were harvested 13 days post fourth leaf emergence. The fresh weight was then measured. The graph contains data from *2x35S:OsMYB54* (left) rice from the first (▲), second (▲) and third (▲) repetition and from *2x35S:OsMYB54 amiRNA* (centre) and *JRC0470:OsMYB54* (right) rice. Error bars represent SEM. n≥3.

4.4. Discussion

When a plant is exposed to a salt stress, the plant will sense the stress and then respond with series of signalling cascades that result in changes in gene expression. The genes code for an array of proteins that form channels and pumps that prevent sodium entry into roots or loading into the xylem, as well as the sequestration of sodium in the vacuole of the cells of both root and shoot. Regulation of genes involved in conferring salt tolerance occurs *via* a network of transcription factors. This project aimed to identify some of those transcription factors.

Altered expression of transcription factors has been shown to markedly affect indicators of salt tolerance; sodium and potassium accumulation and plant fresh weight. These include transcription factors from different families, which may function in the same signalling cascades and whose altered expression yield similar plant phenotypes.

Plants with constitutive overexpression of transcription factor coding sequences were initially selected based on T-DNA copy number. Southern blots were performed to detect the presence of the hygromycin resistance gene as the transcription factor genes could not be used as endogenous copies were already present in the plant genome. The aim was to select transgenic plants with one copy of the T-DNA, however some plants with two or three copies were included when there were not sufficient numbers with a single copy. Plants with a single copy of the T-DNA are selected to ensure that the same transgene is present in the progeny and so that an observed phenotype might be associated with that event. If there are multiple copies present, this association is complicated by additional copies, only one of which may be responsible for any observed phenotype. In additions, multiple insertions of the T-DNA at different sites in the genome can lead to methylation and silencing (Assaad *et al.*, 1993), or they can be in the form of inverted repeats, which can induce silencing (Hobbs *et al.*, 1993). Also, if multiple copies are present, the phenotype seen in the plants could be due to a dosage effect, where more copies of the gene result in higher mRNA levels and enhance the phenotype. A single T-DNA insertion can still result in a dosage-dependent phenotype, as the plants may be homozygous or hemizygous for the T-DNA (Vain *et al.*, 2011). Single T-DNA insertions may also be affected by position in the chromosome (al-Shawi *et al.*, 1990) and by methylation (Pröls & Meyer, 1992). However, in rice, transgene copy number has been reported to have no correlation with protein level or stability, except at very high copy numbers where protein levels decreased (Maqbool & Christou, 1999).

The first salinity tolerance assay (Section 4.3.3) was performed to reduce the total number of plants to be screened in subsequent Na^+ and K^+ accumulation experiments. The screening system used was similar to that employed at the International Rice Research Institute (Gregorio *et al.*, 1997), in which plant health is assessed based on a visual scoring system. Our system had plants arranged in a randomised design, rather than in rows of the same transgenic event. A randomised design aids statistical tests following the experiment, but makes quick visual assessments much more difficult during the experiment. The scoring system is flawed however, as the bias of the observer may change. Bias was controlled by having printed pictures and notes of what plant phenotype constituted each number on the scoring scale. There is also potential for human error.

Three different approaches were used to alter transcription factor expression. These included constitutive overexpression, salt inducible overexpression and amiRNA knockdown. Inducible overexpression should result in the least negative effects on plant health and phenotype (Kasuga *et al.*, 1999; Pino *et al.*, 2007), as the transgene is only expressed in the presence of a stress. Unfortunately the salt inducible promoter, JRC0470, was less than ideal in this experiment, due to low level constitutive expression in the root vasculature and some leaf tissues. Finding a stress-inducible promoter which is not leaky is difficult; even the classic stress-inducible promoter, *Rd29A*, is leaky (Chen *et al.*, 2010). However, promising results were obtained from plants with JRC0470 driving expression of *OsOrphan19* and *OsLUX*.

The other two approaches, constitutive overexpression and amiRNA knockdown, both have positive and negative features. AmiRNA knockdown should give the best indication of the effect of the transcription factor on plant salt tolerance, as these plants have the least side-effects resulting from the construct. There is only an insertional effect, where insertion of the T-DNA may have knocked out or altered the expression of one or more genes. However, knocking down expression of a single transcription factor may not be effective in producing any plant phenotype, considering that transcription factor families often have functional redundancies (Du *et al.*, 2009) (Borevitz *et al.*, 2000; Bouché & Bouchez, 2001; Byrne *et al.*, 2000; Heim *et al.*, 2003; Kalde *et al.*, 2003; Putterill *et al.*, 1995; Zhang *et al.*, 2003). Dominant repression is an alternative strategy that could be used to knock down expression of the target and functionally redundant transcription factors (Hiratsu *et al.*, 2003). AmiRNA knockdown does not result in complete suppression of the target mRNA, but usually gives a range of knockdowns with variably reduced expression. This is useful in identifying gene

function as the severity of the observed plant phenotype may be associated with the variation in mRNA level.

The plants with a dual 35S promoter driving expression of the transcription factor CDS have an insertional effect and also have the effect of the CDS being expressed in all cells of the plant. This imposes a larger metabolic load upon the plant as well expressing downstream genes in inappropriate cell types, which can result in stunted or sterile plants (Gevaudant *et al.*, 2007; Hsieh *et al.*, 2002a; Hsieh *et al.*, 2002b; Romero *et al.*, 1997). Plant sterility was a significant problem in this study for plants with constitutive overexpression of transcription factor CDSs, with delayed flowering and florets often not filling. Overexpression also has the issue that transcription factors often require activation via phosphorylation (Mao *et al.*, 2011; Yang *et al.*, 1999), so increased transcript levels or even protein levels may not produce a plant phenotype.

Each transcription factor will now be discussed.

4.4.1. OsOrphan19

The data show that the transcription factor OsOrphan19 is also involved in plant responses to salinity stress.

Expression analysis showed that the *OsOrphan19* transgene was highly expressed when driven by the 2x35S or salt inducible promoter. Endogenous expression of *OsOrphan19* was visibly reduced in the T0 generation of amiRNA expressing plants, however the amiRNA construct appears to have been silenced in the T1 generation. Semi-quantitative PCR required 40 cycles of amplification for a PCR product to become visible, indicating very low expression. A faint band is also present in the wild type sample of approximately 350bp, whereas the target product is 267bp. Expression analysis should be repeated using qRT-PCR. However, qRT-PCR may not clarify this issue as endogenous expression of *OsOrphan19* is very low in Nipponbare leaf blades at 32-64 copies/µL (Figure 2.1), which is in the range of background for qRT-PCR. Therefore it would be difficult to determine if the amiRNA construct was effective in reducing *OsOrphan19* expression. *OsOrphan19* expression was slightly higher in the salt sensitive cultivar, IR29, at 64-256 copies/µL. Under normal growing conditions, *OsOrphan19* is highly expressed in the seedling root, mature leaf and inflorescence at the mature pollen stage in *O. sativa* ssp. *indica* cv. IR64 (Jain *et al.*, 2007; Winter *et al.*, 2007). Sampling of these tissues or use of IR29 or IR64 for transformations may have more easily demonstrated the effectiveness of the amiRNA construct.

Expression of *OsOrphan19* was also different in wild type Nipponbare plants grown in soil or hydroponics. The Nipponbare plants used for qRT-PCR in Figure 2.1 were grown in hydroponics, as were the plants used for semi-q PCR in the lower panel of Figure 4.28. Expression of *OsOrphan19* was very low in both these experiments, at 32-64 copies/µL for qRT-PCR and a product only being amplified at 40 cycles for semi-q PCR. The Nipponbare plants used for semi-q PCR in the upper panel of Figure 4.28 were grown in soil. *OsOrphan19* transcripts were detectable at 35 cycles, suggesting that *OsOrphan19* is more highly expressed in leaf tissue in soil grown plants. Differences in gene expression between different growth media are not unexpected as different QTL have been identified in soil versus hydroponically grown plants (Ghandilyan *et al.*, 2009). Also, plant responses to salt stress are known to be different in hydroponics compared to soil (Tavakkoli *et al.*, 2010). *OsOrphan19* could be one of the genes involved in these differing responses.

JRC0470 gave slight salt inducibility of *OsOrphan19* in line 6, but there was no noticeable difference for line 4. A small amount of induction was expected from the GUS assay, where plants were analysed up to the three leaf stage. However, to avoid sampling necrotic tissue for genotyping in the Na^+ accumulation experiments, leaf material was taken from the youngest fully emerged leaf blade; the fifth leaf. The expression data would therefore suggest that *JRC0470* is only very mildly inducible in the fifth leaf.

The salinity tolerance assay showed that there was significant variation in plant health and height within each transgenic event and also between events. The plants of lines 8A and 9D were more consistent whereas lines 8C and 9B were more variable. All lines had single T-DNA insertions which should give the least number of undesirable effects.

The results for line 9B in the salinity tolerance assay reflect those in the Na^+ and K^+ accumulation experiments. In the salinity tolerance assay, the plants segregated into two distinct groups of better performing taller plants and poor performing shorter plants. Similarly, in the Na^+ and K^+ accumulation experiments, there were two plants with a low leaf Na^+ concentration and two plants with a high leaf Na^+ concentration. Southern analysis showed that line 9B only contained one copy of the T-DNA, so that does not explain the observed differences. The differences could be explained by the zygosity of the plants. The progeny from the T0 transformant will be homozygous, hemizygous and null for the transgene in a 1:2:1 ratio. Therefore, the effect of the transgene could be dosage-dependent (Vain *et al.*, 2011). Ideally, this experiment should have been performed with single insert homozygous lines. However, there may still be variation in transgene expression between the different transgenic events as the T-DNA genomic location affects expression, as some areas are more transcriptionally active (al-Shawi *et al.*, 1990; Beaujean *et al.*, 1998).

Line 8C performed similarly in the salinity tolerance assay, where plants segregated into two distinct groups of better performing taller plants and poor performing shorter plants. The Na^+ accumulation was however consistently low.

It is possible that overexpressing *OsOrphan19* to a moderate level has a positive influence on salt tolerance and that higher overexpression has a negative influence. Transgene expression in overexpression line 9B is marginally higher than the other overexpression lines. This line also consistently has high Na^+ accumulation and a poor K^+:Na^+ ratio. The other overexpression lines mostly have lower Na^+ and a better K^+:Na^+ ratio.

The amiRNA overexpressing lines have the most consistent effect on indicators of salinity tolerance. When subjected to a salt stress, all lines have increased Na^+ accumulation, a low K^+:Na^+ ratio and reduced fresh weight, indicating that *OsOrphan19* plays a crucial role in plant responses to salinity. When grown in unstressed conditions, the fresh weights of the amiRNA expressing lines were lower, comparable to and higher than wild type plants (Data not shown). This suggests that *OsOrphan19* does not affect plant growth in unstressed conditions.

OsOrphan19 likely affects plant responses to salinity through multiple mechanisms. The homolog *STO* is able to complement calcineurin deficient yeast and also complement another salt sensitive yeast mutant, deficient in a L-type calcium channel (Lippuner *et al.*, 1996; Rodriguez-Franco *et al.*, 2008). Overexpression of *STO* in *A. thaliana* also enhanced salt tolerance (Nagaoka & Takano, 2003). It is therefore possible that OsOrphan19 performs similarly in rice, modulating that K^+:Na^+ ratio as calcineurin does in yeast (Bressan *et al.*, 1998; Mendoza *et al.*, 1996; Mendoza *et al.*, 1994).

OsOrphan19 may also regulate the expression of other transcription factors. The homolog STO binds HPPBF-1, a single MYB DNA-binding domain containing protein, which is nuclear localised and for which mRNA transcripts accumulate upon salt stress treatment, then decrease after two hours (Nagaoka & Takano, 2003). Nagaoka and Takano *et al.* (2003) suggest that STO modulates plant salt stress responses by regulating the nuclear localisation of proteins including HPPBF-1 through dephosphorylation.

STO also interacts with RCD1/CEO1, which moves from the nucleus to cytoplasm and interacts with the SOS1 C-terminal cytoplasmic tail in response to salt stress (Belles-Boix *et al.*, 2000; Jaspers *et al.*, 2009; Katiyar-Agarwal *et al.*, 2006). It is not known whether STO interacts with RCD1/CEO1 in stressed or unstressed conditions. This is therefore a potential link between OsOrphan19 and SOS1 in rice plants.

Plants with altered expression of *OsOrphan19* had differing coleoptile growth rates following germination. The *OsOrphan19* constitutive overexpression and amiRNA knockdown plants had inhibited and enhanced coleoptiles growth rates, respectively. This is in contrast to the homologous transcription factors, STO and STH, in *A. thaliana*. Both *STO* and *STH* RNAi or T-DNA loss-of-function mutants display inhibited hypocotyl growth in red and far-red light, and *STO* mutants also display inhibited hypocotyl growth in blue light (Indorf *et al.*, 2007; Khanna *et al.*, 2006). *STO* overexpresssors have increased hypocotyl growth in red and far-red light and blue light

(Indorf *et al.*, 2007). This suggests that STO and STH function as negative regulators in the light-mediated inhibition of hypocotyl elongation (Indorf *et al.*, 2007; Khanna *et al.*, 2006). Observations from this experiment would suggest that OsOrphan19 performs the opposite function, as a positive regulator in the light-mediated inhibition of coleoptile elongation.

4.4.2. OsEREB67

The results here show that OsEREB67 in involved in plant responses to salinity, affecting Na^+ and K^+ accumulation and plant fresh weight.

Overexpression of *OsEREB67* caused infertility in all but two lines. Only line 10B had sufficient seed for further analysis, for which Southern analysis showed that three copies of the T-DNA were present and expression analysis showed that line 10B had barely detectable expression of the transgene. This was consistent with almost all overexpression lines being sterile; higher expression appears to cause infertility. This phenomenon occurs in *A. thaliana* where constitutive overexpression of *OsEREB67* results in stunted growth, delayed flowering and fewer siliques in 44% of T1 plants (Hu *et al.*, 2008b). The altered phenotypes are associated with higher *OsEREB67* expression, whereas the transgenic plants with normal phenotypes have no detectable expression (Hu *et al.*, 2008b). These phenotypes are similar to *A. thaliana* plants with constitutive overexpression of *ERF1* and *EIN3*, that have constitutive activation of the ethylene-response pathway (Chao *et al.*, 1997; Solano *et al.*, 1998). Constitutive overexpression of *OsEREB67* in *A. thaliana* also up-regulates the expression of the ethylene responsive genes *PDF1.2* and *b-chitinase*, suggesting that *OsEREB67* is involved in ethylene signalling (Hu *et al.*, 2008b). Additionally, the *A. thaliana* homolog, *AtERF1*, may be regulated by AP2, which is involved in floral organ development (Jofuku *et al.*, 1994; Ogawa *et al.*, 2007). There was increased expression of *AtERF1* in *ap2* mutant plants, suggesting that AP2 negatively regulates *AtERF1* (Ogawa *et al.*, 2007). Assuming some functional redundancy between OsEREB67 and AtERF1, this provides a link between OsEREB67 and floral development and may explain the infertility in *OsEREB67* overexpressing plants.

Variable expression of *OsEREB67* in the T1 amiRNA expressing lines suggests that there was silencing of the construct, possibly due to there being multiple insertions of the T-DNA (Assaad *et al.*, 1993). Multiple insertions are possible as Southern analysis was not performed.

The constitutive overexpression line 10B performed well in the salinity tolerance assay, with average to short healthy plants and very little variation between plants. This is consistent with the results of the Na^+ and K^+ accumulation experiments, where line 10B accumulated less Na^+ and had a higher K^+:Na^+ ratio, but lower fresh weight, relative to wild type plants. Decreased expression of *OsEREB67* increases sodium accumulation, lowers the K^+:Na^+ ratio and may also reduce fresh weight. This is interesting in that any

alteration in *OsEREB67* expression negatively impacts plant growth, despite large differences in the K^+:Na^+ ratio depending on whether expression is increased or reduced. The reduction in plant fresh weight in plants constitutively overexpressing *OsEREB67* could be explained by the function of OsEREB67 in ethylene signalling. As mentioned above, constitutive activation of the ethylene response pathway by overexpression of *EIN2* reduces plant fresh weight. NaCl hypersensitivity in the amiRNA expressing plants is consistent with a defective ethylene response as *ein2-5* mutants are hypersensitive to NaCl (Wang *et al.*, 2007). However, *ein2-5* mutants grow normally in unstressed conditions. This suggests that *OsEREB67* is also involved in another signalling pathway affecting plant development.

This may be a jasmonic acid mediated signalling pathway. Both OsEREB67 homologs from *A. thaliana*, are involved in jasmonic-acid mediated pathogen defence signalling (Brown *et al.*, 2003; Chen *et al.*, 2002; McGrath *et al.*, 2005; Oñate-Sánchez & Singh, 2002). Altered expression of *OsEREB67* may therefore affect jasmonic acid levels or jasmonic acid-mediated signalling. Since jasmonates stunt plant growth by inhibiting mitosis (Zhang & Turner, 2008), this may explain the stunted phenotype of plants with altered *OsEREB67* expression. Specifically, OsEREB67 may positively regulate ethylene responses, stunting growth in *OsEREB67* overexpressing plants, and may negatively regulate jasmonate responses, stunting growth in the amiRNA expressing *OsEREB67* knockdown plants.

The evidence presented here and in the literature place OsEREB67 in multiple signalling pathways involving biotic and abiotic stresses, and plant development.

4.4.3. OsMYBR63

The results here show that OsMYBR63 is not clearly involved in plant salinity tolerance. OsMYBR63's homology to the *A. thaliana* transcription factor RVE2 indicates that OsMYBR63 may be involved in plant responses to drought, cold and oxidative stress (Kasukabe *et al.*, 2004; López-Martin *et al.*, 2008; Soitamo *et al.*, 2008; Tran *et al.*, 2007), but we show here that the role of OsMYBR63 is unclear in plant responses to salt in rice.

The two lines with constitutive overexpression of *OsMYBR63* performed differently in the salinity tolerance assay. Line 11A was split into taller better performing plants and shorter poor performing plants, whereas line 11C consisted of average height better performing plants. Since both T0 lines had two T-DNA insertions, variation in the T1 progeny is expected, as they will have either T-DNA insert or both present.

The constitutive overexpression plants had strong expression of the transgene whereas the amiRNA expressing lines appeared to have a semi-functional construct that variably reduced endogenous *OsMYBR63* expression. The differences in Na^+ accumulation and the K^+:Na^+ ratio do not associate with the differences in *OsMYBR63* expression. amiRNA line 15 had high Na^+ accumulation and a low K^+:Na^+ ratio, which was not consistent with the other amiRNA expressing lines. This suggests that insertion of the T-DNA in line 15 may have knocked out a gene involved in regulating Na^+ transport. The sequence flanking the insertion site could be identified using thermal asymmetric interlaced PCR (Qin *et al.*, 2003). More amiRNA expressing lines need to be screened to determine the effect of reduced *OsMYBR63* expression. Alternatively, a new amiRNA construct could be designed as the construct used in this study was not highly effective.

It is possible that *OsMYBR63* does play some role in salt stress responses, but that they have been missed here due to the constructs being transformed into *japonica* rather than *indica* rice. *OsMYBR63* is known to have two splice forms and both are protein coding. However, what is not known is whether the splice forms are expressed similarly in both *indica* and *japonica* rice. The constitutive overexpression construct contained the full length gene with introns, so the splice form expressed or more highly represented would be dependent on the rice cultivar. EST data showed that the second splice form had not been found in *indica* rice and but was present in *japonica* rice. The microarray probesets and qRT-PCR primers did not distinguish between the different splice forms, so the expression level of each splice form is not known. Future experiments would analyse

expression of the two splice forms individually. In the microarray data, *OsMYBR63* had reduced signal intensity in response to salt stress. Therefore it would be expected that the amiRNA constructs would enhance plant salt tolerance and that the overexpression constructs would reduce salt tolerance. However, the overexpression lines had slightly improved salt tolerance and the amiRNA lines had improved or massively reduced tolerance depending on the line. It is possible that Nipponbare rice detects the high level of *OsMYBR63* expression in plants with the constitutive overexpression construct and forces splicing in favour of the second splice form, which may be non-functional. This would explain why overexpression of *OsMYBR63* did not reduce salt tolerance.

Salt inducible lines had reduced fertility, with only one line giving sufficient seed for further analysis. The constitutive overexpression lines and amiRNA expressing lines did not have noticeably reduced fertility. This indicates that differences in spatial and temporal expression of *OsMYBR63* affect plant fertility to a greater extent than constitutive or reduced expression.

The data do not conclusively demonstrate that OsMYBR63 is involved in responses to salinity in rice. Further studies are required using a more effective amiRNA construct and constructs that overexpress each splice form of *OsMYBR63* individually.

4.4.4. OsbHLH17

Altered expression of *OsbHLH17* had inconsistent effects on indicators of plant salinity tolerance. Unfortunately, the constitutive overexpression lines were generated at a late stage in the project and could not be included in salinity tolerance assays, so only the amiRNA overexpressing lines were analysed. These had reduced expression of endogenous *OsbHLH17* in the T0 generation but the transgene appears to have been silenced in the T1 generation. However, it is difficult to accurately determine whether the amiRNA is functional as endogenous gene expression in the leaf blade is approximately 128 copies/µL (Figure 2.4) and required 40 cycles to amplify a product using semiQ PCR, which is at the limit of detection. The efficacy of the amiRNA could have been established more easily using tissue from the root or sheath, where expression is higher.

The large variability in Na^+ accumulation, the K^+:Na^+ ratio and plant fresh weight between the transgenic lines is not associated with similar changes in *OsbHLH17* expression. The variability may be explained by insertion of the T-DNA knocking out a gene involved in regulating Na^+ accumulation for line 14 and a gene regulating plant growth for line 8. All lines did however have lower fresh weights and lower K^+:Na^+ ratios, relative to wild type plants, indicating that reduced expression of *OsbHLH17* does reduce plant salinity tolerance. Altered K^+:Na^+ ratios cannot be directly explained by the OsbHLH17 homologs. The OsbHLH17 homologs, AIB and MYC2 are both involved in ABA signalling and drought stress responses, but neither can be linked to the regulation of Na^+ (Abe *et al.*, 2003; Dombrecht *et al.*, 2007; Li *et al.*, 2007; Lorenzo *et al.*, 2004; Pozo *et al.*, 2008; Yadav *et al.*, 2005). *AtMYC2* overexpression does increase osmotic tolerance and osmotic stress is a component of salinity stress (Abe *et al.*, 2003). The reduced fresh weight and low K^+:Na^+ ratios seen in the amiRNA expressing plants may therefore be due to decreased osmotic stress tolerance. Further experiments should be performed to analyse the effect of mannitol and desiccation stress on plants with altered expression of *OsbHLH17*.

OsbHLH17 is an interesting candidate for further study, as the plants with reduced expression had a stay-green phenotype. A stay-green phenotype is usually associated with improved drought tolerance (Campos *et al.*, 2004; Tollenaar & Wu, 1999; Yan *et al.*, 2004). *OsbHLH17* may therefore have a dual role in drought and salt tolerance. The OsbHLH17 homologs in *A. thaliana* certainly suggest that OsbHLH17 may be involved in drought tolerance (Abe *et al.*, 2003; Li *et al.*, 2007). The stay-green phenotype following water withholding would suggest improved drought tolerance, but those same

plants have reduced salt tolerance, evident in the low K^+:Na^+ ratio and reduced plant fresh weight. As discussed previously in Section 3.4, the stay-green phenotype of the *OsbHLH17* amiRNA expressing lines may be due to *OsbHLH17* being a positive regulator of jasmonate synthesis. Knocking down *OsbHLH17* expression reduces jasmonate levels which reduce the senescence promoting effects to delay senescence. It is possible that *OsbHLH17* is not a good target for improving yield in rice, as a stay-green phenotype has also been found to have a negative correlation with yield (Jiang *et al.*, 2004). Further analysis of transgenic plants with constitutive and salt inducible overexpression of *OsbHLH17* in drought and salinity tolerance assays would need to be performed to determine the role of *OsbHLH17* in plant responses to these stresses. These future experiments would also grow plants through to maturity to assess the effect of abiotic stresses and the stay-green phenotype on yield.

4.4.5. OsLUX

The results here show that OsLUX is involved in regulating plant salinity tolerance.

In the salinity tolerance assay, the two lines constitutively overexpressing *OsLUX* had better performing average height plants and better performing average to tall plants. Some variability was expected as the T0 lines had two T-DNA insertions, so the progeny will have inherited either or both T-DNA inserts.

Increased transgene expression levels were associated with an improvement in the K^+:Na^+ ratio. Plants with salt inducible expression of *OsLUX* had drastically improved K^+:Na^+ ratios, whereas constitutive overexpression marginally improved the K^+:Na^+ ratio and the effect was variable. Reduced expression of *OsLUX* had a highly variable effect on both Na^+ accumulation and the K^+:Na^+ ratio and was associated with expression level. Line 3 had the most variable *OsLUX* expression, ranging from wild type levels to very low levels and the phenotype was most like wild type plants. Lines 1 and 9 with consistently low expression levels had increased Na^+ accumulation and a low K^+:Na^+ ratio.

The exact function of OsLUX in regulating plant salinity tolerance is unknown. However, the stress related genes *DREB1A/CBF3*, *COR15b* and *COR6.6* were found to be regulated by CCA1, a component of the *A. thaliana* circadian clock (Harmer, 2000). Recently, *AtLUX* was shown to bind the *PRR9* promoter and negatively regulate its expression (Helfer *et al.*, 2011). PRR9 and PRR7 negatively regulate *CCA1* expression (Farré *et al.*, 2005; Nakamichi *et al.*, 2005). So, assuming the circadian clocks act similarly between *A. thaliana* and rice, increased *OsLUX* expression may repress *PRR9*, which relieves inhibition of *CCA1*. Increased levels of CCA1 then positively regulate the stress related genes *DREB1A/CBF3*, *COR15b* and *COR6.6* to enhance plant stress tolerance. This line of reasoning is confirmed by another recent paper examining the *prr9-11 prr7-10 prr5-10* triple mutant (*d975*), which would again have relieved inhibition of *CCA1*. The *d975* mutant has increased cold, salt and drought stress tolerance and increased expression of *DREB1A*, *DREB1C* and *DREB2A* (Nakamichi *et al.*, 2009). However, the rice plants in our experiments do not show the typical dwarf phenotype associated with constitutive overexpression of the *DREB* genes (Dubouzet *et al.*, 2003; Ito *et al.*, 2006). Therefore further analysis is required to examine *DREB* gene expression levels to determine whether they are indirectly regulated by OsLUX.

4.4.6. OsMYB54

The results here show that OsMYB54 is likely to be involved in regulating plant salinity tolerance.

In the salinity tolerance assay, lines 23E and 25A were mostly short healthy plants, line 22E had mostly tall healthy plants and line 22C had average height healthy plants and short poor performing plants. These data associate well with the plant fresh weights and with the K^+:Na^+ ratios. Variation may be attributed to there being one to three T-DNA insertions in the T0 transgenic lines. There is not however a direct relationship with *OsMYB54* expression. It would appear that a small amount of *OsMYB54* overexpression enhances growth rate and improves salinity tolerance, and that further increases in expression result in negative effects. amiRNA expressing lines had increased Na^+ accumulation, drastically reduced the K^+:Na^+ ratio and variably reduced fresh weight, which did not associate with expression level. The amiRNA construct appears to have been silenced in the T1 generation, but there is still a salt sensitive phenotype associated with the presence of the T-DNA.

It is particularly difficult to speculate on the function of OsMYB54 in conferring plant salt tolerance. All homologs in both rice and *A. thaliana* have no published information on function or interacting partners but are all included in patents claiming the generation of plants with enhanced tolerance to cold treatment, osmotic stress and other abiotic stresses. Additionally, three years into this project, OsMYB54 was included in the patent WO/2009/127443, entitled “Transcription factors involved in salt stress in plants”. Changes in Na^+ accumulation in plants with altered *OsMYB54* expression suggests that OsMYB54 could be involved in regulating gene expression or the activity of proteins involved in ion transport throughout the plant, such as HKT, NHX or members of the SOS pathway (Haro *et al.*, 2010; Jiang *et al.*, 2010; Mahajan *et al.*, 2008).

4.4.7. Drawback, strengths and future directions

4.4.7.1. Silicate affects Na^+ accumulation

The use of silicate in the growth solution created ongoing problems through almost all growth experiments. A silicate concentration of 0.5mM was toxic to rice and its removal from the growth medium resulted in two-fold or greater Na^+ accumulation in the shoot. This was partially due to the leaf tips dying, which then acts as a wick and increases the apparent Na^+ concentration in the leaf. This resulted in some measured concentrations in excess of 1M Na^+, which is not possible except for some extreme halophytes (Flowers *et al.*, 1977). The removal of silicate also indirectly increases Na^+ uptake. Silicate is an essential component of rice cell walls that increases ion selectivity and reduces Na^+ and Cl^- accumulation in the leaf blade (Bonilla & Tsuchiya, 1998). Using the apoplastic tracer trisodium 3-hydroxy-5,8,10-pyrene trisulphonic acid (PTS), a strong correlation was found between PTS and Na^+ transport, suggesting that apoplastic leakage is a major contributor to shoot Na^+ accumulation under saline conditions in rice plants (Yadav *et al.*, 1996; Yeo *et al.*, 1987). Supplementation of rice growth media with silicate is thought to reduce apoplastic leakage by possibly producing colloidal precipitates of silica in the apoplast and by blocking leakage around emerging lateral roots (Yeo *et al.*, 1999). Lateral roots are a major apoplastic entry site for Na^+ due to the absence of an exodermis, allowing Na^+ to freely enter the stele (Faiyue *et al.*, 2010). Therefore, to prevent excess apoplastic leakage silicate is an essential component of rice growth medium. However, a low silicate concentration should be used. A silicate concentration of 0.05mM gave no toxicity symptoms.

4.4.7.2. Cultivar selection

In retrospect, the salt sensitive cultivar used in the original microarray experiment, *indica* cv. IR29, should have been transformed, rather than the *japonica* cv. Nipponbare. Nipponbare was used because there was an established transformation system, but there are two reasons why it would have been better to use IR29. First, IR29 is a widely used elite cultivar with multiple disease and insect resistance used in breeding programs throughout Asia (Gregorio, 2009; Khush & Brady, 1977). Therefore less effort would be required in the transition from greenhouse to field-based trials and farmer acceptance. Currently, constructs would need to be transformed into IR29 and screening for salt tolerance started again. Second, the endogenous expression level of some of the transcription factors was much lower in Nipponbare than IR29, around the level of background for qRT-PCR. Consequently, checking expression levels using semiQ-PCR,

a less sensitive assay than qRT-PCR, was particularly difficult in the transgenic plants expressing amiRNA constructs, as we were trying to analyse the difference between background and less than background. qRT-PCR could have been used as it is a more sensitive assay, however financial constraints prevented this.

4.4.7.3. Effectiveness of the approach

The approach of using microarray data to find novel transcription factors involved in regulating plant salt tolerance was an effective strategy. Five transcription factors were identified that were found to alter plant salt tolerance. The general approach of examining transcription factor function *in planta* was effective, but was highly susceptible to external influences. The outbreak of two fungal species resulted in a change in growth room that affected temperature, light intensity, day length and humidity. There was also a change in the concentration of silicate in the growth medium, as the system was not optimised prior to project commencement. Calculation of Na^+ accumulation is also susceptible to variation due to the leaf tips dying. These variables presented as the project progressed, so could not be adequately controlled. Future experiments examining transcription factor function *in planta* could be more effective with less variability by using strict quarantine protocols to limit cross-contamination between greenhouses and the outside environment, using an optimised growth medium and harvesting at an earlier stage to limit sampling of leaves with dead tips.

The manipulation of transcription factors is an effective strategy for altering plant characteristics, although crude in its current form. Massively increasing or reducing expression has large and often negative effects upon plant growth or fertility. The approach taken in this study is required initially to identify transcription factors of interest, but it may not be effective in the field. What is required is more subtle manipulation, perhaps using *promoter:gene* combinations from salt tolerant species. Use of the native promoters from the salt tolerant cultivars, FL478, IR63731 or Pokkali in combination with their transcription factor coding sequences may have yielded Nipponbare rice with improved salt tolerance and less negative effects. Future experiments could use *promoter:gene* combinations from these salt tolerant cultivars.

4.4.8. Concluding remarks

Homology to known stress-responsive genes and the results presented here show that the transcription factors *OsOrphan19*, *OsEREB67*, *OsbHLH17*, *OsLUX* and *OsMYB54* are involved in plant salt stress responses. Altering the expression of these transcription

factors has indeed been shown to alter plant salinity tolerance. Further experiments should be performed to determine the role of these transcription factors in plant responses to salt stress. These include identifying interacting proteins using yeast two-hybrid assays and confirming interactions using electrophoretic mobility shift assays. Also, protein-DNA interactions with downstream target promoters could be identified using chromatin immunoprecipitation sequencing. Further characterisation of the transgenic plants with altered expression of these transcription factors is required to assess whether the transcription factors are involved in plant responses to other abiotic stresses. Finally, analysis of total mRNA populations using RNA sequencing should be performed in transgenic plants with improved or reduced salt tolerance, to identify which downstream genes may be responsible for the plant phenotype.

Chapter 5. Appendices

5.1. Oligonucleotides used in PCR

Table 5.1 qRT-PCR primers

The primers with probeset names were designed to amplify sequence targeted by the Affymetrix probesets, usually the 3′ UTR.

Primer name	**Primer Sequence**	**Product size (bp)**
Actin	F 5′- GAAGATCACTGCCTTGCTCC -3′	226
	R 5′- CGATAACAGCTCCTCTTGGC -3′	
α-tubulin	F 5′- TACCGTGCCCTTACTGTTCC -3′	234
	R 5′- CGGTGGAATGTCACAGACAC -3′	
Glyceraldehyde-3-phosphate dehydrogenase(GAP)	F 5′- GGGCTGCTAGCTTCAACATC -3′	190
	R 5′- TTGATTGCAGCCTTGATCTG -3′	
Elongation factor 1B2Os	F 5′- ATCTGGGAAATCATCGGTTCTG -3′	189
	R 5′- AGATCGTCCACAATGGTCATCA -3′	
Peptidylprolyl isomerase (Pplase)	F 5′- CTGGCAAAGAACCTAAACCTCATC -3′	194
	R 5′- TGCTCCTGTGGTGTAACTGCTTT -3′	
OS.49746.1.S1_AT	F 5′- CGCCAACCACTGATCCATTC -3′	254
	R 5′- GCCTTTGTCACGCAGGTAGG -3′	
OS.46956.1.S1_AT	F 5′- CCAACTGTTATCAGTCTTTGAATCC -3′	180
	R 5′- TATACGATGCTTCAGGCTAAAT -3′	
OS.46600.1.S1_AT	F 5′- GTCACCACCGTCGCCGCC -3′	175
	R 5′- CTGGAGCACATCTGGGAAAGTAGG -3′	
OS.26695.1.S1_AT	F 5′- CCACCATCCCCAGGAACAAC -3′	279
	R 5′- GCGTCACCCGAGCCATCTC -3′	
OS.15849.1.S1_S_AT	F 5′- CAGACGCCGACGAGCAAGTAG -3′	281
	R 5′- CACCAGAGCCAATAGAAAATAGAAAAG -3′	
OS.11094.1.A1_AT	F 5′- ACGCCCCAACGGATTGACCAG -3′	236
	R 5′- CCACCACCACGCTACTATCTGCTAGAG -3′	
OS.56210.1.S1_AT	F 5′- GTCCTACCACCTCATCCTCCACG -3′	198
	R 5′- CTTTATCCCACGAGCACGCC -3′	
OS.51307.1.S1_AT	F 5′- TTAGTTTACCGACGATGGAAGTGG -3′	181
	R 5′- GTGGAGAACCGCCGCCC -3′	
OS.10235.1.S1_AT	F 5′- GCACTCCAGGGACTCCAAACC -3′	161
	R 5′- GGAATGATCTGAACATGCTACGG -3′	
OS.18955.1.S1_AT	F 5′- GCAGATGACCGCTCCCCAG -3′	300
	R 5′- ACCAATCCCCGCAAACAAAC -3′	
OS.3397.1.S1_AT	F 5′- CCCGACGACGAGGAGGATT -3′	267
	R 5′- TAAACATAAGAGCAGAGCCTGGAG -3′	
OS.11250.1.S1_AT	F 5′- TACGCAGTAGTATTTTCCGTTTCAG -3′	264
	R 5′- GTGCTTTTCCTGTTTCTGTAGTTTTTG -3′	

Table 5.2 Primers targeting Affymetrix probeset:cDNA combinations

Signal intensity was analysed from the microarray experiment and cDNAs for each probeset were chosen in which the signal was the highest. These primer:cDNA combinations were used to maximise the likelyhood of amplifying a product.

Primers for Affymetrix probeset	cDNA template
Os.56210.1.S1_at	Salt stressed IR63731 shoot
Os.10235.1.S1_at	Control IR63731 root
Os.11250.1.S1_at	Control IR63731 root
Os.18955.1.S1_at	Control IR63731 root
Os.3397.1.S1_at	Control IR63731 root
Os.51307.1.S1_at	Control IR63731 root
Os.11094.1.A1_at	Salt stressed IR63731 root
Os.15849.1.S1_s_at	Salt stressed IR63731 root
Os.26695.1.S1_at	Salt stressed IR63731 root
Os.46956.1.S1_at	Salt stressed IR63731 root
Os.49746.1.S1_at	Salt stressed IR63731 root
Os.46600.1.S1_at	Salt stressed FL478 root

Table 5.3 Subcellular locations were predicted using TargetP

```
### TargetP v1.1 prediction results ###################################
Number of query sequences:  7
Cleavage site predictions included.
Using PLANT networks.
```

Name	Length	cTP	mTP	SP	other	Loc	RC	TPlen
Os02g39360	271	0.024	0.005	0.893	0.245	S	2	27
Os04g46220	318	0.057	0.369	0.050	0.647	–	4	–
Os06g51260.1	451	0.167	0.103	0.136	0.863	–	2	–
Os06g51260.2	348	0.133	0.308	0.002	0.433	–	5	–
Os01g50940	473	0.169	0.046	0.348	0.478	–	5	–
Os01g74020	238	0.249	0.050	0.006	0.900	–	2	–
Os04g42950	239	0.174	0.211	0.038	0.574	–	4	–
cutoff		0.000	0.000	0.000	0.000			

cTP- Chloroplast transit peptide –
mTP- Mitochondrion transit peptide
SP- Secretory pathway
Loc- Prediction of localisation
RC- Reliability class; 1 to 5, where 1 indicates the strongest prediction.
TPlen- Predicted presequence length
(Dyrløv Bendtsen *et al.*, 2004; Emanuelsson *et al.*, 2000).

Table 5.4 Nested primer sets used to amplify transcription factor coding sequences

Primer sets were designed to amplify the complete coding sequence from start to stop codon.

Primer Name		Primer Sequence	
OsOrphan19.Code_F	5′-	ATGAAGATCCAGTGCGACGC	-3′
OsOrphan19.Code_R	5′-	TCAACCAAGATCAGGGACGATG	-3′
OsOrphan19.Code_F1	5′-	TGTTCTTGAGTTGTGTTCTTGGTTG	-3′
OsOrphan19.Code_R1	5′-	CCTCTGGAACGAACTCCTCTTC	-3′
OsEREB67.Code_F	5′-	ATGACGGCGCGAAGCATG	-3′
OsEREB67.Code_R	5′-	TTAGATGACGAGCTGCTCCACG	-3′
OsEREB67.Code_F1	5′-	GGCGAGGTCGGGTCAGC	-3′
OsEREB67.Code_R1	5′-	CATTGCTGGCTTGGTTTGC	-3′
OsEREB67.Code_F2	5′-	GAAGTGTGTAACCAAGTTTGAGACAG	-3′
OsEREB67.Code_R2	5′-	GGGATGCTGCTCGCTTGC	-3′
OsMYBR63.Code_F	5′-	ATGGCTTCCATGCCGCAG	-3′
OsMYBR63.Code_R	5′-	TTATTGAAATTTGCATCTTTTATAAGGC	-3′
OsMYBR63.Code_F1	5′-	AAGCAAGATTTGAACTACTCCTCCTC	-3′
OsMYBR63.Code_R1	5′-	GAAAATACCCGCTTGCCTGC	-3′
OsMYBR63.Code_F2	5′-	GCTTTGCTTCTTCGGTCGC	-3′
OsMYBR63.Code_R2	5′-	GTTCGTGCTCCTACTCTTCTCTG	-3′
OsbHLH17.Code_F	5′-	ATGTCGTGGTCCGAGACGG	-3′
OsbHLH17.Code_R	5′-	TCACGGGGAGGGAGTGGTG	-3′
OsbHLH17.Code_F1	5′-	CGGAGGCGGCGAGCTAG	-3′
OsbHLH17.Code_R1	5′-	TACTAACTTACTGCACTGCACTGTCAC	-3′
OsbHLH17.Code_F2	5′-	GGTTGATTTGTTTGACCGACG	-3′
OsbHLH17.Code_R2	5′-	ATAACAGTTGGCAGCTATACCGTAC	-3′
OsLUX.Code_F	5′-	ATGGGCGAGGAGGCG	-3′
OsLUX.Code_R	5′-	TCAGTGGTTGGCGTGGTGG	-3′
OsLUX.Code_F1	5′-	ATTTGATTGAGAAGAAAATCCGAG	-3′
OsLUX.Code_R1	5′-	GGAAATGAAATGATGATGAGAAGG	-3′
OsLUX.Code_F2	5′-	GGAGGAGGAGGAACGAGTGG	-3′
OsLUX.Code_R2	5′-	CTTCTCTTCTCTTATTGATGAAACACC	-3′
OsMYB54.Code_F	5′-	ATGGCGGCGGCGGAAG	-3′
OsMYB54.Code_R	5′-	CTACCACAGCGTGCATGCTCC	-3′
OsMYB54.Code_F1	5′-	AAGAAGAGAGGTAGCTGGACTGAAG	-3′
OsMYB54.Code_R1	5′-	CGACTACTCTTCTCTCATGGCGTC	-3′

Table 5.5 Primers used in the construction of artificial microRNA expression vectors

Primer sets were generated using the Weigelworld WMD 2 – Web MicroRNA Designer website (http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl) to specifically modify the rice specific vector pNW55 for expressing amiRNAs (Warthmann *et al.*, 2008).

Universal primers	G-4368: CTG CAA GGC GAT TAA GTT GGG TAA C
	G-4369: GCG GAT AAC AAT TTC ACA CAG GAA ACA G
OsOrphan19	I miR-s agTTAAACATAAGAGCGGAGCGTcaggagattcagtttga
	II miR-a tgACGCTCCGCTCTTATGTTTAActgctgctgctacagcc
	III miR*s ctACGCTGCGCACTTATGTTTAAttcctgctgctaggctg
	IV miR*a aaTTAAACATAAGTGCGCAGCGTagagaggcaaaagtgaa
OsEREB67	I miR-s agTTGACGCGAATTATGACCGTTcaggagattcagtttga
	II miR-a tgAACGGTCATAATTCGCGTCAActgctgctgctacagcc
	III miR*s ctAACGGACATTATTCGCGTCAAttcctgctgctaggctg
	IV miR*a aaTTGACGCGAATAATGTCCGTTagagaggcaaaagtgaa
OsMYBR63	I miR-s agTATCGCATTATTGGAACCGCGcaggagattcagtttga
	II miR-a tgCGCGGTTCCAATAATGCGATActgctgctgctacagcc
	III miR*s ctCGCGCTTCGAATAATGCGATAttcctgctgctaggctg
	IV miR*a aaTATCGCATTATTCGAAGCGCGagagaggcaaaagtgaa
OsbHLH17	I miR-s agTACTAACTTACTGCACCGCGCcaggagattcagtttga
	II miR-a tgGCGCGGTGCAGTAAGTTAGTActgctgctgctacagcc
	III miR*s ctGCGCGCTGCTGTAAGTTAGTAttcctgctgctaggctg
	IV miR*a aaTACTAACTTACAGCAGCGCGCagagaggcaaaagtgaa
OsLUX	I miR-s agTGAATTAGGAGTTTACAGTTCcaggagattcagtttga
	II miR-a tgGAACTGTAAACTCCTAATTCActgctgctgctacagcc
	III miR*s ctGAACAGTATACTCCTAATTCAttcctgctgctaggctg
	IV miR*a aaTGAATTAGGAGTATACTGTTCagagaggcaaaagtgaa
OsMYB54	I miR-s agTATTAAAGAAGTGTGACCGATcaggagattcagtttga
	II miR-a tgATCGGTCACACTTCTTTAATActgctgctgctacagcc
	III miR*s ctATCGCTCAGACTTCTTTAATAttcctgctgctaggctg
	IV miR*a aaTATTAAAGAAGTCTGAGCGATagagaggcaaaagtgaa

Table 5.6 Primers used in analysis of transgene expression

Primers were designed to target the 3′ end of each transgene.

OsOrphan19 3′F QPCR3	TTCCGCCAGAGCAAGAAGG
OsEREB67 3′F QPCR2	GCAGCTCGTCATCTAAAAGGGC
OsMYBR63 3′F QPCR1	AGCAACAGCGGAGATGAAGAGG
OsbHLH17 3′F QPCR1	GTCACGCACACGATAATGGTG
OsLUX 3′F QPCR1	CTACCACCACTACCACCACGCC
OsMYB54 3′F QPCR1	GCACGCTGTGGTAGAAGGGC

Table 5.7 Miscellaneous primers

QPCR_JRC0470F	5′-TCTCCACACACTCTTCTTCTCCAC-3′
QPCR_JRC0470R	5′-GCCACCTCCTCCGTCACCTTC-3′
JRC0470_3′F	5′-TCCACTCCTCCGCCGCTC-3′
pMDC32_3165R	5′-CGAACGGATAAACCTTTTCACG-3′
HYG1	5′- GTCGATCGACAGATCCGGTC-3′
HYG2	5′-GGGAGTTTAGCGAGAGCCTG-3′
NOSF	5′-GAATTTCCCCGATCGTTCAAAC-3′
NOSR	5′-GCGGGACTCTAATCATAAAAACC-3′
NOSR2	5′-GTTTGAACGATCGGGGAAATTC-3′
GW1	5′-GTT GCA ACA AAT TGA TAA GCA ATG C-3′
GW3	5′-TTA ATA TAT TGA TAT TTA TAT CAT TTT ACG-3′

5.2. Media

LB: 1% NaCl w/v, 1% tryptone w/v, 0.5% yeast extract w/v, pH 7.0

TE buffer: 10mM Tris pH 8, 1mM EDTA

GTE buffer: 50mM glucose, 25mM Tris-HCl, 10mM Na_2EDTA

NaOH/SDS solution: 0.2M NaOH, 1% SDS

DNA Extraction Buffer: 0.1M Tris-HCl, 0.05M EDTA, 1.25% SDS, pH 8

R40: 40µg/mL RNase A in TE buffer

20X SSC: 3M NaCl, 0.3M Tri-sodium citrate

Sephadex G-100: 300mL 1x TE, 10g Sephadex G-100. Incubated with gentle shaking for 2 hr at 65°C.

Dextran Sulphate: 250g dextran sulphate made up to 1 litre with water.

Salmon sperm DNA: 0.5g salmon sperm DNA, 100mL nanopure water.

Denhardt's III: 2% w/v BSA, 2% w/v Ficoll 400, 2% w/v polyvinyl-pyrrolidone 360

5x HSB: 3M NaCl, 100mM Pipes, 25mM Na_2EDTA, pH 6.8 with 4M NaOH

Pre-hybridization Solution 300mL: 150mL 10x SSC, 105mL water, 30mL Denhardt's III, 15mL salmon sperm DNA (5mg/mL)

Hybridisation Solution 100mL: 5mL water, 30mL 5x HSB, 30mL Denhardt's III, 30mL Dextran sulphate 25%, 5mL salmon sperm DNA (5mg/mL)

6x Ficoll dye: 15% Ficoll type 4000, 0.25% bromphenol blue, 0.25% xylene cyanol FF

Oligo buffer: Primers: 0.3µg/12.5µL, dATP, dTTP and dGTP: 60µM each, 150mM Tris-HCl pH 7.6, 150mM NaCl, 30mM MgCl2, 300µg/mL BSA

Stripping solution 1L: 0.1% SDS (1g), 2 mM EDTA pH 8.0 (0.75g)

GUS staining solution: 50mM Na Phosphate pH7, 10mM EDTA, 0.1% Triton X-100, 2mM potassium ferrocyanide, 2mM potassium ferricyanide, 0.5mg/mL X-Gluc. Note: Na Phosphate pH7 prepared from 1M stock of Na_2HPO_4 and 1M stock of $NaH_2PO_4.H_2O$ autoclaved and mixed 1:1. X-gluc prepared by dissolving 40mg X-gluc in 1mL dimethylformamide, store at -20ºC.

Table 5.8 ACPFG plant nutrient solution

Recipe for cereal nutrient solution used at the Australian Centre for Plant Functional Genomics.

	Salts	Formula Weight	Mass (g) for 1 litre of stock solution	Stock solution concentration (M)	Vol. of stock (mL) for 1L culture	Final concentration (mM)
1	Macronutrients					
	NH_4NO_3	80.0	80.0	1.0	5.0	5.0
	KNO_3	101.1	101.1	1.0		5.0
2	$Ca(NO_3)_2•4H_2O$	236.1	94.4	0.4	5.0	2.0
3	$MgSO_4•7H_2O$	246.5	98.6	0.4	5.0	2.0
	KH_2PO_4	136.1	2.72	0.02		0.1
4	Na_2SiO_3	122.0	112 mL of 4.45 M liquid stock	0.5	1.0	0.5
5	NaFe(III)EDTA	367.1	18.4	0.05	1.0	0.05
6	Micronutrients			(mM)		(μM)
	H_3BO_3	61.8	3.09	50.0	1.0	50.0
	$MnCl_2•4H_2O$	197.9	0.990	5.0		5.0
	$ZnSO_4•7H_2O$	287.5	2.875	10.0		10.0
	$CuSO_4•5H_2O$	249.7	0.125	0.5		0.5
	Na_2MoO_3	242.0	0.024	0.1		0.1

5.3. Statistical tests

5.3.1.1. OsOrphan19

One way analysis of variance was conducted on the fresh weight values for plants with altered expression of *OsOrphan19*.

One Way Analysis of Variance Wednesday, May 11, 2011, 10:47:56 AM

Data source: All E FW stats in 2011_5_11_Statistics for thesis, Na K, FW, ratios.JNB

Normality Test: Passed (P = 0.762)

Equal Variance Test: Passed (P = 0.538)

Group Name	N	Missing	Mean	Std Dev	SEM
E1 FW Nipp	9	0	0.834	0.280	0.0935
E1 FW Null	16	0	1.002	0.353	0.0882
E1 FW 8A	8	0	0.710	0.187	0.0661
E1 FW 9D	10	0	1.315	0.317	0.100

Source of Variation	DF	SS	MS	F	P
Between Groups	3	1.902	0.634	6.789	<0.001
Residual	39	3.643	0.0934		
Total	42	5.545			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.938

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
E1 FW 9D vs. E1 FW 8A	0.605	4.171	<0.001	0.009	Yes
E1 FW 9D vs. E1 FW Nipp	0.481	3.427	0.001	0.010	Yes
E1 FW 9D vs. E1 FW Null	0.313	2.542	0.015	0.013	No
E1 FW Null vs. E1 FW 8A	0.291	2.202	0.034	0.017	No
E1 FW Null vs. E1 FW Nipp	0.168	1.320	0.195	0.025	No
E1 FW Nipp vs. E1 FW 8A	0.123	0.831	0.411	0.050	No

Table 5.9 Artificial microRNA expressing rice plant information

Information on transgenic rice plants overexpressing amiRNA constructs, including, the number of seeds harvested from T0 plants, germination rates for T1 plants and any observations about those T1 plants.

Construct	T0 seeds	T1 germination (%)	Observations day six
Nipponbare grown in CE1	500+	97.5	
OsOrphan19 amiRNA-2	311	100	
OsOrphan19 amiRNA-5	342	100	Early vigour, large plants
OsOrphan19 amiRNA-9	187	100	Early vigour, large plants
OsEREB67 amiRNA-1	250	97.5	Thin shoot, long roots
OsEREB67 amiRNA -8	570	100	
OsEREB67 amiRNA -10	205	100	
OsMYBR63 amiRNA -1	81	95	Thin shoot, long roots
OsMYBR63 amiRNA -2	92	97.5	Thin shoot, long roots
OsMYBR63 amiRNA -15	620	100	All heterogeneous,3 bleached
OsbHLH17 amiRNA -1	231	97.5	
OsbHLH17 amiRNA -8	133	100	
OsbHLH17 amiRNA -14	370	100	
OsLUX amiRNA -1	410	92.5	
OsLUX amiRNA -3	82	100	
OsLUX amiRNA -9	118	92.5	
OsMYB54 amiRNA -2	295	100	
OsMYB54 amiRNA -3	265	95	Small, thin, pale
OsMYB54 amiRNA -4	430	100	

Table 5.10 Transcription factor coding sequence overexpression rice plant information

Information on transgenic rice plants overexpressing transcription factor CDSs, including, the number of seeds harvested from T0 plants, germination rates for T1 plants, any observations about those T1 plants and 100 grain weight.

Construct	T0 seeds	1st -3rd Rep-T1 germination (%)	3rd Rep-Observations day six	100 grain weight
Nipponbare Yanco		96.4		2.109
OsOrphan19-8A	167	87-93.8	Short coleoptile, long seminal root	1.313
OsOrphan19-8C	230	89	Short coleoptile, long seminal root	1.675
OsOrphan19-9B	230	100	Short coleoptile, long seminal root	1.631
OsOrphan19-9D	160	100	Short coleoptile, long seminal root	1.660
OsEREB67-10B	280	51-90	8 large, 12 small	1.731
OsEREB67-10C	23	62	6 huge, 2 small	1.877
OsMYBR63-11A	150	77-94	16 large, 4 small	1.605
OsMYBR63-11C	120	96.4-100		1.424
OsLUX-17C	125	93-95.6	13 large, 1 small	1.67
OsLUX-19C	250	55-73.7		1.893
OsMYB54-22C	280	100		1.922
OsMYB54-22E	106	80-85.2		1.716
OsMYB54-23C	166	79	21 large, 9 small	1.547
OsMYB54-25A	290	97	24 huge, 4 small	1.628
1D		100		1.841

Table 5.11 Salt inducible transcription factor coding sequence rice plant information

Information on transgenic rice plants salt inducibly overexpressing transcription factor CDSs, including, the number of seeds harvested from T0 plants, germination rates for T1 plants, any observations about those T1 plants and 100 grain weight.

Construct	T0 seeds	T1 germination (%)	Observations	100 grain weight
Nipponbare Yanco		100		2.106
JRC0470:OsOrphan19-4	250	100		1.792
JRC0470:OsOrphan19-6	180			1.507
JRC0470:OsOrphan19-11	300	100		1.948
JRC0470:OsLUX-1	190	100		1.608
JRC0470:OsLUX-2	200	100		1.835
JRC0470:OsLUX-12	200	100		2.031

References

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