Modifying sodium transport to improve salinity tolerance of commercial rice cultivars (Oryza sativa L.)

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<td>International Rice Research Institute</td>
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<td>National Center for Biotechnology Information</td>
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<td>nitrate</td>
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<td><em>Oryza sativa</em></td>
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<td>rice vacuolar pyrophosphatase</td>
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<td>phosphate buffered saline</td>
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<td>polymerase chain reaction</td>
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<td>recombinant inbred line</td>
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<td>RO</td>
<td>reverse osmosis</td>
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<td>RT</td>
<td>room temperature</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
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<tr>
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<td>salt overly sensitive</td>
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<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
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<td>T_0</td>
<td>primary rice transformant containing T-DNA</td>
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<td>progeny of T_1</td>
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<td>tris-acetate-EDTA</td>
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<td>transfer deoxyribonucleic acid</td>
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<td>melting temperature, of primers</td>
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<td>upstream activation sequence</td>
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<td>promoter of maize <em>Ubiquitin-1</em></td>
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<td>University California soil mix</td>
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<td>UV</td>
<td>ultraviolet</td>
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<td>V</td>
<td>voltage</td>
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<td>volume per volume</td>
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Abstract

Salinity tolerance in rice is negatively correlated with sodium accumulation in the shoot. Therefore, one approach to improve rice salinity tolerance is through the modification of sodium transport pathways within the plant, either by constitutive or cell type-specific expression of genes encoding proteins important for sodium homeostasis. In rice, work so far has predominately been limited to poorly adapted cultivars or has used technologies incompatible with future breeding programs. It is therefore important to transfer the knowledge obtained from the modification of Na\(^+\) transport processes in other plants and test the validity of this approach in commercially relevant rice cultivars, using compatible technologies for further application of the approach in the field.

Five candidate commercial rice cultivars were selected from Indonesia. The salt tolerance of these rice cultivars were studied in hydroponics. Variation existed in the salinity tolerance mechanisms among the rice cultivars, offering the potential to use different approaches for improving salinity tolerance. *Agrobacterium*-mediated transformation efficiency of the cultivars was evaluated using calli derived from the scutellum of mature seeds. The study revealed only two cultivars, Fatmawati and IR64, could regenerate transgenics.

A non-destructive image based phenotyping protocol was developed for screening rice undergoing salt stress and was used to further examine the salinity tolerance of Fatmawati and IR64. The two cultivars showed differences in both their salinity tolerance and in the salinity tolerance mechanisms they used. Due to the differences in their salinity tolerance and due to their amenability for *Agrobacterium*-mediated transformation, Fatmawati and IR64 were selected for transformation with salinity tolerance genes using constitutive and cell type-specific promoters.

The maize *Ubiquitin-1* and cauliflower mosaic virus 35S promoters were used as constitutive promoters. Cell type-specific promoters were identified from either the literature or rice databases and used to drive the genes in specific cells in the root. The cell type-specific alterations are targeted to minimize net sodium influx into the root from the soil, maximise sodium retrieval from the xylem, or increase sodium compartmentalization in the root tissue.

Rice lines were generated which constitutively expressed the genes encoding the vacuolar H\(^+\)-pyrophosphatases *AtAVP1* and *OsOVP4* and the protein kinase *AtCIPK16*. Transgenic rice lines were also developed which expressed Na\(^+\) transporter *OsHKT1;5* driven by a stelar specific promoter and Na\(^+\) pumping ATPase from *Physcomitrella patens* (*PpENA1*) driven by an epidermal specific promoter. The salinity tolerance of the transgenic rice lines was characterized in the T\(_1\) generation using either non-destructive image based phenotyping or destructive analysis in hydroponic experiments. Results from this study showed that constitutive expression of *AtAVP1* lead to increased biomass of transgenic rice both under salt stress and non-stress conditions. The present study demonstrated the expression of *OsHKT1;5* in the root stele reduced shoot Na\(^+\) accumulation, while the expression of *PpENA1* in the root epidermis reduced root Na\(^+\) concentration. However, the effect of the alteration on the whole plant salinity tolerance of the transgenic rice still requires further characterization. Further assessment of these transgenic lines in later generations is necessary.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Aris Hairmansis and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Aris Hairmansis

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Date
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Finally I would like to thank my parents, my wife and my daughters, Afifah and Hanifah, who have supported me throughout my study with their prayer and patience.
Chapter 1 : Literature Review and Research Aims

1.1. The importance of rice

1.1.1. Rice as a major staple food in the world

Rice (*Oryza sativa* L.) is the staple food for almost half the world’s population. It is grown across an area of approximately 159 million ha and is the most consumed food in the world (GRiSP 2013). Together with maize and wheat, rice supplies more than 42% of the total calories consumed by the human population (GRiSP 2013). About 90% of rice is produced and consumed in Asia (GRiSP 2013).

1.1.2. Rice as a model species of cereals

Rice is a model species for cereals. Rice has the smallest genome size compared to other cereals (Arumuganathan and Earle 1991) and the genome has extensive conserved synteny with other cereals (Moore et al. 1995; Devos 2005). The rice genome was completely sequenced in 2005 by the consortium of the International Rice Genome Sequencing Project (International Rice Genome Sequencing Project 2005). The complete sequence of the rice genome has become an invaluable resource for genomic comparison with other cereals (Paterson et al. 2005; Xu et al. 2005; Cooke et al. 2007).

1.2. The salinity problem in agriculture with emphasis on rice production

Salinity in the soil is caused by the accumulation of soluble salts which mainly is comprised of by different proportions of cations, such as sodium, calcium and magnesium and anions, such as chloride and sulphate (US Salinity Laboratory Staff 1954). Saline soils are associated with soils which have an electrical conductivity (ECe) of the saturation extract of above 4 dS/m (approximately 40 mM NaCl) and an exchangeable-sodium-percentage of less than 15% (US Salinity Laboratory Staff 1954). In most studies, the plant response to salinity stress specifically refers to a soil salinity level which negatively affects the growth and yield of plants. For most crops a salt concentration in...
the soil of greater than about 40 mM NaCl, which can result in an osmotic pressure of about 0.2 MPa, is deemed to be detrimental (Munns and Tester 2008).

Salinity affects a large area of productive agricultural land. It has been estimated that 32 million ha of dryland agriculture and 45 million ha of irrigated farmland are affected by salinity (Munns and Tester 2008). Dryland salinity can be caused both by rising of water tables, which is known as seepage salinity and by accumulation of salt from rainfall and weathering in the solum soil, which is known as transient salinity (Rengasamy 2002, 2006). Irrigated salinity is mainly caused by salt introduced from poor quality irrigation water (Rengasamy 2006). Additionally, salinity is also a problem in coastal areas which are often farmed (Mensvoort et al. 1984; Gregorio et al. 2002; Ismail et al. 2007). Flooding of coastal farmland by sea water, in addition to the concentration of ions in the soil during the dry season exacerbates salt stressed conditions in these farms (Gregorio et al. 2002; Ismail et al. 2007).

Salinity is the second major abiotic stress after drought which hampers rice production (Li and Xu 2007). Rice yield can be reduced by as much as 50% by the addition of 50 mM NaCl (Yeo and Flowers 1986), making it one of the most susceptible crop species (Grattan et al. 2002; Munns and Tester 2008). The effect of salinity stress on rice varies with the plant’s developmental age. Salinity stress seriously affects plant growth at both the seedling and flowering stages, while rice appears to be more tolerant during the germination and vegetative stages (Li and Xu 2007). It has been estimated that about 48 million ha of potentially useful agricultural land in South and South East Asia are unsuitable for growing rice due to saline soils (Ponnamperuma and Bandyopadhyya 1980). In Indonesia alone, 13.2 million ha of potential agricultural land in wetland areas are affected by salinity to some degree. Due to the levels of salinity only 0.5 million ha of the 13.2 million ha of potential land are currently used for rice cultivation. This is approximately 7% of the total rice area of the country (Ponnamperuma and Bandyopadhyya 1980). A secondary problem is the loss of approximately 30,000 ha of rice farming areas in the Aceh province of Indonesia to salinity because of the effects of the tsunami on 26th December 2004 which inundated the land with seawater (McLeod et al. 2010).

### 1.3. Symptoms of salinity stress in plants

Salinity stress affects plants by two different stresses, the first being independent of the shoot Na+ accumulation (osmotic phase) and the second associated with the accumulation
of shoot Na⁺ (ionic phase) (Munns and Tester 2008; Roy et al. 2014). Osmotic stress occurs rapidly when the concentration of salt in the soil reaches the threshold level (about 40 mM), mostly resulting in a reduction of the soil water potential and causing symptoms similar to drought in plants (Munns and Tester 2008; Hasegawa 2013; Roy et al. 2014). Ionic stress, however, occurs only when the salt accumulating in the plant’s shoot reaches toxic concentrations (Munns and Tester 2008; Hasegawa 2013; Roy et al. 2014) and therefore this is not an immediate stress on a newly salt stressed plant. Both stresses lead to a reduction in shoot growth and photosynthesis rate due to the plants growing slower and senescing faster when exposed to salt (Munns and Tester 2008; Hasegawa 2013).

The main effects of osmotic stress in monocotyledonous cereals are the reduction of leaf area as well as the number of tillers, while in dicotyledonous species this water related stress causes smaller sized individual leaves and a decreasing number of branches (Munns and Tester 2008). This occurs due to the decreasing xylem water potential in the elongation zone which is lower than the water potential of peripheral cells (Fricke et al. 2006). Fricke et al. (2006) observed osmotic stress resulted in barley leaves showing a reduction in leaf elongation velocity, an increase in abscisic acid (ABA) production and a reduction in the cytokinin level in the leaf elongation zone. Additionally, the effect of osmotic stress on mature leaves results in a reduction of stomatal conductance which reduces CO₂ assimilation and therefore the rate of photosynthesis (El-Hendawy et al. 2005; James et al. 2008; Rahnama et al. 2010). This reduction of stomatal conductance is strongly correlated with increases in surface leaf temperature and has been used as a useful criterion in the selection of plants with tolerance to osmotic stress (Blum et al. 1982; Sirault et al. 2009; Munns et al. 2010).

Ionic stress results in an earlier onset of senescence of older leaves and can be observed as a yellowing of the leaf tips and margins which expands quickly to all parts of the leaf (Tester and Davenport 2003). The increased senescence is due to high levels of Na⁺ and Cl⁻ which are toxic to metabolism. Both Na⁺ and Cl⁻ have been shown to inhibit plant growth, however, the extent of the toxicity of these ions differs between species. Cl⁻ is found to be more toxic in soybean (Pantalone et al. 1997) and woody perennial plants, such as citrus (Mass 1993; Storey and Walker 1999). However, for most species, Na⁺ is more toxic than Cl⁻ (Munns and Tester 2008).

High concentrations of Na⁺ in the cytosol (above 40 mM) inhibit multiple cellular enzymes and disrupt protein synthesis (Maathuis and Amtmann 1999; Tester and
Davenport 2003). In addition, Tester and Davenport (2003) describe how a high accumulation of Na$^+$ in the shoot can cause further osmotic problems in the cell because of the high accumulation of Na$^+$ in the leaf apoplast which results in water being lost from the cell. Na$^+$ concentrations in the apoplast of rice leaves have been shown to reach as much as 600 mM Na$^+$ when the plant has been grown in 50 mM NaCl for nine days (Flowers et al. 1991).

1.4. Mechanisms of salinity tolerance

A plant’s response to salinity varies greatly both within and between species. It ranges from the very susceptible glycophytes, such as Arabidopsis and rice, to highly tolerant halophytes, such as saltbush (Figure 1-1) (Munns and Tester 2008). Tolerant plants are those which are able to maintain high growth rates and yield when grown under saline conditions, when compared to susceptible plants experiencing the same level of salinity stress (Tester and Davenport 2003). The adaptations of plants to saline environments involve complex mechanisms which are regulated at the single cell level as well as at the whole plant level (Tester and Davenport 2003). Generally, they can be categorized into three main mechanisms; osmotic tolerance, ion exclusion, and ion tissue tolerance (Munns and Tester 2008; Roy et al. 2014). The significant contribution of these three mechanisms to overall salt tolerance varies between different species and even between genotypes within a species (Munns and Tester 2008; Rajendran et al. 2009). However, it has been noticed that plants with two or more different salt tolerance mechanisms are generally more salinity tolerant than those which rely on only one mechanism (Rajendran 2012).
Figure 1-1 The range in salinity tolerance of different plant species.
The level of salinity tolerance is indicated by the ratio of shoot dry weight after exposure
in growth media containing NaCl for at least 3 weeks compared to plant growth in the
control without NaCl. The graph is reproduced from Munns and Tester (2008).

1.4.1. Osmotic stress tolerance

A reduction in plant growth is the immediate response of a plant to salinity stress due to
the osmotic effect, which is independent of the shoot ion accumulation effect (Munns and
Tester 2008; Roy et al. 2014). Clear differences between the osmotic effect and the ion
toxicity effect on plant growth have been found in barley (Munns et al. 1995), wheat
(Munns et al. 1995; Rajendran et al. 2009), maize (Cramer et al. 1994) and rice (Yeo et
al. 1991). It should be noted that once exposed to salt, the osmotic stress continues as long
as the plants are exposed to salt stress (Neumann 1997; Munns and Tester 2008;
Rajendran et al. 2009; Tilbrook and Roy 2013). Variation in osmotic stress tolerance has
been found to exist among genotypes of different species (Neumann 1997; James et al.
2008; Rajendran et al. 2009) which can potentially allow breeders to select for this trait.

While the main components of osmotic stress and osmotic stress tolerance are yet to be
elucidated, a number of adaptations to osmotic stress have been observed. Plants adapt to
osmotic stress through maintaining plant growth, detoxifying reactive oxygen species
(ROS) and controlling cellular homeostasis (Hasegawa et al. 2000; Xiong and Zhu 2002;
Munns 2005). These responses are regulated at the molecular, cellular and whole plant level and are controlled by long distance signalling pathways (Hasegawa et al. 2000; Xiong and Zhu 2002; Munns 2005).

It is suggested that plant growth maintenance and recovery under osmotic stress is controlled by hormonal and secondary messenger signals. Abscisic acid (ABA) may play a central role in the signal as it has an important function in the long distance signalling pathway from root to shoot in drought stress (Zhu 2002; Davies et al. 2005; Munns and Tester 2008). It has been argued that an ABA signal controls leaf development, generates rapid stomatal closure to increase transpiration efficiency and results in smaller and thicker leaves (James et al. 2002a) during salt stress. This is similar to symptoms observed in drought stressed plants (Munns 2005).

In addition to affecting the production of carbohydrates, the decrease in photosynthetic rate caused by osmotic stress can generate oxidative stress because of the increase in reactive oxygen species (ROS), such as hydrogen peroxide and superoxide (Xiong and Zhu 2002; Apel and Hirt 2004; Munns and Tester 2008). Detoxification of ROS can be achieved by the synthesis of antioxidant compounds (e.g. ascorbic acid, glutathione and carotenoids) and ROS scavenging enzymes (e.g. superoxide dismutase, glutathione peroxidase and catalase) (Xiong and Zhu 2002). The plant’s ability to scavenge ROS can be an important determinant in salt stress tolerance (Xiong and Zhu 2002), as shown in salt tolerant Arabidopsis mutants (Tsugane et al. 1999; Miller et al. 2007) and salt tolerant rice (Moradi and Ismail 2007), which have increased ROS scavenging abilities. However, it is also important for the plant to maintain a certain level of ROS in its tissues and cells, as ROS have significant roles in signalling of environmental stresses, such as salt stress, which a plant uses to detect and adapt to the stress (Apel and Hirt 2004; Mittler et al. 2011).

Another mechanism by which plants adapt to osmotic stress is through controlling cellular homeostasis as an osmotic adjustment to reduce cellular osmotic potential (Xiong and Zhu 2002). The osmotic adjustment can be achieved by synthesis of compatible solutes, such as proline, glycine betaine, and mannitol (Hasegawa et al. 2000; Xiong and Zhu 2002). Proline is one of the most intensively characterized osmoprotectants in response to osmotic stress in plants (Delauney and Verma 1993), and several transgenic studies have indicated the positive effects of proline in increasing osmotic tolerance in rice (Zhu
et al. 1998), Arabidopsis (Nanjo et al. 1999; Hong et al. 2000) and soybean (de Ronde et al. 2000).

1.4.2. Sodium exclusion

The ion specific component of salt stress in many monocotyledonous cereals is mainly related to the high accumulation of Na\(^+\) in the leaf blades which has been shown to be negatively correlated with plant growth, as found in durum wheat (Munns et al. 2000), rice (Zhu et al. 2001; Platten et al. 2013) and barley (Garthwaite et al. 2005). A lack of relationship between salt exclusion and salinity tolerance observed in some bread wheat genotypes (El-Hendawy et al. 2005; Genc et al. 2007), however, indicated the important role of the other salinity tolerance mechanisms such as ionic tissue tolerance. The build-up of Na\(^+\) accumulation in the shoot is primarily the result of Na\(^+\) being delivered to the shoot in the transpiration stream carried from the root through the xylem. When the water leaves the leaf blade through the stomata, Na\(^+\) is left behind and thereby builds up in the leaves (Munns 2002). Only a small portion of shoot Na\(^+\) is recirculated from the shoot to the root in the phloem (Munns and Tester 2008) thus it is important for plants to minimise the accumulation of Na\(^+\) in the shoot in the first place.

The control of Na\(^+\) transport to the shoot from the root is determined by four main mechanisms; (i) initial influx of Na\(^+\) from the soil into epidermal and cortical cells of the root, (ii) efflux of Na\(^+\) from cells in the outer root to the soil, (iii) Na\(^+\) loading into the xylem and (iv) retrieval of Na\(^+\) from the xylem before it is transported to the shoot (Tester and Davenport 2003). Figure 1-2, taken from Apse and Blumwald (2007) summarizes radial and longitudinal Na\(^+\) movement in the plant with associated ion transport, demonstrating the importance of multiple proteins which are involved in Na\(^+\) transport. Therefore, to reduce shoot Na\(^+\) accumulation, better control over Na\(^+\) loading into the root xylem is required, either by minimising the amount of Na\(^+\) entering the root or by maximising the retrieval of Na\(^+\) from the xylem in the root (Munns and Tester 2008).
Figure 1-2 Diagram of Na\textsuperscript{+} transport in plants

The diagram was modified from Apse and Blumwald (2007). The diagram illustrates radial transport and longitudinal movement of Na\textsuperscript{+} through a plant. The blue arrows indicate Na\textsuperscript{+} movement; the white arrows indicate proton (H\textsuperscript{+}) movement. The large light blue ellipse indicates vacuoles. The different coloured balls indicate different ion transporters/ ion channels: white balls are plasma membrane Na\textsuperscript{+}/H\textsuperscript{+} antiporters, light green are vacuolar Na\textsuperscript{+}/H\textsuperscript{+} antiporters, blue are cation channels, red are Na\textsuperscript{+}-K\textsuperscript{+} cotransporters and dark green are Na\textsuperscript{+} transporters. Na\textsuperscript{+} transport across the root occurs through radial symplastic transport or/and apoplast (apoplastic pathway). The mechanism of Na\textsuperscript{+} recirculation through the phloem is unclear.
1.4.2.1. Initial influx of Na\textsuperscript{+} into the root

The initial entry of Na\textsuperscript{+} from the soil into the cytosol of the roots is passive and is facilitated by both a concentration gradient and electrochemical potential (Cheeseman 1982; Tester and Davenport 2003; Apse and Blumwald 2007). Several transporters are thought to be involved in initial entry of Na\textsuperscript{+} into the roots, including non-selective cation channels (NSCC), high affinity K\textsuperscript{+} transporters (HKT) and low affinity cation transporters (LCTI) (Tester and Davenport 2003; Apse and Blumwald 2007; Plett and Møller 2010).

The passive influx of Na\textsuperscript{+} into the root cells is mainly mediated by weakly voltage-dependent non-selective cation channels (NSCC) (Davenport and Tester 2000; Demidchik and Maathuis 2007). Several candidate genes have been proposed to regulate these channels including the families of cyclic nucleotide-gated channels (CNGCs) (Leng et al. 2002; Balague et al. 2003; Gobert et al. 2006; Kaplan et al. 2007; Senadheera et al. 2009), and glutamate receptor-like channels (GLRs) (Kim et al. 2001; Davenport 2002; Demidchik et al. 2004; Roy et al. 2008).

Na\textsuperscript{+} influx into the roots might also be facilitated by some members of the high affinity K\textsuperscript{+} transporters (HKT). Phylogenetic analysis has shown the presence of two major clusters of genes in the HKT gene family (Platten et al. 2006), which are characterised depending on whether they have a glycine or serine residue at the selectivity filter of the HKT pore (Maser et al. 2002). Members of subfamily 1 have a serine at the filter and often, but not always, function as Na\textsuperscript{+} transporter, while members of subfamily 2 have a glycine and usually function as a Na\textsuperscript{+}-K\textsuperscript{+} co-transporter (Maser et al. 2002; Platten et al. 2006). Members of the HKT gene subfamily 2 such as TaHKT2;1 in wheat (Schachtman and Schroeder 1994; Rubio et al. 1995), OsHKT2;1 in rice (Garciadeblas et al. 2003; Horie et al. 2007) and HvHKT2;1 in barley (Haro et al. 2005), have been identified as being responsible for Na\textsuperscript{+} influx into the root cytosol.

The low affinity cation transporter (LCTI), initially identified in wheat, leads to elevated cation influx and hypersensitivity to Na\textsuperscript{+} when expressed in yeast (Schachtman et al. 1997; Amtmann et al. 2001) and therefore might also mediate Na\textsuperscript{+} uptake in planta. Studies on this transporter have not yet been done in the plant system (Tester and Davenport, 2003).

Another pathway for the initial entry of Na\textsuperscript{+} into the root is through the apoplast a process known as bypass flow. In general, the apoplastic pathway is restricted by the Casparian
band and suberin lamellae which are developed in exodermal and endodermal cells (Enstone et al. 2002). However, apoplastic movement has been found to be the major pathway of initial Na\(^+\) entry into rice in saline conditions which might be caused by a leakage in the endodermis (Yeo et al. 1987). A strong correlation has been shown between Na\(^+\) transport and the transport of the apoplastic tracer trisodium-8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS) from the outside of the root to the xylem in rice. Genetic variation in bypass flow has been found in rice (Yadav et al. 1996). Faiyue et al. (2010) estimated that about 30% of Na\(^+\) delivered to the shoot of rice cultivar IR36 occurs through this pathway.

1.4.2.2. Efflux of Na\(^+\) from the root to the soil

Na\(^+\) efflux from the outer part of the root back to the soil plays an important role in reducing the toxic effect of Na\(^+\) as it minimises shoot Na\(^+\) accumulation (Tester and Davenport 2003; Munns and Tester 2008; Plett and Møller 2010). Na\(^+\) efflux from the root to the soil is an active process which is energy expensive (Tester and Davenport 2003). Na\(^+\) efflux is therefore probably regulated by Na\(^+\)/H\(^+\) antiporters at the plasma membrane (Tester and Davenport 2003; Apse and Blumwald 2007). Unlike animals and microorganisms, sodium efflux through Na\(^+\) pumps energized by Na\(^+\)-ATPase have not been identified in higher plants (Horie and Schroeder 2004).

In Arabidopsis, the SOS1 (Salt Overly Sensitive 1) gene was mapped using the salt overly sensitive (sos1) mutant (Wu et al. 1996). It has been identified as a plasma membrane Na\(^+\)/H\(^+\) antiporter, which is upregulated by NaCl stress and may function to mediate Na\(^+\) efflux (Shi et al. 2000; Shi et al. 2002). AtSOS1 is expressed in the epidermal cells at the root tip and in parenchyma cells (Shi et al. 2002) and therefore may be involved in Na\(^+\) efflux from the root. In rice, OsSOS1 has been identified as the homolog of AtSOS1 and has also been shown to function as a plasma membrane Na\(^+\)/H\(^+\) exchanger when expressed in yeast (Martinez-Atienza et al. 2007). Constitutive overexpression of AtSOS1 increased the salinity tolerance of transgenic Arabidopsis and reduced Na\(^+\) accumulation in the xylem and the shoot which was probably the result of Na\(^+\) retrieval from the transpiration stream and Na\(^+\) extrusion to the soil (Shi et al. 2003).

The transport activity of SOS1 is regulated by the interaction with the other components of the SOS pathway, which are SOS2 (a serine/threonine protein kinase otherwise known as CIPK24) and SOS3 (a calcium-binding protein, CBL4) (Shi et al. 2000; Qiu et al. 2002). Reconstitution of the Arabidopsis SOS pathways in yeast cells shows that SOS3
activates and recruits SOS2 to the plasma membrane, and then SOS2 activates SOS1 through phosphorylation (Quintero et al. 2002).

There are other possible Na⁺/H⁺ antiporters which may be involved in Na⁺ efflux. The members of the cation/H⁺ exchanger (CHX) family may facilitate Na⁺ efflux (Sze et al. 2004). One of the members of the CHX family in Arabidopsis, ACHX21, has been found to regulate Na⁺ transport and as the gene was expressed in the plasma membrane of endodermal cells, it was suggested to efflux Na⁺ from the endodermis into stelar cells (Hall et al. 2006).

In addition, as previously mentioned, plants do not have Na⁺-ATPase transporters which are found in other organisms such as fungi and mosses. However, use of genetic engineering to incorporate genes encoding these transporters into plants has been successful in improving salinity tolerance. Two fungal type Na⁺-ATPases from a bryophyte, Physcomitrella patens, have been identified, namely PpENA1 and PpENA2 (Benito and Rodriguez-Navarro 2003). Expression analysis in yeast showed that PpENA1 suppressed the sensitivity of a yeast mutant to a high concentration of Na⁺ or K⁺ and was found to function as a Na⁺ pump, while expression of PpENA2 had no effect on the yeast phenotype (Benito and Rodriguez-Navarro 2003). The expression studies in Physcomitrella patens revealed that PpENA1 is up-regulated by salt stress and it has been shown that under 100 mM NaCl stress wild type plants showed a higher ratio of K⁺/Na⁺ compared to ena1 knockout plants (Lunde et al. 2007). Constitutive expression of PpENA1 in rice improved the salinity tolerance of the plant, even though the tolerance mechanism was not directly related to Na⁺ exclusion (Jacobs et al. 2011). However, the specific expression of PpENA1 in the root epidermis and cortex has the potential to increased Na⁺ efflux from the root (Plett and Möller 2010).

### 1.4.2.3. Na⁺ loading into the xylem

Minimization of Na⁺ influx into the transpiration stream of the xylem is crucial to maintain a low accumulation of Na⁺ in the shoot (Tester and Davenport 2003). The energetics of Na⁺ influx into the xylem remains uncertain, however, it is anticipated to involve active transport when Na⁺ concentrations in plant cells are low, and passive transport when cellular Na⁺ concentrations are high during salt stress (Tester and Davenport 2003). The plasma membrane Na⁺/H⁺ antiporter, SOS1, which is expressed in the epidermal cells, is also found to be expressed in parenchyma cells around the xylem of the root (Shi et al. 2002). SOS1 is suggested to have an important role in active loading
of Na\(^+\) into the xylem when *Arabidopsis* is exposed to a mild NaCl stress (Shi et al. 2002). However, when exposed to high concentrations of salinity (100 mM NaCl), SOS1 likely alters its activity limit Na\(^+\) loading into xylem, since the *sos1* mutant plant shows elevated Na\(^+\) accumulation in the shoot (Shi et al. 2002). This is in agreement with the model proposed by Lacan and Durand (1996) that Na\(^+\)/H\(^+\) antiporters act in reverse to exclude Na\(^+\) into the cytosol when Na\(^+\) is high. Otherwise it is also possible that the increased accumulation of Na\(^+\) in the shoot due to the defective function of SOS1 in Na\(^+\) extrusion in the root epidermal cells results in a greater effect than its defective function in the xylem loading (Shi et al. 2002). However, Tester and Davenport (2003) suggested that the increase of shoot Na\(^+\) under high NaCl conditions indicated a common damage symptom of plants and was unlikely to have been caused by the reverse function of a transporter.

### 1.4.2.4. Na\(^+\) retrieval from the xylem

Retrieval of Na\(^+\) from the xylem sap before it reaches the shoot is another important mechanism to reduce Na\(^+\) accumulation in the leaf blade (Tester and Davenport 2003). Many studies showed that several members of the high affinity K\(^+\) transporter (HKT) family are responsible for Na\(^+\) unloading from the xylem (Platten et al. 2006; Horie et al. 2008). These include *AtHKT1;1* in *Arabidopsis* (Sunarpi et al. 2005; Davenport et al. 2007), *OsHKT1;5* (*SKC1*) in rice (Ren et al. 2005) and *TmHKT1;5* in Einkorn wheat, *Triticum monococum* (James et al. 2006a).

*AtHKT1;1* was initially hypothesised to be a Na\(^+\) transporter, which mediates Na\(^+\) influx into roots, as *AtHKT1;1* mutants suppressed the sensitivity of the *SOS* pathway mutants (Rus et al. 2001; Rus et al. 2004). Later, *AtHKT1;1* was suggested to mediate Na\(^+\) recirculation from the shoot to the root by facilitating Na\(^+\) loading into the phloem in the shoot and unloading Na\(^+\) in the roots (Berthomieu et al. 2003). A subsequent study showed that *AtHKT1;1* was expressed in the plasma membrane of xylem parenchyma cells and mediated Na\(^+\) unloading from xylem vessels to xylem parenchyma cells in leaves (Sunarpi et al. 2005). Unidirectional influx measurements using \(^{22}\)Na\(^+\) in the *athkt1;1* mutant confirmed that *AtHKT1;1* is responsible for Na\(^+\) retrieval from the xylem in the root (Davenport et al. 2007) and is neither involved in Na\(^+\) influx (Rus et al. 2001) nor in Na\(^+\) recirculation in the phloem (Berthomieu et al. 2003). Overexpression of *AtHKT1;1* specifically in the stelar cells of *Arabidopsis* decreased Na\(^+\) transfer from the
roots to the shoots and increased salinity tolerance of transgenic *Arabidopsis* (Møller et al. 2009).

*OsHKT1;5*, a member of the HKT family in rice is also expressed in root xylem parenchyma cells and shows a similar function to *AtHKT1;1* (Ren et al. 2005). *OsHKT1;5* functions as a Na⁺ selective transporter and regulates K⁺/Na⁺ homeostasis under salt stress by unloading Na⁺ from the root xylem (Ren et al. 2005).

The two Na⁺ transporter genes from durum wheat, *Nax1* and *Nax2*, have been characterized to regulate Na⁺ retrieval from the xylem (James et al. 2006a). While *Nax2* unloads Na⁺ from the xylem only in the root, *Nax1* unloads Na⁺ from the xylem in the root and shoot resulting in a higher ratio of sheath to blade Na⁺ concentration (James et al. 2006a). The function of *Nax2* is similar to *Kna1* in bread wheat (Gorham 1990; Dubcovsky et al. 1996) and shows similarity to *OsHKT1;5* from rice and *AtHKT1;1* from *Arabidopsis* in unloading Na⁺ from the xylem in the root and maintaining a higher K⁺/Na⁺ ratio in the shoot (Byrt et al. 2007). The Na⁺ transporter *TmHKT1;5-A* is a candidate for *Nax2* and homologous to *TaHKT1;5-D*, a candidate gene for *Kna1* (Byrt et al. 2007). More recently, Munns et al. (2012) confirmed that *TmHKT1;5-A* is located in the *Nax2* locus and is responsible for the shoot exclusion in the wheat relative *Triticum monococcum*. More importantly, introgression of the *Nax2* locus into durum wheat (*Triticum turgidum*) reduced shoot Na⁺ accumulation and increased the yield under saline field conditions (Munns et al. 2012).

### 1.4.3. Tissue tolerance

Plants with tissue tolerance are able to accumulate high concentrations of Na⁺ in their shoots whilst maintaining proper cellular function (Munns and Tester 2008; Rajendran et al. 2009). High accumulation of Na⁺ in the plant tissue can be tolerated at the cellular level through intracellular compartmentalization of Na⁺ (Blumwald 2000; Tester and Davenport 2003; Munns and Tester 2008). Partitioning of Na⁺ into the vacuole is important in maintaining low concentrations of Na⁺ in the cytoplasm and reduces the effects of high levels of Na⁺ on important metabolic processes which mainly occur in the cytoplasm (Blumwald 2000; Tester and Davenport 2003; Munns and Tester 2008). Plants with good tissue tolerance are also able to use the high concentrations of Na⁺ and Cl⁻ in the vacuole to lower the osmotic potential in the leaf, which in turn helps plants to bring more water into their cells, thereby alleviating the symptoms of osmotic stress (Blumwald 2000). However, the accumulation of high concentrations of Na⁺ within the vacuole must
be accompanied by the synthesis of compatible solutes in the cytosol, such as sucrose, proline and glycine betaine, to prevent osmotic imbalance between the cytoplasm and the vacuole (Munns and Tester 2008). Synthesis of compatible solutes is energetically expensive and could potentially result in a growth penalty (Munns and Tester 2008).

The compartmentalization of Na⁺ into vacuoles is regulated by vacuolar Na⁺/H⁺ antiporters (NHX), which are facilitated by the proton gradient produced by the vacuolar H⁺-adenosine triphosphatase (ATPase) and H⁺-inorganic pyrophosphatase (PPase) (Blumwald 2000; Maeshima 2001). ATPase uses the energy from the hydrolysis of ATP, while PPase uses inorganic pyrophosphate as the source of energy (Blumwald 2000; Maeshima 2001). H⁺-ATPases and PPases pump protons into the lumen of the vacuole, generating a proton gradient. This gradient drives Na⁺ transport into the lumen via vacuolar Na⁺/H⁺ antiporters (NHX) (Blumwald 2000).

*AtNHX1* is the first member of the vacuolar Na⁺/H⁺ antiporter family which was cloned from *Arabidopsis* (Gaxiola et al. 1999). However, the extreme sensitivity of the double mutant of *atnhx1* and *atnhx2* to external K⁺ and not to Na⁺ suggested that NHX proteins are associated with the K⁺/H⁺ exchanger in the tonoplast (Bassil et al. 2011; Barragán et al. 2012). While recent evidence has emerged that NHXs may be more involved in the transport of K⁺ than Na⁺, constitutive overexpression of *AtNHX1* increased the salinity tolerance of many species, including *Arabidopsis* (Apse et al. 1999), tomato (Zhang and Blumwald 2001), *Brassica napus* (Zhang et al. 2001), maize (Yin et al. 2004) and wheat (Xue et al. 2004) by improving sequestration of Na⁺ into the vacuole. Overexpression of *OsNHX1*, a vacuolar Na⁺/H⁺ antiporter from rice, has improved salinity tolerance in rice (Fukuda et al. 2004; Chen et al. 2007) and overexpression of *AgNHX1*, a vacuolar Na⁺/H⁺ antiporter from the halophytic plant *Atriplex gmelini*, in rice resulted in strongly salt tolerant plants which could survive in 300 mM NaCl for 3 days (Ohta et al. 2002). Interestingly, Leidi et al. (2010) showed that constitutive overexpression of *AtNHX1* in tomato increased the accumulation of K⁺ in the vacuole instead of Na⁺.

The overexpression of vacuolar H⁺-pyrophosphatases (H⁺-PPase) is hypothesised to increase cation sequestration into the vacuole as the availability of protons in the vacuole increases (Gaxiola et al. 2001). Overexpression of the vacuolar H⁺-PPase *AtAVP1*, enhanced the salinity tolerance of *Arabidopsis* and increased Na⁺ accumulation in the leaves, indicating that the toxic effect of NaCl was reduced by sequestration of the cation into the vacuole (Gaxiola et al. 2001). Similarly, constitutive overexpression of its
homologues from other plant species have significantly increased the salinity tolerance in other transgenic plants, such as overexpression of vacuolar H\textsuperscript{+}-PPase from \textit{Thellungiella halophila} (TsVP) in tobacco (Gao et al. 2006) and overexpression of wheat \textit{TVP1} H\textsuperscript{+}-PPase in \textit{Arabidopsis} (Brini et al. 2007). Subsequently, many studies reported the improvement of salt tolerance by overexpression of vacuolar H\textsuperscript{+}-PPases in different species including alfalfa (Bao et al. 2009a), cotton (Pasapula et al. 2011), rice (Liu et al. 2010b; Kim et al. 2013) and barley (Schilling et al. 2013). However, there is increasing evidence for other roles of vacuolar H\textsuperscript{+}-PPases in addition to vacuolar sequestration (Ferjani et al. 2011; Gaxiola et al. 2012). Overexpression of vacuolar H\textsuperscript{+}-PPases has been shown to increase plant biomass under non-stress conditions (Pasapula et al. 2011; Kim et al. 2013) and improve phosphorus and nitrogen uptake (Yang et al. 2007; Paez-Valencia et al. 2013).

1.5. Modification of Na\textsuperscript{+} transport to increase salinity tolerance

A large number of candidate genes encoding proteins important for Na\textsuperscript{+} homeostasis have been identified and may contribute to salinity tolerance in plants (Tester and Davenport 2003; Hasegawa 2013; Roy et al. 2014). Many studies have shown that modification of Na\textsuperscript{+} transport processes by altering the expression of these genes lead to an improvement in salinity tolerance. The improvement is associated either with an increase in Na\textsuperscript{+} compartmentalization into the vacuoles (tissue tolerance) or a reduction of Na\textsuperscript{+} accumulation in the shoot (Na\textsuperscript{+} exclusion).

Most of the reports on modification of Na\textsuperscript{+} transport used constitutive promoters to express the candidate genes. For example an increase in salt tolerance has been reported using the constitutive expression of the \textit{NHXs} gene family (Apse et al. 1999; Ohta et al. 2002; Fukuda et al. 2004; Chen et al. 2007; Liu et al. 2010b) and vacuolar H\textsuperscript{+}-PPases (Gaxiola et al. 2001; Gao et al. 2006; Bao et al. 2009a; Liu et al. 2010b; Pasapula et al. 2011; Kim et al. 2013; Schilling et al. 2013). As described earlier, while the up-regulation of \textit{NHXs} and vacuolar H\textsuperscript{+}-PPases were targeted to improve Na\textsuperscript{+} sequestration into the vacuole, recent studies indicated other potential roles of \textit{NHXs} (Bassil et al. 2011; Barragán et al. 2012) and vacuolar H\textsuperscript{+}-PPases (Ferjani et al. 2011; Gaxiola et al. 2012), which might contribute to the improvement of plant performance under salt stress.

Improvements in Na\textsuperscript{+} exclusion have been reported as a result of constitutive expression of plasma membrane Na\textsuperscript{+}/H\textsuperscript{+} antiporters, again leading to an increase in salt tolerance (Gao et al. 2003; Shi et al. 2003; Zhao et al. 2006a; Yang et al. 2009). An improvement
in Na\(^+\) exclusion has also been reported by targeting the calcineurin B-like (CBL) interacting protein kinase (CIPK) gene family. The genes are involved in calcium signalling pathways in response to salt stress (Weinl and Kudla 2009). The constitutive expression of \textit{AtCIPK16} lead to reductions in shoot Na\(^+\) in both \textit{Arabidopsis} and barley and lead to the improvement of salinity tolerance of the plants (Roy et al. 2013).

However, since the transport of ions across the plant root involves a number of different cell types, each with specific functions (Tester and Leigh 2001) constitutive expression may not be the best method for expressing all the genes involved in Na\(^+\) transport. Indeed, it is hypothesised that to maximise the favourable movement of ions, it is necessary to alter the expression of certain genes encoding different ion transporters in specific cells types, as constitutive expression of the gene may be detrimental. The study by Møller et al. (2009) showed that when the Na\(^+\) transporter \textit{AtHKT1;1} is specifically overexpressed in the stelar cells, there is a significant increase in salinity tolerance of \textit{Arabidopsis} through unloading Na\(^+\) from the root xylem, thereby reducing the shoot Na\(^+\) accumulation. Importantly, they demonstrated that constitutive expression of \textit{AtHKT1;1} resulted in the increase of shoot Na\(^+\) accumulation. A similar result has been demonstrated in rice where specific expression of \textit{AtHKT1;1} in the cortical cells of rice roots reduced shoot Na\(^+\) concentration and increased salinity tolerance of rice (Plett et al. 2010). Specific expression of \textit{AtHKT1;1} in the cortex has been found to up-regulate the expression of native Na\(^+\) transporter \textit{OsHKT1;5} and increase Na\(^+\) compartmentalization into the vacuoles of cortical cells (Plett et al. 2010).

\subsection{1.6. Progress in the development of salt tolerant rice}

A large effort has been made to develop salt tolerant rice, either through conventional breeding programmes or genetic engineering. However, the progress has been relatively slow, mainly because of the complexity of the mechanism and the lack of efficient screening techniques (Khush 2001; Gregorio et al. 2002; Flowers 2004; Yamaguchi and Blumwald 2005; Mackill 2008; Ismail et al. 2010).

Intensive research has been done to explore natural variation of the salt tolerance within the rice germplasm (Akbar et al. 1972; Yeo et al. 1990; Gregorio et al. 2002; Ahmadi et al. 2011; Negrão et al. 2013; Platten et al. 2013). A number of quantitative trait loci (QTLs) have been identified associated with different physiological traits contributing to
salinity tolerance in rice and concentrate mainly on chromosome 1 (Ismail et al. 2010; Negrão et al. 2011). A major QTL on chromosome 1, SKC1, is involved in the K\(^+\)/Na\(^+\) homeostasis and corresponds to Na\(^+\) transporter OsHKT1;5 (Lin et al. 2004; Ren et al. 2005). Another major QTL involved in the regulation of the Na\(^+\)/K\(^+\) ratio, Saltol, also mapped to chromosome 1 in recombinant inbred lines (RIL) produced from a cross between IR29 and the salt tolerant Pokkali (Bonilla et al. 2002). The Saltol allele is being used to improve salinity tolerance of popular varieties using the marker assisted backcrossing approach (Ismail et al. 2010).

Improvements of salt tolerance in rice through genetic engineering have been reported in a large number of studies. The work so far is dominated by improvement in the synthesis of compatible solutes such as proline (Zhu et al. 1998; Su and Wu 2004; Kumar et al. 2010; Karthikeyan et al. 2011), glycine betaine (Sakamoto and Murata 1998; Mohanty et al. 2002; Su et al. 2006), and trehalose (Garg et al. 2002; Jang et al. 2003; Ge et al. 2008; Redillas et al. 2012). Many studies also reported the generation of salt tolerant transgenic rice by expressing different transcription factors, such as dehydration-responsive element binding (DREB) (Ito et al. 2006; Wang et al. 2008; Mallikarjuna et al. 2011; Datta et al. 2012), C-repeat/dehydration-responsive element binding factors (CBF/DREBs) (Oh et al. 2005; Oh et al. 2007) and NAC (Jeong et al. 2010).

Salt tolerant transgenic rice has also been developed by the modification of Na\(^+\) transport to increase tissue tolerance and Na\(^+\) exclusion. As described earlier, the overexpression of the vacuolar Na\(^+\)/H\(^+\) antiporter NHX1 has been shown to increase tissue tolerance in rice (Ohta et al. 2002; Fukuda et al. 2004; Chen et al. 2007). Improvement in tissue tolerance of rice by Na\(^+\) sequestration into the vacuole was also achieved through constitutive expression of a vacuolar H\(^+\)-PPase (Liu et al. 2010b; Kim et al. 2013).

Using the GAL4 enhancer trap system, Plett et al. (2010) expressed the Na\(^+\) transporter AtHKT1;1 specifically in the root epidermis and root cortex of rice and showed improvement in salt tolerance by reducing Na\(^+\) accumulation in the shoot. A reduction in shoot Na\(^+\) accumulation was also reported by Zhao et al. (2006a) by constitutively expressing the plasma membrane Na\(^+\)/H\(^+\) antiporter from yeast (Schizosaccharomyces pombe), SOD2. Interestingly, in both studies, the reduction of shoot Na\(^+\) appeared to be due to pleiotropic regulation of other genes as a result of the up-regulation of the Na\(^+\) transporter (Zhao et al. 2006a; Plett et al. 2010).
While the improvement of rice salt tolerance using genetic engineering has been shown to be promising, the majority of work so far used *japonica* rice varieties as genetic background and only little attention has been given to *indica* rice varieties. This is possibly because *indica* rice is more recalcitrant to genetic transformation compared to *japonica* rice, particularly using *Agrobacterium*-mediated transformation (Nishimura et al. 2007; Hiei and Komari 2008). Since *indica* rice has a wider geographical distribution in Asia compared to *japonica* rice (Xiong et al. 2011), it is important to extent the application of aforementioned approaches to improve the salinity tolerance of *indica* rice.

### 1.7. Research aims

There have been significant achievements in the study of mechanisms underlying salinity tolerance in plants which have enabled the modification of Na\(^+\) transport pathways either by reducing shoot Na\(^+\) accumulation or compartmentalization of Na\(^+\) in a location where it has a less toxic effect, thus increasing plant salinity tolerance. Furthermore, understanding the role and function of each Na\(^+\) transporter in a particular cell type has shown the importance of targeting the expression of the gene of interest in a cell type-specific manner. In rice, work so far, has predominately been limited to poorly adapted cultivars or has used technologies incompatible with future breeding programmes. It is therefore important to transfer the knowledge obtained from the modification of Na\(^+\) transport processes in other plants and to test the validity of this approach in commercially relevant rice cultivars, using compatible technologies for further application of the approach in the field.

The ultimate aim of this study is to develop salt tolerant commercially relevant rice cultivars by modification of Na\(^+\) transport processes. To achieve this aim, the work will first investigate the salinity tolerance of the candidate commercial relevant cultivars which will be used for the generation of salt tolerant transgenic rice. Furthermore, their efficiency in *Agrobacterium*-mediated transformation will be tested and optimized as the transformation protocol is genotype-dependent (Nishimura et al. 2007).

The availability of an efficient and reliable screening protocol is required in the development of a salt tolerant plant. Therefore, a further aim of this work is to develop a non-destructive salt tolerance screening protocol using image based phenotyping. Non-destructive salt screening will be useful to phenotype transgenic rice as it enables the
characterization of novel transgenic rice lines while allowing the maintenance of transgenic seeds for further generations.

Two approaches will be used to modify Na\(^+\) transport in rice. Firstly, the candidate genes for salt tolerance improvement will be expressed under constitutive promoters such as the cauliflower mosaic virus CaMV35S and maize Ubiquitin-1 promoters. Secondly, the alteration of Na\(^+\) transport will be targeted to specific cell types. Putative cell-type-specific promoters will be identified through this study and will be used to express the candidate genes for salt tolerance improvement in a cell type-specific manner. Transgenic rice generated in this project will be characterized to determine their salinity tolerance.


Chapter 2 : General Materials and Methods

2.1. Rice cultivars

Six rice cultivars were used in this study which consisted of five commercial indica rice cultivars from Indonesia and the japonica cultivar Nipponbare. The indica rice cultivars selected from Indonesia were Fatmawati, IR64, Inpara 5 (IR64-Sub1), Ciherang and Dendang.

Fadmawati is a new plant type of rice (NPT) which has high yield potential (Abdullah et al. 2008). IR64 is a popular rice variety in South Asia and South East Asia. IR64 is also the largest variety grown in Indonesia (Mackill 2008; Brennan and Malabayas 2011). Inpara 5 (IR64-Sub1) is a submergence tolerant rice variety with IR64 as the genetic background (Septiningsih et al. 2009). Ciherang is the second largest variety grown in Indonesia (Brennan and Malabayas 2011). Dendang is an improved variety for tidal swamp areas and has a moderate level of salt tolerance (Suprihatno et al. 2010). The seeds of Indonesian rice cultivars were provided by the Indonesian Centre for Rice Research, Sukamandi, Indonesia. Descriptions of some important traits of Indonesian rice cultivars are presented in Table 2-1.

Nipponbare is a model japonica rice cultivar and has been used by the International Rice Genome Sequencing Project (http://rgp.dna.affrc.go.jp/IRGSP/index.html). Nipponbare has been used as a standard rice cultivar in a variety of abiotic and biotic stress studies and was used in this study for comparison. Nipponbare seed was provided by the Yanco Agricultural Institute, New South Wales Department of Primary Industries (Yanco, Australia).
Table 2-1 Major agronomic traits of the five Indonesian commercial rice cultivars used in this study (Suprihatno et al. 2010)

<table>
<thead>
<tr>
<th>Traits</th>
<th>Fatmawati</th>
<th>IR64</th>
<th>Inpara 5 (IR64-Sub1)</th>
<th>Cierang</th>
<th>Dendang</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maturity (days)</td>
<td>105-115</td>
<td>110-120</td>
<td>115</td>
<td>116-125</td>
<td>123-127</td>
</tr>
<tr>
<td>Plant height (cm)</td>
<td>95-110</td>
<td>115-126</td>
<td>92</td>
<td>107-115</td>
<td>90-100</td>
</tr>
<tr>
<td>Number of productive tiller</td>
<td>8-14</td>
<td>20-35</td>
<td>18</td>
<td>14-17</td>
<td>15-20</td>
</tr>
<tr>
<td>Number of grains per panicle</td>
<td>200-400</td>
<td>NA</td>
<td>102</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1000 grain weight (g)</td>
<td>29</td>
<td>24.1</td>
<td>25</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>Amylose content (%)</td>
<td>23</td>
<td>23</td>
<td>25</td>
<td>23</td>
<td>19.5</td>
</tr>
<tr>
<td>Cooked texture</td>
<td>Soft</td>
<td>Soft</td>
<td>Soft</td>
<td>Soft</td>
<td>Soft</td>
</tr>
<tr>
<td>Yield potential (t/ha)</td>
<td>9.0</td>
<td>6.0</td>
<td>7.2</td>
<td>8.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Yield average (t/ha)</td>
<td>6.0</td>
<td>5.0</td>
<td>4.45</td>
<td>6</td>
<td>4.0</td>
</tr>
<tr>
<td>Pest and disease resistance</td>
<td>NA</td>
<td>NA</td>
<td>Resistant to BPH biotype 1, 2 and moderately resistant to biotype 3. Resistant to bacterial leaf blight (BLB) strain III and moderately tolerant to strain IV.</td>
<td>Resistant to BPH biotype 2 and moderately tolerant to biotype 3. Resistant to BLB strain IV and VIII.</td>
<td>Moderately resistant to BPH biotype 1, 2. Moderately resistant to blast disease.</td>
</tr>
<tr>
<td>Abiotic stress tolerance</td>
<td>NA</td>
<td>NA</td>
<td>Tolerant to complete submergence (up to 14 d) at vegetative stage.</td>
<td>NA</td>
<td>Moderately tolerant to iron toxicity and salinity. Tolerant to aluminium toxicity.</td>
</tr>
<tr>
<td>Target area</td>
<td>Irrigated lowland and medium area.</td>
<td>Irrigated lowland and medium area.</td>
<td>Irrigated lowland, shallow area, and flood prone area.</td>
<td>Irrigated lowland and medium area.</td>
<td>Peat land and acid sulfate area.</td>
</tr>
</tbody>
</table>

NA= no information available
2.2. Rice growth conditions

2.2.1. Growth facilities

Experiments were conducted in both the greenhouse and growth chambers of The Plant Accelerator® (TPA), the University of Adelaide, Waite Campus. The conditions for rice growth were 12 h light/dark, temperatures of 28°C/26°C (day/night), and relative humidity of 70%. During the short day period of winter (June-August) supplementary light was supplied to greenhouse grown plants. Light intensity in the growth chamber was set to a maximum photon irradiance of 700 µmol·m⁻²·s⁻¹. These conditions were designed to emulate rice growing conditions in tropical areas.

2.2.2. Soil mix

University of California (U.C.) soil mix (Matkin and Chandler 1957) was used in all soil based experiments. The soil mix contained sand and peat moss (volume ratio 1.6:1) and fertiliser (1.5 kg Mini Osmocote® per 600 LUC soil base). The pH is adjusted to 6 – 6.5 with a mix of calcium hydroxide and calcium carbonate. The soil mix was supplied by the South Australian Research and Development Institute (SARDI), Waite Campus.

Two types of plastic pots were used for growing rice in soil - white round plastic pots (D150 mm × H190 mm) and green round plastic pots (D140 mm × H140 mm), both with drainage holes in their bases. White plastic pots were used in all soil based salinity tolerance experiments. The pots were placed in deep white plastic saucers (L160 mm × W160 mm × H90 mm) which allowed the application of water and salt from below. Green plastic pots were used during the generation of transgenic rice plants and during seed multiplication. Six green pots were placed in a black watering tray (L500 mm × W400 mm × H90 mm) and water was applied to the plants from the bottom.

2.2.3. Seed treatment and germination

Rice seeds were sorted for uniform size and weight before being dehusked. Surface sterilisation was carried out with a 70% (v/v) ethanol treatment for 1 min followed by a 30 min bleach treatment (30% (v/v) Domestos, sodium hypochlorite 49.9 g/L). The seeds were then rinsed five times with RO water to remove all traces of sodium hypochlorite. Surface sterilised seeds were germinated on sterile wet Whatman™ filter paper (90 mm Ø) (Cat. No. 1001090, GE Healthcare, Buckinghamshire, UK) in round plastic dishes (100 mm x 20 mm) (Cat. No. 430167, Corning®, Corning, NY, USA). The dishes were
sealed using plastic wrap to prevent evaporation and contamination. Seeds were germinated in a growth cabinet (Model MLR-351, Sanyo Electric Co. Ltd) with 12 h light and a constant temperature of 28°C. Uniformly germinated seedlings were transplanted into either soil or hydroponic media after 7 d.

2.2.4. Hydroponic systems

2.2.4.1. Mini hydroponic with MS Media
Rice was grown in ¼ × MS basal salt mixture (Evrard et al. 2012) in a 2 L plastic container (L350 mm x W140 mm x H120 mm). The pH was adjusted to pH 5.7 using 1 M KOH. Rice seedlings were placed on a meshed rack inside the plastic container (Figure 2-1). The nutrient solution was renewed every 7 d. This hydroponic system was used for short-term rice experiments (up to 14 d after germination).

2.2.4.2. Mini hydroponic with ACPFG rice nutrition solution
A 12 L blue plastic container was used in the hydroponics system for the salinity tolerance assays. Rice seedlings were transplanted into 1.5 mL microfuge tubes which had their lids removed and bottom 6 mm of the tubes removed to allow for growth of the roots from the tube into the nutrient solution. The tubes were placed into holes in a white lid suspended above the hydroponic solution so their roots touched the liquid. Each lid container could contain 60 to 96 plants (Figure 2-1). The containers were filled with 11 L nutrient solution (5.0 mM NH₄NO₃, 5.0 mM KNO₃, 2.0 mM Ca(NO₃)₂.4H₂O, 2.0 mM MgSO₄.7H₂O, 0.1 mM KH₂PO₄, 0.05 mM NaFe(III)EDTA, 5.0 µM MnCl₂.4H₂O, 5.0 µM ZnSO₄.7H₂O, 0.5 µM CuSO₄.5H₂O and 0.1 µM Na₂MoO₃). The nutrient solution was modified from Cotsaftis et al. (2011) by removing boron (H₃BO₃) as the boron concentration in the Waite Campus RO water was sufficient for rice growth (6 - 10 µM) (unpublished data, Julie Hayes, ACPFG). The pH was adjusted daily to pH 5.5 using 1 M KOH. The nutrient solution was replaced 7 d after transplantation and then renewed every 3-4 d until experiments were completed. RO water was added regularly to the container to maintain the volume of nutrient solution. The system described allowed rice plants to grow for up to 30 d after germination.
2.3. Destructive measurements of plant biomass and tissue ion concentration

Destructive sampling was performed to determine final plant biomass and to compare classical measures of biomass with data obtained via image analysis. Plant tissue was separated into the youngest fully expanded leaf taken from the main tiller, total shoots and total roots. Root samples were taken from hydroponically grown plants. Roots were rinsed with 10 mM CaCl$_2$ to remove residual NaCl and other nutrients sticking to the root’s surface before being blotted dry in paper towels. Samples were weighed immediately to determine the fresh weight. Dry weights of the tissues were measured after samples were dried in oven at 70°C for a minimum of 24 h.

Tissue Na$^+$ and K$^+$ concentrations were measured in the youngest fully expanded leaf taken from the main tiller and in total roots. Oven dried tissue samples were placed in 50 mL Falcon tubes and digested in 20 mL 1% (v/v) nitric acid (HNO$_3$) for 5 h in a Hot Block (Model SC 154, Environmental Express, Mt Pleasant, SC, USA) at 70°C. The samples were shaken approximately every 30 min to ensure complete digestion. The concentrations of Na$^+$ and K$^+$ were determined using a flame photometer (Model 420; Sherwood Scientific Ltd., Cambridge, UK). Tissue ion concentrations were calculated based on the tissue water content.

![Image of hydroponic systems](image1.png)

**Figure 2-1 Two types of mini hydroponic systems for rice experiments.** The hydroponic system on the left was filled with ¼×MS media and was used for seedlings grown up to 14 d. The system on the right was filled with ACPFG nutrient solution and was used for seedlings grown up to 30 d.
2.4. Molecular Analysis

2.4.1. Genomic DNA extraction

2.4.1.1. Simple genomic DNA extraction

The genomic DNA (gDNA) extraction method described by Edwards et al. (1991) was used to extract rice leaf DNA for routine PCR. Fresh rice leaves were sampled, placed in 2 mL microfuge tubes and then frozen in liquid nitrogen. Frozen leaf tissue was ground using ball bearings with vigorous vortexing. After the ball bearings were removed from the tubes, the samples were mixed with 400 µL extraction buffer (200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS, pH 8) to disrupt cell membranes. The mixture was vortexed for 5 sec and left at room temperature for 1 h. The samples were then centrifuged at 16,100 g for 1 min to separate cell debris. Subsequently, 300 µL of the supernatant was transferred into fresh 1.5 mL microfuge tubes and mixed with 300 µL 100% (v/v) isopropanol to precipitate the DNA. The samples were left at room temperature for 1 h before being centrifuged at 16,100 g for 5 min to pellet the DNA. The DNA pellets were dried by discarding the supernatant and inverting the tubes on paper towels for about 5 min or until dry. The DNA pellets were then dissolved in 50 µL 1×TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C overnight and stored at -20°C until further use.

2.4.1.2. Genomic DNA extraction using phenol-chloroform

High quality gDNA for Southern Blot analysis was extracted using the phenol-chloroform method (Rogowsky et al. 1991; Pallotta et al. 2000). Approximately 200 mg of rice tissue was collected and powdered in a 2 mL microfuge tube using a vortex and ball bearings (Section 2.4.1.1). To extract DNA, 600 µL of extraction buffer (1% (v/v) sarkosyl, 100 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 8.5) were added and the tissue was macerated using a plastic micro pestle. Afterwards, 600 µL of phenol/chloroform/isooamy alcohol (25:24:1) was added and mixed thoroughly by shaking for 5 sec to remove protein in the cell extract. The samples were placed on an orbital mixer for at least 10 min before centrifugation at 16,100 g for 10 min. After that, approximately 550 µL of the upper aqueous phase was carefully transferred into a new 1.5 mL microfuge tube, avoiding the transfer of the white inter phase layer. The phenol extraction step was repeated twice. The samples were gently mixed with 60 µL Na-acetate (3 M, pH 4.8) and 600 µL isopropanol and left at room temperature for 2 min to precipitate the DNA. The
samples were centrifuged at 16,100 g for 2 min to pellet the DNA. The supernatants were removed before adding 1 mL of 70% ethanol to the pellet. The samples were centrifuged for 2 min and the supernatants were discarded. The DNA pellets were dried and finally resuspended in 30 µL of R40 (1 × TE pH 8.0, 40 µg·mL⁻¹ RNase A) at 4°C overnight.

2.4.2. Primer design

Primers were designed for genotyping, promoter cloning and sequencing. Primers were developed using the computer software Vector NTI Advance™ 11.00 (Invitrogen, Mulgrave, VIC, Australia). The primers were verified using Primer BLAST (NCBI) to determine the match with the reference genome. Primers that were used to clone previously published promoters were designed according to the specifications in the original publications.

For amplification of DNA regions of putative cell type-specific promoters, sequences of restriction sites –PmeI and Ascl were incorporated into the 5’ end of the primers to facilitate cloning into the desired destination vectors (Section 2.4.12.3). The sequence CACC was also added to the 5’end of the forward primer to enable directional cloning when using pENTR™/D-TOPO® (Section 2.4.12.1). All primers were synthesized by Geneworks (Adelaide, SA, Australia). The details of the primers used in each experiment are described in the relevant chapter.

2.4.3. Polymerase chain reaction (PCR)

2.4.3.1. General PCR

Platinum®Taq DNA polymerase (Cat. No. 10966, Invitrogen, Mulgrave, VIC, Australia) and GOTaq® Green Master Mix (Cat. No. M7122, Promega, Madison, WI, USA) were used for general PCR. PCRs were performed following the manufacturer’s protocol.

The solution for PCR using Platinum®Taq DNA polymerase was performed in a 25 µL mix containing 1 × PCR buffer, 0.2 mM dNTP mixture, 1.5 mM MgCl₂, 0.2 µM forward primer, 0.2 µM reverse primer, 0.2 µL (5U/µL) Platinum®Taq DNA polymerase, 1 µL template DNA (50-100 ng) and water was added to a final volume of 25µL.

The solution for PCR using GOTaq® Green Master Mix was performed in 25 µL mix containing 1 × GoTaq® Green Master Mix (with 0.2 mM dNTP mixture and 1.5 mM MgCl₂), 0.2 µM forward primer, 0.2 µM reverse primer, 1 µL DNA (50-100 ng) and water to a final volume of 25 µL.
Thermal cycles were carried out in a DNA Engine Tetrad® 2 (Bio-Rad). PCR cycles were started with an initial denaturation at 94°C for 2 min, followed by 30 to 35 cycles of denaturation (94°C for 30 sec), annealing (50-60°C, depending on primer pair, for 30 sec) and extension (72°C for 60 sec per kb of PCR product). PCRs were completed with a final extension at 72°C for 10 min.

### 2.4.3.2. Proofreading PCR

Platinum®Pfx™ DNA polymerase (Cat. No. 11708-013, Invitrogen) was used in PCRs to amplify the promoter regions. PCRs were set up with a 50 µL mix containing 1 × FailSafe™ PCR PreMix G buffer (Cat. No. FSP995G, Epicentre, Madison, WI, USA), 0.6 µM forward primer, 0.6 µM reverse primer, 1 µL (2.5 U/µL) Pfx50™ DNA polymerase and 1 µL genomic DNA (50-100 ng). An initial denaturation of DNA was performed at 94°C for 2 min before 35 cycles of DNA denaturation (94°C for 15 sec), primer annealing (60-68°C, depending on primer pair, for 30 sec) and DNA extension (68°C for 60 sec per kb of PCR product). A final extension at 68°C for 30 min was performed to complete any truncated strands and to add single 3’ A overhangs in all PCR products to facilitate the cloning into entry vectors (Section 2.4.12.1).

### 2.4.4. Gel electrophoresis

DNA products from PCR and restriction digestions were separated by agarose gel electrophoresis. The gels were prepared using 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA), 1% (w/v) agarose (Cat. No. BIO-41026, Bioline, Alexandria, NSW, Australia) and either 0.5 µg·mL⁻¹ ethidium bromide or 0.05 µl·mL⁻¹ SYBR-safe (Cat. No. S33102, Invitrogen, Mulgrave, VIC, Australia) for DNA staining. A 10 × sucrose loading buffer (0.25% (w/v) bromophenol blue dye (Cat. No. B8026-5G, Sigma Aldrich, St. Louis, MO, USA, 0.25% (w/v) xylene cyanol, 40% (w/v) sucrose, dissolved in sterile Mili-Q water) was added to each PCR product before loading onto the gel. DNA marker ladders, either HyperLadder™ 1 kb (Cat. No. BIO-33025, Bioline, Alexandria, NSW, Australia) or HyperLadder™ 100 bp (Cat. No. BIO-33029, Bioline, Alexandria, NSW, Australia) were loaded on the gel to determine DNA size and estimate DNA quantities. Electrophoresis was run at 90-120 volts to separate the DNA. DNA was visualised under a UV transilluminator using a GeneFlash gel documentation system (Syngene, Cambridge, UK).
2.4.5. DNA extraction from agarose gel

DNA fragments of correct size were excised from agarose gels with a scalpel under low UV light exposure and extracted using Isolate PCR and Gel Kit (Cat. No. BIO-52030, Bioline) following the protocol provided by the manufacturer. In brief, the gel slice containing the target PCR product was completely dissolved in 650 µL Gel Solubilizer by heating the sample in a 50°C water bath. The sample was then mixed with 50 µL Binding Optimizer before being transferred to a filter column (Spin Column A) placed in a 2 mL collection tube and centrifuged at 10,000 g for 1 min to bind the DNA onto the column membrane. The filtrate in the Collection tube was discarded. Wash Buffer A (750 µL) was added to the filter column and centrifuged at 10,000 g for 1 min to remove salts. The washing step was repeated to remove all traces of ethanol. Elution buffer (30-50 µL) was then added directly to the filter column membrane and incubated for 1 min at room temperature. The filter column was centrifuged at 10,000 g for 1 min to elute the DNA.

2.4.6. DNA Sequencing

DNA preparation and labelling was performed using the BigDye® Terminator (BDT) v3.1 Cycle Sequencing Kit (Cat. No. 433745, Applied Biosystem, Mulgrave, VIC, Australia). Reactions were performed in a 20 µL mix containing 1 µL template DNA (100 ng), 1 µL primer (20 µM), 2 µL BDT v3.1, 3 µL BDT buffer and nuclease free water to volume. Thermal cycles were performed with the following condition: 96°C for 2 min followed by 30 cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min. Excessive BigDye® Terminator was then removed from the reaction using MgSO₄. The reaction clean-up was performed in a 1.5 mL microfuge tube by adding 75 µL of fresh 0.2 mM MgSO₄ (in 70% (v/v) ethanol) to the sequencing reaction. The reaction was mixed thoroughly by vortexing and incubated at room temperature for a minimum of 15 min to precipitate the DNA. Samples were then centrifuged for 15 min at 16,100 g. The supernatant was removed by pipetting, and samples washed with 70% (v/v) ethanol. Samples were centrifuged for another 15 min at 16,100 g and ethanol was removed from the samples by pipetting or decanting. The samples were air dried at 37°C in an oven or a heat block. Dried samples were submitted to the Australian Genome Research Facility (AGRF) for capillary separation (Waite Campus, Urrbrae, South Australia). DNA sequence analysis and DNA alignment were performed using Vector NTI Advance™ 11.0 (Invitrogen, Mulgrave, VIC, Australia).
2.4.7. Restriction enzyme digestion

Restriction enzyme digestions were performed to: 1) digest genomic DNA for Southern blot analysis (Section 2.4.8), 2) identify plasmids with the correct orientation (Section 2.4.13), and 3) facilitate cloning (Section 2.4.12.3). Restriction enzyme digestions were performed in 20 µl reactions containing 1 × supplied reaction buffer, 1-3 µg DNA, 2 µL BSA (10mg·mL⁻¹) (if required) and 5-10 units restriction enzyme (New England Biolabs®). Reactions were incubated at the recommended temperature for optimum activity (mostly 37°C) for 3-4 h and then heat inactivated at 65°C for 20 min.

2.4.8. Southern blot

Southern blot hybridisation was performed to determine the copy number of the transgene insertion in transgenic rice plants following the protocol described by Sambrook et al. (1989). In brief, 1-3 µg of rice genomic DNA isolated using the phenol-chloroform method (Section 2.4.1.2) was digested using relevant restriction enzymes as described in Section 2.4.7. UltraPure™ Agarose (Cat. No. 16500500, Invitrogen) gels (1% (w/v) agarose, 1 × TAE buffer) were used to separate DNA fragments by electrophoresis in a constant voltage of 35 V overnight. DNA fragments were transferred from the agarose gel to a Hybond N⁺ nylon membrane (Biodyne B, Pall Corporation) using capillary force with 0.4 M NaOH as the transfer buffer. The DNA transfer was set up as depicted in Figure 2-2. After 5 h of transfer, the membrane was rinsed in 2 × SSC (diluted from stock solution 20 × SSC (3 M NaCl, 0.3 M tris-sodium citrate)) and blotted on a Whatman™ filter paper. The PCR-amplified product of the Nos terminator was radiolabelled with [α-\textsuperscript{32}P]dCTP and used as probe for hybridization with the membrane. Excessive, unbound probes were washed off the membrane with 0.2 × SSC, 0.1% SDS at 65°C for 20 min. The membrane was placed in an autoradiography cassette with Fuji medical X-ray film (Fujifilm) and stored at -80°C for 7 d to increase the film sensitivity. The film was developed using a CP1000 automatic film processor (AGFA). Assistance in probe labelling, hybridization and autoradiography were provided by Ms. Margaret Pallotta at ACPFG.
The plastic screen had an opening the size of the gel to ensure transfer buffer would only flow through the gel and not directly from the reservoir into the membrane and paper towels.

2.4.9. RNA extraction

Total RNA from rice tissue was extracted using the TRIZOL® based extraction method (Chomczynski and Sacchi 1987; Simms et al. 1993). TRIZOL® reagent was replaced by TRIZOL-like reagent (38% (v/v) phenol pH 4.3 (Cat. No. P4682, Sigma Aldrich, St. Louis, MO, USA), 12% (w/v) guanidine thiocyanate (Cat. No. G9277, Sigma Aldrich, St. Louis, MO, USA), 7% (w/v) ammonium thiocyanate (Cat. No. 221988, Sigma Aldrich, St. Louis, MO, USA), 3% (w/v) 3 M sodium acetate pH 5.0, 5% (v/v) glycerol). About 50 to 100 mg of frozen and ground rice tissue was placed in 2 mL microfuge tubes (Section 2.4.1.1). Plant material was lysed by adding 1 mL TRIZOL-like reagent. Samples were thoroughly mixed and then incubated at room temperature for 5 min. A 200 µL volume of chloroform was added to the samples which were vigorously shaken to denature nucleoprotein complexes. Samples were incubated at room temperature for 3 min and then centrifuged at 12,100 g for 15 min at 4°C. After centrifugation, the colourless upper aqueous phase containing RNA was transferred into fresh 1.5 mL tubes. A 500 µL volume of isopropanol was added to the aqueous phase and incubated for 10 min at room temperature to precipitate the RNA. Samples were centrifuged at 12,100 g for 10 min at 4°C to pellet RNA. Supernatants were removed and the RNA pellets were washed by adding 1 mL of 75% (v/v) ethanol. Samples were mixed and centrifuged at 7,500 g for 5 min at 4°C. Ethanol was removed with a pipette and the RNA pellets were air dried in a fume hood for about 10 min. The RNA was resuspended in 25 µL of RNase-free water or sterile Mili-Q water. Contaminating DNA in the RNA samples was removed by using the DNA free kit (Cat. No. AM1906, Ambion, Carlsbad, CA, USA) following
the manufacturer’s instructions. In brief, 2.5 µL 10 × DNase I buffer and 1 µL (2 units) DNase I were added to the RNA samples. Samples were then mixed gently and incubated at 37°C for 30 min. A 5 µL volume of resuspended DNase inactivation reagent was added to each sample and mixed. Samples were incubated at room temperature for 2 min and mixed 2-3 times during the incubation. Samples were then centrifuged at 10,000 g for 1 min to fraction the suspension. The clear aqueous phase containing the RNA was transferred to fresh tubes and stored at -80°C. The RNA concentration was measured using a Nano-drop (ND-1000 UV/Vis, NanoDrop Technologies Inc., Wilmington, DE, USA) and the RNA quality was analysed by gel electrophoresis (Section 2.4.4).

2.4.10. Complementary DNA (cDNA) synthesis

First strand cDNA was synthesised using SuperScript™ III reverse transcriptase (Cat. No. 18080, Invitrogen, Mulgrave, VIC, Australia) following the protocol provided by the manufacturer. The cDNA synthesis was performed in a 20 µL volume using PCR tubes. Firstly, 1 µL of oligo (dT)18 (50 µM), 1 µL of 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP (Cat. No. BIO-39026, Bioline, Alexandria, NSW, Australia), 1 µg of total RNA and sterile water to a final volume of 13 µL were mixed by pipetting. The mixture was then incubated for 5 min at 65°C to remove secondary RNA structures and placed immediately on ice for at least 1 min. Afterwards, the samples were briefly spun before adding 4 µL of 5 × first-strand buffer, 1 µL 0.1 M DTT, 1 µL RNaseOUT Recombinant RNase Inhibitor (Cat. No. 10777-019, Invitrogen) and 1 µL SuperScript™ III RT (50 units). The solutions were mixed gently using a pipette and incubated at 50°C for 1 h. The samples were then heated at 70°C for 15 min to terminate the reaction.

2.4.11. Reverse Transcription (RT) PCR

RT PCR was performed to analyse the expression of transgenes and endogenous control genes. The RT PCR was set up in the same manner as the general PCR (Section 2.4.3.1.) using cDNA (Section 2.4.10) as a template.
2.4.12. Gateway® cloning

2.4.12.1. Generation of Gateway® entry vectors containing putative cell type-specific promoters

The putative cell type-specific promoter regions were amplified using proof reading PCR as described in Section 2.4.3.2 and primers listed in Table 6-2. DNA fragments of the amplified promoter regions of correct size were extracted from agarose gels as described in Section 2.4.5. Gel purified PCR products of the putative cell type-specific promoters were cloned into Gateway® entry vectors via TOPO® cloning. Two Gateway® entry vectors were used, pENTR™/Directional-TOPO® (Cat. No. K2400-20, Invitrogen) (Figure 2-3) and pCR®8/GW/TOPO® (Cat. No. K2500-20, Invitrogen) (Figure 2-4). TOPO® cloning reactions were performed following the manufacturer’s protocols. Each reaction contained 1-4 µL PCR product, 1 µL salt solution, 1 µL TOPO® vector and RO water to a final volume of 6 µL. After 5 min incubation at room temperature, 2 µL of the reaction mix was transformed into One Shot® TOP10 E. coli (Invitrogen, included in the kit) following the transformation method described in Section 2.4.13. Kanamycin (100 µg·mL⁻¹) was used as antibiotic to select for E.coli colonies containing the correct plasmid generated from pENTR™/D-TOPO® cloning. Spectinomycin (100 µg·mL⁻¹) was used as antibiotic to select positive colonies containing plasmids generated from pCR®8/GW/TOPO® cloning.

2.4.12.2. DNA Ligation

DNA ligation was used to insert DNA sequences of putative cell type specific promoters into the backbone of Gateway® enabled destination vectors (Section 2.4.12.3). DNA ligations were performed using T4 DNA Ligase (Cat. No. 15224, Invitrogen, Mulgrave, VIC, Australia). Each reaction contained 5 × ligase reaction buffer, 1:3 molecular ratio of vector DNA and insert DNA, and 1 unit T4 DNA Ligase. The reaction was incubated at room temperature for 30 min before being transformed into E. coli for multiplication (Section2.4.13).

2.4.12.3. Generation of Gateway® destination vectors containing putative cell type-specific promoters

To enable the utilization of putative cell type specific promoter in Gateway® cloning, compatible destination vectors were developed carrying putative cell type specific promoters in between Gateway® recombination sites. The destination vector pMDC32
Curtis and Grossniklaus 2003) was used as the backbone of all destination vectors with cell type specific promoters (Figure 2-5). The vector was modified by removing the 2 × 35S CaMV promoter 5' of the CMr gene by performing a double digest with the restriction enzymes PmeI and Ascl (Section 2.4.7). Linearised pMDC32 fragment was separated from the 2 × 35S CaMV promoter fragment by gel electrophoresis (Section 2.4.4). The linear backbone plasmid pMDC32 was isolated from the gel as described in Section 2.4.5.

DNA fragments of putative cell type specific promoters were cut from the relevant entry vector (Section 2.4.12.1) by a double digest with the restriction enzymes PmeI and Ascl (Section 2.4.7). The fragments were separated from entry vector backbone by gel electrophoresis (Section 2.4.4) and isolated from the gel (Section 2.4.5).

DNA fragment of putative cell type specific promoters were ligated to the linearised pMDC32 backbone using T4 DNA Ligase (Section 2.4.12.2). The plasmid product of ligation was transformed into E. coli DB3.1™ which is resistant to ccdB for propagation (Section 2.4.13).

2.4.12.4. LR cloning

LR cloning was used to transfer DNA fragments of promoters or genes of interest from Gateway® entry vectors into destination vectors. LR recombination reactions were performed by adding 1 µL of LR Clonase II enzyme mix (Cat. No. 11791, Invitrogen) to 8 µL of reaction mix, containing 1-7 µL entry clone (50-150 ng/µL), 1 µL destination vector (150 ng/µL) and TE buffer pH 8.0 (to a final volume of 8 µL). The samples were incubated at 25°C for 1 h. Proteinase K solution (1 µL) was added to the samples and incubated at 37°C for 10 min to degrade LR Clonase II and terminate the reaction. A 4 µL sub sample was used for transformation into One Shot® TOP10 E. coli (Section 2.4.13).
Figure 2-3 Map of Gateway® entry vector pENTR\textsuperscript{TM}/D-TOPO® (2580bp).
The vector contains the following components: transcription termination sequences\((rrnBT1\text{ and } T2)\), M13 forward and reverse primer sites for sequencing the insert, Gateway® recombination site\((attL1\text{ and } attL2)\), directional TOPO® cloning site, T7 promoter/ primer site for in vitro transcription and sequencing the insert, kanamycin resistance gene for the selection of the plasmid in \textit{E.coli} (Kan\textsuperscript{R}) and pUC origin of replication (pUC origin) for high copy replication and maintenance in \textit{E. coli}.

Figure 2-4 Map of Gateway® entry vector PCR8/GW/TOPO (2817 bp).
The vector contains the following components: transcription termination sequences\((rrnBT1\text{ and } T2)\), M13 forward and reverse primer sites for sequencing the insert, Gateway® recombination site\((attL1\text{ and } attL2)\), GW1 and GW2 primer sites for sequencing of the insert, TOPO® cloning site for rapid cloning of \textit{Taq}-amplified PCR product, spectinomycin (Spn) promoter to express spectinomycin resistance gene, spectinomycin resistance gene (SpnR) for the selection of the plasmid in \textit{E.coli} and pUC origin of replication (pUC origin) for high copy replication and maintenance of \textit{E. coli}. 
Figure 2-5 Map of Gateway® destination vector pMDC32.
The vector backbone contains the following components: kanamycin resistance gene, origin of replication for *E. coli* (pBR322 origin), and minimal replicon (pVS1) for stable maintenance in *E. coli*. T-DNA cassette contains right border sequence (RB), dual cauliflower mosaic virus 35S promoter (2×35S), Gateway® recombination sequences (attR1 and attR2), chloramphenicol resistance gene (CMr), *ccdB* gene for negative selection of the plasmid, nopaline synthase (*Nos*) terminator, cauliflower mosaic virus 35S promoter (*CaMV35S*), hygromycin resistance gene, cauliflower mosaic virus 35S terminator (*A35S*) and left border sequence (LB).
2.4.13. Plasmid DNA transformation into chemically competent *Escherichia coli* cells

Two types of chemically competent *E. coli* cells were used in experiments. One Shot® TOP10 *E. coli* (Cat. No. K2500-20, Invitrogen, Mulgrave, VIC, Australia) was used for transformation of Gateway® entry vectors and expression vectors resulting from LR recombination. Chemically competent *E. coli* cells DB3.1™ were used for transformation of plasmids containing the *ccdB* gene, such as Gateway® destination vector plasmids. *E. coli* strain DB3.1 is resistant to the *ccdB* gene, which is a lethal gene in other *E. coli* strains (Bernard and Couturier 1992).

Transformations were performed using the heat shock method. In brief, 2 to 4 µL of the reaction mix of cloning product were mixed gently into chemically competent *E. coli* and incubated on ice for 30 min. The cells were incubated at 42°C for 30 sec, to allow entry of the plasmid, and immediately transferred to ice for 5 min. A 250 µL volume of liquid Luria Betani (LB) media (tryptone 10 g·L⁻¹, yeast extract 5 g·L⁻¹, NaCl 5 g·L⁻¹, pH 7.5) was then added and the cells incubated at 37°C for 1 h with shaking. A 50 µL volume of bacterial culture was spread onto LB agar plates containing the correct antibiotic for positive selection. The plates were incubated at 37°C overnight. Positive colonies which were resistant to the antibiotic in the LB agar were selected and cultured in liquid LB media with the correct antibiotic at 37°C overnight. The integration of the correct plasmid DNA was analysed using PCR with 1 µL liquid cell culture as DNA template (Section 2.4.3.1). Positive bulked up cultures were then used for plasmid DNA isolation (Section 2.4.14). The correct orientation of the plasmid was confirmed by restriction digestion (Section 2.4.7) using isolated DNA plasmid (Section 2.4.14). For long term storage, 1 µL of bacterial cultures were mixed with 1 µL glycerol and stored at -80°C.

2.4.14. Isolation of plasmid DNA

Plasmid DNA was isolated using the Bioline Plasmid Mini Kit (Cat. No. BIO-52027, Bioline, Alexandria, NSW, Australia) following the instruction provided by the manufacturer. In brief, 2 mL of bacterial culture were transferred to a 2 mL microfuge tube and centrifuged for 1 min at 16,100 g to pellet the cells. The supernatant was then removed by decanting or pipetting. The pellet was resuspended in Resuspension Buffer (250 µL) and Lysis Buffer P (250 µL) was added to the sample to lyse the cells.
Neutralization Buffer was then added to the sample to precipitate chromosomal DNA and the DNA was separated from cellular components by centrifugation at 16,100 g for 10 min. The upper phase containing the plasmid DNA was then transferred to a filter column (Spin Column P), placed in a 2 mL collection tube and centrifuged at 10,000 g for 1 min to bind the plasmid DNA onto the column membrane. Bound DNA was washed by the addition of 500 µl of Wash Buffer AP and centrifuged at 10,000 g for 1 min. Wash Buffer BP (700 µL) was added to remove salts from the plasmid DNA and samples were centrifuged at 10,000 g for 1 min. Residual ethanol from the Wash Buffer BP was removed by an additional centrifugation for 2 min at 16,100 g. To elute the plasmid DNA from the filter membrane, 30-50 µL of Elution Buffer was added and the sample was incubated at room temperature for 1 min before being centrifuged at 10,000 g for 1 min.

2.5. Data analyses

Descriptive statistical analyses, including means and standard error of means were performed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). Analyses of variance and mean comparisons were performed using GenStat 15th Edition (VSN International, Hemel Hempstead, UK).
Chapter 3: Salinity Tolerance and Transformation Efficiency of Commercially Relevant Rice Cultivars from Indonesia

3.1. Introduction

Rice improvement through conventional breeding has resulted in a significant number of high yielding rice varieties that combine desirable traits including tolerance to biotic and abiotic stress and good grain quality (Khush 2001; Mackill 2008). Further improvement of the existing varieties is a challenge for all rice breeding programs particularly for traits with complex mechanisms such as tolerance to salinity or other abiotic stresses (Khush 2001; Flowers 2004; Mackill 2008). A large effort has been made to elucidate the mechanisms of salinity tolerance and use the knowledge to increase plant salt tolerance. One area that has received extensive research has been the improvement of plant salinity tolerance by modifying sodium (Na\textsuperscript{+}) transport (Munns and Tester 2008; Plett and Møller 2010; Roy et al. 2014).

Many studies have attempted to improve the salinity tolerance of rice through modification of Na\textsuperscript{+} transport processes. However, most of these studies have used the more salt sensitive \textit{japonica} rice as the genetic background. For example, \textit{japonica} rice cultivars were used in salinity tolerance improvement studies with constitutive expression of vacuolar Na\textsuperscript{+}/H\textsuperscript{+} antiporters (Ohta et al. 2002; Fukuda et al. 2004), vacuolar H\textsuperscript{+}-PPase (Kim et al. 2013) and the combination of constitutive expression of vacuolar Na\textsuperscript{+}/H\textsuperscript{+} antiporters and vacuolar H\textsuperscript{+}-PPases (Zhao et al. 2006b; Liu et al. 2010b).

Previous efforts to modify Na\textsuperscript{+} transport to improve salinity tolerance in rice at the ACPFG have so far only used the \textit{japonica} rice cultivar Nipponbare as the genetic background (Plett et al. 2010; Jacobs et al. 2011). Plett et al. (2010) used GAL-enhancer trap lines of Nipponbare to specifically express the \textit{Arabidopsis} Na\textsuperscript{+} transporter \textit{AtHKT1;1} in root cortical cells. Rice expressing \textit{AtHKT1;1} in cortical cells had reduced Na\textsuperscript{+} in the shoot and increased salt tolerance (Plett et al. 2010). Jacobs et al. (2011) constitutively expressed a Na\textsuperscript{+} pumping ATPase from moss (\textit{Physcomitrella patens}), \textit{PpENA1}, in Nipponbare and observed an improved biomass under salt stress. Nipponbare is a model \textit{japonica} rice variety which has been used intensively in a range of rice functional genomics studies and was accepted as the single material for sequencing in the
International Rice Genome Sequencing Project (Sasaki and Burr 2000). The genome of this variety was completely sequenced in 2005 (International Rice Genome Sequencing Project 2005) and has been used as the reference genome not only for rice but also for other cereals (Xu et al. 2005; Cooke et al. 2007).

Nipponbare, like other japonica type varieties also has a relatively high transformation efficiency using Agrobacterium-mediated transformation (Sallaud et al. 2003; Nishimura et al. 2007; Hiei and Komari 2008). It has therefore been used in the development of various genetic materials for gene function and regulatory element studies, such as T-DNA insertion mutagenesis lines (Sallaud et al. 2004) and enhancer trap lines (Yang et al. 2004; Johnson et al. 2005). However, japonica rice, such as Nipponbare, is primarily adapted to temperate regions (Khush 1997); therefore it has limited adoption in tropical areas.

In general, indica rice has a higher level of salinity tolerance when compared to japonica (Lee et al. 2003; Platten et al. 2013). Pokkali and Nona Bokra are two traditional indica rice varieties which were intensively used as donor parents for salinity tolerance in conventional breeding programs (Ismail et al. 2007; Platten et al. 2013). Nevertheless, breeding programs aiming at improving rice salinity tolerance using these two sources have been relatively slow, mainly due to the complexity of the different salinity tolerance mechanisms (Gregorio et al. 2002; Ismail et al. 2007; Mackill 2008). To improve salinity tolerance in rice it is important to use several different strategies to alter transport of Na\(^+\) through the plant. The strategies, which have previously shown promising results in Nipponbare and other japonica rice cultivars, could be potentially adapted to more commercially relevant varieties. The advantage of directly transforming commercially relevant cultivars with genes to help alter Na\(^+\) transport is that it will increase the probability of the new varieties to be accepted and adopted by farmers (Mackill 2008).

In contrast to the intensive genetic engineering efforts to alter Na\(^+\) transport of japonica rice cultivars, little genetic modification has been performed on indica rice, possibly due to the difficulty in the transformation. Indica rice cultivars were more recalcitrant in Agrobacterium-mediated transformation compare to japonica cultivars (Nishimura et al. 2007; Hiei and Komari 2008; Sahoo et al. 2011). A number of protocols have been reported to improve the transformation efficiency of indica rice, including using immature embryo as a starting material, which has been reported as giving highly efficient transformations (Nishimura et al. 2007; Hiei and Komari 2008; Slamet-Loedin et al. 2011).
Other laboratories have reported improving transformation efficiency of *indica* rice using callus from mature seeds (Khanna and Raina 1999; Kumar et al. 2005; Sahoo et al. 2011; Shri et al. 2013). Transformation efficiency, however, is genotype dependent and requires optimization for every individual genotype (Nishimura et al. 2007).

In the first step in the development of salt tolerant commercially relevant rice through modification of Na\(^+\) transport, this chapter aimed to:

1) Test the salinity tolerance of the candidate commercially relevant rice cultivars and to identify the tolerance mechanisms of each cultivar.

2) Test the transformation efficiency of each cultivar and optimize the protocol of *Agrobacterium*-mediated transformation to increase the transformation efficiency.

Results from this chapter will be used as foundation for the selection of rice cultivars that can be used in generation of salinity tolerant rice using constitutive and cell type-specific expression of genes encoding proteins important for Na\(^+\) homeostasis.
3.2. Materials and methods

3.2.1. Rice materials

Five *indica* rice cultivars were selected to determine their salinity tolerance and their suitability for *Agrobacterium*-mediated transformation: Fatmawati, IR64, Inpara 5 (IR64-Sub1), Dendang and Ciherang. The *japonica* rice cultivar Nipponbare was used for comparison in salinity screening experiments. The details of the cultivars were previously described in Section 2.1.1.

3.2.2. Salinity tolerance assay

3.2.2.1. Rice growth conditions

Salt screening was conducted from 23 September 2013 to 22 October 2013 in the greenhouse of The Plant Accelerator (University of Adelaide, Waite Campus, South Australia). The growth conditions were as described in Section 2.2.1. Day length in Adelaide during the experiment ranged from 12 hours to 13 hours. The daily global solar exposure ranged from 6.1 to 32.6 MJ/m² and the average was 24.23 MJ/ m² (Bureau of Meteorology Australia; http://www.bom.gov.au/climate/data/index.shtml).

3.2.2.2. Salt screening

The salt screening experiment was performed in a hydroponics system (Cotsaftis et al. 2011) as described in Section 2.2.4.2. Rice cultivars were exposed to two salt treatments, 0 mM NaCl and 70 mM NaCl. Ten biological replicates were used for each rice cultivar per treatment. Salt stress was started when the majority of rice plants had their 4th leaf fully expanded (17 d after germination). Salt stress treatment was applied in increments of 25 mM NaCl over a 3 d period (20 mM on the last day) until 70 mM NaCl was reached. To maintain Ca²⁺ activity of 1 mM, additional CaCl₂ was supplied. The CaCl₂ concentration to add for each NaCl treatment was determined using Visual MINTEQ ver. 3.0 (Version 3.0, KTH, Department of Land and Water Resources Engineering, Stockholm, Sweden).

Salt stress was maintained for 10 d and the hydroponic solutions in both control and salt treated tanks were renewed every 3-4 d to avoid nutrient deficiency and to prevent development of algae. Rice plants were harvested 10 d after 70 mM NaCl was reached.
3.2.2.3. **Biomass and ion concentration measurement**

Data were collected for fresh weight, dry weight and concentration of Na\(^+\) and K\(^+\) of the youngest fully expanded leaf and whole root. Sampling and ion concentration measurements were carried out as described in **Section 2.3**.

3.2.3. **Plasmid construct**

The binary expression vector pMDC32Ubi:AVP1 (13188 bp) (provided by Dr. Darren Plett, ACPFG) was used in transformations to test efficiency. The plasmid contained the *Arabidopsis thaliana* vacuolar H\(^+\)-pyrophosphatase gene (*AtAVP1*) (Gaxiola et al. 2001). The *AtAVP1*(*At1g15690*) gene was cloned from *A. thaliana* Col-0 (Schilling et al. 2013) and driven by the maize *Ubiquitin-1* promoter (Figure 3-1).

![Figure 3-1 Map of the expression vector pMDC32Ubi:AVP1 containing AtAVP1.](image)

The vector backbone contains kanamycin resistance gene, origin of replication for *E. coli* (pBR322 origin), and minimal replicon (pVS1) for stable maintenance in *E. coli*. T-DNA cassette contains right border sequence (RB), maize *Ubiquitin-1* promoter (*Ubi1*), Gateway\textsuperscript{®} recombination sequences (*attB1* and *attB2*), the AVP1 coding sequence, nopaline synthase (*Nos*) terminator, hygromycin resistance gene, kanamycin resistance gene and left border sequence (LB). *EcoRV* restriction sites were used for digestion in Southern blot analysis.
3.2.4. Preparation of chemically competent *Agrobacterium tumefaciens* cells strain AGL1

The *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) was used in all rice transformations. To increase AGL1 competent cell stocks, 1 mL of AGL1 competent cell was cultured in 200 mL liquid LB (tryptone 10 g·L\(^{-1}\), yeast extract 5 g·L\(^{-1}\), NaCl 5 g·L\(^{-1}\), pH 7.5) with 25 µg·L\(^{-1}\) antibiotic rifampicin and incubated overnight at 28ºC with vigorous agitation until the culture reached an OD\(_{550nm}\) of 0.5-0.8. The culture was then equally divided into four 50 mL falcon tubes and centrifuged to pellet the cells using a Hettich centrifuge (Model Rotanta 460R, Andreas Hettich GmbH & Co.KG, Tuttingen, Germany) at 4500 g for 15 mins at 4ºC. The pellet was then washed with sterile 1×TE (10 mM Tris-HCl, 1 mM EDTA, pH8.0). TE was then removed after centrifugation at 4500 g for 15 mins at 4ºC. The cells were resuspended in 0.1 × the original volume of LB (5 mL for each falcon tube), and aliquoted into 250 µL fractions in 1.5 mL sterile tubes. The cells were snap-frozen in liquid nitrogen and stored at -80ºC.

3.2.5. Plasmid transformation into *Agrobacterium tumefaciens* strain AGL1

The plasmid pMDC32Ubi:AVP1 was transformed into *Agrobacterium tumefaciens* strain AGL1 using the freeze-thaw method (Hofgen and Willmitzer 1988). To insert pMDC32Ubi:AVP1 into the *Agrobacterium* strain AGL1, a 250 µL aliquot of AGL1 cells was thawed on ice prior to transformation. The plasmid DNA of the expression vector (1-5 µg) was then added and gently mixed with the cells. The mixture was incubated on ice for 5 mins before being frozen in liquid nitrogen for 5 mins. The mixture was then transferred to a 37 ºC water bath and incubated for 5 mins. Liquid LB media (1 mL) was then added to the mixture and the cells incubated at room temperature for 2-4 hours, with shaking. The tube was then briefly centrifuged to pellet the cells. The cells were collected and spread on a LB agar plate, containing 25 µg·L\(^{-1}\) of rifampicin and 50 µg·L\(^{-1}\) of kanamycin. The plate was wrapped with aluminium foil to prevent light from entering and incubated at 28ºC for 4 d. Individual colonies were then selected from the plate using a disposable pipette tip and cultured in 10 mL liquid LB containing 25 µg·L\(^{-1}\) rifampicin and 50 µg·L\(^{-1}\) kanamycin. The culture was incubated at 28ºC with gentle shaking for 3 d. The insertion of the expression vector was confirmed by colony PCR, using primers specific for the hygromycin resistance gene (HYG1-F: 5’-TCACTGGCAAACGTGATGGAC-3’ and HYG1-R: 5’-
GGTTTCCACTATCGGCGAGTAC-3’), and 1 µL of the cells as the DNA template (Section 2.4.3.1.) PCR amplification conditions were set as described in Section 2.4.3.1.

3.2.6. **Agrobacterium tumefaciens-mediated transformation**

The rice transformation protocol used in this study was modified from an *Agrobacterium*-mediated transformation procedure which has been routinely used at the ACPFG for Nipponbare transformation. This method was originally derived from the protocol described by Sallaud et al. (2003). The summary of transformation steps is shown in Figure 3-2.

3.2.6.1. **Callus induction**

Embryogenic calli were generated from scutellum of mature seeds of rice cultivars Fatmawati, IR64, Inpara 5, Dendang and Ciherang. Two types of callus induction media were initially compared for callus induction. The first callus media used Chu N6 (Chu 1975) with vitamins (Cat. No. C167, PhytoTechnology Laboratories, Shawnee Mission, KS, USA) as basal salt medium. The second callus media used MS (Cat. No. M524, PhytoTechnology Laboratories, Shawnee Mission, KS, USA) (Murashige and Skoog 1962; Khanna and Raina 1999) supplemented with Gamborg vitamins (Cat. No. G219, PhytoTechnology Laboratories, Shawnee Mission, KS, USA) (Gamborg et al. 1968).

Rice seeds were dehusked and surface sterilized as described in Section 2.2.3 prior to inoculation. A total of 100 seeds of each cultivar were used for callus induction. Ten sterilized seeds were placed into one plastic plate (100mm diameter x 20mm depth) (Cat. No. 430167, Corning®, Corning, NY, USA) containing 50 mL callus induction medium either with Chu N6 or MS as basal salt (Table 3-1). Callus induction medium with Chu N6 was only used in the preliminary experiment to compare the callus induction rate of Indonesian cultivars in Chu N6 and MS. Callus induction medium with MS was used in the rest of the transformations. The seeds were dried in a sterile laminar flow before the plates were sealed using Gladwrap (Glad wrap, Glad Products, Australia) and incubated in the dark at 28 ºC for 4 weeks. Embryogenic calli (small to medium sized) were then released from the zone of shoot emergence and transferred to a new callus induction medium (12-16 calli per plate). The calli were incubated in the dark at 28 ºC for 2 weeks.
3.2.6.2. Inoculation and co-cultivation

An Agrobacterium culture was prepared 3 d before transformation by incubating 10 µL liquid culture of the Agrobacterium strain AGL1 transformed with the expression vector pMDC32Ubi:AVP1 (Section 3.2.5) in 10 mL liquid LB containing the antibiotics rifampicin (25 µg·mL⁻¹) and kanamycin (50 µg·mL⁻¹). The cultures were wrapped with aluminium foil to keep them in the dark and were incubated at 28ºC with shaking.

After 3 d the Agrobacterium culture was pelleted by centrifugation at 4500 g for 10 mins using a Hettich centrifuge. The supernatant was then removed and the Agrobacterium pellet was suspended in 40 mL infection medium (Table 3-1) in a sterile 50 mL falcon tube. The optical density (OD₆₀₀) of the inoculated infection medium was determined and adjusted to ~0.1 using blank infection medium.

Approximately 20-50 embryogenic calli were transferred from callus induction medium to a sterile plastic plate containing blank infection medium and incubated for 5 mins at room temperature. The infection medium was then removed and replaced with infection medium containing Agrobacterium with the desired transformation vector. The calli were incubated in the Agrobacterium culture for 2 mins at room temperature with gentle swirling. The calli were then blotted dry on sterile filter paper and transferred into plates containing 50 mL of co-cultivation medium (Table 3-1) and sealed with glad wrap. The plates were wrapped with aluminium foil and the calli were incubated in the dark at 25ºC for 3 d.
Table 3-1 Composition of media used in Agrobacterium-mediated rice transformation

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callus induction-Chu N6</td>
<td>3.99 g·L⁻¹ Chu N6 with vitamins¹, 100 mg·L⁻¹ myo-inositol², 2 g·L⁻¹ L-proline³, 30 g·L⁻¹ sucrose, 0.3 g·L⁻¹ casein⁴, 2.5 mg·L⁻¹ 2,4-dichlorophenoxyacetic (2,4-D), 2.6 g·L⁻¹ phytagel* (adjusted using 1 M NaOH)</td>
</tr>
<tr>
<td>Callus induction-MS</td>
<td>4.33 g·L⁻¹ MS (⁶), 1 mL·L⁻¹ Gamborg vitamins⁷, 30 g·L⁻¹ sucrose, 0.5 g·L⁻¹ casein, 2 g·L⁻¹ L-proline, 2 mg·L⁻¹ 2,4D, 2.6 g·L⁻¹ phytagel* or gerlite⁸, pH 5.8</td>
</tr>
<tr>
<td>Infection</td>
<td>4.33 g·L⁻¹ MS, 1 mL·L⁻¹ Gamborg vitamins, 68.4 g·L⁻¹ sucrose, 0.7 g·L⁻¹ L-proline, 2.5 mg·L⁻¹ 2,4D, 100 µM acetosyringone⁹, pH 5.2</td>
</tr>
<tr>
<td>Co-cultivation</td>
<td>4.33 g·L⁻¹ MS, 1 mL·L⁻¹ Gamborg vitamins, 30 g·L⁻¹ sucrose, 0.3 g·L⁻¹ casein, 2.5 mg·L⁻¹ 2,4D, 100 µM acetosyringone, 7 g·L⁻¹ agarose¹⁰</td>
</tr>
<tr>
<td>Selection</td>
<td>4.33 g·L⁻¹ MS, 1 mL·L⁻¹ Gamborg vitamins, 30 g·L⁻¹ sucrose, 0.5 g·L⁻¹ casein, 2 g·L⁻¹ L-proline, 2 mg·L⁻¹ 2,4D, 50 mg·L⁻¹ hygromycin¹¹, 400 mg·L⁻¹ cefotaxime¹², 7 g·L⁻¹ agarose, pH 5.2</td>
</tr>
<tr>
<td>Pre-regeneration</td>
<td>4.33 g·L⁻¹ MS, 1 mL·L⁻¹ Gamborg vitamins, 30 g·L⁻¹ sucrose, 0.3 g·L⁻¹ casein, 2.8 g·L⁻¹ L-proline, 0.1 g·L⁻¹ myo-inositol, 0.5 g·L⁻¹ glutamine¹³, 5 mg·L⁻¹ ABA, 2 mg·L⁻¹ BAP, 1 mg·L⁻¹ NAA, 50 mg·L⁻¹ hygromycin, 400 mg·L⁻¹ cefotaxime, 7 g·L⁻¹ agarose, pH 5.2</td>
</tr>
<tr>
<td>Regeneration</td>
<td>4.33 g·L⁻¹ MS, 1 mL·L⁻¹ Gamborg vitamins, 30 g·L⁻¹ sucrose, 0.3 g·L⁻¹ casein, 0.5 g·L⁻¹ L-proline, 0.1 g·L⁻¹ myo-inositol, 0.5 g·L⁻¹ glutamine, 2 mg·L⁻¹ BAP, 1 mg·L⁻¹ NAA, 50 mg·L⁻¹ hygromycin, 7 g·L⁻¹ gerlite, pH 5.8</td>
</tr>
<tr>
<td>Rooting</td>
<td>4.33 g·L⁻¹ MS, 1 mL·L⁻¹ Gamborg vitamins, 50 g·L⁻¹ sucrose, 50 mg·L⁻¹ hygromycin, 7 g·L⁻¹ gerlite, pH 5.8</td>
</tr>
</tbody>
</table>

*Phytagel was only used as gelling agent in the initial experiment to determine callus induction rate. Gerlite was then used in the rest of experiments.

¹Cat. No. C167, PhytoTechnology Laboratories, Shawnee Mission, KS, USA
²Cat. No. I-7508, Sigma Aldrich, St Louis, MO, USA
³Cat. No. P698, PhytoTechnology Laboratories, Shawnee Mission, KS, USA
⁴Cat. No. C184, PhytoTechnology Laboratories, Shawnee Mission, KS, USA
⁵Cat. No. P8169, Sigma Aldrich, St Louis, MO, USA
⁶Cat. No. M524, PhytoTechnology Laboratories, Shawnee Mission, KS, USA
⁷Cat. No. G219, PhytoTechnology Laboratories, Shawnee Mission, KS, USA
⁸Cat. No. G469, PhytoTechnology Laboratories, Shawnee Mission, KS, USA
⁹Cat. No. A104, PhytoTechnology Laboratories, Shawnee Mission, KS, USA
¹⁰Cat. No. BIO-41026, Bioline (Aust), Alexandria, NSW, Australia
¹¹Cat. No. H370, PhytoTechnology Laboratories, Shawnee Mission, KS, USA
¹²Cat. No. C380, PhytoTechnology Laboratories, Shawnee Mission, KS, USA
¹³Cat. No. 49F-0667, Sigma Aldrich, St Louis, MO, USA
3.2.6.3. **Selection of transformed calli**

After 3 days in co-cultivation medium, the calli were transferred to a 50 mL falcon tube and washed five times with RO water containing 400 µg·L⁻¹ cefotaxime to eliminate the *Agrobacteria*. The calli were blotted dry on sterile filter paper and then transferred into selection medium (50 mL) in a plastic plate (100 mm diameter x 20 mm depth) (Table 3-1). Plates were sealed with plastic wrap and incubated in the dark at 28°C for two weeks. During the selection period, the calli were monitored to check for the presence of *Agrobacteria* overgrowth. Calli which showed overgrowth of *Agrobacteria* were removed by dissecting the over-infected calli into two on the plate or by transferring the uninfected calli into a new selection medium. After two weeks in selection medium, the majority of the calli turned brown, however, some calli were covered by tiny white growths which indicated the transformation events. At this stage, all the calli (brown and white calli) were transferred to a new selection media and incubated in the dark at 28 °C for two weeks.

Following the second two weeks in selection media, the white calli were detached from the parental callus and transferred to new selection media to proliferate the transformed calli. Calli derived from one parental callus were grouped together and kept separated to other calli. Lines were drawn on the bottom of selection medium plates to keep track of the different calli. The calli were incubated in the dark for a further two weeks at 28°C. Proliferated calli were then moved to new selection medium for another two weeks in the dark at 28°C before being transferred to pre-regeneration medium (callus maturation process).

3.2.6.4. **Maturation of transformed calli**

After the eight week selection process, putative transgenic calli, which were yellow/white in colour, with a compact shape, were transferred to pre-regeneration medium (Table 3-1) to induce shoot primordia. Calli were incubated at 28°C in the dark for 7 d.

3.2.6.5. **Regeneration of transformed calli**

Calli from pre-regeneration medium were transferred to regeneration medium (Table 3-1) to regenerate shoots. Calli were initially incubated in the dark at 28°C for 2 d before being transferred to a growth cabinet (Model MLR-351, Sanyo Electric Co. Ltd) with 12 hours
light/dark at 28°C to promote shoot growth. Calli were incubated in the regeneration medium for five weeks or until the shoot emerged.

3.2.6.6. Root development

Shoots regenerated from putative transformed calli were excised from their callus. The upper two-third portion of the shoot (and any roots which had formed naturally) were discarded using a sterile scalpel before the remaining plantlet was transferred to rooting medium (Table 3-1) in a 1 L plastic jar. Three individual shoots were collected from each parental callus and placed in one plastic jar. The plastic jars were sealed with the lids. Plantlets were incubated at 28 °C in a growth cabinet under 12 h light/dark for four weeks or until the shoots reaching the lid.

3.2.6.7. Acclimation and cultivation of transgenic T₀ plants

Regenerated plantlets were removed from the rooting medium for further acclimation. Only one plantlet from each tube (one plant per callus) was used for further analysis. The shoot and root of the plantlets were trimmed leaving approximately 2 cm of shoot and 1 cm root. Plantlets were planted in rehydrated jiffy peat pots (Cat. No. 32170142, Jiffy Products International, Stage, Norway), placed in a plastic tray with standing RO water and covered with a plastic dome to maintain high humidity. The trays were placed in a greenhouse as described in Section 2.2.2. The plantlets were grown in the jiffy pots for two weeks before being transplanted to a UC Davis soil mix in a green plastic pot and grown for 90 to 120 d (depending on the genotypes and growth condition) until maturity (Section 2.2.1).

3.2.7. Molecular confirmation of the transgene

A leaf sample of the T₀ plant from each transformation event was collected for DNA extraction as described in Section 2.4.1. The presence of the transgene was confirmed by PCR analysis (Section 2.4.3.1) using AtAVP1 specific forward (AVP1-F: 5’-TGTGTTTGACCCCTAAAGTTATTAC-3’) and reverse (AVP1-R: 5’-TGGCTCTGAACCCTTGTGTC-3’) primers, with an annealing temperature of 57°C and an extension time of 1 min. The copy number of each transformation event was determined using Southern blot analysis using the restriction enzyme EcoRV (Section 2.4.8).
Figure 3-2 Summary of *Agrobacterium*-mediated transformation steps for Indonesian rice cultivars.
The process from callus induction until seed harvest from T₀ plants takes approximately 40 to 44 weeks.
3.3. Results

3.3.1. Salinity tolerance of Indonesian rice cultivars

The salinity tolerance during the early vegetative stage of five rice cultivars from Indonesia were studied and compared to the model rice cultivar Nipponbare in a hydroponics system. The rice cultivars were grown either under a moderate salt stress (70 mM NaCl) or in 0 mM NaCl. Salt stress was imposed in increments starting when the 4th leaf was fully expanded (17 d after germination) and plants were harvested 10 d after the 70 mM NaCl treatment was reached. The salt stressed rice showed obvious reductions in plant size when compared to those grown in 0 mM NaCl. Little leaf senescence, which is an expected symptom of ionic stress, was observed in the leaf tips of the stressed plants (Figure 3-3).

The response of different rice cultivars to salt stress was determined using destructive measurements including biomass and tissue Na$^+$ and K$^+$ concentrations (Figure 3-4 to Figure 3-7). Analysis of variance was performed to examine for significant effects of cultivars, salt treatments and/or their interaction on biomass and ion concentration (Table 3-2). The result indicated there were significant differences among cultivar and salt stress treatment in all measurements. The effect of interaction between cultivar and salt stress was not significant in the biomass measurement, but significant for all ion concentration traits (Table 3-2).
Figure 3-3 Representative examples of individuals from six rice cultivars grown in hydroponic experiment under (A) 0 mM NaCl and (B) 70 mM NaCl. Salt stress was imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Photographs were taken 10 days after reaching 70 mM NaCl. A blue 30 cm ruler is shown for scale.
Table 3-2 Summary of analysis of variance for biomass and ion concentration traits in salinity tolerance experiment

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Shoot dry weight</th>
<th>Root dry weight</th>
<th>Total dry weight</th>
<th>Shoot Na⁺</th>
<th>Root Na⁺</th>
<th>Shoot K⁺</th>
<th>Root K⁺</th>
<th>Ratio shoot K⁺/Na⁺</th>
<th>Ratio root K⁺/Na⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Salt stress</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Cultivar x salt stress</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

** = significant at \( P<0.01 \); ns = not significant

The five commercial rice cultivars from Indonesia exhibited a higher biomass production when compared to Nipponbare, under both 0 mM and 70 mM NaCl conditions (Figure 3-4). In addition, there were differences between the Indonesian cultivars, with Dendang, Fatmawati and IR64 showing greater dry weights when compared to Inpara 5 and Ciherang under both 0 mM and 70 mM salt stress.

Salinity tolerance can frequently be defined as the ability to maintain biomass production under salt stress, therefore, the biomass of each cultivar grown under 70 mM NaCl was compared to the same cultivar grown in 0 mM NaCl (Table 3-3). Fatmawati had the highest salinity tolerance among the cultivars studied, maintaining 80% of its biomass when grown in 70 mM NaCl. IR64 and Dendang showed similar degrees of salinity tolerance to each other (about 74%) while the salinity tolerance of Nipponbare (69%) was comparable to Inpara 5 (68%). Rice cultivar Ciherang showed the lowest level of salinity tolerance compared to all cultivars, approximately 65% (Table 3-3).

The concentrations of Na⁺ and K⁺ in shoot and root were determined 10 d after rice plants were exposed to 70 mM NaCl. As predicted, the Na⁺ concentration in shoot and root of salt stressed plants significantly increased compared to non-salt stressed plants (Figure 3-5). Under 0 mM NaCl conditions, there were no significant differences in the shoot and root Na⁺ concentration in any of the cultivars. Under salt stress, the shoot Na⁺ of the cultivars Fatmawati (28 mM), Inpara 5 (29 mM) and Dendang (32 mM) were significantly lower than the others. IR64 and Nipponbare showed a similar level of shoot Na⁺ (about 39 mM), while Ciherang showed the highest shoot Na⁺ (47 mM) compared to the other rice cultivars.
All rice cultivars showed very high concentrations of Na\(^+\) in the root under salt treatment (ranging from 63 mM to 93 mM), with some cultivars accumulating higher concentrations than the external Na\(^+\) concentration. In contrast to the concentration of Na\(^+\) in the shoot, Fatmawati showed the highest root Na\(^+\) (94 mM) compared to other rice cultivars. The root Na\(^+\) of Nipponbare (64 mM) was the lowest amongst all others.

The concentration of K\(^+\) in the shoots and roots of the six rice cultivars were significantly different both in normal and salt stress conditions. Salt stress caused significant reductions in shoot and root K\(^+\) concentrations (Figure 3-6). Among the rice cultivars, Fatmawati and Ciherang showed the largest reduction in shoot K\(^+\) under salt stress compared to 0 mM NaCl conditions. The cultivars IR64, Inpara 5 and Nipponbare retained the same degree of K\(^+\) concentration in the shoot after exposure to 70 mM NaCl (ranging from 256 mM to 267 mM) (Figure 3-6). Fatmawati was the cultivar with the lowest root K\(^+\) concentration (71 mM) under salt stress compared to the other Indonesian rice cultivars, having a similar level of concentration to Nipponbare (76 mM) (Figure 3-6).

Salt stress significantly reduced the ratio of K\(^+\)/Na\(^+\) of the rice shoot and roots. Under normal conditions, Dendang showed the highest ratio of K\(^+\)/Na\(^+\) both in shoot and root compared to the other cultivars. Under salt stress, these ratios were very low and there were no significant differences among the rice cultivars (Figure 3-7).
Figure 3-4 (A) Shoot dry weight, (B) root dry weight and (C) total dry weight per plant of six rice cultivars grown in hydroponics under 0 mM and 70 mM NaCl. Salt stress was imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. Values are the means ± SEM (n=10).
Table 3-3 Ratio of total dry biomass of six rice cultivars grown under 70 mM and 0 mM NaCl.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Total dry weight grown in 70 mM NaCl ÷ total dry weight grown in 0 mM NaCl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatmawati</td>
<td>80.13</td>
</tr>
<tr>
<td>IR64</td>
<td>74.13</td>
</tr>
<tr>
<td>Inpara 5</td>
<td>68.32</td>
</tr>
<tr>
<td>Dendang</td>
<td>73.28</td>
</tr>
<tr>
<td>Ciherang</td>
<td>64.85</td>
</tr>
<tr>
<td>Nipponbare</td>
<td>69.11</td>
</tr>
</tbody>
</table>
Figure 3-5 Na⁺ concentration in the (A) shoot and (B) root of six rice cultivars grown in hydroponics under 0 mM and 70 mM NaCl treatments.

Salt stress was imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. Values are the means ± SEM (n=10). Data points with the same letter are not significantly different according to LSD (0.05) test.
Salt stress was imposed in 25 mM increments over a 3-day period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 days after germination). Rice plants were harvested 10 days after 70 mM NaCl was reached. The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. Values are the means ± SEM (n=10). Data points with the same letter are not significantly different according to LSD (0.05) test.
Figure 3-7 Ratio of K⁺/Na⁺ concentration in (A) the shoot and (B) root of six rice cultivars grown in hydroponics under 0 mM and 70 mM NaCl treatments.
Salt stress was imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Plants were harvested 10 d after 70 mM NaCl was reached. The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. Values are the means ± SEM (n=10). Data points with the same letter are not significantly different according to LSD (0.05) test.
3.3.2. *Agrobacterium*-mediated transformation of Indonesian rice cultivars

The transformation efficiency of five *indica* rice cultivars using *Agrobacterium*-mediated transformation was determined. All cultivars were transformed using *Agrobacterium tumefaciens* strain AGL1 carrying a binary plasmid pMDC32Ubi:AVP1. The transformation method used callus derived from scutellum of mature seeds and was modified from the transformation protocol which was routinely used at ACPFG for *japonica* rice Nipponbare (Sallaud et al. 2003).

Before transformation, two preliminary experiments were carried out to improve the callus induction rate of the rice cultivars. Two different basal salts were compared for callus induction medium for four *indica* rice cultivars: Fatmawati, IR64, Ciherang and Dendang (Table 3-4). Inpara 5 was not included in the first experiment due to seed vigour problems of the original imported seed from Indonesia. In both experiments, the use of MS as basal salts in the callus induction medium increased callus development rate of all four rice cultivars when compared to using the N6 salts. In particular, noticeable improvements in callus development were shown for Ciherang, IR64 and Dendang when using MS in the induction medium (Table 3-4). MS therefore replaced N6 in all media for subsequent rice transformation experiments.

Table 3-4 Callus development rate of five Indonesian rice cultivars induced from scutellum using MS and N6 as basal salt

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>MS&lt;sup&gt;(1)&lt;/sup&gt;</th>
<th>N6&lt;sup&gt;(1)&lt;/sup&gt;</th>
<th>MS&lt;sup&gt;(2)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatmawati</td>
<td>60.0</td>
<td>52.0</td>
<td>51</td>
</tr>
<tr>
<td>IR64</td>
<td>50.0</td>
<td>22.5</td>
<td>52</td>
</tr>
<tr>
<td>Inpara 5</td>
<td>na</td>
<td>na</td>
<td>53</td>
</tr>
<tr>
<td>Ciherang</td>
<td>50.0</td>
<td>2.0</td>
<td>15</td>
</tr>
<tr>
<td>Dendang</td>
<td>16.0</td>
<td>0.0</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>(1)</sup> Seed from Indonesia  
<sup>(2)</sup> Seed produced in the greenhouse on the University of Adelaide’s Waite Campus  
na = not available due to poor germination rate

To test the transformation efficiency of the cultivars using plasmid pMDC32Ubi:AVP1, calli from mature seed of the five rice cultivars from Indonesia were generated again using callus induction medium with MS. Even though the callus development rate of Fatmawati, IR64 and Inpara 5 were comparable (Table 3-4), the quality of the calli
generated from scutellum of these rice cultivars was different. Calli from Fatmawati were drier and more compact compared to calli from IR64 and Inpara 5 (Figure 3-8). Calli from IR64, Inpara 5, Ciherang and Dendang were wet and had many rhizogenic regions.

Primary calli from the scutellum of the rice seed were transferred to new callus induction medium to proliferate the calli for a further 2 weeks (Figure 3-9). Fatmawati showed faster proliferation compared to the other cultivars and its callus was also more compact, nodular shaped, with a yellowish colour. On the other hand, calli from the other cultivars developed slowly and their textures were soft and wet.

Figure 3-8 Calli developed from scutellum of five Indonesian rice cultivars.
The rice seeds were placed in callus induction media with MS as the basal salt for 4 weeks in the dark at 28°C.
The most compact and white or yellowish calli from the proliferation process from each of the five cultivars were then selected for transformation. Calli were inoculated with *Agrobacterium* strain AGL1 containing the pMDC32Ubi:AVP1. Transformed calli were screened in selection medium using the antibiotic hygromycin. Cefotaxime was also used in the selection process to eliminate the remaining AGL1 after the transformation process. Calli were sub-cultured in selection medium 4 times for 2 weeks each. During this process putative transformed calli remained white, while non-transformed calli were brown and turned black. By the second sub-culture in selection media, almost all the calli from Ciherang and Dendang were turning brown and black. Transformed calli were easily picked from Fatmawati, IR64 and Inpara5 (Figure 3-10). These calli were sub-cultured again until the 4th selection (Figure 3-11). After 4 sub-cultures in selection medium, 38 out of 80 Fatmawati calli (47.5%) had survived, while 11 calli from both IR64 and Inpara5 survived (14%) (Table 3-5).
Figure 3-10 Calli of five Indonesian cultivars after the 2nd selection using selection medium with hygromycin and cefotaxime. Non-transformed calli turned brown and black, while putative transformed calli form white and yellowish calli. Lines on the plate bottom were used to separate calli from different parent calli.
Figure 3-11 Calli of four Indonesian cultivars after the 4th selection using selection medium with hygromycin and cefotaxime. Non-transformed calli turned brown and black, while putative transformed calli form white and yellowish calli. Lines on the plate bottom were used to separate calli from different parent calli.
Table 3-5 Summary of *Agrobacterium*-mediated transformation of five Indonesian cultivars using *Agrobacterium* strain AGL1 carrying expression vector pMDC32Ubi:AVP1

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Number of co-cultivated calli</th>
<th>Number of survived calli after 4th selection</th>
<th>Number of regenerated shoots</th>
<th>Number of surviving T₀ plants</th>
<th>Number of positive T₀ plants (1)</th>
<th>Transformation efficiency (%) (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatmawati</td>
<td>80</td>
<td>38</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>13.75</td>
</tr>
<tr>
<td>IR64</td>
<td>80</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1.25</td>
</tr>
<tr>
<td>Inpara5</td>
<td>80</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ciherang</td>
<td>48</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dendang</td>
<td>24</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(1) Confirmed by PCR and Southern blot analysis
(2) Transformation efficiency = number of positive T₀ plants/number of co-cultivated calli (%)

Hygromycin resistant calli were transferred to regeneration medium to promote shoot development (Figure 3-12, Table 3-5). Only Fatmawati and IR64 showed regenerated shoots, while calli from other cultivars either stopped developing or died. All regenerative shoots from Fatmawati calli developed into transgenic plants, while only 1 transgenic plant of IR64 was produced. Over all, Fatmawati showed 13.75% transformation efficiency, while IR64 had a transformation efficiency of 1.25%. The other three rice cultivars did not produce transgenic plants with the chosen protocol (Table 3-5).

The insertion of the transgene was confirmed by PCR using *AtAVP1* gene specific primers and Southern blot analysis using a probe against the *Nos* terminator of the plasmid. Southern blot analysis also confirmed all transgenic lines generated were independent events (Figure 3-13). The number of T-DNA insertions in transgenic Fatmawati varied from 1 to 6, while the single IR64 transgenic line was found to carry a single T-DNA insertion (Table 3-6). Almost all transgenic lines produced were fertile, with only two transgenic lines found to be completely sterile (0% fertility) and 1 line with very low fertility (5.84%) (Table 3-6). All fertile transgenic lines showed a normal phenotype similar to the wild type Fatmawati or IR64 when grown in the greenhouse.
Figure 3-12 Examples of the steps to go from Fatmawati calli to plants. (A) Rooting medium, (B) acclimation in 1 L plastic jar, (C) growth of $T_0$ transgenic seedlings in Jiffy pots and (D) regenerated plants prior to harvesting. Transgenic rice lines were generated by Agrobacterium-mediated transformation and constitutively expressing $AtAVP1$. 
Figure 3-13 Southern blot analysis of T₀ plants of transgenic Fatmawati constitutively expressing AtAVP1 (Line 1-11) and transgenic IR64 constitutively expressing AVP1 (Line 12). Restriction enzyme EcoRV was used to digest gDNA of the transgenic plants. PCR-amplified product of the Nos terminator was radiolabelled with [α-³²P]dCTP and used as probe.

Table 3-6 Number of T-DNA insertion based on Southern blot and grain fertility of T₀ transgenic rice Fatmawati and IR64 overexpressing AVP1 gene

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>Number of T-DNA insertion</th>
<th>Grain fertility (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatmawati-AVP1-1</td>
<td>2</td>
<td>67.67</td>
</tr>
<tr>
<td>Fatmawati-AVP1-2</td>
<td>2</td>
<td>0.00</td>
</tr>
<tr>
<td>Fatmawati-AVP1-3</td>
<td>1</td>
<td>5.84</td>
</tr>
<tr>
<td>Fatmawati-AVP1-4</td>
<td>1</td>
<td>59.40</td>
</tr>
<tr>
<td>Fatmawati-AVP1-5</td>
<td>6</td>
<td>46.56</td>
</tr>
<tr>
<td>Fatmawati-AVP1-6</td>
<td>1</td>
<td>32.83</td>
</tr>
<tr>
<td>Fatmawati-AVP1-7</td>
<td>1</td>
<td>31.46</td>
</tr>
<tr>
<td>Fatmawati-AVP1-8</td>
<td>4</td>
<td>47.19</td>
</tr>
<tr>
<td>Fatmawati-AVP1-9</td>
<td>2</td>
<td>49.21</td>
</tr>
<tr>
<td>Fatmawati-AVP1-10</td>
<td>2</td>
<td>0.00</td>
</tr>
<tr>
<td>Fatmawati-AVP1-11</td>
<td>1</td>
<td>68.21</td>
</tr>
<tr>
<td>IR64-AVP1-1</td>
<td>1</td>
<td>91.78</td>
</tr>
</tbody>
</table>

*Grain fertility= Percentage of fertile grain/total grain of T₀ plants
3.4. Discussion

3.4.1. Five rice cultivars selected from Indonesia for generation of salt tolerant transgenic rice

Five commercially relevant rice cultivars were imported from Indonesia as candidate materials to generate salt tolerant rice. The cultivars were selected based on their popularity in Indonesia and after discussion with a rice breeder from the Indonesian Centre for Rice Research (personal communication with Dr Suwarno, Indonesian Centre for Rice Research). Each cultivar has unique and important traits:

Fatmawati is a new plant type (NPT) rice which was released in Indonesia in 2003 (Abdullah et al. 2008). NPT rice has a novel morphological architecture and was designed to increase the yield potential of rice (Khush 1995). The NPT rice breeding program attempted to modify plant architecture of modern rice varieties by reducing the number of tillers, increasing the number of grains per panicle and increasing stem sturdiness (Khush 1995; Khush 2005). Improvement of salinity tolerance of this cultivar will add to the value of the cultivar and allow the cultivation of the cultivar in unfavourable environments.

IR64 is known as ‘mega variety’ because it has been grown in million hectares of paddy field in Asia (Mackill 2008). The variety was developed by the International Rice Research Institute (IRRI) and has been released as commercial variety in 13 countries throughout Asia and Africa (Khush and Virk 2005). In Indonesia alone, IR64 had been grown on more than 47 million ha since 1985 and has become the most popular variety in the country (Brennan and Malabayas 2011). IR64 has a moderate level of salinity tolerance and has been used intensively in many studies for abiotic stress tolerance, investigating aspects of salinity (Ueda et al. 2006; Castillo et al. 2007; Nakhoda et al. 2012; Platten et al. 2013), drought (Yadav et al. 1997; Lenka et al. 2011) and submergence (Septiningsih et al. 2009; Fukao et al. 2011) stress. The variety also has many desirable traits, such as resistance to brown plant hopper and bacterial leaf blight, which are two main biotic constraints in rice cultivation (Khush and Virk 2005).
Inpara 5 (IR64-Sub1) is an improvement of IR64 with an introgression of the submergence tolerant gene Sub1 (Septiningsih et al. 2009; Bailey-Serres et al. 2010; Mackill et al. 2012). It was developed through backcross breeding and has an almost identical genome to IR64 (Septiningsih et al. 2009). The variety was released for flash flood prone areas. As in rice fields, submergence and salinity can occur in the same area, particularly in the coastal regions, pyramiding salinity tolerance and submergence tolerance traits has the potential to increase rice yield stability (Gregorio et al. 2013).

Ciherang is the second largest variety grown in Indonesia after IR64 (Brennan and Malabayas 2011). It was released in 2000 and by 2009 had been grown in more than 19.1 million ha in Indonesia (Brennan and Malabayas 2011). The variety was developed by the Indonesian Centre for Rice Research and has good resistance to brown plant hopper and bacterial leaf blight (Suprihatno et al. 2010).

Dendang is a moderately salt tolerant variety which was released in 1999 (Suprihatno et al. 2010). The variety was mainly targeted for tidal swamp areas where rice production is hampered by diverse soil problems, such as salinity, iron toxicity and aluminium toxicity (Driessen and Sudjadi 1984). It is expected that a genetic modification approach could increase the salt tolerance of this cultivar beyond its current tolerance level.

Using genetic engineering to modify elite rice cultivars which are widely grown in Indonesia is expected to accelerate the development of new salt tolerant rice lines. In addition, the insertion of a transgene into different rice cultivars (which have different tolerance mechanisms) will give valuable information on the mode of action of the gene in different genetic backgrounds (Yamaguchi and Blumwald 2005).

3.4.2. Salinity tolerance of rice cultivars correlates with vigour

The salinity tolerance of candidate cultivars for the generation of salt tolerant transgenic rice was evaluated as the basis for further improvement. A moderate salt stress of 70 mM NaCl was chosen instead of higher salt stresses to allow physiological studies of the response, rather than just the “death” response of the plant. Survival assays using high salt concentrations are still widely used especially in breeding programs to screen large populations of rice (Gregorio et al. 1997; Platten et al. 2013), even though rice is known to be a salt sensitive crop. However, screening with high levels of stress does not allow the exploration of physiological mechanisms underlying the tolerance.
Results from this study showed that the biomass of different rice cultivars under salt stress depended on the innate vigour of each cultivar. Cultivars such as Fatmawati, IR64 and Dendang which showed the largest biomass production under non-saline conditions also had higher total dry weight under salt stress compared to Ciherang or Inpara 5 (Figure 3-4). Yeo et al. (1990) demonstrated that vigour was the most correlated physiological trait with the rice survival under salt stress from a large scale salt screening involving 200 rice genotypes. However, it should be noted that this is a trend and not an absolute fact - Nipponbare which exhibits lower biomass production compared to Ciherang has a greater level of tolerance (Figure 3-4, Table 3-3) indicating there are other tolerance mechanisms employed by the cultivar, such as Na+ exclusion.

3.4.3. Different mechanisms exist related to tissue Na+ concentration

The shoot Na+ concentration of the six rice cultivars studied showed significant variation. Fatmawati and Dendang which have the greatest salinity tolerance, compared to the other cultivars, have the lowest shoot Na+ concentrations, suggesting they have an improved ability to exclude Na+ from the shoot (Ren et al. 2005; Platten et al. 2013). Interestingly, Fatmawati has much higher root Na+ concentrations than Dendang, potentially suggesting that Fatmawati limits the transfer of Na+ from the root to the shoot, potentially using Na+ transporters such as HKTs (Ren et al. 2005; Platten et al. 2013), while Dendang may be better at limiting uptake of Na+ from the soil. A similar observation was found in tolerant cultivar Pokkali, which showed a high Na+ concentration in roots while having low Na+ in the shoot, potentially the result of Na+ exclusion from the shoot (Cotsaftis et al. 2011). It is also possible that the low concentration of shoot Na+ in these cultivars is due to their large biomass which increased Na+ dilution in their shoot tissue (Yeo et al. 1990; Platten et al. 2013).

Interestingly, IR64 which showed a similar degree of salt tolerance to that of both Fatmawati and Dendang has a higher shoot Na+ concentration, which may indicate IR64 has better tissue tolerance mechanisms in the shoot. This observation was supported by the senescence data from image based phenotyping (Chapter 4), which showed that even though shoot Na+ was higher in IR64 than Fatmawati, IR64 showed a smaller percentage of leaf senescence. Plants adapt to high concentrations of Na+ in their tissue by different mechanisms including partitioning the Na+ into the vacuole, accumulating compatible solutes and increasing the activity of enzymes responsible for reactive oxygen species (ROS) detoxifications (Munns and Tester 2008; Roy et al. 2014). The Na+
compartmentalization into the vacuole is facilitated by vacuolar Na\(^+\)/H\(^+\) antiporters as a result of electrochemical gradients of protons generated by the vacuolar H\(^+\)-ATPase and H\(^+\)-PPase (Blumwald et al. 2000). It is important to analyse the intracellular compartmentalization of Na\(^+\) in IR64 to understand the tissue tolerance mechanism of this cultivar (Blumwald and Poole 1985; Garbarino and DuPont 1989; James et al. 2006b).

The levels of tissue K\(^+\) were also significantly different among cultivars, both under non-saline and salt stressed conditions. Of particular interest are the differences between the two tolerant cultivars, Fatmawati and IR64. IR64, which accumulates higher Na\(^+\) in the shoot compared to Fatmawati, also accumulates higher K\(^+\) in the shoot, and thus IR64 is better able to maintain K\(^+\)/Na\(^+\) ratios in the shoot which may also contribute to the overall salinity tolerance of the cultivar (Asch et al. 2000).

3.4.4. Potential Na\(^+\) transport modification strategies to improve salt tolerance in rice

The variation in shoot and root Na\(^+\) status among the six rice cultivars shows they use different strategies to deal with Na\(^+\) accumulation in the shoot. The differences between the cultivars also suggest that strategies for genetic engineering of salinity tolerance in rice may depend on the cultivar in question.

For the shoot excluding cultivars, such as Fatmawati and Dendang, increasing the ability of the plant to compartmentalize Na\(^+\) in vacuoles in the shoot may improve the level of salinity tolerance in these cultivars, if they ever were grown in an environment where Na\(^+\) levels were so high their natural excluding mechanism would no longer be sufficient. Two strategies can be employed to increase Na\(^+\) sequestration into the vacuole (Munns and Tester 2008; Plett and Møller 2010). First, it can be achieved by overexpressing NHX encoding a vacuolar Na\(^+\)/H\(^+\) antiporter which facilitates the translocation of Na\(^+\) into the vacuole in exchange for H\(^+\) (Blumwald 2000). Constitutive expression of OsNHX1 from rice (Fukuda et al. 2004; Chen et al. 2007) and AgNHX1 from the halophytic plant Atriplex gmelini (Ohta et al. 2002) lead to the improvement of salinity tolerance in rice. Unfortunately, transgenic rice overexpressing OsNHX1 also showed alterations in plant development, such as delayed flowering and growth (Chen et al. 2007). Analyses on the single knockout mutant of AtNHX1 (Apse et al. 2003) and double knockout mutant of AtNHX1 and AtNHX2 (Bassil et al. 2011) indicated that AtNHX1 is not only important in the Na\(^+\) and K\(^+\) homeostasis, but also influences developmental processes.
Na\(^{+}\) sequestration into the vacuole can also be improved by constitutively expressing *AtAVP1*, the gene encoding a vacuolar H\(^{+}\)-pyrophosphatase, which generates a proton gradient across the tonoplast important for Na\(^{+}\)/H\(^{+}\) exchange (Gaxiola et al. 2001). Many studies have shown a significant improvement in salt tolerance by overexpressing this gene or its homologue in many species including *Arabidopsis* (Gaxiola et al. 2001), tomato (Park et al. 2005), alfalfa (Bao et al. 2009b), cotton (Pasapula et al. 2011) and barley (Schilling et al. 2013). More importantly, the constitutive expression of the gene also enhanced salinity tolerance in rice (Liu et al. 2010b; Kim et al. 2013).

Reducing the root concentration of Na\(^{+}\) in Fatmawati could also be an area of improvement – turning Fatmawati more salt tolerant. Root growth is less affected by salt stress compare to leaves (Rodriguez et al. 1997; Munns 2002). However, long term effects of Na\(^{+}\) accumulation in the roots might be detrimental. For example, accumulation of salt in the leaf apoplast caused cell dehydration which resulted in leaf damage (Flowers et al. 1991). It is of interest to understand where Na\(^{+}\) accumulated in the roots of Fatmawati, whether it is in the apoplast (Muhling and Lauchli 2002), the cytoplasm (Halperin and Lynch 2003) or vacuoles (Garbarino and DuPont 1989).

Expression of genes responsible for Na\(^{+}\) efflux specifically in the outer part of rice roots has the potential to reduce the accumulation of Na\(^{+}\) in the root. One potential gene for this strategy is the Na\(^{+}\) pumping ATPase from the moss *Physcomitrella patens* (*PpEna1*) (Lunde et al. 2007; Jacobs et al. 2011). Constitutive expression of *PpEna1* in rice Nipponbare under the *CaMV35S* promoter resulted in improved salinity tolerance (Jacobs et al. 2011). More specific control of this gene by expressing it in root epidermal cells (Plett and Møller 2010) might increase the salinity tolerance of Fatmawati.

For tissue tolerant cultivars such as IR64, reducing Na\(^{+}\) transport from the root to the shoot can be a potential approach to pyramid favourable traits. Work in *Arabidopsis* has shown that specific expression of the Na\(^{+}\) transporter *AtHKT1;1* in root xylem parenchyma cells significantly reduced shoot Na\(^{+}\) concentration and improved salinity tolerance (Møller et al. 2009). Specific expression of *AtHKT1;1* in the root cortex also lead to the reduction of shoot Na\(^{+}\) and improved salinity tolerance of rice Nipponbare, even though the mechanism was not directly related to the retrieval of Na\(^{+}\) from the xylem (Plett et al. 2010). It will be interesting to transfer this approach to cultivars such as IR64 to test whether it will further improve the salinity tolerance.
3.4.5. Transformation efficiency of Indonesian rice cultivars

A preliminary experiment has been conducted to study the efficiency of Agrobacterium-mediated transformation in five commercial cultivars from Indonesia. Agrobacterium-mediated transformation was chosen to generate salt tolerant transgenic rice because of its advantages over particle bombardment transformation, which was also available at the ACPF. In rice, Agrobacterium transformation results in transgenic plants with a lower number of T-DNA inserts, more intact transgenes and more stable gene expression compared to particle bombardment (Dai et al. 2001; Hiei and Komari 2008). To date, there were numerous reports on the use of Agrobacterium-mediated transformation to generate transgenic rice for functional genomics studies or crop improvement. Hiei and Komari (2008) reported that more than 80% of rice transformation genetic studies used Agrobacterium-mediated transformation.

The availability of actively growing tissue as a starting material for transformation is the first critical point when using Agrobacterium-mediated transformation (Birch 1997; Shrawat and Lörz 2006; Nishimura et al. 2007; Hiei and Komari 2008). Callus derived from scutellum of mature seeds was found to be the best tissue for Agrobacterium-mediated transformation (Hiei et al. 1994). For recalcitrant rice such as the indica group, high transformation efficiency was mostly achieved using immature embryos as a starting material (Nishimura et al. 2007; Hiei and Komari 2008; Slamet-Loedin et al. 2014). However, preparation of immature embryos is challenging because it needs actively growing rice plants throughout the year with different developmental stages of seed to ensure the correct stage of embryo can be used for transformation (Hiei and Komari 2008). Due to this difficulty, Agrobacterium-mediated transformation in this study used callus from mature seeds as the starting material. The protocol described by Sallaud et al. (2003) was used as the primary reference. The protocol has been routinely used at the ACPF and all reagents and equipment to support the protocol were available.

Modification of the medium was made by replacing N6 with MS for basal salts (Table 3-1 and Table 3-4). The main difference between N6 and MS is the composition of their inorganic nitrogen (Armstrong and Green 1985). An initial experiment showed that medium with MS gave higher callus induction rates of Indonesian rice cultivars compared to N6 (Table 3-4). Other studies with indica rice also preferred MS over N6 in their medium for callus induction (Khanna and Raina 1999; Kumar et al. 2005; Sahoo et al. 2011; Sahoo and Tuteja 2012). The callus induction response of rice cultivars was
genotype dependent and in many cases callus culture techniques were not reproducible in different laboratories (Hiei and Komari 2008).

The method used in this study gave a variable rate of callus induction of Indonesian cultivars (Table 3-4). Fatmawati, IR64 and Inpara 5 had higher callus induction (>50%) and also better callus proliferation compared to Ciherang and Dendang. The quality of calli developed were variable and Fatmawati exhibited better calli compared to the others which was indicated by dry, compact, yellowish and nodular calli as characteristic of embryogenic calli (Rueb et al. 1994). In contrast, calli from other cultivars were wet and soft with many rhizogenic regions, which were an indication of non-embryogenic calli (Rueb et al. 1994; Sahoo et al. 2011). Further modifications of media for callus culture were not carried out because of the time constraints of this project. In subsequent experiments medium with MS as basal salt was continuously used. To obtain sufficient calli for transformation, the number of calli were increased by increasing the number of seed inoculated.

Overall, the efficiency of Agrobacterium mediated transformation of Indonesian rice cultivars was low, mainly as a consequence of the low level of regeneration. In Fatmawati, only 28% of putative transformed calli (after 4th selection medium) regenerated shoots, and only 18% in IR64. None of the putative transformed calli of Inpara 5, Ciherang and Dendang regenerated shoots (Table 3-5). The low regeneration ability has been previously reported as a major constraint in indica rice transformation (Khanna and Raina 1999; Nishimura et al. 2007). A range of factors contribute to the regeneration ability of rice during transformation including the genotype, quality of calli, media composition, gelling agent, composition of growth regulators, antibiotic concentrations and the length of exposure to antibiotics (Abe and Futsuhara 1986; Khanna and Raina 1999; Shrawat and Lörz 2006; Nishimura et al. 2007). However, it was not possible to make adjustments to these factors to increase the regeneration of all cultivars in this study because of time limitations.

Overall, transgenic commercially relevant indica rice cultivars Fatmawati and IR64 have been successfully generated using the Agrobacterium-mediated transformation even though the efficiencies were low. Fertile and single T-DNA insert transgenic rice lines have been successfully generated expressing AtAVP1 driven by the Ubiquitin-1 promoter. The protocol described in this chapter was subsequently used to transform other
expression vectors to generate salt tolerant transgenic rice and to study regulatory elements for putative cell type-specific expression.

3.5. Summary

This chapter highlights the variation of (i) salinity tolerance of Indonesian rice cultivars and (ii) their transformation efficiency using Agrobacterium-mediated transformation. Variation existed in the salinity tolerance mechanisms among the rice cultivars, offering the potential to use different approaches for improving salinity tolerance.

Results from Agrobacterium-mediated transformation studies revealed that only two cultivars, Fatmawati and IR64, could be transformed with the described protocol. Fertile and low copy number transgenic rice plants have been successfully generated using this technique. The developed protocol and the two rice cultivars, Fatmawati and IR64, will be used in the subsequent transformation work to generate transgenic rice for salt tolerance improvement and for promoter studies using reporter genes.
Chapter 4 : Image-Based Phenotyping for Non-Destructive Screening of Different Salinity Tolerance Traits in Rice

Introduction
This chapter contains a manuscript to be submitted for review to the Rice Journal. The chapter is formatted according to the author’s instructions by the journal.
STATEMENT OF AUTHORSHIP

Image-based phenotyping for non-destructive screening of different salinity tolerance traits in rice

Manuscript submitted to Rice journal.

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Image-based phenotyping for non-destructive screening of different salinity tolerance traits in rice

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Abstract

Background

Soil salinity is an abiotic stress wide spread in rice producing areas, limiting both plant growth and yield. The development of salt-tolerant rice requires efficient and high-throughput screening techniques to identify promising lines for salt affected areas. Advances made in image-based phenotyping techniques provide an opportunity to use non-destructive imaging to screen for salinity tolerance traits in a wide range of germplasm in a reliable, quantitative and efficient way. However, the application of image-based phenotyping in the development of salt-tolerant rice remains limited. The aim of this study was to establish a screening protocol for quantifying traits hypothesised to contribute to salinity tolerance in rice using high-throughput image analysis.

Results

A non-destructive image-based phenotyping protocol to assess salinity tolerance traits of rice has been established in this study. The response of rice to different levels of salt stress was quantified over time based on total shoot area and senescent shoot area, calculated from colour and fluorescence images. The response of rice to moderate salt stress (50 mM NaCl) could be clearly distinguished from the control as indicated by the reduced increase of shoot area. The salt concentrations used had only a small effect on the growth of rice during the initial phase of stress, the shoot Na$^+$ accumulation independent phase termed the ‘osmotic stress’ phase. However, after 20 d of treatment, the shoot area of salt stressed plants was dramatically reduced compared with non-stressed plants. This was accompanied by a significant increase in the concentration of Na$^+$ in the shoot. Variation in the senescent area of the cultivars IR64 and Fatmawati in response to a high concentration of Na$^+$ in the shoot indicates variation in tissue tolerance mechanisms between the cultivars.
Conclusions

Image analysis has the potential to be used for high-throughput screening procedures in the development of salt-tolerant rice. The ability of image analysis to discriminate between different tolerance mechanisms makes it a useful tool for genetic and physiological studies to elucidate salinity tolerance mechanisms in rice. The technique has the potential for identifying the genetic basis of these mechanisms and assisting in pyramiding different tolerance mechanisms into breeding lines.

Keywords

Rice (*Oryza sativa* L.), salinity tolerance, phenotyping, image analysis

Background

Salinity is a major abiotic stress that threatens the sustainability of global rice production. Rice yield can be reduced significantly by the addition of as little as 50 mM NaCl (Yeo and Flowers 1986), making it one of the crop species most susceptible to salt stress (Grattan et al. 2002; Munns and Tester 2008). It has been estimated that about 48 million ha of potentially useful agricultural land is unusable for growing rice in Southern Asia and South East Asia due to saline soils (Ponnamperuma and Bandyopadhya 1980). The cultivation of salt-tolerant rice is important to maintain the sustainability of rice production in such areas. However, progress with breeding programmes to develop salt-tolerant rice has been slow (Gregorio et al. 2002; Flowers 2004; Yamaguchi and Blumwald 2005). One of the limiting factors in the breeding of salt tolerant rice is the availability of efficient and reliable screening techniques to select tolerant plants (Gregorio et al. 2002).

A number of screening methods for different physiological traits have been used to measure salinity tolerance in rice, including shoot weight (Yeo et al. 1990; Aslam et al. 1993), shoot Na⁺ concentration, the ratio of shoot Na⁺/K⁺ (Yeo et al. 1988; Gregorio and Senadhira 1993; Asch et al. 2000), leaf injury and survival rate (Yeo et al. 1990;
Gregorio et al. 1997), leaf area (Akita and Cabuslay 1990; Zeng et al. 2003) and bypass flow in the root (Faiyue et al. 2012). Of these traits, shoot weight was shown to be closely related to overall plant performance (Yeo et al. 1990) and to the performance of the plant in the field (Aslam et al. 1993). However, most protocols that measure plant biomass are destructive, thus making it difficult to measure dynamic responses in plant growth in response to salt application and to collect seed from the individuals being measured. Recent developments in image-based phenotyping have enabled the non-destructive assessment of plant responses to salinity over time and allows determination of shoot biomass measurements without having to harvest the whole plant (Rajendran et al. 2009; Furbank and Tester 2011; Berger et al. 2012b).

When plants are exposed to salt, their growth immediately slows due to the shoot ion independent stress, the so-called osmotic component of salt stress, and plants produce fewer tillers (Munns and Tester 2008; Rajendran et al. 2009; Horie et al. 2012). Over time, Na$^+$ and Cl$^-$ will accumulate to toxic concentrations in the shoot, resulting in premature leaf senescence and death – the ionic component of salt stress (Tester and Davenport 2003; Munns and Tester 2008; Munns 2010; Horie et al. 2012). Importantly, image-based phenotyping can differentiate between the effects of the osmotic and ionic components of salt stress in growing plants. This can be done by measuring growth responses immediately after salt application, before the accumulation of toxic concentrations of ions in the shoot. This allows for at least some dissection of salinity tolerance mechanisms (Rajendran et al. 2009; Sirault et al. 2009).

Several studies have used image based phenotyping to measure salinity tolerance in crops, in particular wheat and barley (Rajendran et al. 2009; Sirault et al. 2009; Harris et al. 2010), where digital colour images were used to quantify plant biomass, leaf area and health (Rajendran et al. 2009; Harris et al. 2010; Golzarian et al. 2011). The measurement of senescent leaf area in combination with the measurement of shoot Na$^+$
concentration enabled the quantification of shoot tissue tolerance in salt stressed einkorn wheat (*Triticum monococcum*) (Rajendran et al. 2009). Infrared thermography has also been used to measure leaf temperature, as a surrogate for stomatal conductance, to screen the osmotic tolerance of barley and durum wheat seedlings (Sirault et al. 2009). To our knowledge, there has been no report to date on using image-based phenotyping to assess salinity tolerance in rice. In the current study, high-throughput image acquisition and analysis was used to study the salinity tolerance traits of two rice cultivars (IR64 and Fatmawati) under different levels of salt stress. The use of this technology for screening individual salt tolerance traits in rice, as well as whole plant salt tolerance, is demonstrated here. These methods can now be used in genetic studies to inform breeding programs of approaches to improve the salinity tolerance of rice.

**Results and Discussion**

**Imaging as a surrogate for rice shoot biomass measurements**

Non-destructive imaging of plants allows multiple measurements of plant growth and plant health on the same individual over time, without having to harvest plant material for analysis (Rajendran et al. 2009; Furbank and Tester 2011; Berger et al. 2012b; Fiorani and Schurr 2013). Non-destructive imaging is important when measuring the dynamic response of individual plants to the onset of an environmental stress such as salinity and water deficit. It is also important to use non-destructive techniques when plants are unique, such as early generation transgenics, which need to be phenotyped but also maintained for seed collection. Key to the success of this technique is that the measurements obtained are quantitative, quick to obtain and a good surrogate for important traits, such as determination of plant biomass.

A number of studies have used projected shoot area to estimate the shoot biomass of different crops, such as wheat and barley under conditions of salinity stress (Rajendran et al. 2009; Harris et al. 2010; Golzarian et al. 2011).
To determine whether shoot biomass of rice plants correlated with the measurements of projected shoot area, and is therefore a quantifiable parameter that can be used to measure plant biomass, RGB images were obtained of plants that had been exposed to various salt stress levels for 20 d (Figure 1) before the fresh weight of each plant was determined by destructive harvest. The projected shoot area was calculated based on two side view images (at 90° from each other) and one top view image (Figure 1). There was a strong positive correlation between the projected shoot area obtained by image analysis and shoot fresh weight in the two rice cultivars, Fatmawati ($R^2=0.97$) and IR64 ($R^2=0.98$) (Figure 2) and there was no indication of any deviation from a linear relationship even at the highest biomasses measured in this experiment. Projected shoot area is therefore a suitable surrogate for rice shoot biomass up to six weeks of age and 24 g of shoot fresh weight (Figure 2).

**IR64 and Fatmawati differ in their response to salt stress**

IR64 and Fatmawati were grown in soil under moderate (0, 50, 75 and 100 mM NaCl) and high (0, 100, 150 and 200 mM NaCl) salt stress. Digital images were taken through time, at 0, 10, and 20 d after salt application. Measurements of plant growth are important to permit an understanding of the physiological mechanisms underlying the plant response to salt stress over time (Munns 2010), particularly to separate the effects of the osmotic and ionic stress components, which dominate plant growth at different times (Munns and Tester 2008).

Interestingly, under these growth conditions there is no apparent reduction in biomass production immediately after salt application (between days 0 and 10), suggesting little response to the osmotic stress component of salt stress in the growing rice plants (Figure 3 and Figure 4). This finding differs to the responses observed in other cereal species, such as einkorn wheat (*T. monococcum*) (Rajendran et al. 2009), durum wheat (James et al. 2008; Rahnama et al. 2010) and bread wheat (Rahnama et al. 2011), where
an immediate response to salt application can be seen - the shoot ion independent stress (osmotic stress) (Munns and Tester 2008). This early reduction in plant growth is not observed in either IR64 or Fatmawati, suggesting these cultivars have good levels of osmotic tolerance, and that later reductions in growth are likely to be due to ionic stress leading to an increase in premature senescence. A similar observation was reported by Moradi and Ismail (2007), where a significant reduction in growth in the seedling stage of rice only became visible after 2 weeks of salt stress. The authors hypothesised that salt tolerant rice plants have the ability to control their stomatal conductance during the initial stress and recover their growth immediately through an acclimation process (Moradi and Ismail 2007). It is well known that IR64 is a moderately salt tolerant rice variety and has been used intensively in salinity tolerance studies (Asch et al. 2000; Ueda et al. 2006; Castillo et al. 2007; Nakhoda et al. 2012), however, no information is available on the salinity tolerance of Fatmawati.

After 20 d of salt treatment, however, the projected shoot area of salt stressed rice was clearly reduced compared with that of the non-stressed plants, even at 50 mM NaCl. The reduction in shoot area was more pronounced under the higher concentrations of salt with the shoot area of Fatmawati and IR64 reduced by 37% and 30%, respectively, after 20 d of growth in 100 mM NaCl when compared with non-stressed plants (Figure 3). There was a slight difference in response of rice plants to 100 mM NaCl treatment between two subsequent experiments, which might be attributed to different soil batches being used (Figure 3 and Figure 4). In the second experiment (high salt stress; Figure 4), after 20 d of growth under 100 mM NaCl the shoot area of Fatmawati and IR64 was reduced by 49% and 54% compared to control plants, respectively (Figure 4). However, the 0 mM NaCl plants in the second experiment (Figure 4) grew bigger than in the first experiment (low salt stress; Figure 3) for both Fatmawati and IR64, while the plants grown in 100 mM NaCl reached a similar size in both experiments. The increase in
biomass reduction is therefore primarily a result of the control plants growing bigger in the second experiment. The late reduction in growth rate in response to salinity in both experiments suggests that the plants experience ionic stress, which can be determined by measurements of leaf senescence and leaf ion concentration.

**Fatmawati exhibits greater shoot senescence under salinity stress than IR64**

A common method for determining rates of senescence and leaf injury is the visual scoring of symptoms, classifying individuals on an integer scale from zero to an arbitrary upper value (Gregorio et al. 1997; Negrão et al. 2011). However, this process is subjective, and can vary depending on the person making the observations. In this study, fluorescence image analysis was used to objectively quantify the degree of leaf senescence of salt stressed plants over time. No difference in senescent area could be observed between stressed and control plants 10 d after salt application. However, the total senescent area was found to increase dramatically by 20 d (Figure 5).

The cultivar Fatmawati appears to be significantly more salt sensitive than IR64, showing considerable shoot senescence (23%) when exposed to 200 mM NaCl for 20 d (Figure 5A). IR64, in contrast, exhibited little shoot senescence (4%), even under very high NaCl concentrations (Figure 5B). While there was an increase in shoot senescent area that corresponded to increasing salt concentrations, both cultivars had little senescence at moderate salinity levels (Figure 5A and Figure 5B).

**IR64 plants accumulate sodium in the shoot while exhibiting low levels of shoot senescence**

The concentrations of Na$^+$ and K$^+$ in the youngest, fully expanded leaf of both IR64 and Fatmawati were measured after 20 d of salt application. Leaf Na$^+$ concentrations of the stressed plants increased as expected with increasing external NaCl concentration (Table 1 and Table 2). The amount of Na$^+$ in the leaf was cultivar dependent, with IR64 accumulating more leaf Na$^+$ than Fatmawati (Table 1 and Table 2). However, despite
accumulating lower concentrations of Na\(^+\) in the leaf, Fatmawati was found to have higher levels of shoot senescence (Table 2 and Figure 5). This suggests that IR64 may have more efficient Na\(^+\) tissue tolerance mechanisms, such as the ability to accumulate Na\(^+\) in the vacuoles of leaf cells (Garbarino and DuPont 1989; Blumwald et al. 2000). Sub-cellular measurements of ion accumulation and whole plant/tissue ion fluxes will help elucidate the mechanisms involved (James et al. 2006b; Møller et al. 2009; Plett et al. 2010). Interestingly, the concentrations of shoot K\(^+\) in the stressed plants were higher than those of the control plants, perhaps suggesting that rice attempts to maintain as high a K\(^+\) concentration as possible during salt stress. However, shoot K\(^+\)/Na\(^+\) ratios in the stressed plants were significantly lower than those of the non-stressed plants (Table 1 and Table 2).

It was not possible to measure Na\(^+\) and K\(^+\) concentrations in the last fully expanded leaf of Fatmawati plants grown in 200 mM NaCl due to the high level of senescence (Table 2 and Figure 5).

**A role for non-destructive phenotyping in screening for rice salinity tolerance**

Automation of the phenotyping process in combination with automated plant handling and watering allows large numbers of plants to be screened efficiently with limited handling. Entire populations of plants can be grown in soil media, emulating field conditions (at least for the earlier stages of growth), thus facilitating the transfer of knowledge from controlled environment to growth conditions in the field. An increasing number of phenotyping facilities are now accessible globally, such as the Australian Plant Phenomics Facility (http://www.plantphenomics.org.au), used in this study, or the centres of the European Plant Phenomics Network (http://www.plant-phenotyping-network.eu). The process described here has the potential to be scaled to phenotype large numbers of rice breeding lines and mapping populations allowing the evaluation of the effect of different salt tolerant mechanisms on plant growth and yield. Screening
of hundreds of mapping lines and/or rice accessions for bi-parental or association mapping studies can now be done relatively quickly for traits that require time course measurements of growth. The use of such populations has the potential to identify the underlying genetic mechanisms of salinity tolerance in a forward genetics screen.

**Conclusions**

An efficient and high-throughput screening protocol to select salt-tolerant rice is required to accelerate the development of salt-tolerant rice cultivars. A non-destructive image-based phenotyping method to analyse the responses of rice to different levels of salinity stress has been developed and revealed differences in the effects of salt stress in two cultivars of rice, IR64 and Fatmawati. Use of non-destructive imaging technologies, such as those described here, in combination with measurements of tissue ion concentration, allow the differentiation between the ionic and osmotic components of salt stress in growing rice. This will enable the identification of new traits and sources of salinity tolerance genes that can be used to pyramid different salinity tolerance mechanisms into elite rice breeding lines.

**Methods**

**Plant material and growth conditions**

Two Indica rice cultivars, IR64 and Fatmawati, were used in this study. IR64 was developed at the International Rice Research Institute (IRRI) in the Philippines and is known as a “mega variety” because of its wide adoption and cultivation in large areas (Mackill 2008). Fatmawati is a new plant type of rice from Indonesia (Abdullah et al. 2008) which has new morphological traits, such as fewer tillers, sturdy culms and higher number of seed per panicle compared to traditional rice cultivars (Khush 1995; Abdullah et al. 2008). IR64 and Fatmawati seeds were obtained from the Indonesian Centre for Rice Research, Sukamandi, Indonesia.
Rice seeds were sorted for uniform size and then dehusked and surface sterilised with 70% (v/v) ethanol for one min followed by a 30 min bleach treatment (30% (v/v) commercial Domestos bleach solution, sodium hypochlorite 49.9 g/L). The seeds were then washed with RO water five times to remove all traces of bleach. Surface sterilised seeds were germinated by placing them on sterile wet filter paper in dishes. The dishes were sealed to prevent evaporation and contamination and placed in a growth chamber with 12 h light and a constant temperature of 28°C. After seven days, uniformly germinated seedlings were transferred into white plastic pots (150 mm diameter × 200 mm height) containing 3 kg dry University of California (U.C.) soil mix (Matkin and Chandler 1957), composed of sand and peat moss (volume ratio 1.6:1) and fertiliser (1.5 kg Mini Osmocote® per 600 litre UC soil base). The soil surface was covered by blue plastic pellets to reduce development of algae, reduce evaporation and to provide a favourable background colour for image analysis. Three rice seedlings were transplanted into each pot but were thinned to a single plant per pot after one week. The plastic pots had drainage holes in their base, allowing watering from underneath (Figure 1A). The pots were placed in a deep white plastic saucer (160 mm x 160 mm x 90 mm) to which a volume of 600 mL of water was applied to each pot via the saucer. Water levels were monitored daily by weighing the pots using a digital scale, and pots were adjusted to the target weight by adding water to maintain a constant salt concentration in each pot.

The rice plants were placed in a growth chamber (Conviron, Model PGC20) at The Plant Accelerator® (Australian Plant Phenomics Facility, University of Adelaide, Adelaide, Australia) with a 12hr/12hr day/night cycle and a maximum photon irradiance of 700 µmol/m²/s. Humidity was maintained at 70% with day/night temperatures of 28°C/26°C, respectively.
Salt treatment

Two separate salt experiments (moderate salt stress and high salt stress) were carried out sequentially in 2012. The moderate salt experiment was conducted from 15 February to 26 March 2012 and the high salt experiment was from 23 March to 3 May 2012. In the moderate salt stress experiment, the rice cultivars were subjected to four levels of salt, 0 mM, 50 mM, 75 mM and 100 mM NaCl. In the high salt stress experiment the salt levels were 0 mM, 100 mM, 150 mM and 200 mM NaCl. Salt treatments were imposed 14 d after seedling transplantation in 50 mM increments every 12 h until the desired level was reached to minimise the osmotic shock to the plants. For each increment, 60 mL of a 0.5 M NaCl solution was applied to the saucer in which the pots sat. Additional CaCl$_2$ was added to prevent Na$^+$ induced Ca$^{2+}$ deficiency with the ratio of Na$^+$:Ca$^{2+}$ molar concentration of 30:1 (3.3 mM CaCl$_2$ for 100 mM NaCl (Genc et al. 2010)). The concentrations of NaCl in the soil were maintained at constant levels by watering each pot to weight, as described in the previous section.

Image capture and image analysis

Shoot images were taken using the LemnaTec 3D Sc analyzer system (LemnaTec GmbH, Aachen, Germany) at The Plant Accelerator®. The pots were manually loaded onto the conveyer belt and automatically moved to the image capture stations. Three colour images (RGB images) were taken per plant, two from the side at 90° from each other and one from the top. Three fluorescent images were taken in a separate imaging chamber with blue light excitation (400nm to 500nm) and a colour camera with a 500nm high pass filter capturing fluorescence emission from 500nm to 750nm. After image capture, all images were analysed using the LemnaTec Grid software package (LemnaTec GmbH, Aachen, Germany).

In brief, the plant was separated from the imaging background using a nearest-neighbour colour classification. Noise was removed from the images using erosion and
dilation steps as well as a size filter. Subsequently, all objects identified as being part of the plant were composed to one single object. The colour images were used to measure size and height of the object (Berger et al. 2012a). The summed area of all three images per plant was used as an approximation for shoot biomass (Rajendran et al. 2009; Golzarian et al. 2011; Berger et al. 2012a). The top view fluorescent images were used to quantify the level of shoot senescence. After object separation from the background and noise reduction, nearest-neighbour colour classification was used to separate the shoot into healthy leaf area (red chlorophyll fluorescence) and senescent leaf area (yellow fluorescence; Figure 1D). The level of senescence was calculated as the percentage of senescence pixels relative to total shoot area.

**Measurement of shoot biomass and shoot ion concentration**

Shoots were harvested 20 d after salt application and the fresh weight was measured using a digital scale. Leaf tissue for shoot Na\(^{+}\) and K\(^{+}\) measurements were taken from the youngest fully expanded leaf at 20 d after salt application. The leaves were weighed immediately after harvest to determine their fresh weight and then dried in an oven at 70°C for 24 hours. The dry weight was measured to determine the tissue water content. Dried leaf samples were placed in 50 mL Falcon tubes and digested in 20 mL 1% (v/v) nitric acid (HNO\(_3\)) for 5 h in a heat block at 70°C. The samples were shaken every hour to ensure complete digestion. The concentrations of Na\(^{+}\) and K\(^{+}\) were determined using a flame photometer (model 420; Sherwood Scientific Ltd., Cambridge, UK).
Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

AH carried out the experiments. SR, BB, MT conceived and supervised the experiments. AH and BB analysed the data. AH, BB and SR wrote the manuscript. All authors read and commented on the manuscript.

Acknowledgments

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References

The list of references of this manuscript is included in the thesis references.
Table 1. Shoot Na\(^+\) and K\(^+\) accumulation and K\(^+\)/Na\(^+\) ratio of rice cv. Fatmawati and IR64 at 20 d after application of a moderate salt stress (50, 75 and 100 mM NaCl).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Salt stress (mM NaCl)</th>
<th>Na(^+) (mM)</th>
<th>K(^+) (mM)</th>
<th>Shoot K(^+)/Na(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatmawati</td>
<td>0</td>
<td>1.57 ± 0.12 a</td>
<td>297.35 ± 6.60 a</td>
<td>196.82 ± 16.57</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.39 ± 0.25 b</td>
<td>343.02 ± 6.61 b</td>
<td>150.98 ± 14.09</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>3.31 ± 0.16 c</td>
<td>362.03 ± 5.63 bc</td>
<td>110.95 ± 5.37</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.35 ± 0.22 c</td>
<td>365.59 ± 7.16 c</td>
<td>111.75 ± 6.55</td>
</tr>
<tr>
<td>IR64</td>
<td>0</td>
<td>2.54 ± 0.13 b</td>
<td>345.07 ± 7.43 b</td>
<td>137.44 ± 5.12</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.77 ± 0.33 d</td>
<td>357.31 ± 10.03 bc</td>
<td>76.66 ± 4.35</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>5.67 ± 0.40 e</td>
<td>365.41 ± 3.48 c</td>
<td>66.36 ± 4.69</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6.06 ± 0.35 e</td>
<td>385.54 ± 7.21 d</td>
<td>65.04 ± 4.28</td>
</tr>
</tbody>
</table>

F-test Cultivar (C) ** NS ** **
Salt stress (S) ** NS **
C x S ** * NS

Values are the means ± SE (n=7). Values in the same column followed by the same letter are not significantly different according to LSD (0.05) test. ** and * = significant at P < 0.01 and 0.05, respectively. ns= not significant
Table 2. Shoot Na\(^+\) and K\(^+\) accumulation and K\(^+/\)Na\(^+\) ratio of rice cv. Fatmawati and IR64 at 20 d after application of a high salt stress (100, 150 and 200 mM NaCl).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Salt stress (mM NaCl)</th>
<th>Na(^+) (mM)</th>
<th>K(^+) (mM)</th>
<th>Shoot K(^+)/Na(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatmawati</td>
<td>0</td>
<td>1.30 ± 0.07</td>
<td>265.30 ± 5.53</td>
<td>208.32 ± 12.28 b</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.81 ± 0.09</td>
<td>326.12 ± 8.19</td>
<td>85.81 ± 2.14 c</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>8.21 ± 0.99</td>
<td>346.34 ± 9.76</td>
<td>46.32 ± 6.06 d</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>IR64</td>
<td>0</td>
<td>0.63 ± 0.06</td>
<td>284.44 ± 4.80</td>
<td>465.21 ± 47.99 a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5.91 ± 0.40</td>
<td>320.00 ± 6.96</td>
<td>55.38 ± 4.80 cd</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>11.62 ± 1.06</td>
<td>325.68 ± 6.01</td>
<td>29.11 ± 2.46 d</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>12.45 ± 1.33</td>
<td>366.38 ± 11.05</td>
<td>31.24 ± 2.86 d</td>
</tr>
</tbody>
</table>

F-test

<table>
<thead>
<tr>
<th>F-test</th>
<th>Cultivar (C)</th>
<th>Salt stress (S)</th>
<th>C x S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>**</td>
<td>**</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ± SE (n=7). NA= data not available due to the high level of senescence in this cultivar. Values in the same column followed by the same letter are not significantly different according to LSD (0.05) test. ** = significant at \( P < 0.01 \). ns= not significant.
Figure 1. Example images of rice cv. Fatmawati taken 20 d after salt application.

Salt stress was imposed two weeks after transplantation. From left to right: image of rice treated with 0 mM, 100 mM, 150 mM and 200 mM NaCl. (A) Side view RGB images of rice plants. (B) Identified object after image analysis for size measurement. (C) Top view RGB images of the same plants shown in A. (D) Corresponding fluorescent images taken from above. (E) Colour classified images derived from fluorescence images in D, where green depicts healthy leaves and purple indicates senescent areas. Bars = 10 cm.
Figure 2. Relationship between projected shoot areas and shoot fresh weight of rice. A positive linear relationship was observed in both rice cv. Fatmawati ($R^2=0.97$, $n=56$) and IR64 ($R^2=0.98$, $n=56$). The projected shoot area of rice cv. Fatmawati (blue) and IR64 (red) growing under moderate and high salt stress levels were obtained 20 d after salt application prior to harvest for total shoot fresh weight. The circles represent salt stressed samples (50, 75, 100, 150 and 200 mM NaCl), the triangles represent non-stressed samples.
Figure 3. Projected shoot area of rice cv. (A) Fatmawati and (B) IR64 in moderate salt stress. The salt levels were 0 mM, 50 mM, 75 mM and 100 mM NaCl, imposed two weeks after transplantation. Values are the means ± SEM (n=7).
Figure 4. Projected shoot area of rice cv. (A) Fatmawati and (B) IR64 in high salt stress. The salt levels were 0 mM, 100 mM, 150 mM and 200 mM NaCl, imposed two weeks after transplantation. Values are the means ± SEM (n=7).
Figure 5. Percentage of senescent area of rice cv. (A) Fatmawati and (B) IR64 in high salt stress. The salt levels were 0 mM, 100 mM, 150 mM and 200 mM NaCl, imposed two weeks after transplantation. Senescent area was determined through colour classification from the top view fluorescent images. The results are presented as the percentage of senescent pixels area to the total shoot area of the top view image.
Chapter 5 : Constitutive Expression of Candidate Genes for Improving Salt Tolerance

5.1. Introduction

Plants cope with high salt concentrations in soil using multiple mechanisms to tolerate both the osmotic and ionic stresses imposed on them (Tester and Davenport 2003; Apse and Blumwald 2007; Munns and Tester 2008). To reduce the ionic effect of the salt in the shoots, plants rely on mechanisms at the tissue and cellular levels, including: excluding Na\(^+\) from the cells, compartmentalizing Na\(^+\) in the vacuole and reducing the amount of Na\(^+\) in the transpirational stream that brings water from the root to the shoot (Tester and Davenport 2003; Apse and Blumwald 2007). These processes can involve various transporters and signalling elements located in different regions within the plant (Tester and Davenport 2003; Apse and Blumwald 2007).

The advances in functional characterization of Na\(^+\) transporters and the signalling elements responsible for Na\(^+\) homeostasis allow the use of genetic engineering to improve plant salt tolerance. Many reports show the effectiveness of altering Na\(^+\) transporters to improve salt tolerance. While for particular Na\(^+\) transporters, such as \(HKT1;1\), the expression of the gene in a specific cell type is necessary to improve salt tolerance (Møller et al. 2009; Plett et al. 2010), for many other genes, studies show that an improvement in salinity tolerance can be achieved using constitutive expression systems. An increase in salt tolerance was effectively achieved in a variety of plant species by the constitutive expression of a number of ion transporters and/or proton pumps: plasma membrane Na\(^+\)/H\(^+\) antiporters (Gao et al. 2003; Shi et al. 2003; Zhao et al. 2006a; Yang et al. 2009), vacuolar Na\(^+\)/H\(^+\) antiporters (Apse et al. 1999; Ohta et al. 2002; Fukuda et al. 2004; Chen et al. 2007; Liu et al. 2010b), vacuolar H\(^+\)-pumping pyrophosphatases (Gaxiola et al. 2001; Gao et al. 2006; Bao et al. 2009a; Liu et al. 2010b; Pasapula et al. 2011; Kim et al. 2013; Schilling et al. 2013) and Na\(^+\)-ATPases (Kong et al. 2008; Jacobs et al. 2011; Kishimoto et al. 2013).

Improvements in plant salt tolerance using a constitutive expression system have also been shown to be effective using the calcineurin B-like (CBL) interacting protein kinases
(CIPK) gene family. CIPKs are known to be involved in calcium signalling pathways in response to salt stress (Weinl and Kudla 2009). The constitutive expression of CIPK genes improved the salt tolerance of Arabidopsis (Roy et al. 2013), rice (Xiang et al. 2007), tomato (Huertas et al. 2012; Deng et al. 2013), and barley (Roy et al. 2013).

In comparison to other cereals, genetic engineering efforts to improve salt tolerance by manipulating Na⁺ transport in rice are more advanced. This is facilitated, amongst other things, by having an efficient transformation system in rice. However, the work so far has been limited to non-commercial rice cultivars, mainly from sub species japonica, due to their high efficiency in transformation. There has been little investigation as to whether the same approaches can be used to improve commercially relevant rice. Therefore, it is necessary to transfer this approach into more commercial rice cultivars to bring this promising technology to the field.

In this study, four candidate genes were selected for constitutive expression including type I vacuolar H⁺-pumping pyrophosphatase (H⁺-PPase) from Arabidopsis (AtAVP1) and from rice (OsOVP4), a calcineurin B-like (CBL) interacting protein kinase (CIPK) from Arabidopsis (AtCIPK16) and a Na⁺-ATPase from moss Physcomitrella patens (PpENA1). Fatmawati and IR64 were used as the genetic background as these cultivars exhibited higher transformation efficiency in Agrobacterium–mediated transformation compared to other commercial cultivars (Chapter 3). These two cultivars also showed a good level of salt tolerance with different mechanisms related to Na⁺ transport (Chapter 3 and Chapter 4) and were therefore ideal for testing the effectiveness of the transgene and comparing the gene effect in a different genetic background.

It is hypothesized that constitutive expression of these genes in commercial rice Fatmawati and IR64 could increase the salt tolerance of the cultivars above their current level of tolerance. To test this hypothesis, the study in this chapter aimed to:

1. Develop transgenic commercially relevant rice cultivars constitutively expressing candidate genes for salt tolerance.
2. Characterize the genotype and assess salinity tolerance of the T₁ transgenic lines.
Three consecutive experiments were designed to characterize transgenic rice constitutively expressing candidate genes for salt tolerance:

- Experiment 1: Characterization of transgenic Fatmawati constitutively expressing *AtAVP1* and *OsOVP4*.
- Experiment 2: Characterization of transgenic Fatmawati constitutively expressing *PpENA1* and *AtCIPK16*.
- Experiment 3: Characterization of transgenic IR64 constitutively expressing *AtAVP1* and *PpENA1*. 
5.2. Methods

5.2.1. Rice materials

Rice cultivars Fatmawati and IR64 were used as genetic background for generation of transgenic rice constitutively expressing candidate genes for salt tolerance improvement. The details of the two cultivars are described in Section 2.1.

5.2.2. Salt tolerance assay

5.2.2.1. Rice growth conditions

The phenotyping of transgenic rice lines was conducted in the greenhouse of The Plant Accelerator, Waite Campus, the University of Adelaide. The growth conditions were set up as described in Section 2.2.1. The specific conditions in each experiment are described in Table 5-1. Supplementary lights were supplied in Experiments 2 and 3 to maintain 12 hours day length.

Table 5-1 Day length and daily global solar exposure in Adelaide during the experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Date</th>
<th>Day length (hours)</th>
<th>Daily global solar exposure (monthly mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>5 February-28 March 2013</td>
<td>12-14</td>
<td>20.15 MJ·m⁻²</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>20 July-18 August 2013</td>
<td>10-11</td>
<td>10.7 MJ·m⁻²</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>27 August-25 September 2013</td>
<td>11-12</td>
<td>18.0 MJ·m⁻²</td>
</tr>
</tbody>
</table>

¹http://www.ga.gov.au/bin/geodesy/run/sunrisenset

5.2.2.2. Screening for salinity tolerance

Salt screening in Experiment 1 was performed in soil with two salt treatments: 0 mM and 100 mM NaCl. Non-destructive image analysis was used for phenotyping. The soil experiment system, salt application and image analysis were set up as described earlier in Chapter 4. Twelve to twenty T1 transgenic plants (including nulls) were grown in individual pots as biological replicates for each line.

The subsequent salt screenings (Experiment 2 and Experiment 3) were performed using hydroponics because of space limitations in the GMO physical containment facility for image analysis in The Plant Accelerator. The hydroponic salt screening in Experiments 2 and 3 was carried out as described in Section 2.2.4 and Section 3.2.2.2. Two salt treatments were used in the hydroponic system: 0 mM and 70 mM NaCl. Salt treatments were applied as described in Section 3.2.2.2. Twenty T1 transgenic plants (including nulls) were grown as biological replicates. Sampling for biomass and tissue ion concentration measurements were carried out as described in Section 2.3.

Separate hydroponic experiments were set up for determining the expression of the transgenes in the leaves and roots of transgenic and wild type plants.

5.2.3. Agrobacterium-mediated rice transformation

5.2.3.1. Plasmid constructs

Four candidate genes for improving salt tolerance in rice were expressed constitutively: Arabidopsis vacuolar H+-PPase (AtAVP1) (Gaxiola et al. 2001), rice vacuolar H+-PPase (OsOVP4) (Liu et al. 2010a; Plett et al. 2010), Na+ pumping ATPase from Physcomitrella patens (PpENA1) (Jacobs et al. 2011) and calcineurin B-like (CBL)-interacting protein kinase from Arabidopsis (AtCIPK16) (Roy et al. 2013). The maize Ubiquitin-1 (Ubi-1) and cauliflower mosaic virus (CaMV) 35S promoters were used to constitutively express the genes.

Ubi:AtAVP1 construct

The binary expression vector, pMDC32Ubi:AVP1 (13188 bp) (provided by Dr. Darren Plett, ACPFG), was used to express AtAVP1 under the control of Ubiquitin-1 promoter. The details of the construct are described in Section 3.2.3.
**35S:PpENA1 construct**

The binary expression vector, pAJ55 (provided by Dr. Andrew Jacobs, ACPFG), was used to constitutively express *PpENA1* under control of the *CaMV35S* promoter (Figure 5-1). *PpENA1* (2938bp) was cloned from protonemal tissue of *Physcomitrella patens* (Jacobs et al. 2011).

![Diagram of pAJ55 vector](image)

**Figure 5-1 Map of the expression vector pAJ55 containing PpENA1.**

The vector backbone contains the following components: kanamycin resistance gene, origin of replication for *E. coli* (pBR322 origin), and minimal replicon (pVS1) for stable maintenance in *E. coli*. The T-DNA cassette contains right border sequence (RB), the dual cauliflower mosaic virus 35S promoter (2×35S), Gateway® recombination sequences (*attB1* and *attB2*), the *PpENA1* coding sequence, the nopaline synthase (*Nos*) terminator, the hygromycin resistance gene and left border sequence (LB). The *BamHI* restriction site was used for digestion in Southern Blot analysis.
**Ubi:OsOVP4 construct**

The binary expression system, pMDC32Ubi:OVP4, was developed in this study to express *OsOVP4* (*Os02g0802500*) under the control of the maize *Ubiquitin-1* promoter. The Gateway®- enabled entry vector containing *OsOVP4* (pCR8:OVP4) was provided by Ms. Jodie Kretschmer and Dr. Andrew Jacobs (ACPFG). cDNA of *OsOVP4* was cloned from the root tissue of salt stressed Nipponbare. The entry vector, pCR8:OVP4, was cloned into the Gateway®-compatible destination vector pMDC32Ubi (provided by Dr. Darren Plett), through LR recombination as described in Section 2.4.12. The resultant expression vector (13164bp) is shown in Figure 5-2.

**Figure 5-2 Map of the expression vector pMDC32Ubi:OVP4 containing OsOVP4 (Os02g0802500).**

The vector backbone contains the following components: kanamycin resistance gene, the origin of replication for *E. coli* (pBR322 origin), and the minimal replicon (pVS1) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), maize *Ubiquitin-1* promoter (*Ubi-1*), Gateway® recombination sequences (*attB1* and *attB2*), the *OsOVP4* coding sequence, the nopaline synthase (*Nos*) terminator, the hygromycin resistance gene, and left border sequence (LB). The *EcoRV* restriction site was used for digestion in Southern Blot analysis.
The binary expression system, Ubi:CIPK16, was developed in this study to express AtCIPK16 (At2g25090) under the control of the constitutive promoter, maize Ubiquitin-1. The AtCIPK16 gene (2069bp) was cloned from Arabidopsis gDNA (Roy et al. 2013). The Gateway® enabled entry vector, pCR8 containing AtCIPK16 (Roy et al. 2013), was recombined into the Gateway® compatible destination vector, pMDC32Ubi (provided by Dr. Darren Plett), using Gateway LR recombination as described in Section 2.4.12. The resultant expression vector is shown in Figure 5-3.

**Figure 5-3 Map of the expression vector Ubi:CIPK16 containing AtCIPK16.**
The vector backbone contains the following components: kanamycin resistance gene, the origin of replication for *E. coli* (pBR322 origin), and the minimal replicon (pVS1) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), maize Ubiquitin-1 promoter (*Ubi1*), the Gateway® recombination sequences (attB1 and attB2), the AtCIPK16 coding sequence, the nopaline synthase (*Nos*) terminator, the hygromycin resistance gene and left border sequence (LB). The *HindIII* restriction site was used for digestion in Southern Blot analysis.
5.2.3.2. Rice transformation

The *Agrobacterium*-mediated rice transformations were carried out using the protocol described in Section 3.2.6.

5.2.4. Molecular characterization

Genomic DNA was extracted from T₀ and T₁ transgenic rice as described in Section 2.4.1. The presence of the transgene was confirmed by PCR (Section 2.4.3) using gene specific primers and primers amplifying the hygromycin (Hyg) resistance gene (Table 5-2). Specific primers were designed to distinguish between the native *OsOVP4* and the transgene *OsOVP4* by having a reverse primer anneal across the region between the end of the gene and the *Nos* terminator in the vector. Southern Blot analysis (Section 2.4.8), using a probe against the *Nos* terminator, was performed to determine the number of T-DNA insertions. Appropriate restriction enzymes were selected according to the sequence of the constructs (Figure 3-1, Figure 5-1, Figure 5-2, Figure 5-3).

Total RNA was extracted from salt stressed plants (Section 5.2.2.2) as described in Section 2.4.9. The RNA was then used for cDNA synthesis (Section 2.4.10). The expression of the transgene was analysed using reverse transcriptase (RT) PCR using the cDNA as template (Section 2.4.11) and amplified using gene specific primers (Table 5-2). The glyceraldehyde 3-phosphate dehydrogenase gene from rice (*OsGAPdh*) (Table 5-2) was used as the internal control gene for both PCR and RT-PCR.
Table 5-2 Primers used in genotyping of transgenic rice constitutively expressing candidate genes for salt tolerance improvement

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVP1-F</td>
<td>TGTTTTGACCCCTAAAGTTATC</td>
</tr>
<tr>
<td>AVP1-R</td>
<td>TGGCTCTGAACCCCTTTTGTC</td>
</tr>
<tr>
<td>CIPK16-F</td>
<td>TGATGTGATGAATTGGAAAGCG</td>
</tr>
<tr>
<td>CIPK16-R</td>
<td>ACTCTCAAGATTGCTTTGTGCCG</td>
</tr>
<tr>
<td>OVP4-NOS-F</td>
<td>TTCGCCACGCACGGAGGA</td>
</tr>
<tr>
<td>OVP4-NOS-R</td>
<td>AAGACCCGGCAACAGGATCAA</td>
</tr>
<tr>
<td>PpENA1F5</td>
<td>CCTACATGCTCCTCAGCATTT</td>
</tr>
<tr>
<td>PpENA1R5</td>
<td>GGACAAAGTCGCCAACAGTCT</td>
</tr>
<tr>
<td>OsGAPdh-F</td>
<td>GGGCTGCTAGCTTCAACATC</td>
</tr>
<tr>
<td>OsGAPdh-R</td>
<td>TTGATTGCAGCCTTGATCTAG</td>
</tr>
<tr>
<td>Hyg-F</td>
<td>TCACTGGCAAAACTGTGATGGAC</td>
</tr>
<tr>
<td>Hyg-R</td>
<td>GGTTCACATAGCGCGAGTAC</td>
</tr>
</tbody>
</table>
5.3. Results

5.3.1. Generation of transgenic rice constitutively expressing candidate genes for salt tolerance

Four candidate genes were expressed constitutively to determine if they could improve the salt tolerance of either Fatmawati and/or IR64. The number of fertile transgenic rice lines which were generated through Agrobacterium-mediated transformation can be found in Table 5-3. Unfortunately, despite up to three rounds of transformation attempts the number of fertile transgenic rice lines generated for phenotyping was limited for most of the constructs, in part due to the low transformation efficiency of both Fatmawati and IR64.

The integration of the transgene of interest was confirmed using both PCR and Southern Blot analysis. Ten independent T₁ transgenic rice lines constitutively expressing different genes for salt tolerance were selected for salt screening using either non-destructive image based phenotyping or hydroponic experiments (Table 5-4). Ideally for each gene and genetic background a minimum of three independent transformation events, each with only one T-DNA insertion, would have been selected to verify the effect of the transgene on the plant phenotype. Unfortunately, due to the low success in generating fertile, positive transgenic plants, the lines were selected based on the availability of seed for performing the screening.
Table 5-3 Summary of the results of *Agrobacterium*-mediated transformation of candidate genes for salt tolerance driven by constitutive promoters

<table>
<thead>
<tr>
<th>Genetic background</th>
<th>Promoter-gene combination</th>
<th>Number of transformation attempts</th>
<th>Number of transformed calli</th>
<th>Number of independent transgenic lines</th>
<th>Number of fertile transgenic lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatmawati</td>
<td>Ubi:AtAVP1</td>
<td>3</td>
<td>214</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Ubi:OsOVP4</td>
<td>1</td>
<td>75</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>35S:PpENA1</td>
<td>3</td>
<td>220</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ubi:AtCIPK16</td>
<td>1</td>
<td>75</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>IR64</td>
<td>Ubi:AtAVP1</td>
<td>3</td>
<td>210</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Ubi:OsOVP4</td>
<td>1</td>
<td>75</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>35S:PpENA1</td>
<td>3</td>
<td>210</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Ubi:AtCIPK16</td>
<td>1</td>
<td>75</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5-4 Selected T1 transgenic lines for salt tolerance screening

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Transgenic lines</th>
<th>Genetic background</th>
<th>Promoter-gene combination</th>
<th>Number of T-DNA insertion in line in T0 generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>R1</td>
<td>Fatmawati</td>
<td>Ubi:AtAVP1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>R38</td>
<td>Fatmawati</td>
<td>Ubi:AtAVP1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R36</td>
<td>Fatmawati</td>
<td>Ubi:OsOVP4</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>R5</td>
<td>Fatmawati</td>
<td>35S:PpENA1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R44</td>
<td>Fatmawati</td>
<td>35S:PpENA1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R45</td>
<td>Fatmawati</td>
<td>35S:PpENA1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R50</td>
<td>Fatmawati</td>
<td>Ubi:CIPK16</td>
<td>6</td>
</tr>
<tr>
<td>III</td>
<td>R4</td>
<td>IR64</td>
<td>Ubi:AtAVP1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R6</td>
<td>IR64</td>
<td>35S:PpENA1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R56</td>
<td>IR64</td>
<td>35S:PpENA1</td>
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</tr>
</tbody>
</table>
5.3.2. Characterization of transgenic Fatmawati constitutively expressing *AtAVP1* and *OsOVP4*

5.3.2.1. Molecular characterization of transgenic rice Fatmawati constitutively expressing *AtAVP1* and *OsOVP4*

Two T$_1$ transgenic rice Fatmawati lines constitutively expressing *AtAVP1* (R1 and R38) and one T$_1$ transgenic Fatmawati line constitutively expressing *OsOVP4* (R36) were selected to determine if the salt tolerance of commercially relevant rice could be improved by constitutive expression of vacuolar H$^+${-}PPases. The number of T-DNA insertions in the transgenic lines was analysed using Southern Blot analysis in the T$_0$ generation. The Fatmawati *AtAVP1* lines, R1 and R38, had 6 and 2 transgene copies, respectively. The Fatmawati *OsOVP4* line R36 had a single insertion (Table 5-4). The integration of the transgenes was also confirmed by PCR using gene specific primers both in the T$_0$ and T$_1$ generations.

RT-PCR analyses were performed to test the expression of the transgenes in the T$_1$ generation. Total RNA were extracted from leaf tissue of salt stressed plants and were used to synthesize cDNA on which PCR was performed. The analyses confirmed the expression of *AtAVP1* in transgenic Fatmawati (Figure 5-4). The expression of *OsOVP4* driven by the *Ubiquitin-1* promoter was also confirmed using primers amplifying the sequence between *OsOVP4* and the *Nos* terminator to differentiate from endogenous *OsOVP4* (Figure 5-5). As a negative control, the expression of the transgene in wild type Fatmawati was also determined. As expected neither *AtAVP1* nor transgene specific *OsOVP4* transcripts could be detected in untransformed Fatmawati (Figure 5-4, Figure 5-5).
Figure 5-4 Expression analysis using RT-PCR of \textit{AtAVP1} in the shoot of T\textsubscript{1} transgenic Fatmawati constitutively expressing \textit{AtAVP1} (R1 and R38) and wild type Fatmawati

The cDNAs were synthesized from total RNA extracted from leaf tissue of rice plants grown under 100 mM NaCl. The rice plants were grown in soil with salt treatments imposed 50 mM increments over a two days period two weeks after transplantation (21 d after germination). Leaf samples were taken 30 d after 100 mM NaCl was reached. Four plants were used as biological replicates. PCR was performed with 32 cycles using \textit{AtAVP1} gene specific primers and \textit{OsGAPdh} was used as the internal control gene. Fatmawati was included as a control to show the PCR was specific for the transgene. (-) = negative control (water used in PCR instead of DNA).

Figure 5-5 Expression analysis using RT-PCR of \textit{OsOVP4} in the shoot of T\textsubscript{1} transgenic Fatmawati constitutively expressing \textit{OsOVP4} (R36) and wild type Fatmawati

The cDNAs were synthesized from total RNA extracted from leaf tissue of rice plants grown under 100 mM NaCl. The rice plants were grown in soil with salt treatments imposed 50 mM increments over a two days period two weeks after transplantation (21 d after germination). Leaf samples were taken 30 d after 100 mM NaCl was reached. Four plants were used as biological replicates. PCR was performed with 32 cycles using \textit{OsOVP4-Nos} terminator specific primers and \textit{OsGAPdh} was used as the internal control gene. Fatmawati was included as a control to show the PCR was specific for the transgene. (-) = negative control (water used in PCR instead of DNA)
5.3.2.2. Salt screening transgenic rice Fatmawati constitutively expressing AtAVP1 and OsOVP4

5.3.2.2.1. Constitutive expression of AtAVP1 increased projected shoot area of transgenic Fatmawati

Image based phenotyping was used to screen for salinity tolerance of soil grown transgenic Fatmawati constitutively expressing AtAVP1 and OsOVP4. Two weeks after transplantation, the transgenic rice plants and null segregants were subjected to two salt stress treatments: 0 mM and 100 mM NaCl. Digital images were taken at 0, 10, 20, and 30 d after salt application. The projected shoot area of individual plants was determined from colour (RGB) images from two side view images and one top view image (Chapter 4).

The number of biological replicates for each line varied due to segregation in the T1 plants. The nulls which came from the lines produced during the same transformation round were combined. There were no null segregants from the transgenic rice line R1 (Ubi:AVP1_1) grown under salt stress treatment, presumably due to the large number of T-DNA inserts in this line. It was important to keep the different transformation events separate during assessment as transgenic rice plants and nulls from the first round of transformation exhibited a larger projected shoot area than from the second round transformation (Figure 5-6).

The differences in projected shoot area between 0 mM and 100 mM NaCl treatments were obvious 10 d after 100 mM NaCl application. The trends continued to 30 d after salt application (Figure 5-6).

Under control conditions (0 mM NaCl), the two transgenic Fatmawati lines constitutively expressing AtAVP1 (R1 and R38) showed larger shoot area compared to the nulls at 10 and 20 d after salt application. Fatmawati constitutively expressing AtAVP1 showed 10-15% biomass increase compared to the nulls when grown under control conditions at 10 d after salt application, however, by day 30 the growth of the null plants had caught up with the transgenics.

Under salt stress (100 mM NaCl), transgenic Fatmawati constitutively expressing AtAVP1 showed a larger shoot area compared to the nulls. The differences of projected shoot area between transgenic line R38 (Ubi:AVP1_2) and nulls under stress conditions at 10, 20 and 30 d after salt treatment were 25, 58 and 94 cm², respectively (Figure 5-6).
It was not possible to compare the projected shoot area of transgenic R1 (*Ubi:AVP1_I*) to the nulls because the null segregants from the same transformation round were not available under salt stress treatment.

Transgenic Fatmawati constitutively expressing *OsOVP4* had a smaller shoot area compared to the nulls, both under control and salt stress conditions.

The ratio of projected shoot area of the transgenic and nulls in salt stress and control conditions was determined to assess the salinity tolerance of the rice plants (Table 5-5). The salinity tolerance of transgenic Fatmawati lines constitutively expressing *AtAVP1* (R1 and R38) were similar to the nulls at 10 d after salt treatment and 20 d after salt treatment (Table 5-5). However, the salinity tolerance level of the transgenic *AtAVP1* was elevated at 30 d after salt treatment. At 30 d after salt treatment, the transgenic *AtAVP1* lines R1 and R38 retained 58% and 54% of the shoot area, respectively, while the nulls retained 49% of the shoot area (Table 5-5). The salinity tolerance of the *OsOVP4* line was lower than the nulls at 20 and 30 d after salt treatment.
Figure 5-6 Projected shoot area of T1 transgenic rice Fatmawati constitutively expressing vacuolar H\(^+\)-PPases and nulls grown under 0 mM NaCl (blue bar) and 100 mM NaCl (red bar).

The rice plants were grown in soil with salt treatments imposed in 50 mM increments over 2 d period, two weeks after transplantation (21 d after germination). RGB image data were taken at (A) 0, (B) 10, (C) 20 and (D) 30 d after 100 mM NaCl was reached. The vertical dashed lines separate transgenic rice lines and nulls from different rounds of transformations. Nulls were derived from segregation of transgenic rice lines at the T1 generation. Nulls of transgenic rice lines from the same transformation round (R38 and R36) were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
Table 5-5 Relative projected shoot area of T1 transgenic rice Fatmawati constitutively expressing vacuolar H\(^+\)-PPases and nulls grown under 100 mM NaCl compared to the lines grown under 0 mM NaCl at 10, 20 and 30 d after salt applications.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Projected shoot area under 100 mM NaCl ÷ projected shoot area under 0 mM NaCl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 d</td>
</tr>
<tr>
<td>Nulls (R1)</td>
<td>NA</td>
</tr>
<tr>
<td>R1 (Ubi:AVP1_1)</td>
<td>58</td>
</tr>
<tr>
<td>Nulls (R38+R36)</td>
<td>60</td>
</tr>
<tr>
<td>R38 (Ubi:AVP1_2)</td>
<td>59</td>
</tr>
<tr>
<td>R36 (Ubi:OVP4)</td>
<td>60</td>
</tr>
</tbody>
</table>

NA= not available

5.3.2.2.2. The tissue ion concentrations of transgenic Fatmawati constitutively expressing \(AtAVP1\) and \(OsOVP4\) were similar to the nulls

The concentrations of shoot Na\(^+\) and K\(^+\) of transgenic Fatmawati constitutively expressing \(AtAVP1\) and \(OsOVP4\) were determined from the youngest, fully expanded leaf at 20 d after salt treatment. Salt treatment increased both Na\(^+\) and K\(^+\) concentrations in the shoot. There were no clear differences in shoot Na\(^+\) and K\(^+\) concentrations between transgenics constitutively expressing vacuolar H\(^+\)-PPases and the nulls (Figure 5-7).
Figure 5-7 Concentration of (A) shoot Na⁺, (B) shoot K⁺ and (C) ratio of shoot K⁺/Na⁺ of T₁ transgenic rice Fatmawati constitutively expressing vacuolar H⁺-PPases and nulls grown under 0 mM NaCl (blue bar) and 100 mM NaCl (red bar). The rice plants were grown in soil with salt treatments imposed in 50 mM increments over 2 d period, two weeks after transplantation (21 d after germination). Ion concentrations were measured in the youngest, fully expanded leaf 20 d after 100 mM NaCl was reached. The vertical dashed lines separate transgenic rice lines and nulls from different round of transformations. Nulls were derived from segregation of transgenic rice lines at the T₁ generation. Nulls of transgenic rice lines from the same transformation round (R38 and R36) were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
5.3.3. Characterization of transgenic Fatmawati transformed with *PpENA1* and *AtCIPK16* under the control of constitutive promoters

5.3.3.1. *PpENA1* expression could not be detected in transgenic Fatmawati carrying 35S:*PpENA1* insert

Three transgenic Fatmawati lines transformed with the 35S:*PpENA1* construct (R5, R44 and R45) were selected for salinity tolerance screening. The integration of the transgene in these lines was confirmed using Southern Blot analysis in the T₀ generation (Table 5-4, Figure 5-8). The presence of *PpENA1* in the T₀ and T₁ generations was also confirmed by PCR on genomic DNA using gene specific primers.

RT-PCR was performed to determine whether transcripts of *PpENA1* were present in the T₁ generation of transgenic Fatmawati. The results showed that despite the presence of the transgene, *PpENA1* was not expressed in either the shoot or root of transgenic Fatmawati. No expression product could be detected at 35 cycles of RT-PCR (Figure 5-9).

![Figure 5-8 Southern Blot analysis of five T₀ transgenic rice transformed with *PpENA1* using a probe against the Nos terminator.](image)

The DNA fragments of the three transgenic Fatmawati carrying 35S:*PpENA1* insert (R5, R44, and R45) and two transgenic IR64 carrying 35S:*PpEna1* insert (R6 and R56) are indicated by arrows. The restriction enzyme *BamHI* was used for gDNA digestion.
Figure 5-9 Molecular analyses of T₁ transgenic Fatmawati carrying 35S:PpENA1 insert.

(A) The presence of PpENA1 in transgenic Fatmawati was confirmed by PCR using gDNA as the template. (B) RT-PCR analyses were performed on RNA extracted from the same plants to determine whether PpENA1 was expressed in leaf and (C) root tissue of the plants grown under 70 mM NaCl treatment. The rice plants were grown in hydroponics with salt treatment imposed in 25 mM increments over 3 d period (20 mM on the last day), starting when the 4th leaf was fully expanded (18 d after germination). Leaf and root tissue were sampled 10 d after 70 mM NaCl was reached. Three plants were used as biological replicates. PCR and RT-PCR were performed with 35 cycles using PpENA1 specific primers and OsGAPdh was used as the internal control gene. (-) = negative control (water used in PCR instead of DNA); (+) = positive control (plasmid containing PpENA1 was used as a source of DNA)
5.3.3.2. *AtCIPK16* was constitutively expressed in transgenic rice under the control of the *Ubiquitin-1* promoter.

One transgenic Fatmawati constitutively expressing *AtCIPK16* was successfully generated using *Agrobacterium*-mediated transformation. The transgenic line was designated as R50. Southern Blot analysis and PCR in the T₀ generation confirmed the insertion of *AtCIPK16*. Six T-DNA insertions were detected in the *AtCIPK16* line using *HindIII* as the restriction enzyme and *Nos* terminator as the probe (Table 5-4). The expression of the *AtCIPK16* transgene in rice R50 was also confirmed using 32 cycles of RT-PCR both in shoots and roots (Figure 5-10).

![Figure 5-10 Expression analysis using RT-PCR of *AtCIPK16* in the shoot and roots of T₁ transgenic Fatmawati constitutively expressing *AtCIPK16* (R50) and wild type Fatmawati.](image)

The cDNAs were synthesized from total RNA extracted from leaf and root tissue of the plants grown under 70 mM NaCl treatment. The rice plants were grown in hydroponics with salt treatment imposed in 25 mM increments over 3 d period (20 mM on the last day), starting when the 4th leaf was fully expanded (18 d after germination). Leaf and root tissue were sampled 10 d after 70 mM NaCl was reached. Three plants were used as biological replicates. PCR was performed in 32 cycles using *AtCIPK16* specific primers and *OsGAPdh* was used as the internal control gene. (-) = negative control (water used in PCR instead of DNA)
5.3.3.3. Segregation of T-DNA insertions of transgenic Fatmawati constitutively expressing *AtCIPK16* in the T<sub>1</sub> generation

Southern Blot analysis was performed to analyse the T-DNA insertion patterns of T<sub>1</sub> transgenic rice Fatmawati, constitutively expressing *AtCIPK16* (R50). There were variations in the number of T-DNA insertions in the T<sub>1</sub> generation with some plants having fewer copy numbers (2-3 copies) than the primary transgenic (T<sub>0</sub>) (Figure 5-11). Seeds from the T<sub>1</sub> generation with low copy number have been collected for further characterization.

![Southern Blot analysis of T<sub>1</sub> and T<sub>0</sub> plants of transgenic rice Fatmawati constitutively expressing *AtCIPK16*. T<sub>1</sub> lines with lower copy number than T<sub>0</sub> are indicated by the asterisks. Restriction enzyme *HindIII* was used to digest gDNA of the transgenic plants. PCR-amplified product of *Nos* terminator was radiolabelled with [α-<sup>32</sup>P]dCTP and used as a probe.](image)

Figure 5-11 Southern Blot analysis of T<sub>1</sub> and T<sub>0</sub> plants of transgenic rice Fatmawati constitutively expressing *AtCIPK16*. T<sub>1</sub> lines with lower copy number than T<sub>0</sub> are indicated by the asterisks. Restriction enzyme *HindIII* was used to digest gDNA of the transgenic plants. PCR-amplified product of *Nos* terminator was radiolabelled with [α-<sup>32</sup>P]dCTP and used as a probe.
5.3.3.4. Salinity tolerance of transgenic Fatmawati carrying 35S:PpENAI and Ubi:AtCIPK16 inserts

Since the automated imaging system of The Plant Accelerator had been booked for quarantine experiments by another user in the second half of 2013 and was therefore unavailable for non-quarantine plants, an alternative method had to be used for subsequent salt stress screenings. Salinity tolerance of T1 transgenic Fatmawati transformed with PpENAI and AtCIPK16 under the control of constitutive promoters was carried out in hydroponics. The transgenic rice lines along with the nulls were subjected to moderate salt stress of 70 mM NaCl which started when the 4th leaf was fully expanded (18 d after germination). The salt treatment was applied in 25 mM increments (20 mM on the last day) over a 3 d period. The plants were harvested 10 d after 70 mM NaCl was reached.

The transgenic Fatmawati lines carrying 35S:PpENAI (R44 and R45), showed comparable biomass to the null segregants under both control and salt stress conditions (Figure 5-12). Unfortunately, it was difficult to compare the biomass of another 35S:PpENAI (R5) to the nulls because of the limitation of the number of nulls derived from the same transformation round (Figure 5-12).

The transgenic rice Fatmawati constitutively expressing AtCIPK16 had lower dry biomass compared to the nulls both under control and salt stress conditions (Figure 5-12).

The ratio of biomass in salt stress and control conditions was calculated to determine the salinity tolerance of each line. Transgenic Fatmawati carrying 35S:PpENAI (line R44) showed a slightly lower level of salt tolerance (81%) compared to nulls (82%), while the 35S:PpENAI line R45 had higher salt tolerance (86%) compared to the nulls (Table 5-6). A slight improvement in salt tolerance was also observed in line R50 constitutively expressing AtCIPK16 (84%) when compared to the nulls (Table 5-6).
5.3.3.5. Tissue ion concentration of transgenic rice Fatmawati carrying 35S:PpENA1 and Ubi:AtCIPK16 inserts

The three transgenic rice Fatmawati carrying 35S:PpENA1 (R5, R44 and R45) showed variation in their shoot and root Na$^+$ concentration after salt stress (Figure 5-13). Line R44 had higher shoot Na$^+$ concentration (52 mM) compared to nulls (37 mM), while R5 had lower Na$^+$ concentration (11 mM) (Figure 5-13). In contrast, the root Na$^+$ concentration of R44 (70 mM) was lower than that of the nulls (74 mM), while R5 demonstrated higher Na$^+$ concentration in the root (77 mM).

Transgenic Fatmawati constitutively expressing AtCIPK16 (R50) showed higher shoot Na$^+$ concentration (53 mM) compared to the nulls (37 mM). The root Na$^+$ concentration of this line was comparable to the nulls (about 74 mM) (Figure 5-13).

Salt stress of 70 mM NaCl clearly reduced shoot K$^+$ concentrations of the nulls and transgenic Fatmawati constitutively expressing AtCIPK16, but not the transgenic 35S:PpENA1 lines (Figure 5-14). All three transgenic 35S:PpENA1 lines had similar shoot K$^+$ concentrations in control and salt stress conditions. Under salt stress conditions, the shoot K$^+$ concentrations of transgenic 35S:PpENA1 were higher than the nulls (Figure 5-14).

Transgenic Fatmawati constitutively expressing AtCIPK16 showed higher shoot K$^+$ and root K$^+$ concentrations than the nulls, under both normal and salt stress conditions (Figure 5-14).

Salt stress reduced the ratio of K$^+$/Na$^+$ both in shoots and roots of the transgenic rice lines and nulls (Figure 5-15). While the ratio of shoot K$^+$/Na$^+$ of the transgenic lines varied under 0 mM NaCl, the ratios under 70 mM NaCl were almost similar (Figure 5-15). Under non-salt conditions, the ratios of shoot K$^+$/Na$^+$ of transgenic Ubi:AtCIPK16 (R50) and two 35S:PpENA1 lines (R5 and R45) were higher than the nulls (Figure 5-15).
Figure 5-12 (A) Shoot dry weight, (B) root dry weight and (C) total dry weight of transgenic Fatmawati carrying 35S::PpENA1 and Ubi::AtCIPK16 and the nulls grown under 0 mM and 70 mM NaCl.

The rice plants were grown in hydroponics with salt treatments imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (18 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. The vertical dashed lines separate transgenic rice lines and nulls from different rounds of transformation. Nulls were derived from segregation of transgenic rice lines at the T1 generation. Nulls of transgenic rice lines from the same transformation round (R44, R45 and R50) were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
Table 5-6 Relative total dry weight of transgenic Fatmawati lines carrying 35S:PpENA1, Ubi:AtCIPK16 and nulls grown under 70 mM NaCl compared to the same lines grown under 0 mM NaCl

<table>
<thead>
<tr>
<th>Lines</th>
<th>Total dry weight under 70 mM NaCl ÷ total dry weight under 0 mM NaCl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nulls (R5)</td>
<td>NA</td>
</tr>
<tr>
<td>R5 (35S:ENA1_1)</td>
<td>81</td>
</tr>
<tr>
<td>Nulls (R44+R45+R50)</td>
<td>82</td>
</tr>
<tr>
<td>R44 (35S:ENA1_2)</td>
<td>81</td>
</tr>
<tr>
<td>R45 (35S:ENA1_3)</td>
<td>86</td>
</tr>
<tr>
<td>R50 (Ubi:CIPK16)</td>
<td>84</td>
</tr>
<tr>
<td>NA= not available</td>
<td></td>
</tr>
</tbody>
</table>

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Figure 5-13 Concentration of Na\(^+\) in (A) shoot and (B) root of transgenic Fatmawati carrying 35S:PpENA1 and Ubi:AtCIPK16 and the nulls grown under 0 mM and 70 mM NaCl.

The rice plants were grown in hydroponics with salt treatments imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4\(^{th}\) leaf was fully expanded (18 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. The vertical dashed lines separate transgenic rice lines and nulls from different rounds of transformation. Nulls were derived from segregation of transgenic rice lines at the T\(_1\) generation. Nulls of transgenic rice lines from the same transformation round (R44, R45 and R50) were combined. Values are the means ± SEM. \(n\) = the number of biological replicates for each line.
Figure 5-14 Concentration of K⁺ in (A) shoot and (B) root of transgenic Fatmawati carrying 35S:PpENA1 and Ubi:AtCIPK16 and the nulls grown under 0 mM and 70 mM NaCl.

The rice plants were grown in hydroponics with salt treatments imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (18 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. The dashed separate transgenic rice lines and nulls from different rounds of transformation. Nulls were derived from segregation of transgenic rice lines at the T₁ generation. Nulls of transgenic rice lines from the same transformation round (R44, R45 and R50) were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
Figure 5-15 Ratio of (A) shoot $K^+$/Na$^+$ and (B) root $K^+$/Na$^+$ of transgenic Fatmawati carrying 35S:$PpENA1$ and Ubi:AtCIPK16 and the nulls grown under 0 mM and 70 mM NaCl.

The rice plants were grown in hydroponics with salt treatments imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (18 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. The vertical dashed separate transgenic rice lines and nulls from different rounds of transformation. Nulls were derived from segregation of transgenic rice lines at the T1 generation. Nulls of transgenic rice lines from the same transformation round (R44, R45 and R50) were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
5.3.4. Characterization of transgenic IR64 transformed with \textit{AtAVP1} and \textit{PpENA1} under the control of constitutive promoters

5.3.4.1. \textit{PpENA1} expression could not be detected in transgenic IR64 carrying the 35S:\textit{PpENA1} insert

Two fertile independent transgenic IR64 lines carrying 35S:\textit{PpENA1} (R6 and R56) were developed from different transformation rounds (Table 5-3). The integration of T-DNA carrying \textit{PpENA1} under the control of the 35S promoter was confirmed by PCR and Southern Blot analysis in the T\textsubscript{0} generation. Both R6 and R56 had a single insertion of T-DNA based on Southern Blot analysis (Table 5-4 and Figure 5-8). However, similar to the transgenic Fatmawati with the 35S:\textit{PpENA1} insert, the expression of \textit{PpENA1} in T\textsubscript{1} transgenic IR64 could not be detected through RT-PCR, both in shoots and roots.

5.3.4.2. Constitutive expression of \textit{AtAVP1} in transgenic IR64

One transgenic IR64 line constitutively expressing \textit{AtAVP1} (R4) was selected for performing a salt tolerance assay on. The integration of \textit{AtAVP1} in the IR64 background was confirmed by PCR and Southern Blot in the T\textsubscript{0} generation (Table 5-4). Expression analysis was performed in the T\textsubscript{1} generation using cDNA synthesized from total RNA of salt stressed plants. RT-PCR results show \textit{AtAVP1} was expressed constitutively both in shoots and roots of transgenic IR64 (R4) (Figure 5-16).

Figure 5-16 Expression analysis using RT-PCR of \textit{AtAVP1} in the shoot and roots of T\textsubscript{1} transgenic IR64 constitutively expressing \textit{AtAVP1} (R4) and wild type IR64.

The cDNAs were synthesized from total RNA extracted from leaf and root tissue of the plants grown under 70 mM NaCl treatment. The rice plants were grown in hydroponics with salt treatment imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4\textsuperscript{th} leaf was fully expanded (17 d after germination). Leaf and root tissue were sampled 10 d after 70 mM NaCl was reached. Three plants were used as biological replicates. PCR was performed in 32 cycles using \textit{AtAVP1} specific primers and \textit{OsGAPdh} was used as the internal control gene. (-) = negative control (water used in PCR instead of DNA)
5.3.4.3. Salinity tolerance of transgenic IR64 carrying *Ubi:AtAVP1* and *35S:PpENA1*

Transgenic rice lines transformed with *AtAVP1* and *PpENA1* under the control of constitutive promoters were evaluated together in a salt screening experiment using the hydroponic system. The transgenic rice lines were grown in moderate salt stress of 70 mM NaCl and control conditions (0 mM NaCl). Salt stress was applied in 25 mM increments (last increment 20 mM) over a 3 d period, starting when the 4th leaf of the rice plants was fully expanded (approximately 17 d after germination). The plants were harvested after 10 d exposure to 70 mM NaCl.

Under control conditions (0 mM NaCl), transgenic IR64 carrying *Ubi:AtAVP1* and *35S:PpENA1* showed larger dry biomass compared to the nulls (Figure 5-17). The transgenic plants produced larger shoots and roots compared to the nulls (Figure 5-17). In particular, IR64 constitutively expressing *AtAVP1* showed 22% increase in total biomass compared to the nulls under control condition.

Under stress conditions (70 mM NaCl), the transgenic rice carrying *Ubi:AtAVP1* and *35S:PpENA1* produced similar amounts of dry biomass to the nulls (Figure 5-17). In terms of the ability of the rice plants to retain their relative biomass under salt stress, all transgenic rice plants showed lower relative dry weight retention compared to the nulls due to the size of control grown plants. Transgenic IR64 constitutively expressing *AtAVP1* (R4) retained only 60% of the total biomass under salt stress, while the nulls were able to retain 75% of the total biomass under salt stress compared to non-stress conditions. Similarly, the transgenic rice harbouring the *PpENA1* gene, (R6 and R56), retained about 61% of their biomass under salt stress (Table 5-7).
Figure 5-17 Shoot dry weight (A), root dry weight (B) and total dry weight (C) of transgenic IR64 carrying Ubi:AtAVP1 and 35S:PpENA1 and the nulls grown under 0 mM and 70 mM NaCl.

The rice plants were grown in hydroponics with salt treatments imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. The dashed vertical line separate transgenic rice lines and nulls from different rounds of transformation. Nulls were derived from segregation of transgenic rice lines at the T1 generation. Nulls of transgenic rice lines from the same transformation round, (R4 and R6), were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
Table 5-7 Relative total dry weight of transgenic IR64 lines constitutively expressing *AtAVP1*, *PpENA1* and nulls grown under 70 mM NaCl compared to the lines grown under 0 mM NaCl

<table>
<thead>
<tr>
<th>Lines</th>
<th>Total dry weight under 70 mM NaCl ÷ total dry weight under 0 mM NaCl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null (R4+R6)</td>
<td>75</td>
</tr>
<tr>
<td>R4 (Ubi:AVP1)</td>
<td>61</td>
</tr>
<tr>
<td>R6 (35S:ENA1_1)</td>
<td>61</td>
</tr>
<tr>
<td>Null (R56)</td>
<td>82</td>
</tr>
<tr>
<td>R56 (35S:ENA1_2)</td>
<td>61</td>
</tr>
</tbody>
</table>
5.3.4.4. Tissue ion concentration of transgenic IR64 carrying *Ubi:AtAVP1* and *35S:PpENA1*

Under control conditions, the concentration of Na\(^+\) in the shoots and roots of all the rice plants were similar, while under salt stress, the accumulation of tissue Na\(^+\) varied among the transgenic lines.

Under salt stress, transgenic rice constitutively expressing *AtAVP1* (R4) showed a higher concentration of Na\(^+\) in the shoot (48 mM) compared to the nulls (40 mM), while the *AtAVP1* rice line showed a lower root Na\(^+\) concentration (69 mM) compared to nulls (72 mM) (Figure 5-18). The trend was similar to transgenic *PpENA1* R56, but different to another *PpENA1* line, R6. Transgenic rice line R6 had a lower Na\(^+\) concentration in the shoot and a higher concentration in roots compared to the nulls (Figure 5-18).

There were no clear differences in shoot K\(^+\) concentration between transgenic IR64 and the nulls both under salt stress and control conditions (Figure 5-19). However, the ratio of shoot K\(^+\)/Na\(^+\) in transgenic IR64 carrying *Ubi:AtAVP1* and *35S:PpENA1* was lower than in the nulls (Figure 5-20).
Figure 5-18 Concentration of Na⁺ in (A) shoot and (B) root of transgenic IR64 carrying Ubi:AtAVP1 and 35S:PpENA1 and the nulls grown under 0 mM and 70 mM NaCl.

The rice plants were grown in hydroponics with salt treatments imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. The vertical dashed lines separate transgenic rice lines and nulls from different rounds of transformation. Nulls were derived from segregation of transgenic rice lines at the T1 generation. Nulls of transgenic rice lines from the same transformation round, (R4 and R6), were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
Figure 5-19 Concentration of K⁺ in (A) shoot and (B) root of transgenic IR64 carrying Ubi:AtAVP1 and 35S:PpENA1 and the nulls grown under 0 mM and 70 mM NaCl.

The rice plants were grown in hydroponics with salt treatments imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. The vertical dashed lines separate transgenic rice lines and nulls from different rounds of transformation. Nulls were derived from segregation of transgenic rice lines at the T₁ generation. Nulls of transgenic rice lines from the same transformation round (R4 and R6) were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
Figure 5-20 (A) and (B) ratio shoot K⁺/Na⁺, (C) and (D) ratio root K⁺/Na⁺ of transgenic IR64 carrying Ubi:AtAVP1 and 35S:PpENA1 and the nulls grown under 0 mM (blue bar) and 70 mM NaCl (red bar). The rice plants were grown in hydroponics with salt treatments imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Rice plants were harvested 10 d after 70 mM NaCl was reached. The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. The vertical dashed lines separate transgenic rice lines and nulls from different rounds of transformation. Nulls were derived from segregation of transgenic rice lines at the T₁ generation. Nulls of transgenic rice lines from the same transformation round (R4 and R6) were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
5.4. Discussion

5.4.1. Limitation of the number of independent transformation events

*Agrobacterium*-mediated transformation has been used to generate transgenic commercial rice constitutively expressing candidate genes for salt tolerance improvement. Whilst fertile transgenic events with low copy number of T-DNA insertion have been successfully developed, the low transformation efficiency of commercial *indica* rice limited the number of independent lines for salt screening. For some constructs only one independent line was available for phenotyping (Table 5-3, Table 5-4). As a number of factors possibly affects the expression pattern of transgenes including the integration sites (positional effects) (Peach and Velten 1991; Matzke and Matzke 1998; van Leeuwen et al. 2001), the number of transgene insertions (Gendloff et al. 1990; Hobbs et al. 1993; Flavell 1994), the integrity of the integrated transgene (Kohli et al. 1999) and RNA silencing (Schubert et al. 2004), several independent lines are ideally used for phenotyping to give more rigorous results as the expression of a transgene might vary among transformation events. Therefore, while some trends might be described in the above study, it is not possible to make definitive conclusions on the effect of constitutively expressing these genes in rice without further transgenic lines being generated. Despite performing up to three rounds of transformations it was not possible to prepare three independent lines for each gene and cultivar combination during the course of the PhD. It should be noted that the transformation process used in this study took approximately 10 months from callus induction until T₀ transgenic seed was obtained (Chapter 3).

An attempt was made during the course of this study to increase the transformation efficiency of *Agrobacterium*-mediated rice transformation, particularly for IR64, by visiting the International Rice Research Institute to learn a more efficient technique using immature embryos as starting material, as described in (Slamet-Loedin et al. 2014). Unfortunately, a regular supply of the correct embryo stage could not be ensured in Adelaide. Supplemental lighting and temperature control were not sufficient for efficient rice growth during the South Australian winter, with a shortened photoperiod and low night time temperatures. It would therefore be worthwhile in the future to use the immature embryo-based transformation for *indica* rice to increase the number of transgenic events, if the correct embryo stage can be obtained on a regular basis. This
requires the availability of an optimal growth condition for growing rice throughout the year in South Australia – procedures which will have to be more thoroughly developed.

Due to the small number of successful transformants produced in this study, it was not possible to be selective of the lines to study. Usually, transgenic lines with only single T-DNA insertions are characterized because multiple insertions of T-DNA might confound the effect of the gene expression (Hobbs et al. 1993). Moreover, multiple copies of T-DNA could lead to gene silencing (Flavell 1994) and might destruct the expression of native genes as a result of the excision of the multiple integration sites (Yao et al. 2006). Typically in the ACPFG, 15-20 barley transformation events are generated for each construct, ensuring at least 5-8 single insert lines for further characterization. This is the result of years of optimization of the barley transformation protocol – a procedure now required here for the indica rice. Notably, for transgenic Fatmawati constitutively expressing AtCIPK16 (R50), which had 6 copies of T-DNA inserted, Southern Blot analysis on the T1 transgenic revealed segregation of the T-DNA insertions into lower copy number with a different integration site (Figure 5-11). It is necessary to characterize the segregants with a lower copy number to test whether the number of insertions and the integration sites had effects on the current results.

5.4.2. Rice response to salt stress differed between hydroponic and soil screening

Two different screening techniques were used in this study to characterize the salt tolerance of transgenic rice. The first experiment used soil based phenotyping and employed image analysis to quantify the response of rice to 0 mM and 100 mM NaCl treatments. The remainder of the experiments used hydroponics with 0 and 70 mM NaCl treatments. The first experiment was established based on the results from Chapter 4 in which 100 mM NaCl treatment gave moderate stress on rice cultivars Fatmawati and IR64, and the effects can be clearly differentiated from 0 mM NaCl treatment. The hydroponic system used in the second and third experiments was based on the method described by Cotsaftis et al. (2011). A similar screening protocol was also used to screen the wild type rice from Indonesia (Chapter 3) and was able to discriminate different tolerance mechanisms of the Indonesian rice cultivars. Ideally, all of the phenotyping of the transgenic rice would have been performed using non-destructive image-based phenotyping, to allow the measurement of the dynamic response of the transgenics to salt stress while maintaining the seeds for the next generation. However, lack of space in the physical containment facility for image analysis of transgenic materials in The Plant
Accelerator, due to other bookings at the time, hindered the application of the technique in the second and third experiments. It was therefore necessary to use hydroponics for these studies instead.

Regardless of the differences in the salt concentrations and the climate conditions in the two screening protocols, there were clear differences in the responses of rice cultivars to the salt treatments in these two methods, particularly the relation between biomass reduction and tissue ion concentrations. The nulls from transgenic Fatmawati can be used for comparison as these nulls were used in both types of experiment (Table 5-5 and Table 5-6). The reduction of plant biomass in Fatmawati nulls was higher in the soil experiment compared to the hydroponic experiment after 10 d grown under salt stress. In the soil experiment, 100 mM NaCl treatment for 10 d reduced the biomass of Fatmawati nulls by up to about 40% (Table 5-5). While in the hydroponic system, 70 mM NaCl treatment for 10 d reduced the biomass of Fatmawati nulls by only 20% (Table 5-6). This suggests that depending on the screening system used, the plants were exposed to different amounts of the various stress components involved in salt stress.

The immediate reduction of plant growth under salt stress is associated with the osmotic effect of salt stress (Munns and Tester 2008). The reduction is independent of the amount of Na\(^+\) accumulation in the shoot and is regulated by long distance signals (Munns and Tester 2008). These results suggested that rice plants in the soil-based experiment suffered more from osmotic stress compared to the hydroponic experiment.

The measured tissue ion concentration supported the hypothesis that the growth reduction was independent of the Na\(^+\) accumulation in the shoot. Despite the fact that the accumulation of shoot Na\(^+\) concentration in the hydroponic experiment was much higher than in the soil experiment, the growth reduction in the hydroponic experiment was lower than in the soil experiment. When grown under 100 mM NaCl treatment in soil for 20 d, the Fatmawati nulls only accumulated approximately 2.5 mM Na\(^+\) in their youngest, fully expanded leaf (Figure 5-7). However, when grown under 70 mM NaCl for 10 d in the hydroponic experiment, the youngest, fully expanded leaf of Fatmawati nulls accumulated up to 40 mM Na\(^+\) (Figure 5-13).

Another interesting observation was the difference in shoot K\(^+\) concentration of salt stressed rice plants. In the soil-based experiment, the shoot K\(^+\) concentrations of salt treated plants were higher than the control plants (Figure 5-7), while in hydroponics, the shoot K\(^+\) concentration of salt-stressed plants was lower than in control plants (Figure
5-14), as commonly reported for salt stressed plants (Yeo et al. 1991; Fricke et al. 1996; Ren et al. 2005; Genc et al. 2007). High concentrations of shoot K⁺ in rice during the soil experiment could be hypothesized to occur because the rice plants experienced higher osmotic stress compared to the hydroponic experiment and the rice plants used K⁺ as cationic osmolyte (Fricke et al. 2006). Another possibility may be that the high osmotic stress experienced by soil-grown plants induced an up-regulation of K⁺ channels (Su et al. 2001; Pilot et al. 2003) and/or K⁺ transporters (Su et al. 2002), thus increasing the K⁺ uptake capacity of the plants. Further studies are important to determine the differences in physiological response of rice to salt stress in hydroponic and soil experiments involving more rice genotypes. More importantly, the association between the results observed from hydroponic and soil experiments with the performance of rice in the saline field need to be studied, as in barley the result from these two screening methods appear to differently correlate to the field performance (Tavakkoli et al. 2012).

5.4.3. Constitutive expression of *AtAVP1* increased the biomass of transgenic Fatmawati and IR64

Na⁺ sequestration in vacuoles is an important process for salt tolerance in many plant species (Munns and Tester 2008; Gaxiola et al. 2012). Accumulated evidence has shown that constitutive expression of type I vacuolar H⁺-PPase from *Arabidopsis* (*AtAVP1*) and its homologues increased salinity tolerance in a range of species including *Arabidopsis* (Gaxiola et al. 2001), tobacco (Gao et al. 2006), alfalfa (Bao et al. 2009a), cotton (Pasapula et al. 2011), barley (Schilling et al. 2013) and rice (Liu et al. 2010b; Kim et al. 2013); however, not always through improving Na⁺ sequestration (Schilling et al. 2013).

In this study, *AtAVP1* was constitutively expressed in two commercial *indica* rice cultivars, Fatmawati and IR64, under the control of the maize *Ubiquitin-1* promoter. To our knowledge, there have not been any reports on the utilization of this gene in improving salinity tolerance of elite *indica* rice cultivars. The majority of the work in rice has so far used *japonica* rice as the genetic background (Zhao et al. 2006b; Yang et al. 2007; Liu et al. 2010b; Kim et al. 2013). It should be noted that the salt tolerance level of *japonica* rice is generally lower than *indica* rice (Lee et al. 2003; Platten et al. 2013). The results from Chapter 3 also indicated that Fatmawati and IR64 have higher levels of salt tolerance compared to *japonica* rice Nipponbare. It was hypothesized that constitutive expression of *AtAVP1* could further increase the tolerance of commercial rice cultivars from the current level of tolerance.
The constitutive expression of \textit{AtAVP1} in Fatmawati and IR64 was confirmed by RT-PCR (Figure 5-4, Figure 5-16). Constitutive expression of \textit{AtAVP1} in Fatmawati led to a small increase in biomass under salt stress (Figure 5-6). However, the gene expression did not improve the biomass of IR64 under salt stress (Figure 5-17). Fatmawati and IR64 apparently have different mechanisms of sodium transport in response to salt stress (Chapter 3). Fatmawati is a typical Na\textsuperscript{+} excluder, which maintains low Na\textsuperscript{+} in the shoot to reduce the ionic effect of the salt. IR64 is likely to be a tissue tolerant cultivar, which can effectively manage the accumulation of Na\textsuperscript{+} in the shoot. The differences in the response of Fatmawati \textit{AtAVP1} and IR64 \textit{AtAVP1} might therefore be related to the different salinity tolerance mechanisms of the two genetic backgrounds. Improvements in vacuolar sodium sequestration might be more effective in increasing salinity tolerance of an excluding cultivar such as Fatmawati compared to a tissue-tolerant cultivar such as IR64, which is likely to already have good levels of vacuolar acidification and Na\textsuperscript{+} sequestration.

Results from this study were also quite different to previous results when \textit{japonica} rice cultivars were used as genetic background (Zhao et al. 2006b; Liu et al. 2010b; Kim et al. 2013) or other in species (Gaxiola et al. 2001; Bao et al. 2009a; Pasapula et al. 2011; Schilling et al. 2013), which showed significant improvement in plant biomass under salt stress as a result of over-expression of \textit{AtAVP1}. The good level of salinity tolerance of the two cultivars might cause difficulty in observing a significant improvement. Another possible explanation is that only T\textsubscript{1} plants were phenoyyped in this study. The transgenic T\textsubscript{1} generation is not ideal for phenotyping as it may include hemizygous plants which lack transgene stability (James et al. 2002b) and the seed quality may be low as it is produced from the primary transgenic plants. The improvement in the phenotype of the T\textsubscript{2} and later generations compared to the T\textsubscript{1} generation has also been observed in transgenic barley constitutively expressing \textit{AtAVP1} (Schilling et.al. unpublished data).

Interestingly, constitutive expression of \textit{AtAVP1} apparently increased the biomass of both transgenic Fatmawati and IR64 under non-stressed conditions (0 mM NaCl) (Figure 5-6 and Figure 5-17). Constitutive expression of \textit{AtAVP1} increased the shoot area of transgenic Fatmawati by 10-15\% and by 22\% for total dry weight of transgenic IR64 compared to their nulls (Figure 5-6 and Figure 5-17). Nevertheless, if the salinity tolerance is defined as the ability of the plants to maintain their yield under stress relative to the non-stress condition, there was no salt tolerance improvement in the \textit{AtAVP1} transgenic lines (Table 5-5, Table 5-7).
The increased biomass of the transgenic plants under control conditions was unexpected, but consistent with results from other plant species with constitutive expression of vacuolar H\(^{+}\)-PPase (Gaxiola et al. 2012; Schilling et al. 2013). *Arabidopsis* constitutively expressing *AtAVP1* produced larger leaf area than the wild type as the result of an increase in cell numbers (Li et al. 2005; Gonzalez et al. 2010). This phenotype was associated with an altered organ development due to the increase of auxin transport in transgenic *Arabidopsis* over-expressing *AtAVP1* (Li et al. 2005). Similarly, rice overexpressing *AtAVP1* showed increased biomass under non-stressed conditions as a result of an improvement in photosynthetic activity compared to the wild type (Kim et al. 2013). Increased biomass shown by overexpression of *AtAVP1* possibly was also associated with the improvement of nutrient uptake, particularly phosphorus and nitrogen (Yang et al. 2007; Paez-Valencia et al. 2013). Improvement in nutrient uptake was mediated by the increased in rhizosphere acidification and root proliferation (Yang et al. 2007; Paez-Valencia et al. 2013). In addition, improvement in root proliferation of *AtAVP1* overexpression plants also increased the plants’ capacity to take up water, particularly in water deficit conditions (Park et al. 2005).

The improvement in biomass as the result of up-regulation of *AtAVP1* was also suggested as the result of an improved sucrose transport (Gaxiola et al. 2012). A study using rice suspension cells showed up-regulation of vacuolar H\(^{+}\)-PPase during a sucrose starvation period (Wang et al. 2007). In other studies, constitutive expression of *AtAVP1* in *Arabidopsis* up-regulated genes involved in sucrose transport and sucrose synthesis (Gonzalez et al. 2010; Gaxiola et al. 2012).

Moreover, a biomass increase observed in transgenic plants overexpressing vacuolar H\(^{+}\)-PPase has been hypothesized to be associated with improvements in seedling vigour (Schilling et al. 2013). It has been shown recently that *AtAVP1* potentially has a significant role in the hydrolysis of inorganic pyrophosphate (PPi) in the cytosol (Ferjani et al. 2011). A suppression of PPi levels in the cytosol is necessary to increase gluconeogenesis, which is important in organ development in the post-germination stages (Ferjani et al. 2011).

The various phenotypes demonstrated by the over expression of *AtAVP1* also led to the hypothesis that the protein has different functions in different tissues (Gaxiola et al. 2012). Therefore, it is necessary to express the gene in specific cell types to study the effect of the gene in different tissues.
It is also important to test whether the AtAVP1 lines generated in this study have improved tolerance for other abiotic stresses. This is significant because many studies have indicated the role of vacuolar H\(^+\)-PPase in improving tolerance to other abiotic stresses, such as P\(_i\) deficiency (Yang et al. 2007), NO\(_3^-\) starvation (Paez-Valencia et al. 2013), drought (Gaxiola et al. 2001; Park et al. 2005; Pasapula et al. 2011) and cold (Zhang et al. 2011). Interestingly, Zhang et al. (2011) showed that constitutive expression of OsOVP1 (the AtAVP1 homologue from rice) improved cold tolerance in rice but not salt tolerance. This result suggests that there might be useful phenotypes from the current transgenic rice constitutively expressing AtAVP1 other than salt tolerance. Ideally, these studies would be performed on multiple independent lines in the T\(_2\) or later generation.

5.4.4. Constitutive expression of OsOVP4 negatively affected rice growth

There are six vacuolar H\(^+\)-PPases which have been identified from rice including OsOVP1, OsOVP2 (Sakakibara et al. 1996), OsOVP3, OsOVP4, OsOVP5 (Choura and Rebai 2005) and OsOVP6 (Liu et al. 2010a). OsOVP4 (Os02g55890) was identified by sequence homology with OsOVP1 and OsOVP2 (Choura and Rebai 2005). The deduced amino acid sequence of OsOVP4 has 74% identity with OsOVP1 (Liu et al. 2010a). The expression of OsOVP4 was found to be significantly increased in transgenic rice expressing AtHKT1;1, in the root cortex under salt stress (Plett et al. 2010). It was hypothesized that the up-regulation of OsOVP4 led to an increase of Na\(^+\) sequestration capacity of the root cells and improved the salinity tolerance by reducing the amount of Na\(^+\) transported to the shoot (Plett et al. 2010). To test whether OsOVP4 can increase salinity tolerance, OsOVP4 constitutive expression lines were developed. Unfortunately, only one transgenic line constitutively expressing OsOVP4 could be generated from Agrobacterium-mediated transformation using 75 calli (Table 5-3). Hence, the characterization of OsOVP4 constitutive expression is limited to a single line.

The salt screening of the T\(_1\) generation of this line showed a negative effect of the constitutive expression of the gene in rice (Figure 5-6). The biomass of the OsOVP4 T\(_1\) lines was lower than that of the nulls both in non-stress and saline conditions. It was difficult to conclude the effect of constitutive expression of the gene on overall rice performance since the work was only performed with a single transformation event. However, Liu et al. (2010a) showed that transcript levels of OsOVP4 across rice tissues were significantly lower than transcription of its isoforms OsOVP1 and OsOVP6. It might suggest that rice plants do not need a high expression level of this gene in their regulatory
network, hence constitutive expression of the gene might have negative effects. However, it will be interesting to test the effect of up-regulating the gene in a specific cell type, such as the root cortex. Gaxiola et al. (2012) hypothesized that up-regulation of vacuolar H+-PPase from Arabidopsis AtAVP1 has differing effects in different tissues. This may also apply to the OsOVP4 gene. It has been shown that the expression level of OsOVP4 in roots increased upon the overexpression of the Na+-transporter, AtHKT1;1, in the rice cortex, suggesting the important role of OsOVP4 in Na+ sequestration in cortical cells, thus reducing the Na+ accumulation in the shoot (Plett et al. 2010).

5.4.5. Constitutive expression of AtCIPK16 increased both shoot Na+ and K+

Calcineurin B-like (CBL) proteins and CBL-interacting kinases (CIPKs) form complex calcium (Ca2+) signalling networks which are involved in the plant adaptation process in response to various abiotic stresses, including salt (Weinl and Kudla 2009). Ten CBLs and 26 CIPKs have been identified in Arabidopsis (Kolukisaoglu et al. 2004; Weinl and Kudla 2009), while 10 CBLs and 30 CIPKs have been identified in rice (Kolukisaoglu et al. 2004; Xiang et al. 2007). One of the CIPKs from Arabidopsis, AtCIPK16, has been shown to play a role in Na+ exclusion in Arabidopsis (Roy et al. 2013). The expression of AtCIPK16 was up-regulated under salt stress (Roy et al. 2013). Its homologue in rice, OsCIPK16, also showed up-regulation upon salt treatment (Xiang et al. 2007), suggesting a similar function of CIPK16 both in Arabidopsis and rice. Importantly, constitutive expression of this gene in barley reduced shoot Na+ accumulation in the plants under salt stress, thus improving salinity tolerance of the cereal (Roy et al. 2013).

In this study, AtCIPK16 was constitutively expressed in the rice cultivar Fatmawati under the control of the maize Ubiquitin-1 promoter. Only one transgenic Fatmawati constitutively expressing AtCIPK16 was regenerated in this study and it harboured six T-DNA insertions in the genome – therefore it was not an optimal line to study. The constitutive expression of the gene has been confirmed both in the shoot and root. Unexpectedly, Fatmawati rice constitutively expressing AtCIPK16 (R50) accumulated higher shoot Na+ compared to the nulls under salt stress (Figure 5-13), in contrary to the results seen in Arabidopsis and barley (Roy et al. 2013). The biomass of the line was also lower than that of the nulls, both under stress and control conditions (Figure 5-12). There are several possible reasons for the observed results: the difference in effect in this study may be due to AtCIPK16 interacting with different native CBL proteins in rice; there may
have been complications with a large number of T-DNA insertions; and/or only one independent line was characterized.

For a particular CBL-CIPK complex, such as SOS2-SOS3 (CIPK24-CBL4), the regulation was shown to be conserved between *Arabidopsis* and rice (Martinez-Atienza et al. 2007). However, several studies demonstrated that AtCIPK24 (SOS2) was also able to interact with different CBLs in *Arabidopsis*, forming alternative complexes (Kolukisaoglu et al. 2004; Kim et al. 2007; Weinl and Kudla 2009). For instance, AtCIPK24 interacted with CBL10 and formed a complex which may regulate vacuolar Na\(^+\) sequestration (Kim et al. 2007).

Thus, the results from this study may suggest that *AtCIPK16* in rice might interact with different rice CBLs and may therefore target different downstream proteins, which are not involved in reducing shoot Na\(^+\) accumulation. Interestingly, Fatmawati constitutively expressing *AtCIPK16* showed elevated shoot K\(^+\) compared to the nulls both in salt stress and non-stress conditions. A study by Lee et al. (2007), demonstrated that AtCIPK16 could form a complex with AtCBL1, which was then able to activate the potassium transporter AKT1 in *Arabidopsis*. It is possible that enhanced K\(^+\) uptake showed by Fatmawati constitutively expressing *AtCIPK16* is caused by the up-regulation of the rice homologue of *AKT1* as a result of the interaction between AtCIPK16 with different rice CBLs. Quantitative measurement of the expression of the rice *AKT1* in transgenic plants through qRT-PCR might answer this question. Therefore, further study is important to investigate the pleiotropic effect of the constitutive expression of *AtCIPK16* in rice under different salt stress regimes.

However, it should be noted that the transgenic Fatmawati *AtCIPK16* line used in this study had multiple T-DNA insertions. Even though copy number has been shown not to affect the expression of genes in rice (Maqbool and Christou 1999), multiple insertions of T-DNA may cause gene silencing (Flavell 1994; Stam et al. 1997) and may affect the expression of indigenous genes due to the rearrangement of multiple integration sites (Yao et al. 2006). It is necessary to characterize the lines segregating for the inserts in later generations, particularly using *AtCIPK16* lines with a lower copy number (Figure 5-11). It also again should be noted that there was only one independent transformation event for this construct, and ideally a minimum of three independent events should be characterized before any firm conclusions are made about the role of *AtCIPK16* and rice salt tolerance.
5.4.6. Lack of expression in transgenic Fatmawati and IR64 transformed with 
*PpENA1* under the control of 35S promoter

The sodium pumping ATPase *PpENA1* was identified from the bryophyte *Physcomitrella patens*. It has similarity to the fungal-type ENA-ATPase but is absent in flowering plants (Benito and Rodriguez-Navarro 2003). Results from both heterologous systems and *in planta* studies showed that *PpENA1* acted as a Na\(^+\) pump, which excluded Na\(^+\) from cells and maintained a high K\(^+\)/Na\(^+\) ratio in media with high external Na\(^+\) (Benito and Rodriguez-Navarro 2003; Lunde et al. 2007). Constitutive expression of *PpENA1* under the 35S promoter has improved salt tolerance of rice Nipponbare under 50 mM NaCl stress (Jacobs et al. 2011). Interestingly, the salt tolerance improvement was not related to Na\(^+\) exclusion as the tissue Na\(^+\) concentration of transgenic *PpENA1* was similar to that in the control plants (Jacobs et al. 2011).

In this study, an expression vector containing *PpENA1* driven by the 35S promoter was transformed into two commercial cultivars, Fatmawati and IR64, to test the effectiveness of the gene in improving salt tolerance in a different rice genetic background. Whilst *PpENA1* had successfully integrated in the genome of Fatmawati and IR64, RT-PCR expression analysis on five transgenic rice harbouring *PpENA1* failed to detect the expression of the gene in all the transgenic lines characterized, both in their shoot and root tissues (Figure 5-9).

The lack of expression of *PpENA1* in transgenic rice might be due to gene-silencing which is a common problem in transformation genetics (Stam et al. 1997; Matzke and Matzke 1998; Vaucheret et al. 1998). Gene-silencing can be caused by many factors including positional effect, transgene copy number or truncated inserts (Peach and Velten 1991; Hobbs et al. 1993; Matzke and Matzke 1998; Kohli et al. 1999; van Leeuwen et al. 2001; Schubert et al. 2004). The mechanism of gene silencing is generally mediated by two types of gene silencing: transcriptional gene silencing, reducing the amount of transcript being produced and post-transcriptional gene silencing, leading to rapid degradation of the produced transcripts (Stam et al. 1997). It is curious, however, that out of the four genes studied transformed into rice, *PpENA1* was the only one where there were issues of expression, suggesting that the issue is something unique to overexpressing *PpENA1* in rice.

It should be noted that many primary transgenic plants (T\(_0\)) were produced in the study by Jacobs et al. (2011) which had minimal expression of *PpENA1* based on real-time
PCR, suggesting a common gene-silencing problem in plants constitutively expressing *PpENA1*. In higher plants, Na\(^+\) exclusions from the cytoplasm is facilitated by two Na\(^+\)/H\(^+\) antiporters, *SOS1* in the plasma membrane (Shi et al. 2000) and *NHX1* in the tonoplast (Apse et al. 1999). These two Na\(^+\)/H\(^+\) antiporters are conserved across species including rice (Fukuda et al. 2004; Martinez-Atienza et al. 2007). *PpENA1* has a similar function to the *SOS1* Na\(^+\)/H\(^+\) antiporter (Benito and Rodriguez-Navarro 2003), however, uses ATP to generate the energy to transport Na\(^+\). It is possible that expression of *PpENA1* under the strong and constitutive promoter 35S resulted in excessive transcript levels of the gene with a similar function which could be detrimental to the rice plants. This situation possibly triggered RNA silencing mediated by an RNA sensing mechanism to eliminate excessive transcript as a genome defence mechanism, particularly since *PpENA1* is not a gene native to rice (Schubert et al. 2004). Plett (2008) compared the expression level of *PpENA1* in rice Nipponbare under a constitutive promoter (35S) and cell-type specific promoters (epidermal and cortex). It was surprising that the expression of *PpENA1* under a cell-type-specific promoter was ten times higher than under a constitutive promoter (Plett 2008). Furthermore, result from the study in Chapter 6 which shows the expression of *PpENA1* under the control of the epidermal specific promoter supported the hypothesis that gene silencing in transgenic *PpENA1* in the current study might be caused by the excessive transcript level of *PpENA1*.

5.5. Summary

This chapter highlights the results of constitutive expression of *AtAVP1, OsOVP4, AtCIPK16* and *PpENA1* in Fatmawati and IR64 as attempts to improve salinity tolerance of commercially relevant cultivars. Whilst significant salt tolerance improvement by constitutive expression of these genes could not be observed in this study, several interesting phenotypes have been observed.

Constitutive expression of *AtAVP1* appeared to improve the biomass of transgenic Fatmawati, but not IR64, under salt stress compared to the nulls. Furthermore, the constitutive expression of *AtAVP1* improved the biomass of both Fatmawati and IR64 under normal conditions.

Constitutive expression of *AtCIPK16* appeared to increase both shoot Na\(^+\) and K\(^+\) accumulation in transgenic rice, suggesting an interaction of AtCIPK16 with different native CBLs in rice, which activate different ion transporters.
The lack of salt tolerance phenotype observed in the transgenic lines in this study may also be explained by the difficulty in increasing the salinity tolerance of cultivars which already have good levels of salt tolerance, making the improvement beyond their current tolerance level more difficult.

However, before firm conclusions can be made about the effect of constitutive expression of these four genes in rice, more independent transformation events are necessary and further characterization of T2 individuals is required. Therefore, it is the recommendation of this chapter that further assessment should be carried out on these transgenic lines in later generations.
Chapter 6 : Cell Type-Specific Expression of Candidate Genes for Improving Salinity Tolerance

6.1. Introduction

Plant adaptation to a high concentration of Na\(^+\) involves various adaptation processes both at the cellular and whole plant level (Tester and Davenport 2003). At the cellular level the tolerance mechanism is similar in all plant cells, keeping Na\(^+\) from accumulating to high levels in the cytosol and involves processes, such as Na\(^+\) sequestration into the vacuole or efflux of Na\(^+\) out of the cell across the plasma membrane. At the whole plant level, different mechanisms in different cell-types are required for a coordinated transport of Na\(^+\) throughout a plant to reduce Na\(^+\) accumulation in vital tissues and cells (Tester and Davenport 2003). Some cells may predominately want to exclude Na\(^+\), while others will actively import Na\(^+\) into them and lock the Na\(^+\) away in the vacuole, stopping it reaching vital cell types. Therefore, the manipulation of specific genes responsible for Na\(^+\) transport within the plant should be targeted to specific cell types instead of using constitutive expression in all cells (Tester and Davenport 2003; Plett and Møller 2010). This was demonstrated in the manipulation of the Na\(^+\) transporter AtHKT1;1 in Arabidopsis where specific expression of the gene in root stelar cells led to the significant reduction of shoot Na\(^+\) and increased salt tolerance of Arabidopsis. Constitutive expression of AtHKT1;1 had a detrimental effect on the plant (Møller et al. 2009).

A coordinated strategy can be applied to manipulate Na\(^+\) transport within a plant involving the expression of different genes in different cell types. They include minimizing the initial influx of Na\(^+\) into the root, maximizing the Na\(^+\) efflux from the outer part of the root to the soil, minimizing Na\(^+\) loading into the xylem, maximizing Na\(^+\) retrieval from stelar cells and maximizing Na\(^+\) compartmentalisation where Na\(^+\) has less negative effects, such as in old leaves or in the root (Tester and Davenport 2003; Plett and Møller 2010). However, little work has been done so far to test the effectiveness of spatial manipulation of Na\(^+\) transport in different cell types. In Arabidopsis, the work is limited to the stelar expression of AtHKT1;1 as described in Møller et al. (2009), and the work by Plett et al. (2010), which showed a reduction in shoot Na\(^+\) accumulation as the result of the specific expression of AtHKT1;1 in epidermal and cortical cells of Arabidopsis. Plett et al. (2010) also demonstrated that cell type-specific expression of AtHKT1;1 in
root cortical cells of rice could reduce Na\(^+\) accumulation in the rice shoot and increase salinity tolerance of rice cultivar Nipponbare.

Spatial control of the transgene can be achieved by using an enhancer trap system, such as GAL4 enhancer trap lines which are available in Arabidopsis (Haseloff 1999) and rice (Johnson et al. 2005). The rice GAL4 enhancer trap lines have been used to express AtHKT1;1 in cortical cells of rice Nipponbare to improve the salt tolerance of the cultivar (Plett et al. 2010). However, the application of the cell type-specific alteration using this type of population is limited to the genetic background in which the enhancer trap system was developed. To test the effectiveness of cell type-specific modification of Na\(^+\) transport in more commercially-relevant rice cultivars, it is necessary to use a cell type-specific promoter to drive the expression of candidate genes for improving salinity tolerance as it would take time to 1) develop enhancer lines in that cultivar and 2) screen the enhancer lines for the desired cellular expression. However, to drive gene expression in a specific cell type using native plant promoters depends on the availability of cell type-specific promoters. Once a cell type-specific promoter has been identified, this approach can be efficiently transferred into different genetic backgrounds, either by genetic transformation or crossing.

Using the current scientific literature as well as the rice expression profile database and plant promoter database it is possible to identify putative promoters for driving a gene in a specific cell. Furthermore, the availability of the Gateway\(^\circledR\) cloning system means the generation of gene constructs for the manipulation of gene expression in different cell types can be done in a more efficient way compared to conventional cloning technology (Curtis and Grossniklaus 2003). Once the cell type-specific promoter has been identified and cloned into the Gateway\(^\circledR\) system, different candidate genes can be easily inserted into the destination vector.

In this study, cell type-specific alteration of Na\(^+\) transport was used as an approach to improve salinity tolerance of two commercially relevant rice cultivars from Indonesia, Fatmawati and IR64. Putative cell type-specific promoters were identified from the literature and rice expression profile databases. The promoters were used to drive the expression of different candidate genes for salt tolerance in specific cell types. The cell type-specific alterations of Na\(^+\) transport were targeted to: 1) increase Na\(^+\) efflux from root to the soil, 2) maximize Na\(^+\) retrieval from the xylem, or 3) increase Na\(^+\) compartmentalization in the root. A range of promoter-gene combination were developed
utilizing the Gateway® cloning system and employing different strategies to minimize the toxic effect of Na⁺ in rice. To increase Na⁺ efflux from outer part of the roots, the *Physcomitrella patens* Na⁺-ATPase (*PpENA1*) and *Arabidopsis* Na⁺/H⁺ antiporter (*AtSOS1*) genes were each fused to epidermal specific promoters. To maximize Na⁺ retrieval from xylem, the rice Na⁺ transporter (*OsHKT1;5*) was expressed under the control of a stelar specific promoter. To increase Na⁺ sequestration in the root, vacuolar H⁺-PPase from *Arabidopsis* (*AtAVP1*) and rice (*OsOVP4*) genes were targeted to be expressed specifically in the root cortex. It is hypothesized that these cell type-specific expression approaches could be used to improve salt tolerance of the commercial rice cultivars.

To test the hypothesis this study aimed to:

1. Identify putative root epidermal, cortical and stelar specific promoters.
2. Generate transgenic rice of Fatmawati and IR64 which express candidate genes for salinity tolerance improvement under cell type-specific promoters.
3. Characterize transgenic rice of Fatmawati and IR64 which express candidate genes for salinity tolerance improvement under cell type-specific promoters.
6.2. Methods

6.2.1. Plant Materials

6.2.2.1. Rice cultivars

The *japonica* rice cultivar, Nipponbare, was used as the genomic DNA source to amplify a putative cell type-specific promoter region. Genomic DNA of rice cultivar Nipponbare was kindly provided by Ms Margaret Pallotta (ACPFG).

The Indonesian commercial *indica* rice cultivars, Fatmawati and IR64, were used as genetic background in *Agrobacterium*-mediated transformation.

The details of the rice cultivars used are described in Section 2.1.1 and Section 3.4.1.

6.2.2.2. *Arabidopsis thaliana*

The putative cell type-specific promoters from *Arabidopsis* were cloned from *Arabidopsis thaliana* ecotype Columbia-0 (Col-0). The genomic DNA of *Arabidopsis* Col-0 was kindly provided by Dr. Bo Li (ACPFG).

6.2.2. Cloning of putative cell type-specific promoters

6.2.2.1. Identification of putative cell type-specific promoters

Putative cell type-specific promoters were identified from the literature and rice databases (Table 6-1). The Rice Expression Profile Database (RiceXPro) (http://ricexpro.dna.affrc.go.jp/) (Sato et al. 2011) and the plant promoter database (PPDB) (http://ppdb.agr.gifu-u.ac.jp/ppdb/cgi-bin/index.cgi) (Yamamoto and Obokata 2008) were used as the main database references. The DNA sequences of the promoter regions were obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Two putative root epidermal promoters were identified, one from *Arabidopsis, AtPHT1;1* (At5g43350) (Koyama et al. 2005) and the other from rice, *OsAnt1* (Os09g0440300) (Shrawat et al. 2008; Good et al. 2009).
Putative root cortical promoters were identified from Arabidopsis \((At1g62500 \text{ (Co2)})\) (Heidstra et al. 2004) and rice \((Os10g0552200)\). The promoter of the gene \(Os06g0250600\) was selected as putative promoter for driving stelar-specific expression.

The root-specific promoter from rice, \(OsRCc3 \text{ (Os02g0662000)}\) (Xu et al. 1995; Flasinski et al. 2009; Jeong et al. 2010), was selected as a candidate promoter for organ specific expression in the root.

### 6.2.2.2. Primer design

Primers were designed for promoter amplification and genotyping (Section 2.4.2). Primers for amplification of the promoter region were designed as described in Section 2.4.2. Primers which were used to clone previously published promoters were designed according to the original papers describing the promoter cloning.

Primers PHT1F and PHT1R were designed to amplify the putative promoter region of 3888 bp 5′ of the start codon of \(AtPHT1;1\) (Table 6-2). Primers OsAnt1PmeF and OsAnt1AscR were used to amplify the putative promoter region of \(OsAnt1\) (975 bp) (Table 6-2). Primers Co2F and Co2R were designed to amplify 550 bp of the putative promoter of \(At1g62500\), while primer pairs OsCORPmeF and OsCORAscR were designed to amplify 1800 bp putative promoter region of \(Os10g0552200\) (Table 6-2). Primers OSST2PmeF and OSST2AscR were designed to amplify 1000 bp of the putative promoter region of \(Os06g0250600\) (Table 6-2). Primers RCC3PMEF and RCC3R2 were designed to amplify 1943 bp of the putative promoter region of \(OsRCc3\) (Table 6-2).

Due to the size of the promoters being amplified, additional primers were also designed to aid the complete sequencing of cloned promoters. The list of primers for promoter region amplification and sequencing is presented in Table 6-2. Primers for genotyping transgenic rice are listed in Table 6-3.
6.2.2.3. Amplification of promoter regions

The DNA sequences of putative cell type-specific promoters were amplified using Proofreading PCR (Section 2.4.3.2). The PCR products were separated by agarose gel electrophoresis (Section 2.4.4). The putative promoter DNA fragment (Table 6-1) of the correct size was extracted from agarose gels as described in Section 2.4.5.

<table>
<thead>
<tr>
<th>Putative promoter</th>
<th>Size (bp)</th>
<th>DNA source</th>
<th>Target expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAtPHT1;1</td>
<td>3888</td>
<td>Arabidopsis Col-0</td>
<td>Root epidermis</td>
<td>(Koyama et al. 2005)</td>
</tr>
<tr>
<td>pOsAnt1</td>
<td>975</td>
<td>Rice Nipponbare</td>
<td>Root epidermis</td>
<td>(Shrawat et al. 2008; Good et al. 2009)</td>
</tr>
<tr>
<td>pAt1g62500 (Co2)</td>
<td>550</td>
<td>Arabidopsis Col-0</td>
<td>Root cortex</td>
<td>(Birnbaum et al. 2003; Heidstra et al. 2004)</td>
</tr>
<tr>
<td>pOs10g0552200</td>
<td>1800</td>
<td>Rice Nipponbare</td>
<td>Root cortex</td>
<td>RiceXpro, PPDB</td>
</tr>
<tr>
<td>pOs06g0250600</td>
<td>1000</td>
<td>Rice Nipponbare</td>
<td>Root stele</td>
<td>RiceXpro, PPDB</td>
</tr>
<tr>
<td>pOsRCC3</td>
<td>1943</td>
<td>Rice Nipponbare</td>
<td>Root</td>
<td>(Xu et al. 1995; Flasinski et al. 2009; Jeong et al. 2010)</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence (5’-3’)</td>
<td>Intended purpose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
<td>------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHT1F</td>
<td>CACC GTTTAAACGAAAGGGTAATGTGTAAGG</td>
<td>Amplifying promoter pAtPHT1:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHT1R</td>
<td>CAGGCCGCGCCTTCTTAGCTTATACTACAACG</td>
<td>Amplifying promoter pAtPHT1:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHTF2</td>
<td>TAAATCGATCATAACAAACTCTTAGG</td>
<td>Sequencing promoter pAtPHT1:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHTF3</td>
<td>GTTATAACCTTTTGGCTGTAC</td>
<td>Sequencing promoter pAtPHT1:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHTF4</td>
<td>TGATGGGATGAGTCTACTGAC</td>
<td>Sequencing promoter pAtPHT1:1</td>
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<td></td>
</tr>
<tr>
<td>PHTF5</td>
<td>AAGCAGTGATCATAAGCAGTAC</td>
<td>Sequencing promoter pAtPHT1:1</td>
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<td></td>
</tr>
<tr>
<td>PHTF7</td>
<td>GAGCGAGTGATCAGTAC</td>
<td>Sequencing promoter pAtPHT1:1</td>
<td></td>
<td></td>
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<tr>
<td>OsAnt1PmeF</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOsAnt1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OsAnt1AscR</td>
<td>CAGGCCGCGCCTGAGATGAGATTGAG TAGTAC</td>
<td>Amplifying promoter pOsAnt1</td>
<td></td>
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<tr>
<td>PMDC32</td>
<td>GCAATGCTTATTGACACCA</td>
<td>Amplifying promoter pOsAnt1</td>
<td></td>
<td></td>
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<tr>
<td>OsAnt1Seq1</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOsAnt1</td>
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<td>OsCORPmeF</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOs10g0552200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OsCORAscR</td>
<td>CAGGCCGCGCCTGAGATGAGATTGAG TAGTAC</td>
<td>Amplifying promoter pOs10g0552200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OsCORSeq1</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOs10g0552200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OsCORSeq2</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOs10g0552200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co2F</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pCo2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co2R</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pCo2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSST2PmeF</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOs06g0250600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSST2AscR</td>
<td>CAGGCCGCGCCTGAGATGAGATTGAG TAGTAC</td>
<td>Amplifying promoter pOs06g0250600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMDC32</td>
<td>GCAATGCTTATTGACACCA</td>
<td>Amplifying promoter pOs06g0250600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OsST2Seq1</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOs06g0250600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCC3PMEF</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOsRCc3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCC3R2</td>
<td>CAGGCCGCGCCTGAGATGAGATTGAG TAGTAC</td>
<td>Amplifying promoter pOsRCc3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCC3SEQF1</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOsRCc3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCC3SEQF2</td>
<td>CACCGTATTAACAGGAAGTGATTTATTGCTG</td>
<td>Amplifying promoter pOsRCc3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GW1</td>
<td>GTCGCAACAAATTGATGAGCAGAATGC</td>
<td>Sequencing promoter in entry vector pCR®/GW/TOPO®</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GW2</td>
<td>GTCGCAACAAATTGATGAGCAGAATGC</td>
<td>Sequencing promoter in entry vector pCR®/GW/TOPO®</td>
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<td>M13Forward</td>
<td>GTAAACAGACGGCAAG</td>
<td>Sequencing promoter in entry vector pENTR™/Directional-TOPO®</td>
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<tr>
<td>M13Reverse</td>
<td>CAGAAGCAGCAGCTATGAC</td>
<td>Sequencing promoter in entry vector pENTR™/Directional-TOPO®</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6-3 Primers for genotyping transgenic rice

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Intended purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PpENA1F5</td>
<td>CCTACATGCTCCTCGCATTT</td>
<td>Gene specific primer for <em>PpENA1</em></td>
</tr>
<tr>
<td>PpENA1R5</td>
<td>GGACAAGTGCACACAGCTCT</td>
<td>Gene specific primer for <em>PpENA1</em></td>
</tr>
<tr>
<td>NOS-F*</td>
<td>AGTAACATAGATGACACCCGCG</td>
<td>Gene specific primer for <em>OsHKT1;5-Nos</em></td>
</tr>
<tr>
<td>OsHKT-R*</td>
<td>TACACCCTTTGCTACCTGTC</td>
<td>Gene specific primer for <em>OsHKT1;5-Nos</em></td>
</tr>
<tr>
<td>AVP1-F</td>
<td>TGTTTTGACCCTAAAGTTATC</td>
<td>Gene specific primer for <em>AtAVP1</em></td>
</tr>
<tr>
<td>AVP1-R</td>
<td>TGGCTCTGAACCCCTTGTGTC</td>
<td>Gene specific primer for <em>AtAVP1</em></td>
</tr>
<tr>
<td>OVP4-NOS-F*</td>
<td>TCGCCACGCACGGAGGA</td>
<td>Gene specific primer for <em>OsOVP4-Nos</em></td>
</tr>
<tr>
<td>OVP4-NOS-R*</td>
<td>AAGACCGGCAACAGATTCAA</td>
<td>Gene specific primer for <em>OsOVP4-Nos</em></td>
</tr>
<tr>
<td>GFPiF</td>
<td>TCAAGGAGGACGGAACATC</td>
<td>Gene specific primer for <em>GFP</em></td>
</tr>
<tr>
<td>GFPiR</td>
<td>AAAGGGCAAGATGTGTTGAC</td>
<td>Gene specific primer for <em>GFP</em></td>
</tr>
<tr>
<td>GUS-F</td>
<td>ATGTTACGTCTGTAGAAACCC</td>
<td>Gene specific primer for <em>GUS</em></td>
</tr>
<tr>
<td>GUS-sR</td>
<td>AGACTTCCGCCTGAATCCAG</td>
<td>Gene specific primer for <em>GUS</em></td>
</tr>
<tr>
<td>HYGF</td>
<td>TCACTGCGAAACTGATGGAC</td>
<td>Gene specific primer for <em>Hyg</em></td>
</tr>
<tr>
<td>HYGR</td>
<td>GGTTCCTACTACGCGGAGTAC</td>
<td>Gene specific primer for <em>Hyg</em></td>
</tr>
<tr>
<td>OsGAPdh-F</td>
<td>GGGCTGCTAGCTTCAACATC</td>
<td>Primer for internal control gene</td>
</tr>
<tr>
<td>OsGAPdh-R</td>
<td>TTGATTGCGACTTGATCTG</td>
<td>Primer for internal control gene</td>
</tr>
</tbody>
</table>

* These primers were used to specifically amplify the transgene and not the native rice gene.
6.2.2.4. Generation of Gateway® entry vectors

The PCR product of the putative cell type-specific promoter (Section 6.2.2.3) was cloned into the Gateway® entry vector through TOPO® cloning as described in Section 2.4.12.1. The promoter of AtPHT1;1 was cloned into pENTR™/Directional-TOPO® (Cat. No. K2400-20, Invitrogen, Mulgrave, VIC, Australia) (Figure 2-3), while other promoters were cloned into pCR®8/GW/TOPO® (Cat. No. K2500-20, Invitrogen, Mulgrave, VIC, Australia) (Figure 2-4). The TOPO cloning reaction was then transformed into One Shot® TOP10 E. coli (Section 2.4.13). The plasmid entry vector was isolated from E. coli as described in Section 2.4.14. The DNA sequence of the promoter region was analysed by DNA sequencing as described in Section 2.4.6.

6.2.2.5. Modification of a Gateway® destination vector for the cell type-specific expression system

The putative cell type-specific promoters were cloned into the vector backbone of Gateway® destination vector pMDC32 (Figure 2-5) using restriction sites PmeI and Ascl, replacing the duplicate CaMV 35S promoter (2×35S) which is used to drive the expression of genes inserted into the Gateway cloning site (Section 2.4.12.3).

6.2.2.6. LR cloning

6.2.2.6.1. Promoter-reporter gene constructs

The destination vectors, pMDC162, containing the β-glucuronidase gene (UidA) (Curtis and Grossniklaus 2003) and pMDC204, containing green fluorescent protein gene (mgfp6) (http://botserv1.uzh.ch/home/grossnik/curtisvector/index_2.html), were used for promoter-reporter gene constructs to determine the localization of the expression of the cell type-specific promoters.

The Gateway® entry vectors containing the putative cell type-specific promoters (Section 6.2.2.4) were cloned into pMDC162 and pMDC204 through LR recombination as described in Section 2.4.12.2.
Figure 6-1 Map of the destination vectors (A) pMDC162 containing the \( \beta \)-glucuronidase gene (\( U_{id}A \)) and (B) pMDC204 containing the green fluorescent protein gene (\( mgf6 \)).

The vector backbone contains the following components: kanamycin resistance gene, the origin of replication for \( E. \ coli \) (pBR322 origin), and the minimal replicon (pVS1) for stable maintenance in \( E. \ coli \). The T-DNA cassette contains the right border sequence (RB), Gateway® recombination sequences (\( attR1 \) and \( attR2 \)), the chloramphenicol resistance gene, the \( ccdB \) gene for negative selection of the plasmid, the nopaline synthase (\( Nos \)) terminator, the cauliflower mosaic virus 35S promoter (\( CaMV \ 35S \)), the hygromycin resistance gene, the cauliflower mosaic virus 35S terminator (A35S) and the left border sequence (LB).
6.2.2.6.2. Promoter-gene of interest expression constructs

Five candidate genes for salinity tolerance improvement were selected for the cell type-specific expression system including *Arabidopsis* vacuolar H\(^+\)-PPase (*AtAVP1*) (Gaxiola et al. 2001), rice vacuolar H\(^+\)-PPase (*OsOVP4*) (Liu et al. 2010a; Plett et al. 2010), *Arabidopsis* plasma membrane Na\(^+\)/H\(^+\)-antiporter (*AtSOS1*) (Shi et al. 2000), *Physcomitrella patens* Na\(^+\)-ATPase (*PpENA1*) (Jacobs et al. 2011) and rice Na\(^+\) transporter (*OsHKT1;5*) (Ren et al. 2005; Platten et al. 2006).

The Gateway\textsuperscript{®} entry vectors containing *AtAVP1*, *AtSOS1* and *OsHKT1;5* coding sequences were provided by Dr Darren Plett (ACPFG).

The Gateway\textsuperscript{®} entry vectors containing *OsOVP4* and *PpENA1* coding sequences were provided by Dr Andrew Jacobs (ACPFG).

To express the candidate genes in specific cell types of the rice roots, the Gateway\textsuperscript{®} entry vectors containing the candidate genes were cloned by LR recombination (Section 2.4.12.2) into modified pMDC32 destination vectors which contained the appropriate cell-type-specific promoters instead of the 2\(\times\)35S promoter (Section 6.2.2.5). The promoter-gene expression constructs were recombined as detailed in Table 6-4.

### Table 6-4 Promoter-gene combination for cell type specific expression

<table>
<thead>
<tr>
<th>Putative promoter</th>
<th>Gene of interest</th>
<th>Target expression</th>
<th>Aims</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAtPHT1;1</td>
<td>PpENA1, AtSOS1</td>
<td>Root epidermis</td>
<td>Increase Na(^+) efflux to soil</td>
</tr>
<tr>
<td>pOsAnt1</td>
<td>PpENA1, AtSOS1</td>
<td>Root epidermis</td>
<td>Increase Na(^+) efflux to soil</td>
</tr>
<tr>
<td>pAtIg62500</td>
<td>AtAVP1, OsOVP4</td>
<td>Root cortex</td>
<td>Increase Na(^+) storage in root cortex</td>
</tr>
<tr>
<td>pOs10g0552200</td>
<td>AtAVP1, OsOVP4</td>
<td>Root cortex</td>
<td>Increase Na(^+) storage in root cortex</td>
</tr>
<tr>
<td>pOs06g0250600</td>
<td>OsHKT1;5</td>
<td>Root stele</td>
<td>Increase Na(^+) exclusion from shoot</td>
</tr>
<tr>
<td>pOsRCc3</td>
<td>PpENA1</td>
<td>Root</td>
<td>Increase Na(^+) efflux to soil</td>
</tr>
<tr>
<td>pOsRCc3</td>
<td>AtAVP1, OsOVP4</td>
<td>Root</td>
<td>Increase Na(^+) storage in root</td>
</tr>
<tr>
<td>pOsRCc3</td>
<td>OsHKT1;5</td>
<td>Root</td>
<td>Increase Na(^+) exclusion from shoot</td>
</tr>
</tbody>
</table>
6.2.3. Plasmid DNA transformation into chemically competent *E. coli* cells

Plasmid DNA resulting from LR cloning was transformed into chemically competent *E. coli* cells as described in Section 2.4.13. The plasmid was isolated from the cells as described in Section 2.4.14.

6.2.4. Transformation of expression vector plasmids into *Agrobacterium tumefaciens*

*Agrobacterium tumefaciens* strain AGL1 was used for the rice transformation. The expression vector plasmid was transformed into AGL1 using the freeze-thaw method as described in Section 3.2.5.

6.2.5. *Agrobacterium*-mediated rice transformation

*Agrobacterium*-mediated rice transformation was performed as described in Section 3.2.6. The transformation protocol with MS as basal salt was used as it resulted in a better transformation efficiency than Chu N6 (Chapter 3).

6.2.6. Molecular analysis of the transgenic rice

To test for presence or absence of the T-DNA insert, genomic DNA was extracted from T₀ and T₁ transgenic rice plants as described in Section 2.4.1. The presence of the transgene in T₀ and T₁ was analysed by PCR (Section 2.4.3.1) using transgene specific primers and primers for the hygromycin (*Hyg*) resistance gene (Table 6-3). Southern Blot analysis was performed to determine the number of T-DNA insertions in T₀ lines (Section 2.4.8).

Transgene expression was analysed using RT-PCR. For this, total RNA of the transgenic lines and the wild type (Section 6.2.7) was extracted as described in Section 2.4.9. The cDNA synthesis was performed as described in Section 2.4.10. The expression of the transgene was tested by RT-PCR (Section 2.4.11) using the cDNA as a template and primers specific to the transgene (Table 6-3). The glyceraldehyde 3-phosphate dehydrogenase gene from rice (*OsGAPdh*) (Table 6-3) was used as the internal control gene for both PCR and RT-PCR.
6.2.7. Salinity tolerance assay

6.2.2.1. Salinity stress assay for transgenic rice expressing candidate genes for salt tolerance driven by a cell type-specific promoter

A hydroponic salinity assay was used to phenotype transgenic rice expressing candidate genes for improving salinity tolerance driven by a cell type-specific promoter. The experiment was conducted in the greenhouses of The Plant Accelerator, Waite Campus, South Australia from 13 September 2013 to 12 October 2013 under natural light. The day length during the experiment ranged from 12 to 13 h (http://www.ga.gov.au/bin/geodesy/run/sunrisenset). The average daily global solar exposure was 21.7 MJ·m\(^{-2}\) (http://www.bom.gov.au/climate/data/index.shtml). The growth conditions and salt screening protocol were set up as described in Section 5.2.2.

Measurements of plant biomass and tissue ion concentration were performed as described in Section 2.3.

6.2.2.2. Salinity stress assay for transgenic rice expressing reporter genes driven by a cell type-specific promoter

The seeds of transgenic rice expressing the reporter genes *UidA* or *GFP* driven by cell type-specific promoters were germinated on sterile wet filter paper in plastic plates for 7 d (Section 2.2.3.) and transferred into mini hydroponic with ¼ MS media (Section 2.2.4.1). Leaf samples were taken from transgenic rice plants during transplantation for DNA extraction (Section 2.4.1.1) to determine the positive transgenic segregants.

Salt treatments were started 5 d after transplantation (12 d after germination) by replacing the hydroponic solution with new ¼ MS containing 50 mM NaCl and 1.65 mM CaCl\(_2\). On the following day, the hydroponic solution was replaced with new ¼ MS containing 100 mM NaCl and 3.3 mM CaCl\(_2\).

After 24 h grown under 100 mM NaCl treatment, the seminal roots of the rice plants were cut and used either for GUS staining (Section 6.2.2.1) or GFP observation (Section 6.2.2.3). Leaf and root of the salt stressed plants were also sampled at the same time for RNA extraction.
6.2.8. Analysis of the promoter activity in promoter-reporter gene lines

6.2.2.1. Histochemical GUS staining

Histochemical GUS staining was performed on the T₀ and T₁ transgenic plants. Leaf and root tissue for GUS staining of T₀ transgenic plants were sampled from plantlets generated from rooting media before they were transferred into soil (Section 3.2.6.7).

To determine whether the salt stress affects the promoter activity, GUS staining was performed on the roots of salt stress transgenic T₁ plants (Section 6.2.2.2).

GUS staining was carried out following the protocol of Jefferson et al. (1987) with minor modifications. In brief, the plant tissue was submerged either in Cellstar® 6 well culture plates (Cat. No. 657160, Greiner bio-one, West Anaheim, CA, USA) or 10 mL yellow cap tubes (Cat. No. 62.9924.284, SARSTEDT, Ingle Farm, South Australia) containing sufficient GUS solution for full submergence (0.5 mg·mL⁻¹ X-Gluconide (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronic acid, cyclohexammonium salt; Cat. No. G1281C1, Gold Biotechnology, St Louis, MO, USA) (dissolved in dimethylformamide), 50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide). Samples were incubated in the dark at 37°C for 1 h to 24 h depending on the GUS activity. The GUS solution was then removed and replaced with 25% ethanol (v/v) for 1 h followed by 50% ethanol (v/v) for 1 h before being fixed in 75% ethanol (v/v) to remove the chlorophyll.

The GUS-stained intact plant images were photographed using a digital camera Nikon D5100. Close-up images were taken using a Leica MZ FLIII Fluorescence Stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland). Images were taken using a Leica DC300F digital camera and analysed using the IM50 software version 1.20 (Leica Microsystems).

6.2.2.2. Cross section analysis of GUS-stained plant material

For root cross section analysis, the GUS-stained sample was washed with RO water and fixed in 5% (v/v) glutaraldehyde (in 0.1 M sodium phosphate buffer, pH6.8) at 4°C overnight. The sample was then washed three times with 1 × phosphate buffered saline (PBS) for 10 min. After that, the sample was embedded in 1% (w/v) agarose (in 1 × PBS). The embedded sample was cut into a small triangular shape (sized to fit into a 0.2 mL PCR tube). The sample was dehydrated using an ethanol series of 50% (v/v), 70% (v/v),
90% (v/v) and 100% ethanol with an interval of 10-15 min. The sample was then processed for acrylic resin embedding using Technovit 7100 (Cat. No. 64709003, Heraeus Kulzer, Wehrheim, Germany). In brief, the sample was infiltrated by 25% (v/v) Technovit solution A (100 mL Technovit 7100, 1 pack Hardener I and 2.5 mL PEG400) in 100% ethanol for 1 h. The solution was changed with 50% and 75 % (v/v) Technovit solution A (in 100% ethanol), for 1 h each. The solution was then replaced with 100% Technovit solution A and incubated overnight at 4°C. The sample was polymerized in a PCR tube using the mix of 15 mL Technovit solution A and 1 mL Hardener II at room temperature.

Root cross-sectioning was performed using a Leica rotary microtome (RM2265, Leica Biosystems, Nussloch, Germany). The root sample was sectioned into 10 µm thick slices using a glass knife and the cross sections of the root were then placed on a glass slide. DPX mountant (Cat. No. 44581, Sigma Aldrich, St. Louis, MO, USA) was layered on the cross-section samples before they were covered with a cover slide for microscopy analysis. The cross-section image were taken using a Leica AS LMD Laser dissection microscope (Leica Microsystems) and were analysed using IM1000 Leica software (Leica Microsystems).

6.2.2.3. **GFP analysis using a stereomicroscope**

GFP fluorescence in transgenic rice expressing *GFP* under a cell type-specific promoter was analysed in the T₀ and T₁ generations. The sample for GFP observation in the T₀ generation was taken from transgenic plantlets generated from rooting media before being transferred into soil.

The GFP fluorescence was analysed in the T₁ generation using tissue samples from salt stressed plants grown in hydroponics (Section 6.2.2.2).

GFP fluorescence was observed using a Leica MZ FLIII Fluorescence Stereo Microscope with a GFP3 filter set (excitation filter 470/40 nm, barrier filter BP520/50 nm). Images were taken using a DC300F digital camera and analysed using IM50 software version 1.20 (Leica Microsystems).
6.3. Results

6.3.1. Identification of putative root cell type-specific promoters from literature and rice databases

6.3.1.1. Putative root epidermal promoters

Two promoters were identified from the literature with high activity reported in rice root epidermal cells. These were the promoter of a phosphate transporter from *Arabidopsis thaliana* (*pAtPHT1;1*) (Koyama et al. 2005) and an antiquitin from rice (*pOsAnt1*) (Shrawat et al. 2008).

Histochemical GUS analysis of *pAtPHT1;1:UidA* plants showed that the promoter drives *UidA* expression primarily in roots in both *Arabidopsis* and rice (Koyama et al. 2005). The expression of *UidA* under the control of the *pAtPHT1;1* promoter was stronger in rice root epidermal cells compared to other parts of the rice roots (Koyama et al. 2005). The activity of the *pAtPHT1;1* was induced by phosphate starvation. However, under normal soil conditions with available fertilizer, the expression level of the promoter was as strong as the *CaMV 35S* promoter (Koyama et al. 2005).

*pOsAnt1* encodes the antiquitin protein, a member of the aldehyde dehydrogenase gene family (Shrawat et al. 2008). GUS staining analysis showed that the *pOsAnt1* drives *UidA* expression in the rice roots and showed a stronger expression in root epidermal cells compared to other root cells (Shrawat et al. 2008).

6.3.1.2. Putative root cortical promoters

Two putative root cortical promoters were selected for this study: the promoter of *At1g62500* (cortex specific transcript (*Co2*)) from *Arabidopsis thaliana* and *Os10g0552200* from rice. The promoter of *At1g62500* (*pCo2*) from *Arabidopsis* has been demonstrated to have strong activity specifically in the root cortex of *Arabidopsis* (Birnbaum et al. 2003; Heidstra et al. 2004). Based on The Arabidopsis Information Resource (TAIR), *At1g62500* is involved in lipid transport (http://arabidopsis.org/servlets/TairObject?id=137519&type=locus). *Os10g0552200* from rice has been predicted to have a similar function to *At1g62500* and based on the Rice Expression Profile Database (RiceXPro), the gene is mainly expressed in roots (http://ricexpro.dna.affrc.go.jp/field-development.php?featurenum=39467) (Figure 6-2).
The promoter is therefore likely to be active in the root cortex. However, the gene expression profile of *Os10g0552200* ([http://ricexpro.dna.affrc.go.jp](http://ricexpro.dna.affrc.go.jp)) in roots had a very low signal intensity (less than 50) (Figure 6-3) (Takehisa et al. 2012), making difficulty to determine the tissue specific localization of *Os10g0552200*.

**Figure 6-2** The spatio-temporal expression profile of *Os10g0552200* in specific tissues and organs at different growth stages of rice.

The graph was downloaded from the Rice Expression Profile Database (RiceXPro, [http://ricexpro.dna.affrc.go.jp/](http://ricexpro.dna.affrc.go.jp/)). The expression profile data was derived from different tissues and organ types at different developmental stages of field grown rice Nipponbare. Rice Nipponbare seedlings were transplanted to the paddy field 30 d after germination. Vegetative_12:00 and vegetative_00:00 correspond to the sampling time at 27 d and 28 d after transplanting. Reproductive_12:00 and reproductive_00:00 correspond to the sampling time 76 d and 77 d after transplanting. Different colours of bars indicate the different tissues and organs. The expression profile is presented as raw data resulting from hybridization using the Agilent one-colour (Cy3) microarray-based gene analysis system. The detailed description of the diagram features is available on [http://ricexpro.dna.affrc.go.jp/GGEP/sample-list.php](http://ricexpro.dna.affrc.go.jp/GGEP/sample-list.php)
Figure 6-3 The gene expression profile of Os10g0552200 in rice roots in the cultivar Nipponbare.
The graph was downloaded from the Rice Expression Profile Database (RiceXpro, http://ricexpro.dna.affrc.go.jp/). The sample was from a 10 d-old rice seedling and root sections were separated using laser microdissection (Takehisa et al. 2012). The expression profile is presented in raw data resulting from hybridization using the Agilent one-colour (Cy3) microarray-based gene analysis system. The root cross section figures below the graph represent the tissue type (highlighted in blue) from which the sample was taken.
6.3.1.3. Putative root stelar promoter

The promoter of *Os06g0250600* was selected as a putative promoter for stelar-specific expression. The gene encodes a K\(^+\) channel in rice (RiceXPro, http://ricexpro.dna.affrc.go.jp/). The expression profile of the gene showed that the gene is mainly expressed in rice roots during the vegetative stage (Figure 6-4). The gene is also expressed in leaf blades but with lower intensity than in the roots (Figure 6-4). Based on the root gene expression profile analysis available in the RiceXpro database, *Os06g0250600* was mainly expressed in the inner part of the root (Figure 6-5) (Takehisa et al. 2012).

6.3.1.4. Putative root specific promoter

The promoter of *OsRCc3* (root specific protein, *Os02g0662000*) (Xu et al. 1995; Flasinski et al. 2009; Jeong et al. 2010) was selected as the putative root specific promoter to express the candidate gene for salt tolerance improvement. Transgenic rice expressing a transcription factor *OsNAC10* (*NAM (No Apical Meristem), ATAF1-2, and CUC2 (Cup-Shaped Cotyledon)*) under the control of the *OsRCc3* promoter showed larger root stele, cortex and epidermis compared to the transgenic rice constitutively expressing *OsNAC10* and the non-transgenic rice plants, as a result of an increase in the cell number (Jeong et al. 2010).
Figure 6-4 The spatio-temporal profile of *Os06g0250600* in specific tissues and organs at different growth stages of rice.

The graph was downloaded from the Rice Expression Profile Database (RiceXPro, http://ricexpro.dna.affrc.go.jp/). The expression profile data was derived from different tissues and organ types at different developmental stages of field grown rice Nipponbare. Rice Nipponbare seedlings were transplanted to the paddy field 30 d after germination. Vegetative_12:00 and vegetative 00:00 correspond to the sampling time at 27 d and 28 d after transplanting. Reproductive_12:00 and reproductive_00:00 correspond to the sampling time 76 d and 77 d after transplanting. The different coloured bars indicate different tissues and organs. The expression profile is presented as raw data resulting from hybridization using the Agilent one-colour (Cy3) microarray-based gene analysis system. The detailed description of the diagram features is available on http://ricexpro.dna.affrc.go.jp/GGEP/sample-list.php
Figure 6-5 The gene expression profile of *Os06g0250600* in rice roots in the cultivar Nipponbare.

The graph was downloaded from the Rice Expression Profile Database (RiceXpro, http://ricexpro.dna.affrc.go.jp/). The sample was from a 10 d-old rice seedling and root sections were separated using laser microdissection (Takehisa et al. 2012). The expression profile is presented in raw data resulting from hybridization using the Agilent one-colour (Cy3) microarray-based gene analysis system. The root cross section figures below the graph represent the tissue type (highlighted in blue) from which the sample was taken.
6.3.2. Cloning of putative cell type-specific promoters and vector constructions

6.3.2.1. Cloning of putative cell type specific promoters

Six putative promoters have been cloned for cell type specific expression of candidate gene for salt tolerance (Table 6-1). Two putative promoters, *pAtPHT1;1* (3888 bp) and *pAt1g62500* (*pCo2*) (550 bp), were cloned from *Arabidopsis thaliana* Col-0. Four putative promoters were cloned from japonica rice Nipponbare, including *pOsAnt1* (975 bp), *pOs10g0552200* (1800 bp), *pOs06g0250600* (1000 bp), and *pOsRCc3* (1943 bp).

The amplified promoter region of *pAtPHT1;1* was cloned into the Gateway® entry vector pENTR™/Directional-TOPO® (Figure 6-6). The other five promoters were cloned into the pCR8/GW/TOPO entry vector to enable the Gateway® cloning reaction (Supplementary Figure 1 to 5). Sequencing of the putative promoter regions confirmed their alignment to the reference genome of Nipponbare (NCBI), except for the sequence of putative promoter region of *OsRCc3*. The sequencing of the amplified product of the promoter region of *OsRCc3* showed a 4 bp deletion in the sequence (866 bp-869 bp upstream from the start codon) when compared to the reference genome (Supplementary Figure 6).
Figure 6-6 Map of Gateway® entry vector pENTR/D-TOPO+ProAtPHT1;1

The vector contains the transcription termination sequences (rrnB T1 and T2), the M13 forward and reverse primer site for sequencing the insert, the Gateway® recombination site (attL1 and attL2), the directional TOPO® cloning site, the putative promoter of AtPHT1;1, the T7 promoter and T7 primer site for in vitro transcription and sequencing the insert, the kanamycin resistance gene for selection of the plasmid in E. coli (Kan(R)) and the pUC origin of replication (pUC origin) for high copy replication and maintenance in E. coli.
6.3.2.2. Development of Gateway enabled destination vector containing putative cell type specific promoter

Six Gateway® enabled destination cassettes were developed containing the putative cell type specific promoters. The cassettes were generated using Gateway® destination vector pMDC32 as the source for the vector backbone. In each cassette, the putative cell type specific promoter was inserted between the restriction sites PmeI and AscI in pMDC32 replacing the 2×35S promoter. The diagram of new Gateway® enabled destination vector containing putative epidermal promoter pAtPHT1;1 (pMDC32_AtPHT1;1) is shown in Figure 6-7. The map of other Gateway® enabled destination vector with other putative cell type specific promoter are available in Supplementary Figures 7 to 11.

**Figure 6-7 Map of the destination vector pMDC32_AtPHT1;1.**
The vector backbone contains the following components: kanamycin resistance gene, the origin of replication for *E. coli* (pBR322 origin), and the minimal replicon (pVS1) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), the putative promoter of *AtPHT1;1*, the Gateway® recombination sequences (*attR1* and *attR2*), the chloramphenicol resistance gene (*CMr*), the *ccdB* gene for negative selection of the plasmid, the nopaline synthase (*Nos*) terminator, the cauliflower mosaic virus 35S promoter (*CaMV35S*), the hygromycin resistance gene, the cauliflower mosaic virus 35S terminator (*A35S*) and the left border sequence (LB).
6.3.2.3. Generation expression vector for promoter localization studies

Two reporter genes, *UidA* and *GFP*, were used to study the localization of the cell type specific promoters. Gateway® expression vectors were developed to express *UidA* and *GFP* under the control of putative cell type specific promoters. The putative cell type specific promoters from Gateway® entry vector were cloned into both pMDC162 and pMDC204 through LR recombination to express *UidA* and *GFP*, respectively. The resultant expression vectors for promoter localization studies are listed in Table 6-5.

**Table 6-5 Summary of the expression vectors generated for promoter localization studies**

<table>
<thead>
<tr>
<th>Vector resulting from LR cloning</th>
<th>Entry vector</th>
<th>Destination vector</th>
<th>Intended purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAH01</td>
<td>pENTR/D-TOPO+ProAtPHT1;1</td>
<td>pMDC162</td>
<td>Testing <em>pAtPHT1;1</em> as root epidermal specific promoter using <em>UidA</em></td>
</tr>
<tr>
<td>pAH02</td>
<td>pENTR/D-TOPO+ProAtPHT1;1</td>
<td>pMDC204</td>
<td>Testing <em>pAtPHT1;1</em> as root epidermal specific promoter using <em>GFP</em></td>
</tr>
<tr>
<td>pAH15</td>
<td>pCR8-OsAnt1</td>
<td>pMDC162</td>
<td>Testing <em>pOsAnt1</em> as root epidermal specific promoter using <em>UidA</em></td>
</tr>
<tr>
<td>pAH16</td>
<td>pCR8-OsAnt1</td>
<td>pMDC204</td>
<td>Testing <em>pOsAnt1</em> as root epidermal specific promoter using <em>GFP</em></td>
</tr>
<tr>
<td>pAH03</td>
<td>pCR8-ProOs10g0552200</td>
<td>pMDC162</td>
<td>Testing <em>pOs10g0552200</em> as root cortical specific promoter using <em>UidA</em></td>
</tr>
<tr>
<td>pAH04</td>
<td>pCR8-ProOs10g0552200</td>
<td>pMDC204</td>
<td>Testing <em>pOs10g0552200</em> as root cortical specific promoter using <em>GFP</em></td>
</tr>
<tr>
<td>pAH07</td>
<td>pCR8-Co2</td>
<td>pMDC162</td>
<td>Testing <em>pCo2</em> as root cortical specific promoter using <em>UidA</em></td>
</tr>
<tr>
<td>pAH08</td>
<td>pCR8-Co2</td>
<td>pMDC204</td>
<td>Testing <em>pCo2</em> as root cortical specific promoter using <em>GFP</em></td>
</tr>
<tr>
<td>pAH05</td>
<td>pCR8-Os06g0250600</td>
<td>pMDC162</td>
<td>Testing <em>pOs06g0250600</em> as root stelar specific promoter using <em>UidA</em></td>
</tr>
<tr>
<td>pAH06</td>
<td>pCR8-Os06g0250600</td>
<td>pMDC204</td>
<td>Testing <em>pOs06g0250600</em> as root stelar specific promoter using <em>GFP</em></td>
</tr>
<tr>
<td>pAH20</td>
<td>pCR8-OsRCc3</td>
<td>pMDC162</td>
<td>Testing <em>pOsRCc3</em> as root specific promoter using <em>UidA</em></td>
</tr>
<tr>
<td>pAH21</td>
<td>pCR8-OsRCc3</td>
<td>pMDC204</td>
<td>Testing <em>pOsRCc3</em> as root specific promoter using <em>GFP</em></td>
</tr>
</tbody>
</table>
6.3.2.4. **Generation vectors for cell type specific promoter-gene of interest expression**

In parallel with the promoter localization studies, the putative cell type-specific promoters were used to drive the expression of candidate genes for salt tolerance. Expression vectors were developed by using LR cloning to insert the candidate gene of interest from the Gateway® entry vector into the Gateway® enabled destination vectors containing cell type-specific promoters (Table 6-6).

To increase Na⁺ efflux from root to soil, Na⁺-ATPase from *Physcomitrella patens* (*PpENA1*) and plasma membrane Na⁺/H⁺ antiporter (*AtSOS1*) were cloned under the control of putative epidermal promoters *pAtPHT1;1* and *pOsAnt1* (Table 6-6).

Four expression vectors were developed to express vacuolar H⁺-PPases from *Arabidopsis thaliana* (*AtAVP1*) and rice (*OsOVP4*) under the control of two putative cortical specific promoters, *pOs10g0552200* and *pCo2*. These promoter-gene combinations were aimed to increase Na⁺ compartmentalization in the root cortex (Table 6-6).

The putative stelar promoter *pOs06g0250600* was used to drive the expression of Na⁺ transporter (*OsHKT1;5*) from salt tolerant rice cultivar Pokkali in attempt to increase Na⁺ retrieval from the xylem. A Gateway® expression vector was generated by LR recombination between the Gateway® entry vector containing *OsHKT1;5* and the Gateway® destination vector containing putative stelar specific promoter *pOs06g0250600* (Table 6-6).

A final set of expression vectors were developed by the recombination of root specific promoter (*pOsRCc3*) with *AtAVP1*, *OsOVP4* as attempts to increase Na⁺ storage in the root. The promoter was also used to drive *OsHKT1;5* and *PpENA1* to test whether an organ specific promoter can be used to increase Na⁺ exclusion from shoot to root and increase Na⁺ efflux from root to soil, respectively (Table 6-6).
Table 6-6 Summary of the expression vectors generated for cell type-specific expression systems

<table>
<thead>
<tr>
<th>Vector resulting from LR cloning</th>
<th>Entry vector(^1)</th>
<th>Destination vector</th>
<th>Target expression</th>
<th>Target improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAH09</td>
<td>pENTR/D-TOPO[PpEna1]</td>
<td>pMDC32_AtPHT1:1</td>
<td>Root epidermal specific expression of <em>PpENA1</em></td>
<td>Increasing Na(^+) efflux to soil</td>
</tr>
<tr>
<td>pAH10</td>
<td>pCR8/AtSOS1</td>
<td>pMDC32_AtPHT1:1</td>
<td>Root epidermal specific expression of <em>AtSOS1</em></td>
<td></td>
</tr>
<tr>
<td>pAH17</td>
<td>pENTR/D-TOPO[PpEna1]</td>
<td>pMDC32OsAnt1</td>
<td>Root epidermal specific expression of <em>PpENA1</em></td>
<td></td>
</tr>
<tr>
<td>pAH18</td>
<td>pCR8/AtSOS1</td>
<td>pMDC32OsAnt1</td>
<td>Root epidermal specific expression of <em>AtSOS1</em></td>
<td></td>
</tr>
<tr>
<td>pAH11</td>
<td>pCR8-AtAVP1</td>
<td>pMDC32-ProOs10g0552200</td>
<td>Cortical specific expression of <em>AtAVP1</em></td>
<td>Increasing Na(^+) storage in root</td>
</tr>
<tr>
<td>pAH12</td>
<td>pCR8-OsOVP4</td>
<td>pMDC32-ProOs10g0552200</td>
<td>Cortical specific expression of <em>OsOVP4</em></td>
<td></td>
</tr>
<tr>
<td>pAH13</td>
<td>pCR8-AtAVP1</td>
<td>pMDC32Co2</td>
<td>Cortical specific expression of <em>AtAVP1</em></td>
<td></td>
</tr>
<tr>
<td>pAH14</td>
<td>pCR8-OsOVP4</td>
<td>pMDC32Co2</td>
<td>Cortical specific expression of <em>OsOVP4</em></td>
<td></td>
</tr>
<tr>
<td>pAH23</td>
<td>pCR8-AtAVP1</td>
<td>pCR8-OsRCc3</td>
<td>Root specific expression of <em>AtAVP1</em></td>
<td></td>
</tr>
<tr>
<td>pAH24</td>
<td>pCR8-OsOVP4</td>
<td>pCR8-OsRCc3</td>
<td>Root specific expression of <em>OsOVP4</em></td>
<td></td>
</tr>
<tr>
<td>pAH19</td>
<td>pCR8-OsHKT1;5(P8)</td>
<td>pMDC32-ProOs06g0250600</td>
<td>Root stelar specific expression of <em>OsHKT1;5</em></td>
<td>Increasing Na(^+) retrieval from shoot</td>
</tr>
<tr>
<td>pAH22</td>
<td>pCR8-OsHKT1;5(P8)</td>
<td>pMDC32-ProOs10g0552200</td>
<td>Root stelar specific expression of <em>OsHKT1;5</em></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Entry vectors pENTR/D-TOPO[PpEna1] and pCR8-OsOVP4 were provided by Dr. Andrew Jacobs (ACPFG). Entry vectors pCR8-AtAVP1, pCR8/AtSOS1 and pCR8-OsHKT1;5(P8) were provided by Dr. Darren Plett (ACPFG).
6.3.3. Generation of transgenic rice with cell type-specific expression system

Attempts were made to transform all the expression vectors presented in Table 6-5 and Table 6-6 into Fatmawati and IR64 using Agrobacterium-mediated transformation. An exception applied only to the plasmid vector pAH17 (OsAnt1:PpENA1) because the plasmid could not be transformed into the Agrobacterium strain AGL1. However, not all constructs were successfully introduced into the rice cultivars (Table 6-7 and Table 6-8). A total of 18 out of 24 constructs were transformed into rice cultivar Fatmawati, however the number of independent lines for each construct varied and mostly only resulted in a single independent event (Table 6-7). Only four out of 24 constructs were transformed into the IR64 genetic background despite numerous attempts to transform them all (Table 6-8).

Table 6-7 Summary of transgenic Fatmawati generated for cell type-specific expression

<table>
<thead>
<tr>
<th>Vector</th>
<th>Promoter-gene combination</th>
<th>Number of transformation attempts</th>
<th>Total number of calli transformed</th>
<th>Number of independent lines</th>
<th>Number of fertile transgenic lines</th>
</tr>
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<tbody>
<tr>
<td>pAH02</td>
<td>pAtPHT1;1:GFP</td>
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<td>120</td>
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<td>1</td>
</tr>
<tr>
<td>pAH03</td>
<td>pOs10g0552200:UidA</td>
<td>1</td>
<td>45</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>pAH04</td>
<td>pOs10g0552200:GFP</td>
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<td>45</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
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<td>pOs06g0250600:UidA</td>
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<td>150</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>pAH06</td>
<td>pOs06g0250600:GFP</td>
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<td>150</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>pAH07</td>
<td>pCo2:UidA</td>
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<td>75</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pAH08</td>
<td>pCo2:GFP</td>
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<td>75</td>
<td>6</td>
<td>6</td>
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<tr>
<td>pAH09</td>
<td>pAtPHT1;1:PsENA1</td>
<td>2</td>
<td>150</td>
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<td>1</td>
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<tr>
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<td>pAtPHT1;1:PsSOS1</td>
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<td>1</td>
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<tr>
<td>pAH11</td>
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<td>225</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pAH12</td>
<td>pOs10g0552200:OsOVP4</td>
<td>3</td>
<td>225</td>
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<td>1</td>
</tr>
<tr>
<td>pAH14</td>
<td>pCo2:OsOVP4</td>
<td>1</td>
<td>75</td>
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<td>1</td>
</tr>
<tr>
<td>pAH15</td>
<td>pOsAnt1:UidA</td>
<td>2</td>
<td>150</td>
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<tr>
<td>pAH16</td>
<td>pOsAnt1:GFP</td>
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<td>150</td>
<td>5</td>
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<tr>
<td>pAH18</td>
<td>pOsAnt1:PsSOS1</td>
<td>2</td>
<td>150</td>
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<td>2</td>
</tr>
<tr>
<td>pAH20</td>
<td>pOsRCc3:UidA</td>
<td>1</td>
<td>75</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pAH21</td>
<td>pOsRCc3:GFP</td>
<td>1</td>
<td>75</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pAH24</td>
<td>pOsRCc3:OsOVP4</td>
<td>2</td>
<td>150</td>
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</tr>
</tbody>
</table>
Table 6-8 Summary of transgenic IR64 generated for cell type-specific expression

<table>
<thead>
<tr>
<th>Vector</th>
<th>Promoter-gene combination</th>
<th>Number of transformation attempts</th>
<th>Total number of calli transformed</th>
<th>Number of independent lines</th>
<th>Number of fertile transgenic lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAH09</td>
<td>pAtPHT1;1:PpENA1</td>
<td>2</td>
<td>150</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>pAH11</td>
<td>pOs10g0552200:AtAVP1</td>
<td>2</td>
<td>150</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>pAH19</td>
<td>pOs06g0250600:OsHKT1;5</td>
<td>2</td>
<td>150</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pAH20</td>
<td>pOsRCc3:UidA</td>
<td>1</td>
<td>75</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

6.3.4. Expression of reporter genes under the control of putative cell type-specific promoters

6.3.4.1. Promoter pOs10g0552200 drove UidA expression in root phloem

Five independent lines of transgenic Fatmawati have been generated expressing UidA under control of the putative cortical promoter pOs10g0552200 (Table 6-7). Histochemical GUS staining on salt stressed rice tissue confirmed the expression of UidA under the control of pOs10g0552200 (Figure 6-8). Promoter pOs10g0552200 mainly directed the UidA expression in the inner part of the roots (Figure 6-8 B and Figure 6-8 C). The GUS activity was also present in the leaf tip of the transgenic seedling (Figure 6-8B). Cross section root analysis showed that the promoter specifically drove UidA expression in the phloem cells (Figure 6-8D and Figure 6-8 E).
Figure 6-8 Histochemical GUS staining of *pOs10g0552200:UidA* seedlings

(A) GUS staining on 7 d-old rice seedlings of WT Fatmawati as negative control and (B) transgenic Fatmawati *pOs10g0552200:UidA*, which showed strong GUS activity in roots and weak activity in the leaf tip. (C) GUS staining on roots of 14 d old transgenic rice Fatmawati *pOs10g0552200:UidA* grown under 100 mM NaCl. Rice plants were grown in mini hydroponics with ¼ × MS media. Salt stress was imposed in 50 mM NaCl increments over a 2 d period starting from 12 d after germination. Roots were sampled 24 h after 100 mM NaCl treatment was reached. Roots were incubated in GUS staining solution for 16 h. (D) Cross section of the salt stressed roots with 20× and (E) 40× magnification.
6.3.4.2. **Promoter pOsRCc3 drove UidA expression mainly in the root cortex and endodermis**

The expression of UidA under the control of the putative promoter pOsRCc3 in transgenic Fatmawati was confirmed by histochemical GUS staining (Figure 6-9). Histochemical GUS staining was performed on the root tissue of salt stressed rice. GUS activity was found to be very strong and could be detected after 1 h of GUS staining (Figure 6-9). The UidA expression was specifically detected in the root and not in the shoot (Figure 6-9 A). Cross section analysis revealed that the GUS activity was present in all parts of the root and it tended to be stronger in the endodermal and cortical areas (Figure 6-9 B and C).

**Figure 6-9 Histochemical GUS staining pOsRCc3:UidA seedlings**
(A) GUS staining on 7 d rice seedlings of transgenic Fatmawati pOsRCc3:UidA showed strong activity only in the root. (B) GUS staining on roots of 14 d old transgenic rice Fatmawati pOsRCc3:UidA grown under 100 mM NaCl. Rice plants were grown in mini hydroponics with ¼ × MS media. Salt stress was imposed in 50 mM NaCl increments over 2 d period starting from 12 d after germination. Roots were sampled 24 h after 100 mM NaCl treatment was reached. Roots were incubated in the GUS staining solution for 1 h. (C) Cross section of the salt stress root at 40× magnification.
6.3.4.3. The putative epidermal promoters *pAtPHT1;1* and *pOsAnt1* drove *GFP* expression both in shoot and root

A single event of transgenic Fatmawati was generated expressing the reporter gene *GFP* driven by the putative epidermal promoter *pAtPHT1;1* (Table 6-7). Observations of GFP fluorescence in this line in the T₀ and T₁ generations were not conclusive as GFP fluorescence could not be differentiated from the autofluorescence in the wild type Fatmawati (data not shown).

RT-PCR was conducted to determine the expression of *GFP* under the control of *pAtPHT1;1* at the molecular level in the T₁ generation. Total RNA was extracted from salt stressed rice plants and used to synthesize cDNA for RT-PCR. The result showed that the promoter *pAtPHT1;1* was able to express *GFP*, however the expression was present both in shoot and root (Figure 6-10).

![Figure 6-10 Expression analysis using RT-PCR of GFP driven by putative epidermal promoters pAtPHT1;1 and pOsAnt1.](image)

The cDNAs were synthesized from total RNA extracted from leaf and root tissue of T₁ rice plants grown under 100 mM NaCl. Rice plants were grown in mini hydroponic with ¼ × MS media. Salt stress was imposed in 50 mM NaCl increments over 2 d period starting from 12 d after germination. Leaf and root tissue were harvested 24 h after 100 mM NaCl treatment was reached. Three plants from one transgenic line *pAtPHT1;1:GFP*, three plants from three independent transgenic lines *pOsAnt1:GFP*, and two WT Fatmawati plants were used as biological replicates. PCR was performed in 35 cycles using *GFP* specific primers. *OsGAPdh* was used as the internal control gene. (-) = negative control (water used in PCR instead of DNA); (+) = positive control (plasmid containing *GFP* was used as DNA source)
6.3.4.4. The putative epidermal promoter *pOsAnt1* drove *GFP* and *UidA* expression both in shoot and roots

A total of five independent transgenic Fatmawati lines have been generated expressing *GFP* under the putative epidermal promoter *pOsAnt1* (Table 6-7). GFP fluorescence in the transgenic lines could not be observed in both the T<sub>0</sub> and T<sub>1</sub> generations under a stereomicroscope (data not shown). However, the expression of *GFP* was confirmed at the molecular level through RT-PCR analysis (Figure 6-10). The result showed that the promoter *pOsAnt1* drove *GFP* expression both in shoot and root of salt stressed transgenic rice plants (Figure 6-10).

Two transgenic rice Fatmawati lines have been generated expressing *UidA* under the *OsAnt1* promoter (Table 6-7). Histochemical GUS staining was performed to detect GUS activity in these two transgenic lines, in both the T<sub>0</sub> and T<sub>1</sub> generations. However, GUS activity was not detectable after 24 h of staining. To test the expression of the reporter gene, total RNA of these two transgenic lines was extracted and used for cDNA synthesis. RT-PCR analysis using cDNA as the DNA template showed that *UidA* was expressed in these two transgenic lines and did not express in the wild type, indicating that *pOsAnt1* was able to drive the gene expression (Figure 6-11). However, the expression was not limited to the root as the DNA band was also present in the shoot (Figure 6-11).
Figure 6-11 Expression analysis using RT-PCR of $UidA$ driven by putative epidermal promoter $pOsAnt1$.

The cDNAs were synthesized from total RNA extracted from leaf and root tissue of T$_1$ rice plants grown under 100 mM NaCl. Rice plants were grown in mini hydroponic with ¼ × MS media. Salt stress was imposed in 50 mM NaCl increments over 2 d period starting from 12 d after germination. Leaf and root tissue were harvested 24 h after 100 mM NaCl treatment was reached. Two plants from two transgenic lines $pOsAnt1:UidA$, and two WT Fatmawati plants were used as biological replicates. PCR was performed with 35 cycles using $UidA$ specific primers. $OsGAPdh$ was used as the internal control gene. (-) = negative control (water used in PCR instead of DNA); (+) = positive control (plasmid containing $UidA$ was used as DNA source).
6.3.4.5. The putative stelar promoter pOs06g0250600 drove UidA expression mainly in the shoot and less in the root

Three independent transgenic lines in the Fatmawati genetic background have been generated expressing UidA under the control of putative stelar promoter pOs06g0250600 (Table 6-5). GUS activity was not observed after the tissue of these transgenic lines was infiltrated by GUS staining solution for 24 h. However, the expression of UidA under the putative stelar promoter pOs06g0250600 was confirmed by RT-PCR analysis (Figure 6-12). Apparently, UidA expression driven by the pOs06g0250600 was stronger in the shoot compared to the root (Figure 6-12).

![Figure 6-12](image_url)

**Figure 6-12 Expression analysis using RT-PCR of UidA driven by putative stelar promoter pOs06g0250600.**

The cDNAs were synthesized from total RNA extracted from leaf and root tissue of T1 rice plants grown under 100 mM NaCl. Rice plants were grown in mini hydroponic with ¼ × MS media. Salt stress was imposed in 50 mM NaCl increments over 2 d period starting from 12 d after germination. Leaf and root tissue were harvested 24 h after 100 mM NaCl treatment was reached. Three plants from three independent transgenic lines of pOs06g0250600:UidA and two WT Fatmawati plants were used as biological replicates. PCR was performed with 35 cycles using UidA specific primers. OsGAPdh was used as the internal control gene. (-) = negative control (water used in PCR instead of DNA); (+) = positive control (plasmid containing UidA was used as a source of DNA).
The summary of the expression pattern of GFP and UidA under the control of putative cell type specific promoters is shown in Table 6-9. GUS activity was only observed in transgenic pOs10g0552200:UidA and pOsRCc3:UidA (Table 6-9). While the GFP signal or GUS activity in other transgenic lines with promoter-reporter gene constructs could not be observed, the presence of the reporter gene transcripts in these lines has been confirmed by RT-PCR (Table 6-9).

Table 6-9 Summary of the reporter genes activity under the control of the putative cell type-specific promoters

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Predicted expression</th>
<th>GFP expression</th>
<th>GUS expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAtPHT1;1</td>
<td>Root epidermis</td>
<td>No GFP signal</td>
<td>Not tested*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transcript present in the root and shoot</td>
<td></td>
</tr>
<tr>
<td>pOsAnt1</td>
<td>Root epidermis</td>
<td>No GFP signal</td>
<td>No GUS activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transcript present in the root and shoot</td>
<td>Transcript present in the root and shoot</td>
</tr>
<tr>
<td>pAt1g62500</td>
<td>Root cortex</td>
<td>Not tested**</td>
<td>Not tested*</td>
</tr>
<tr>
<td>pOs10g0552200</td>
<td>Root cortex</td>
<td>Not tested**</td>
<td>GUS activity mainly present in the root phloem and little present in leaf tip</td>
</tr>
<tr>
<td>pOs06g0250600</td>
<td>Root stele</td>
<td>Not tested**</td>
<td>No GUS activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transcript present mainly in the shoot and less in the root</td>
<td></td>
</tr>
<tr>
<td>pOsRCc3</td>
<td>Root</td>
<td>Not tested**</td>
<td>GUS activity present in root, mainly in cortex and endodermis</td>
</tr>
</tbody>
</table>

Samples were not tested due to *) the inability to generate transgenic plants containing these constructs or **) time limitation.
6.3.4. Characterization of transgenic rice expressing candidate genes for salt tolerance in specific cell types

Four transgenic rice lines have been generated carrying four different genes for improving salt tolerance under a cell type-specific promoter:

1. Line R32 (construct pAH09): Transgenic IR64 carrying *PpENA1* under control of the putative epidermal promoter *pAtPHT1;1*
2. Line R48 (construct pAH19): Transgenic IR64 carrying *OsHKT1;5* under control of the putative stelar promoter *pOs06g0250600*
3. Line R52 (construct pAH11): Transgenic Fatmawati carrying *AtAVP1* under control of the phloem specific promoter *pOs10g0552200*
4. Line R57 (construct pAH24): Transgenic Fatmawati carrying *OsOVP4* under control of the root specific promoter *pOsRCc3*

The integration of the transgene in lines R32, R48, and R57 was confirmed by PCR and Southern Blot analysis in the T₀ generation, while the integration of the transgene in line R52 was only confirmed by PCR. These four transgenic lines were selected for a salinity tolerance assay in the T₁ generation using the hydroponic system. Unfortunately, while phenotyping of lines R52 and R57 took place (Supplementary Table 1), it was not possible to adequately genotype them as the presence of the transgene in the T₁ populations could not be determined. Therefore, only the results from characterization of lines R32 and R48 will be described in the following sections.

6.3.4.1. Molecular analysis of transgenic IR64 expressing *PpENA1* driven by the promoter *pAtPHT1;1*

Transgenic rice IR64 (line R32) has been generated expressing *PpENA1* driven by promoter *pAtPHT1;1*. Southern Blot analysis showed the *pAtPHT1;1:PpENA1* transgenic line has a single T-DNA insert (Figure 6-13).

The expression of *PpENA1* in line 32 was confirmed by RT-PCR (Figure 6-14). The expression of *PpENA1* under control of *pAtPHT1;1* was stronger in the shoot compared to the root, where only faint transcript levels were detected. The transgene expression was present under both salt stress and non-stress conditions and there was no apparent difference in expression levels between the treatments (Figure 6-14). The expression of *PpENA1* in both shoot and root is consistent with the *pAtPHT1;1:GFP* expression pattern (Figure 6-10).
Figure 6-13 Southern Blot analysis of one T<sub>0</sub> transgenic IR64 pAtPHT1;1:PpENA1 (line R32).
The DNA fragments of line R32 is indicated by an arrow. The DNA of the wild type IR64 was used as a negative control. PCR-amplified product of the Nos terminator was radiolabelled with [α-<sup>32</sup>P]dCTP and used as probe. The restriction enzyme BamHI was used for DNA digestion.
Figure 6-14 Expression analysis using RT-PCR of PpENA1 in the shoot and root of T1 transgenic IR64 expressing PpENA1 driven by pAtPHT1;1 and the wild type IR64.

The cDNAs were synthesized from total RNA extracted from leaf and root tissue of rice plants grown under (A) 70 mM NaCl and (B) 0 mM NaCl for 10 d. Rice plants were grown in hydroponic and salt stress was imposed in 25 mM NaCl increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Leaf and root tissues were harvested 10 d after the 70 mM NaCl treatment was reached. Three plants were used as biological replicates. PCR was performed with 35 cycles using PpENA1 specific primers and OsGAPdh was used as the internal control gene. (-) = negative control (water used in PCR instead of DNA); (+) = positive control (plasmid containing PpENA1 was used as DNA source).
6.3.4.2. Molecular analysis of transgenic IR64 expressing OsHKT1;5 driven by pOs06g0250600

Transgenic IR64 (line R48) was developed expressing OsHKT1;5 under control of the promoter pOs06g0250600. Southern Blot analysis on the primary transgenic (T₀) revealed the transgenic line R48 had two copies of T-DNA inserted (Figure 6-15). The expression of the OsHKT1;5 transgene under the control of pOs06g0250600 was confirmed through RT-PCR using the primers which amplified the region between transgene OsHKT1;5 and the Nos terminator (Figure 6-16). The results showed that the expression was primarily in the shoot and not the root, consistent with results from the promoter/reporter gene analyses (Figure 6-12). Transcripts were present under both salt stress and non-stress conditions in shoot tissue (Figure 6-16).

![Southern Blot analysis of one T₀ transgenic IR64 pOs06g0250600:OsHKT1;5 (line R48).](image)

The DNA fragments of line R48 are indicated by an arrow. The DNA of the wild type IR64 was used as a negative control. PCR-amplified product of the Nos terminator was radiolabelled with [α-³²P]dCTP and used as probe. The restriction enzyme BamHI was used for DNA digestion.
Figure 6-16 Expression analysis using RT-PCR of *OsHKT1;5* in the shoot and root of T$_1$ transgenic rice IR64 expressing *OsHKT1;5* driven by the promoter pOs06g0250600 and the wild type IR64.

The cDNAs were synthesized from total RNA extracted from leaf and root tissue of T$_1$ rice plants grown under (A) 70 mM NaCl and (B) 0 mM NaCl for 10 d. Rice plants were grown in hydroponic and salt stress was imposed in 25 mM NaCl increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Leaf and root tissues were harvested 10 d after the 70 mM NaCl treatment was reached. Three plants were used as biological replicates. PCR was performed with 35 cycles using *OsHKT1;5-Nos* specific primers and *OsGAPdh* was used as the internal control gene. (-) = negative control (water used in PCR instead of DNA); (+) = positive control (plasmid containing *OsHKT1;5-Nos* was used as DNA source).
6.3.4.3. Salt screening of transgenic IR64 expressing \textit{PpENA1} and \textit{OsHKT1}:5 driven by putative cell type-specific promoters

A salt screening experiment of T\textsubscript{1} transgenic IR64 expressing \textit{PpENA1} driven by the \textit{pAtPHT1}:1 promoter (R32) and T\textsubscript{1} transgenic IR64 expressing \textit{OsHKT1}:5 driven by the \textit{pOs06g0250600} promoter (R48) were conducted in hydroponics. The null segregants from both events (R32 and R48) were combined as the lines were developed in the same round of transformation. The transgenic lines and nulls were screened under two conditions: 70 mM NaCl and 0 mM NaCl. The salt was applied in 25 mM increments starting when the 4\textsuperscript{th} leaf was fully expanded. The plant biomass and tissue ion concentration were measured 10 d after the 70 mM NaCl treatment had been achieved.

6.3.4.4. Expression of \textit{PpENA1} driven by \textit{pAtPHT1}:1 increased the biomass of IR64 under control and salt stress

The dry biomass of transgenic IR64 expressing \textit{PpENA1} under the control of \textit{pAtPHT1}:1 (line R32) was slightly higher than the nulls, both in non-stress and salt stress conditions (Figure 6-17). The transgenic R32 showed a biomass improvement both in shoot and root (Figure 6-17 A and Figure 6-17 B). Under control conditions, transgenic R32 had about 18% higher total biomass compared to the nulls, while under salt stress conditions the total biomass of R32 was about 12% higher than the nulls (Figure 6-17 C). However, when comparing the effect of salt on the biomass reduction between transgenic R32 and the nulls, the transgenic had a greater relative biomass reduction (Table 6-10). Salinity treatment with 70 mM NaCl reduced biomass of the nulls by about 20% and by about 25% in transgenic line R32 (Table 6-10).

6.3.4.5. Expression of \textit{OsHKT1}:5 driven by \textit{pOs06g0250600} had minimal effects on the biomass of IR64 under salt stress

The total dry biomass of transgenic IR64 expressing \textit{OsHKT1}:5 driven by the promoter \textit{pOs06g0250600} (line R48) under control conditions was comparable to the nulls (Figure 6-17). Under salt stress, the total biomass of the transgenic R48 was slightly lower compared to the nulls (Figure 6-17). The relative biomass of R48 in salt stress conditions compared to normal conditions was about 75% and was lower than the nulls with a relative biomass of 80% (Table 6-10).

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Figure 6-17 Biomass measurements of transgenic IR64 with cell-type specific expression of salinity tolerance genes.

(A) Shoot dry weight, (B) root dry weight and (C) total dry weight of transgenic rice IR64 expressing \textit{PpENA1} driven by promoter \textit{pAtPHT1;1} (R32) and \textit{OsHKT1;5} driven by promoter \textit{pOs06g0250600} (R48) and nulls grown under 0 mM and 70 mM NaCl for 10 d. The rice plants were grown in hydroponics with salt treatment imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). Rice plants were harvested 10 d after the 70 mM NaCl treatment had been reached. Nulls were derived from segregation of transgenic rice lines at the T\textsubscript{1} generation. Nulls of transgenic rice lines R32 and R48 were combined. Values are the means ± SEM. \(n\) = the number of biological replicates for each line.
Table 6-10 Relative total dry biomass of nulls and transgenic IR64 grown under 70 mM NaCl compared to 0 mM NaCl conditions

<table>
<thead>
<tr>
<th>Lines</th>
<th>Total dry weight under 70 mM NaCl ÷ total dry weight under 0 mM NaCl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nulls</td>
<td>79.93</td>
</tr>
<tr>
<td>R32 (IR64_ AtPHT1;1:PpENA1)</td>
<td>75.56</td>
</tr>
<tr>
<td>R48 (IR64_ Os06g0250600: OsHKT1;5)</td>
<td>75.31</td>
</tr>
</tbody>
</table>

6.3.4.6. Tissue ion concentration of transgenic IR64 expressing PpENA1 and OsHKT1;5 driven by cell type-specific promoters

6.3.4.6.1. Expression of PpENA1 driven by pAtPHT1;1 reduced root Na⁺ in IR64

Under salt stress of 70 mM NaCl, the transgenic IR64 expressing PpENA1 driven by pAtPHT1;1 (line R32) showed a lower concentration of root Na⁺ (74.9±1.6 mM) compared to the nulls (79.2±2.3 mM) (Figure 6-18). However, the shoot Na⁺ concentration of the transgenic R32 (33.5±3.1 mM) was similar to the nulls (33.7±2.6 mM).

The expression of PpENA1 driven by pAtPHT1;1 increased the shoot K⁺ concentration of transgenic IR64 (268.6±4.7 mM) compared to the nulls (243.2±13.1 mM) (Figure 6-19A). The expression of PpENA1 reduced the root K⁺ of the transgenic (86.3±2.4 mM) compared to the nulls (97.6±1.9 mM) (Figure 6-19B).

As a result, transgenic IR64 expressing PpENA1 driven by pAtPHT1;1 (R32) maintained a higher ratio of K⁺/Na⁺ in the shoot (K⁺/Na⁺ = 8.9±0.8) compared to the nulls (K⁺/Na⁺ = 7.6±0.9) (Figure 6-20 A) when grown in 70 mM NaCl. The ratio of K⁺/Na⁺ in the roots of transgenic R32 and nulls were similar under salt stress conditions (Figure 6-20 B).

Under control conditions, pAtPHT1;1:PpENA1 plants (R32) maintained a higher ratio of K⁺/Na⁺ in the root (K⁺/Na⁺ = 260±20) compared to the nulls (K⁺/Na⁺ = 216±21) (Figure 6-20B). Whilst under the same conditions, the ratio of K⁺/Na⁺ in the shoot of the transgenic line 32 (K⁺/Na⁺ = 68.9±10.4) was slightly lower than in the nulls (K⁺/Na⁺ = 75.1±12.6) (Figure 6-20A).
6.3.4.6.2. The expression of OsHKT1;5 driven by pOs06g0250600 reduced shoot Na$^+$ and increased root Na$^+$ concentration of IR64

Under salt stress conditions, transgenic IR64 expressing OsHKT1;5 under the control of promoter pOs06g0250600 (R48) had marginally lower shoot Na$^+$ concentration (29.7±2.4 mM) compared to the nulls (33.7±2.6 mM) (Figure 6-18A) – a reduction of 12%. In contrast, the root Na$^+$ concentration of the transgenic R48 (90.7±1.3 mM) was about 15% higher than in the nulls (79.2±2.3 mM) (Figure 6-18B).

The shoot K$^+$ concentration of transgenic R48 under salt stress was similar to the nulls. However, plants expressing OsHKT1;5 under the control of pOs06g0250600 had reduced K$^+$ concentration in the root (Figure 6-19A). Under salt stress, the root K$^+$ concentration of transgenic R48 was 87.5 mM, while the concentration of root K$^+$ of the nulls was 97.6 mM (Figure 6-19B).

Interestingly, the expression of OsHKT1;5 under the control of pOs06g0250600 increased the ratio of shoot K$^+/Na^+$ of the transgenic under both control and salt stress conditions (Figure 6-20). However, the expression of OsHKT1;5 decreased the ratio of root K$^+/Na^+$ under both control and salt stress conditions. Under salt stress the ratio of shoot K$^+/Na^+$ of the transgenic R48 was 8.9 compared to 7.5 in the nulls, while the ratio of root K$^+/Na^+$ of the transgenic R48 was 0.96 and 1.23 in the nulls.
Figure 6-18 Na⁺ concentration in shoots and roots of transgenic lines R32 and R48

(A) Shoot Na⁺ and (B) root Na⁺ concentration of T₁ transgenic IR64 expressing PpENA1 driven by pAtPHT1;1 (line R32), OsHKT1;5 driven by the pOs06g0250600 (lines R48) and the nulls grown under 0 mM and 70 mM NaCl for 10 d. The rice plants were grown in hydroponics with salt treatment imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. Rice plants were harvested 10 d after the 70 mM NaCl treatment had been reached. Nulls were derived from segregation of transgenic rice lines at the T₁ generation. Nulls of transgenic rice lines R32 and R48 were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
Figure 6-19 K+ concentration in shoots and roots of transgenic lines R32 and R48

(A) Shoot K+ and (B) root K+ concentration of T₁ transgenic IR64 expressing PpENA1 driven by pAtPHT1;1 (line R32), OsHKT1;5 driven by the pOs06g0250600 (lines R48) and the nulls grown under 0 mM and 70 mM NaCl for 10 d. The rice plants were grown in hydroponics with salt treatment imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. Rice plants were harvested 10 d after the 70 mM NaCl treatment had been reached. Nulls were derived from segregation of transgenic rice lines at the T₁ generation. Nulls of transgenic rice lines R32 and R48 were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
Figure 6-20 Ratio of K⁺/Na⁺ concentration in shoots and roots of transgenic lines R32 and R48

(A) Ratio of shoot K⁺/Na⁺ and (B) root K⁺/Na⁺ concentrations of T₁ transgenic IR64 expressing *PpENA1* driven by *pAtPHT1;1* (line R32), *OsHKT1;5* driven by *pOs06g0250600* (lines R48) and the nulls grown under 0 mM and 70 mM NaCl for 10 d. The rice plants were grown in hydroponics with salt treatment imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. Rice plants were harvested 10 d after the 70 mM NaCl treatment had been reached. Nulls were derived from segregation of transgenic rice lines at the T₁ generation. Nulls of transgenic rice lines R32 and R48 were combined. Values are the means ± SEM. n = the number of biological replicates for each line.
6.4. Discussion

6.4.1. Availability of a suitable promoter as the prerequisite for the utilization of a cell type-specific expression system

The application of a cell type-specific expression system to salt tolerance improvement depends highly on the availability of a promoter which can drive the expression of candidate genes for salt tolerance improvement in a specific cell type. Therefore, the initial work in this study was focused on the identification of promoters with activity in specific cell types of the rice root. The candidate promoters were identified from literature and from a publicly available rice database. The rice gene expression database, Rice Expression Profile Database (RiceXPro) (http://ricexpro.dna.affrc.go.jp/) (Sato et al. 2011; Takehisa et al. 2012; Sato et al. 2013) was used mainly as the source for promoter location search in this study. The database provides spatio-temporal gene expression profiles of japonica rice Nipponbare in different organs and tissues at different developmental stages (Sato et al. 2011; Sato et al. 2013) and root gene expression profiles of the rice Nipponbare (Takehisa et al. 2012).

Two promoters have been identified from the literature which drove UidA expression mainly in the rice root epidermis, the promoter of OsAnt1 (Shrawat et al. 2008; Good et al. 2009) and the promoter of AtPHT1;1(Koyama et al. 2005). The epidermal specific promoter is important to drive the expression of the Na⁺ transporter in the outer part of the root to increase Na⁺ efflux to the soil (Tester and Davenport 2003; Plett and Møller 2010). In this study, the promoters of OsAnt1 and AtPHT1;1 were cloned as candidates for the epidermal specific promoters to drive the expression of the plasma membrane Na⁺/H⁺ antiporter AtSOS1 (Shi et al. 2002) and Na⁺-pumping ATPase PpENA1 (Jacobs et al. 2011).

Transgenic rice lines expressing reporter genes GFP and UidA under the putative epidermal promoters pOsAnt1 and pAtPHT1;1 were generated in this study to confirm the tissue specificity of the promoter (Table 6-5). Expression analysis through RT-PCR showed that pAtPHT1;1 and pOsAnt1 were able to drive the expression of the reporter genes (Figure 6-10, Figure 6-11). Unfortunately, the phenotype of GFP fluorescence and GUS staining could not be observed in transgenic Fatmawati lines transformed with these reporter genes under the control of both pAtPHT1;1 promoter and pOsAnt1 promoter.
Therefore, the specific localization of the promoters could not be confirmed on a cellular or tissue level. It is likely that the transcript level driven by these promoters was too low and the GFP fluorescence and GUS activity were difficult to detect. Alternatively, the visualisation of GFP fluorescence or GUS staining may be difficult to observe if they are present in cell types with a large vacuolated region which represses the cytosol to the side of the cells, as would be expected in root cortical cells.

The expression of reporter genes driven by \textit{pAtPHT1;1} observed in this study to some extent differed from the result of Koyama et al. (2005) who showed that the \textit{pAtPHT1;1} strongly expressed \textit{UidA} in the root epidermis. In this study, the promoter of \textit{AtPHT1;1} was only tested using \textit{GFP} instead of \textit{UidA} as transgenic rice with a \textit{pAtPHT1;1:UidA} construct could not be generated. While no fluorescence of GFP could be observed RT-PCR did confirm the expression of the \textit{gfp} gene. Issues with observing GFP fluorescence may have been due to the high autofluorescence background signal and the sensitivity of the GFP protein to light and temperature (de Ruijter et al. 2003). Both of these factors may explain why transcripts could be observed but no fluorescence. Furthermore, if the promoter driving \textit{GFP} is weak then there will be low GFP fluorescence. As GUS staining is based on enzymatic activity there is an amplification of signal (Mantis and Tague 2000), this may explain why Koyama et al. (2005) observed tissue specific GUS staining while no GFP fluorescence was observed in this study. It also should be noted that only one transgenic Fatmawati was generated in this study which expressed \textit{GFP} under the control of \textit{pAtPHT1;1}. The chromosomal position of the site of the T-DNA insertion could also affect the expression level of the reporter gene (Matzke and Matzke 1998; van Leeuwen et al. 2001; de Ruijter et al. 2003) resulting in the differences observed here and between other studies. Therefore, more independent lines need to be generated and analysed to confirm the localisation of the promoter activity.

As with the \textit{pAtPHT1;1} promoter, the expression pattern of the other putative epidermal promoter \textit{pOsAnt1} could not be confirmed in this study. Shrawat et al. (2008) reported a strong GUS activity under the control of \textit{pOsAnt1} in transgenic Nipponbare which can be observed after 15 min of \textit{GUS} staining. In the present study, the two independent transgenic Fatmawati which expressed \textit{UidA} under the control of \textit{pOsAnt1} did not show any GUS activity, even after 24 h staining. As above, the difference in the genetic background of the transgenic (Scott et al. 1998) and the difference in integration sites of the transgene (Matzke and Matzke 1998; van Leeuwen et al. 2001) might cause the differences in expression level of the same gene under the same promoter.
Two putative promoters were cloned as candidates for a cortical specific promoter, including the promoter of \textit{At1g62500} (Co2) (Birnbaum et al. 2003; Heidstra et al. 2004) and the promoter of \textit{Os10g0552200}. A cortical specific promoter is required to manipulate the Na\(^+\) transport within the plant to increase the Na\(^+\) storage capacity of the root cortex. The promoter of \textit{At1g62500} (Co2) has been known to be expressed in root cortical cells of \textit{Arabidopsis} (Birnbaum et al. 2003; Heidstra et al. 2004; Mustroph et al. 2009). \textit{At1g62500} encodes a lipid transfer protein and is also involved in seed storage (http://www.arabidopsis.org/index.jsp). \textit{Os10g0552200} from rice showed a similar function to \textit{At1g62500} in lipid transport and seed storage (http://ricexpro.dna.affrc.go.jp/gene-search.php). It was expected that the promoter of \textit{Os10g0552200} would be a good candidate for root cortical specific expression. To confirm the localization of \textit{pAt1g62500} (Co2) and \textit{pOs10g0552200}, these promoters were used to drive the expression of \textit{UidA} and \textit{GFP}. In parallel, vector constructs were developed using these putative cortical promoters to drive the expression of vacuolar H\(^+\)-PPase from \textit{Arabidopsis} (\textit{AtAVP1}) and from rice (\textit{OsOVP4}) (Table 6-6), aiming to increase cortical storage of Na\(^+\) in the rice roots via sequestration of Na\(^+\) into vacuoles of cortex cells. This is hypothesised to reduce the amount of Na\(^+\) translocation to the shoot. Ideally, it would be best to determine the tissue/cell type specific expression of the putative promoters using the reporter genes \textit{GFP} and \textit{UidA} before attempting to produce lines driving the expression of candidate genes of interest. However, the low transformation rate and the pressure of time in a PhD meant that these transformations had to occur simultaneously.

Transgenic Fatmawati carrying \textit{UidA} and \textit{GFP} under these two putative cortical promoters were developed in this study. However, due to time limitations, further characterization was only conducted on transgenic lines with the \textit{pOs10g0552200:UidA} construct. GUS staining analysis on transgenic Fatmawati \textit{pOs10g0552200:UidA} confirmed that the promoter is primarily active in the inner part of the root (Figure 6-8 B and C). Interestingly, cross section analysis of the stained roots revealed that the promoter specifically expressed the \textit{UidA} in the root phloem and not the cortex (Figure 6-8D and E). The initial aim of cloning of the promoter \textit{pOs10g0552200} was to use the promoter to increase cortical storage of Na\(^+\) in the roots by expressing vacuolar H\(^+\)-PPase in the root cortex. However, the identification of the promoter \textit{Os10g0552200} as a phloem specific promoter became an interesting finding as it can be used to drive the expression of vacuolar H\(^+\)-PPase in the sieve element of the roots. It is hypothesized that over-
expression of *AtAVP1* has different effects in different tissues (Gaxiola et al. 2012). Paez-Valencia et al. (2011) reported the expression of type I H\(^+\)-PPase in the plasma membrane of the sieve element-companion cell complex of *Arabidopsis*. It is hypothesized that in phloem cells the H\(^+\)-PPase may localize to the plasma membrane and act as PPI synthases instead as a PPase. This in turn may increase the activity of plasma membrane ATPases thus generating a proton gradient which facilitates sucrose transport from shoot to the root (Gaxiola et al. 2012). This reverse function of H\(^+\)-PPase and more sugar in the roots might explain the improvement in root biomass of transgenic plants constitutively expressing vacuolar H\(^+\)-PPase which lead to the improvement in nutrient and water uptake (Park et al. 2005; Yang et al. 2007; Gaxiola et al. 2012; Paez-Valencia et al. 2013). Therefore, it is interesting to test this hypothesis by expressing vacuolar H\(^+\)-PPases specifically in phloem cells using the promoter *pOs10g0552200*.

A stelar specific promoter was required to drive the expression of Na\(^+\) transporters, such as *OsHKT1;5* to increase Na\(^+\) retrieval from the transpiration stream back into the root. The promoter of *Os06g0250600* was cloned as a putative stelar specific promoter for this purpose. *Os06g0250600* encodes a K\(^+\) channel in rice (RiceXPro, [http://ricexpro.dna.affrc.go.jp/](http://ricexpro.dna.affrc.go.jp/)). The spatio-temporal expression profile of the gene (RiceXPro, [http://ricexpro.dna.affrc.go.jp/](http://ricexpro.dna.affrc.go.jp/)) indicated that *Os06g0250600* has stronger expression in the root compared to leaves (Figure 6-4) (Sato et al. 2011; Sato et al. 2013). Based on the root gene expression profile database, the gene is mainly expressed in the inner part of the rice root (Figure 6-5) (Takehisa et al. 2012). To confirm the localization of the promoter *Os06g0250600*, the construct *pOs06g0250600:UidA* was developed and transformed into rice Fatmawati. The expression of *UidA* under the control of *pOs06g0250600* was confirmed through RT-PCR analysis. However, GUS activity in the transgenic *pOs06g0250600:UidA* could not be detected, possibly because of the low activity of *pOs06g0250600*.

The root specific promoter *pOsRCc3*, (Xu et al. 1995; Flasinski et al. 2009; Jeong et al. 2010) was also cloned and used in this study. Reporter gene analysis showed that the promoter drives *UidA* expression in all parts of the root with higher activity in the inner part of the root (cortex and endodermis) (Figure 6-9). Constructs were developed to express the vacuolar H\(^+\)-PPases *AtAVP1* and *OsOVP4* using the *OsRCc3* promoter to increase vacuolar compartmentalization of Na\(^+\) in the root. In addition, the promoter was also used to drive the expression of *OsHKT1;5* as a previous study showed that expression of the Na\(^+\) transporter *AtHKT1:1* in cortical cells of rice improved salt tolerance.
potentially due to a pleotropic upregulation of the native OsHKT1;5 in stelar cells (Plett et al. 2010).

6.4.2. Cell type-specific expression of PpENA1 increased the biomass of salt stressed IR64

In Chapter 5, transgenic rice was developed to express the Na⁺ pump-ATPase from Physcomitrella patens (PpENA1) under the control of constitutive promoter 35S. However, the expression of PpENA1 driven by this strong and constitutive promoter could not be detected in three independent transgenic Fatmawati 35S:PpENA1 lines and two transgenic IR64 35S:PpENA1 lines (Section 5.3.3 and Section 5.3.4). It is hypothesized that the use of the 35S promoter induces gene silencing of PpENA1 and therefore expression of the gene under a cell type-specific promoter may be more successful.

In this study a single transgenic IR64 was generated to express PpENA1 under the control of the promoter pAtPHT1;1 (Line R32). RT-PCR analysis confirmed that pAtPHT1;1 was able to drive PpENA1 expression (Figure 6-14). While results from a single transformation event should be treated with caution, the result is in line with the study by Plett (2008) where the author reported that the PpENA1 expression was stronger under cell-type-specific expression compared to constitutive expression. However, the results from RT-PCR analyses in this study indicated that the intensity of PpENA1 expression under the control of pAtPHT1;1 was very low (Figure 6-14) when compared to the expression of the reporter gene (GFP) driven by the same promoter (Figure 6-10). The low transcript level shown by PpENA1 might be caused by a post transcriptional modification in the expression of the gene in plants (Koziel et al. 1996). In future, optimised expression of this moss gene may possibly be improved through codon optimisation to suit codon usage of rice (Koziel et al. 1996; Rouwendal et al. 1997).

Transgenic IR64 expressing PpENA1 driven by pAtPHT1;1 showed an increase in biomass under salt stress. The improvement of the biomass apparently was not related to the concentration of Na⁺ in the shoot as the concentration was similar between the transgenic PpENA1 and the nulls. Nevertheless, the root Na⁺ concentration of transgenic PpENA1 was lower than that of the nulls. Even though the precise location of pAtPHT1;1 expression could not be confirmed in this study, Koyama et al. (2005) showed that the promoter is mainly active in the root epidermis. If PpENA1 was primarily expressed in
the outer layer of the root in line R32 this could lead to the observed reduction of Na⁺ in the root.

Interestingly, the expression of *PpENA1* also led to the increase of biomass under normal conditions. It is likely that the improvement of biomass of transgenic *PpENA1* under salt stress is related to the ability of the plant to maintain the growth, referred to as the osmotic tolerance mechanism (Munns and Tester 2008). In addition, Jacobs et al. (2007) reported that transgenic rice lines constitutively expressing *PpENA1* showed significant alteration in their metabolite profile compared to the nulls. Of particular importance was the significant increase in some metabolite involved in the production of osmo-protectants such as ethanolamine, glyceric acid and quinic acid, in transgenic *PpENA1* compared to the nulls (Jacobs et al. 2007; Jacobs et al. 2011). However, this hypothesis should be tested by analysing the metabolic profiles of the current transgenic IR64 *PpENA1*.

6.4.3. Cell type-specific expression of *OsHKT1;5* reduced shoot Na⁺ concentration of IR64

The sodium transporter *OsHKT1;5* is known to be involved in Na⁺ retrieval from the shoot to the root in rice and contributes to the salinity tolerance of rice cultivars including Nona Bokra and Pokkali (Ren et al. 2005; Thomson et al. 2010; Cotsaftis et al. 2011). The gene is mainly expressed in the xylem parenchyma (Ren et al. 2005). In *Arabidopsis*, it was shown that cell type-specific expression of *AtHKT1;1* (the homolog of *OsHKT1;5*) in the stele significantly reduced shoot Na⁺ of the transgenic line and increased the salt tolerance (Møller et al. 2009). In this study, *OsHKT1;5* from salt tolerant rice Pokkali was expressed in the popular rice cultivar IR64 under control of the putative stelar promoter *pOs06g0250600*. The expression of *OsHKT1;5* driven by *pOs06g0250600* reduced the shoot Na⁺ concentration of transgenic IR64 and at the same time increased the accumulation of Na⁺ in the root (Figure 6-18), results similar to those observed in other studies (Møller et al. 2009). Whether this was due to an improvement in removing Na⁺ from the root xylem or facilitating improved recirculation of Na⁺ from the shoot to the root (as higher *OsHKT1;5* gene expression was detected in the shoot) remains to be seen. Notably, the alteration of Na⁺ in this cultivar did not improve the salinity tolerance level of IR64 line R48 under the tested condition, potentially due to IR64 being more of an ion tolerator than an ion excluder.

As described in Chapter 3, IR64 was suggested to have a tissue tolerance mechanism to adapt to salt stress. It was hypothesized that increasing Na⁺ retrieval from the shoot to the
root in this cultivar might further improve the salinity tolerance of the cultivar. However, the result from this study showed that the reduction of Na\textsuperscript{+} concentration in the shoot of this cultivar as a result of cell type-specific expression of OsHKT1;5 had no effect on the level of salt tolerance. It is possible that the accumulated shoot Na\textsuperscript{+} concentration in IR64 under moderate stress of 70 mM NaCl is still tolerated by the cultivar, therefore the reduction of Na\textsuperscript{+} concentration caused by the expression of OsHKT1;5 has less effect on the plant. It has been shown recently that the introgression of TmHKT1;5-A, a Na\textsuperscript{+} transporter from Triticum monococcum, into durum wheat appeared to only increase the yield in high saline fields, where the accumulation of shoot Na\textsuperscript{+} was higher than the critical level tolerated by the plant, while the effect of the gene was not significant under low and moderate saline field conditions (Munns et al. 2012). It will prove interesting to test the salt tolerance of the transgenic IR64 pOs06g0250600:OsHKT1;5 at a higher level of salt stress, e.g. 150 mM NaCl, to determine the effect of Na\textsuperscript{+} exclusion in a tissue tolerant cultivar such as IR64. In addition, it is also important to further characterize the transgenic developed in this study in later generations using a more reliable screening protocol such as the image-based phenotyping screening method described earlier to perform time course studies. In addition, it will be interesting to see if the reduced Na\textsuperscript{+} accumulation in the shoot phenotype could be transferred to Fatmawati, where it might have a bigger effect, since Fatmawati appears to have a lower tissue tolerance than IR64 (Chapter 3 and Chapter 4).

6.4.4. Limitation of the number of independent transgenic events

As discussed in the Chapter 5, the low transformation efficiency of indica rice Fatmawati and IR64 using callus from mature seeds limited the production of independent transgenic lines for phenotyping. In the present study, only one transgenic event each was generated carrying pAtPHT1;1:PpENA1 and pOs06g0250600:OsHKT1;5. Ideally, more than one independent insertion event of each construct should be characterized to make a clear claim about the effect of the gene. Therefore, most of the journals need at least two, ideally three, independent transformation events to confirm the effect of the gene on the phenotype. Nevertheless, the phenotypes which have been observed in the present study coincided with the results gained from the model species. However, it is important in the future to generate more independent lines to confirm the results.

Interestingly, while most scientific journals require two to three independent transformation events for publication, the large agricultural companies such as DuPont
and Monsanto now often patent promising individual transformation events instead of genes (Scott Whitmore, patent attorney, Phillips Ormonde Fitzpatrick, Adelaide, personal communication). While the validation of the effect of the gene on the phenotype is far more important than the end phenotype for publications, the final product is more important for industry. This might be also applied to the delivery process of the promising transgenic lines from this study. Therefore, a single transformation event with an enhanced phenotype generated from the present study will still be an invaluable genetic resource in breeding programmes.

6.5. Summary

This chapter highlights the identification and validation of cell-type-specific promoters which can be used to drive the expression of different candidate genes to improve salinity tolerance in a cell type-specific manner. Cell type-specific expression of \textit{PpENA1} using the epidermal specific promoter \textit{pAtPHT1;1} resulted in the reduction of root \text{Na}\textsuperscript{+} concentration of rice cultivar IR64, while cell type-specific expression of \textit{OsHKT1;5} under the stelar specific promoter reduced the shoot \text{Na}\textsuperscript{+} concentration of IR64. While the \text{Na}\textsuperscript{+} status of the transgenic has been altered with the cell type-specific expression of transport genes, the effect on the biomass improvement of the plant remains to be further studied. Moreover, the different effects showed by the expression of \textit{PpENA1} in the outer part of the root and the expression of \textit{OsHKT1;1} in the stele leads to the possibility of expressing these two genes simultaneously in different cell types to minimize the accumulation of \text{Na}\textsuperscript{+} in the plant. Further assessment in the later generations is important to test the phenotypes resulting from the \text{Na}\textsuperscript{+} transport alteration.
Chapter 7 : General Discussion

7.1. Summary of accomplished work

The aim of this study was to develop salinity tolerant rice by modification of Na⁺ transport within the plant. To address this aim, a number of genetic and physiological studies were carried out. Significant outcomes have been achieved, generating a number of improved genetic resources, improving the understanding of the salt tolerance mechanisms in rice and potentially contributing to the success of a breeding program for salt tolerant rice.

7.1.1. Variation in salinity tolerance mechanisms in rice

In Chapter 3, a variation in the salt tolerance mechanisms within elite rice cultivars was revealed, particularly in their ability to manage Na⁺ within the plants. This information is important in designing an improvement strategy for each cultivar. It was found that tolerant cultivars such as Fatmawati and Dendang adapt to high external Na⁺ by preventing the accumulation of Na⁺ in the shoot, which is known as the Na⁺ exclusion mechanism, while an elite salt tolerant cultivar, IR64, accumulated Na⁺ in the shoot, suggesting that a tissue tolerance mechanism is employed by this cultivar. To this point, these variations in salt tolerance mechanisms in the different backgrounds will permit strategies in breeding programmes to pyramid different salt tolerance traits together, potentially enhancing the overall tolerance of the plants and improving yield. The incorporation of IR64’s tissue tolerance trait into a variety such as Dendang will provide a “backup” salinity tolerant strategy if external salt levels swamp Dendang’s native exclusion mechanisms.

A question is also raised, based on the results in Chapter 3 of whether a salt susceptible japonica rice cultivar such as Nipponbare remains a good model for determining the correct strategies for engineering salt tolerance improvement. It has been shown that the level of salt tolerance of Nipponbare is much lower than that of elite indica rice cultivars and results from Chapters 5 and 6 show that expression of a gene which enhanced salinity tolerance in Nipponbare may not necessary have the same effect in indica rice. Therefore despite the complete genome information about Nipponbare being available and its high transformation efficiency, the salt tolerance improvement which potentially could be gained by genetic modification using this cultivar might be insignificant compared to the
salt tolerance of the elite rice cultivars. It may only be possible to make Nipponbare as tolerant as, for example IR64, so why try to improve the tolerance of Nipponbare, when you could just grow IR64 instead? A much better strategy would be to focus on improving the already good salt tolerance strategies in *indica* rice.

### 7.1.2. Transformation efficiency of commercially relevant rice cultivars

In an attempt to use commercially relevant rice cultivars in genetic engineering to improve their salt tolerance, optimized *Agrobacterium*-mediated transformation of these cultivars was developed using callus from the scutellum of mature seeds as the starting material (Chapter 3). Two cultivars, Fatmawati and IR64, were amenable to the transformation protocol.

As highlighted in Chapters 5 and 6, the relatively low transformation efficiency of IR64 and Fatmawati (compared to Nipponbare) had a severe effect on the number of transgenic lines which could be studied in this project. IR64 is a very popular variety and grown in millions of ha across Asia. The popularity of IR64 makes the cultivar a favourable genetic background for improving abiotic stress tolerance mechanisms including submergence tolerance (Septiningsih et al. 2009), drought tolerance (Uga et al. 2013), phosphorus deficiency tolerance (Chin et al. 2011) and salinity tolerance (Nakhoda et al. 2012; Gregorio et al. 2013). While it is possible to transform IR64 and Fatmawati with genes of interest in the present study, it is necessary to further improve the transformation and selection processes to produce the required number of transgenic lines to allow the critical evaluation of the performance of transgenic plants which have been transformed with a specific gene encoding a tolerance mechanism. The aforementioned transformation of immature embryos is one technique that could potentially improve transformation efficiency, along with further optimisations of growth solutions and conditions used in this study.

### 7.1.3. Non-destructive image based phenotyping for salt screening

The availability of a reliable and efficient screening protocol is necessary to study the physiological traits important for salt tolerance and to select salt tolerant rice lines. It is argued that one of the limiting factors which slow the progress of the development of salt tolerant rice is the lack of an efficient and reliable screening protocol to select tolerant plants (Gregorio et al. 2002; Faiyue et al. 2012). In Chapter 4, a non-destructive image based phenotyping screening protocol for rice salinity tolerance was developed. This protocol, in combination with the tissue ion concentration measurement, enabled the
differentiation between the ionic and the osmotic components of salt stressed rice. In addition to being able to measure the osmotic component of salt stress, a non-destructive screening protocol also allows the maintenance of the individual rice plants, allowing them to produce seed. This is of particular importance as it is to be used for phenotyping early generations of transgenic rice where there are usually a limited number of seeds or it is required to save a specific plant from a transformation event.

While the use of colour image for measurement of projected shoot area has been demonstrated to be an accurate surrogate for plant biomass, the utilization of fluorescence imaging to determine leaf senescent needs more careful consideration for the respective experimental context. Results from the present study indicated that salt screening using soil as a media result in a lower ionic effect during salt stress when compare to hydroponic experiments. This is particularly true under moderate salt stress, a level that represent the majority of agricultural land. Under moderate stress the development of leaf senescent area appeared to be slow until 30 d after salt application (Chapter 4) and detection of salt induced senescence was confounded by noise detected at leaf edges of both salt stressed and control plants. Only when high salt concentrations were used the degree of leaf senescence is high enough for easy differentiation by fluorescence imaging. However, screening of rice plants under high stress may only be suitable for genetic studies to identify traits for tissue tolerance and may not be applicable for routine breeding programmes as it is not representative of actual field conditions.

It might be worthwhile to perform moderate salt stress assays for longer periods to test whether the application of fluorescence imaging could be used to quantify the development of leaf senescence over time as the result of the ionic effect. However, it should be noted that the critical stages of salt stress on rice are during seedling growth and the reproductive stages, while the plant tends to be more tolerant during the vegetative stage (Heenan et al. 1988; Grattan et al. 2002; Ismail et al. 2010).

7.1.4. Altered phenotypes as the results of modification of Na$^+$ transport

Transgenic commercial rice cultivars have been generated in this study expressing different candidate genes for salinity tolerance improvement under constitutive and cell type specific promoters. Favourable phenotypes as the result of constitutive expression were observed in transgenic rice constitutively expressing AtAVP1 and AtCIPK16. However, constitutive expression of OsOVP4 and PpENA1 seems to be deleterious, since either a negative phenotype was observed or the transgene could not be expressed at all.
Results from the present study indicated that constitutive expression of \textit{OsOVP4} lead to a growth penalty in rice. Similarly, constitutive expression may not be the best option to manipulate Na$^+$ transport in rice using Na$^+$-ATPase, \textit{PpENA1}. None of the five transgenic rice lines transformed with \textit{35:PPENA1} in this study showed \textit{PPENA1} transcript, suggesting the presence of a gene silencing regulation as the result of constitutive expression of \textit{PpENA1}. In contrast, \textit{PpENA1} transcript was present when it was expressed under the control of the epidermal specific promoter \textit{PAtPHT1;1} and lead to a reduction of root Na$^+$ in transgenic rice (Chapter 6), hence indicating the importance of cell type specific alteration for \textit{PpENA1}. In future, it would be worthwhile to test whether cell-type specific expression of \textit{OsOVP4}, such as cortical specific expression, may result in salt tolerance improvement. The use of promoter \textit{PRcc3} which has been shown predominantly drive \textit{UidA} expression in the root cortex and endodermis (Chapter 6) may be a good option to drive \textit{OsOVP4} expression. A construct for this promoter-gene combination is already available for future work.

Cell type specific expression was also demonstrated to be effective to increase Na$^+$ exclusion by upregulating \textit{OsHKT1;5} (Chapter 6). The present study indicated that expression of \textit{OsHKT1;5} using a hypothesised root stellar specific promoter reduced the shoot Na$^+$ in transgenic IR64. While the effect of improving Na$^+$ exclusion in the tissue tolerant IR64 needs to be further characterized, this study validated the importance of the cell type specific expression approach which has been demonstrated in the model species \textit{Arabidopsis} (Møller et al. 2009). As described in Chapter 6, it is important to test salinity tolerance of transgenic IR64 with cell type specific expression of \textit{OsHKT1;5} under higher level of salt stress, as the experiments performed in this study may not have stressed the plants enough. It has been shown in durum wheat containing \textit{TmHKT1;5-A} that the gene expression appear to increase the yield only under high saline field conditions (Munns et al. 2012).

\textbf{7.1.5. Identification of novel cell type specific promoter}

In Chapter 6, six putative cell type-specific promoters were identified and cloned to drive reporter genes and candidate genes for salt tolerance improvement. For this purpose, a large number of Gateway® cassettes have been constructed. The use of the Gateway® system should enable a more efficient utilization of the cell type-specific promoters in future cloning (Curtis and Grossniklaus 2003).
A novel promoter from rice, *pOs10g0552200*, was identified to be specifically expressed in the root phloem. Recently, it was hypothesized that *AtAVP1* may have an important role in sucrose transport as *AtAVP1* may function as a PPI synthase instead of a PPase on the plasma membrane of phloem cells (Gaxiola et al. 2012). Thus promoter *pOs10g0552200* has the potential to be used to express *AtAVP1* specifically in the rice phloem to improve root growth which is important for water and nutrient uptake. Moreover, the promoter could also be used to drive the expression of sucrose transporters to increase carbon partitioning into roots as has been shown in *Arabidopsis* expressing sucrose transporter, *AtSUC2*, specifically in the phloem (Srivastava et al. 2009).

Nevertheless, the precise localization of some cell type-specific promoters used in this project has not been confirmed using the reporter genes. This may be due to the low transcript number caused by the promoters or due to the limited number of independent transgenic lines available for characterization. It is important to confirm the location of gene expression before concluding the effect of gene expression on the Na⁺ transport alteration. Utilization of the mRNA *in situ* localization techniques, such as *in situ* PCRs and RT-PCR using tissue sampled from laser microdissection (Møller et al. 2009) will help clarify the expression location.

### 7.2. Future work

While significant outcomes have been achieved, several questions still remain to be answered and a number of transgenic rice lines developed in this study have yet to be characterized due to time constraints.

#### 7.2.1. Improvement of the salinity tolerance screening protocol, transgenic generation and the number of independent lines

Non-destructive image based phenotyping for salt screening for rice has been developed in this study. The original goal of this project was to make use of this non-destructive screening technique to phenotype T₁ and T₂ transgenic rice produced by this project. Unfortunately, the low transformation efficiency encountered in the project significantly delayed the material which was to be tested and this section of the project was not completed. A lack of phenotype on the overall salt tolerance improvement in transgenic rice which was observed in many lines during the study maybe due to the fact that screenings were performed on the T₁ generation. The T₁ generation of transgenic plants
are not ideal for phenotyping as they lack transgene stability and their seed quality is low, since they are produced from primary transgenic plants. This is why many of the studies in the literature use later generations of transgenics for phenotyping. It certainly would be worthwhile phenotyping the T2 generation of the rice plants produced in this project using a non-destructive technique to identify whether or not there were improvements in the plant’s salinity tolerance in a more stable generation of transgenic lines. This is important to determine the effect of the transgene expression on the phenotype whether it improves the Na⁺ exclusion, tissue tolerance or osmotic tolerance (Rajendran et al. 2009), which will be difficult to perform with destructive measurements alone.

The limited number of independent transgenic lines achieved in this project is also a major drawback in the characterization of the transgenics. Most of the transformations in this study generated only single event transgenic lines. This is not ideal as the positional effect of the transgene could not be analysed. While increasing independent lines through repeated transformation rounds is feasible, it will require additional time and labour. Alternatively, the number of independent lines could be increased by segregation of some transgenic lines with multiple T-DNA inserts. For example, Southern Blot analysis of the T1 transgenic Fatmawati constitutively expressing AtCIPK16 showed that this line had 6 T-DNA insertions spread throughout its DNA. With sufficient genetic distance, these inserts will segregate in the following generations, with the offspring inheriting different AtCIPK16 integration sites (Chapter 5). These segregants with AtCIPK16 present in different regions of their DNA are almost identical to independent transformation events (at a genetic level) and thus the phenotyping of these segregants could determine whether the AtCIPK16 gene is responsible for the phenotype, or if the phenotype is due to a positional effect of the T-DNA insertion site.

7.2.2. Investigation of the tissue tolerance mechanism in rice

Studies on the salinity tolerance mechanism in rice have so far concentrated mainly on the Na⁺ exclusion mechanism. This is probably because the phenotyping of this trait is relatively easy compared to other mechanisms (osmotic tolerance and tissue tolerance) (Roy et al. 2014). The results from the present study indicate the presence of a tissue tolerance mechanism in rice cultivar IR64 (Chapter 3 and Chapter 4). This finding needs to be further characterized to understand how the tissue tolerance in this cultivar is regulated. Firstly, it is important to know in which part of the cells the Na⁺ is deposited. Tissue ion content, as determined by flame photometer measurement is a simple
measurement to analyse Na\(^+\) concentration in plant tissue, however to determine sub-cellular localization of the Na\(^+\), more detailed analysis should be performed. This may include such methods as cryo-scanning electron microscopy (SEM) and X-ray micro analysis (James et al. 2006b), unidirectional \(^{22}\)Na\(^+\) fluxes (Plett et al. 2010), or fluorescence quenching by the acridine orange assay (Blumwald and Poole 1985). It is also necessary to further characterize the genetic mechanism underlying tissue tolerance in IR64. Mapping populations which involve IR64 as the parent, such as the double haploid IR64/Azucena developed by Guiderdoni et al. (1992) or the rice diversity panel (http://www.ricediversity.org/index.cfm) might be useful to determine the underlying genetic mechanisms for tissue tolerance in IR64. With the development of image based phenotyping for salt screening (Chapter 3) and accurate tissue Na\(^+\) measurement, it is now possible to phenotype a tissue tolerance trait which could facilitate the identification of the gene responsible for that trait (Rajendran et al. 2009).

It would also be worthwhile revisiting the hypothesis that constitutive expression of vacuolar H\(^+\)-PPase in transgenic rice may improve tissue tolerance. Roles other than improved tissue tolerance have been proposed for vacuolar H\(^+\)-PPase (Ferjani et al. 2011; Gaxiola et al. 2012), which might contribute to the improvement of plant growth in response to salt stress. These other roles of H\(^+\) PPases are interesting as the present study indicated the improvement of rice biomass under normal (non-salt stressed) conditions as a result of constitutive expression of \textit{AtAVP1}. According to the proposed model by Ferjani et al. (2011), this biomass increase may be due to growth improvement in the very early stages of plant development. \textit{AtAVP1} has been shown to have a significance role in the removal of cytosolic PPI, which is needed during gluconeogenesis (Ferjani et al. 2011) – removal of PPI from the cytosol appears to speed up sucrose remobilisation and increase seedling growth. More recently, using non-destructive image analysis Schilling et al. (2013) demonstrated that transgenic barley constitutively expressing \textit{AtAVP1} had a higher growth rate compare to the nulls only in early growth stages, again suggesting a role for \textit{AtAVP1} in improving seedling vigour. It will be worthwhile to determine the growth rate of transgenic \textit{AtAVP1} rice immediately post germination stages to test whether these plants also have improved seedling vigour. Utilization of non-destructive image based phenotyping to characterize early vigour development of transgenic rice seedlings may help to provide accurate measurements.

Another hypothesis proposed by Gaxiola et al. (2012) suggested the improvement in plant biomass of transgenic \textit{AtAVP1} might be the result of a reverse of function of \textit{AtAVP1} in
sieve elements where it behaves as a plasma membrane PPI synthase thereby supporting the activity of the plasma membrane H⁺-ATPases in increasing the proton gradient in the phloem which is required for the activity of the Suc/H⁺ symporter. Analysis on the transcript levels of rice sucrose transporters (Aoki et al. 2003) and quantification of the sucrose profile of transgenic rice as the result of constitutive expression of AtAVP1 are necessary to elucidate this potential mechanism.

Nevertheless, it is also important to analyse whether the constitutive expression of AtAVP1 in Fatmawati and IR64 increases Na⁺ sequestration into the vacuole through subcellular localization analyses as described earlier to test the original proposal of the function of AtAVP1 in generating a H⁺ gradient across the tonoplast (Gaxiola et al. 2001). Results from the present study showed no indication of differences in tissue Na⁺ concentration of transgenic AtAVP1 and nulls in the generations tested.

7.2.3. Alternative strategies for targeting the expression of Na⁺ transporter

The present study indicated that the expression of PpENA1 in the outer part of the rice root may not be sufficient to reduce the accumulation of Na⁺ in the shoot (Chapter 6). This raised the question of whether the improvement of Na⁺ efflux in the outer part of the root is an effective strategy for rice. It has been well demonstrated in rice that the Na⁺ influx into the root is largely facilitated by an apoplastic bypass flow (Yeo et al. 1987; Krishnamurthy et al. 2009; Faiyue et al. 2010). It has been shown that the hydraulic conductivity of the outer part of the root is much larger than in the inner part of the root (endodermis/stele) (Ranathunge et al. 2003), suggesting that apoplastic flow is larger in the outer part of the root and less in the inner part (Plett 2008). It is likely that improvement in Na⁺ efflux will be more effective if it is targeted to the inner part of the root such as in endodermal cells rather than in outer root cells where it is fighting a continuous battle against incoming Na⁺. It therefore may be hypothesised that more significant effects on shoot Na⁺ levels would be seen in transgenic plants where loading of Na⁺ into the xylem is reduced or retrieval from the xylem is enhanced, such as by stelar specific expression of HKTs (Ren et al. 2005; Møller et al. 2009; Munns et al. 2012). A second approach may be to modify the rice root structure to reduce bypass flow (Faiyue et al. 2010). However, the effect of reducing bypass flow to minimise Na⁺ entry may inhibit the uptake of other valuable nutrients which are also taken up by bypass flow, therefore this needs to be studied further. It might be possible that the large bypass flow
in rice is perhaps an adaptive strategy to allow nutrient uptake with minimal transport across membranes.

In addition, other tissues of the plant may be more suited for altering Na\(^+\) transport, rather than focusing on enhancing efflux from the root to the soil. Evidence from durum wheat suggests that expression of \(TmHKT1;4\text{-}A2\) (\(Nax1\)) in leaf sheaths may remove any remaining Na\(^+\) from the transpiration stream and keep it locked in the sheath away from the leaf blade (James et al. 2006a; Huang et al. 2008; James et al. 2011). In rice, \(OsHKT1;4\) is also highly expressed in the leaf sheath and is hypothesized to have a similar function to \(TmHKT1;4\text{-}A2\) in Na\(^+\) transfer from leaf blade to leaf sheath (Sundstrom 2011; Cotsaftis et al. 2012). Furthermore, localization of another member of the \(HKT\) family in rice, \(OsHKT1;3\), in bulliform cells of mature leaves (the cells which are involved in leaf rolling) may indicate potential role of \(OsHKT1;3\) in reducing Na\(^+\) concentration in mesophyll cells by transporting excess Na\(^+\) into bulliform cells (Jabnoune et al. 2009; Sundstrom 2011). Identifying promoters which could drive the expression of genes in specific cell types in these tissues would be important as well as further physiological studies to investigate these phenomena further.

7.2.4. Phenotyping transgenic rice constitutively expressing \(AtAVP1\) for other abiotic stresses

The constitutive expression of vacuolar H\(^+\)-PPase has been reported to cause increased plant tolerance to different abiotic stresses including drought (Gaxiola et al. 2001; Bao et al. 2009a; Liu et al. 2010b; Pasapula et al. 2011), cold (Zhang et al. 2011), phosphate deficiency (Yang et al. 2007) and nitrogen deficiency (Paez-Valencia et al. 2013). Further characterization of transgenic Fatmawati and IR64 constitutively expressing \(AtAVP1\) exposed to these abiotic stresses will be extremely beneficial since they commonly occur in rice fields in different regions. It is worthwhile to test the phenotype of transgenic \(AtAVP1\) rice in different abiotic stress experiments such as experiments with low phosphorus, a low water potential, or with different nitrogen regimes.

7.2.5. Improving Na\(^+\) transport by expressing multiple genes in different cell types

The results of this study have shown that specific expression of \(OsHKT1;5\) in stelar cells reduced shoot Na\(^+\) accumulation and increased root Na\(^+\), while cell type-specific expression of \(PpENA1\) reduced root Na\(^+\) accumulation in rice. It would be interesting to study the effect of combining these two strategies to minimize Na\(^+\) accumulation in both shoot and root. It can be hypothesized that coordinating multiple genes for salt tolerance
specifically expressed in different cell types could enhance the salt tolerance compared to just altering the expression of a single gene. Work on a multiple cell type-specific expression system to improve salt tolerance is currently underway in Arabidopsis using dual enhancer trap lines (Gordon Wellman, ACPFG, unpublished data). With the genetic material in rice generated in this study, it is now possible to test the hypothesis of improved shoot Na\(^+\) exclusion through combination of multiple transport processes in cereals. Crossing transgenic IR64 specifically expressing PpENA1 in epidermal cells with IR64 expressing OsHKT1;1 in the stele is expected to minimize the amount of Na\(^+\) entering the root and therefore reduce the amount of Na\(^+\) reaching inner part of the root (stele). The remaining Na\(^+\) in the inner part of the root will be more effectively retrieved by expression of OsHKT1;5 preventing the accumulation of Na\(^+\) in the shoot. The same strategy could be transferred to Fatmawati, where it might have a stronger effect, given the lower apparent tissue tolerance of Fatmawati compared to IR64.

### 7.2.6. Planning for a confined field trial of transgenic rice lines

The ultimate aim of this project was to develop salt tolerant rice which can be adopted by farmers in salt affected areas to stabilize rice production. The seeds of transgenic rice developed in this project are now being advanced to a later generation to obtain homozygous lines and to multiply the seeds for further characterization. While phenotyping in a controlled environment will be continued to better understand the effect of the transgene, phenotyping in the rice field is also planned. The first plan is to propose a confined field trial in a salt-affected paddy field in Indonesia to evaluate the transgenic rice lines developed in this study. A confined field trial for transgenic rice has been done previously in Indonesia to evaluate transgenic rice expressing cry1AB for stem borer resistance (Deswina 2009). In addition, the Indonesian Centre for Rice Research (ICRR) is currently collaborating with IRRI in transferring the Golden Rice trait into popular Indonesian varieties (IRRI 2014), indicating the feasibility of conducting field research for transgenic rice in Indonesia. Field trials of salt tolerant transgenic rice will be targeted to irrigated lowland rice in coastal areas and rainfed lowland rice in tidal swamp areas. Characterization of the field trial sites is an important step before conducting the experiment. The soil salinity level of the field trial sites can be characterized using an EM38 electromagnetic induction meter as well as analyses of soil cores collected from the sites. More importantly, the evaluation of the transgenic lines in the field will allow direct evaluation of the effect of the genetic engineering on grain yield the most important aspect to a farmer.
7.3. Conclusion

The development of salt tolerance in rice is an important strategy to maintain rice production in salt-affected areas worldwide. In this project, a genetic engineering approach has been taken in an attempt to develop salt tolerant commercially relevant rice cultivars by manipulating Na\(^+\) transport within the plant. A number of transgenic rice plants expressing genes for salt tolerance under the control of either constitutive or cell type-specific promoters have been generated. While the effect of Na\(^+\) alteration to the overall rice salinity tolerance requires further assessment, the transgenic lines developed in this study have shown several promising phenotypes either related to the biomass improvement or alteration of tissue ion concentration. Results from this study also increase the understanding of the physiological mechanisms of salt tolerance in rice which is important in designing future salt tolerant rice lines. More importantly, as the transgenic rice lines developed in this study have the genetic background of commercially relevant rice cultivars, the materials will be ‘a ready to use’ genetic resource for delivery to the farmer as well as a valuable genetic stock to improve salinity tolerance of other rice cultivars.
Supplementary Materials
Supplementary Figure 1 Map of Gateway® entry vector pCR8-OsAnt1

The vector contains the transcription termination sequences (rrnB T1 and T2), the M13 forward and reverse primer site for sequencing the insert, the Gateway® recombination site (attL1 and attL2), the GW1 and GW2 primers site for sequencing the insert, the TOPO® cloning site, the putative promoter pOsAnt1, the spectinomycin (Spn) promoter to express the spectinomycin resistance gene, the spectinomycin resistance gene (SpnR) for selection of the plasmid in E.coli and the pUC origin of replication (pUC origin) for high copy replication and maintenance in E. coli.
Supplementary Figure 2 Map of Gateway® entry vector pCR8-Co2

The vector contains the transcription termination sequences \((rrnB)\text{T1 and T2}\), the M13 forward and reverse primer site for sequencing the insert, the Gateway® recombination site \((attL1 and attL2)\), the GW1 and GW2 primers site for sequencing the insert, the TOPO® cloning site, the putative promoter \(pCo2\), the spectinomycin \((Spn)\) promoter to express the spectinomycin resistance gene, the spectinomycin resistance gene \((SpnR)\) for selection of the plasmid in \(E.coli\) and the pUC origin of replication (pUC origin) for high copy replication and maintenance in \(E. coli\).
Supplementary Figure 3 Map of Gateway® entry vector pCR8-ProOs10g0552200

The vector contains the transcription termination sequences (rrnB T1 and T2), the M13 forward and reverse primer site for sequencing the insert, the Gateway® recombination site (attL1 and attL2), the GW1 and GW2 primers site for sequencing the insert, the TOPO® cloning site, the putative promoter pOs10g0552200, the spectinomycin (Spn) promoter to express the spectinomycin resistance gene, the spectinomycin resistance gene (SpnR) for selection of the plasmid in E.coli and the pUC origin of replication (pUC origin) for high copy replication and maintenance in E. coli.
Supplementary Figure 4 Map of Gateway® entry vector pCR8-ProOs06g0250600

The vector contains the transcription termination sequences (rrnB T1 and T2), the M13 forward and reverse primer site for sequencing the insert, the Gateway® recombination site (attL1 and attL2), the GW1 and GW2 primers site for sequencing the insert, the TOPO® cloning site, the putative promoter pOs06g0250600, the spectinomycin (Spn) promoter to express the spectinomycin resistance gene, the spectinomycin resistance gene (SpnR) for selection of the plasmid in E.coli and the pUC origin of replication (pUC origin) for high copy replication and maintenance in E. coli.
The vector contains the transcription termination sequences (rrnBT1 and T2), the M13 forward and reverse primer site for sequencing the insert, the Gateway® recombination site (attL1 and attL2), the GW1 and GW2 primers site for sequencing the insert, the TOPO® cloning site, the putative promoter pRCc3, the spectinomycin (Spn) promoter to express the spectinomycin resistance gene, the spectinomycin resistance gene (SpnR) for selection of the plasmid in E.coli and the pUC origin of replication (pUC origin) for high copy replication and maintenance in E. coli.
Supplementary Figure 6 Deletion in the cloned nucleotide sequence of the putative promoter region of *OsRCC3*

The deleted region is indicated in bold and underlined. The upper nucleotide sequence is the putative promoter region of rice cultivar Nipponbare based on the NCBI database. The lower nucleotide sequence is the sequence of the cloned putative promoter OsRCC3 in the Gateway entry vector pCR8-OsRCC3. The numbers indicate the position of nucleotides relative to the start codon (ATG).
**Supplementary Figure 7 Map of the destination vector pMDC32OsAnt1**

The vector backbone contains the kanamycin resistance gene, the origin of replication for *E. coli* (pBR322 origin), and the minimal replicon (pVS1) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), the putative promoter of *pOsAnt1*, the Gateway® recombination sequences (*attR1* and *attR2*), the chloramphenicol resistance gene (*CMr*), the *ccdB* gene for negative selection of the plasmid, the nopaline synthase (*Nos*) terminator, the cauliflower mosaic virus 35S promoter (*CaMV35S*), the hygromycin resistance gene, the cauliflower mosaic virus 35S terminator (*A35S*) and the left border sequence (LB).
Supplementary Figure 8 Map of the destination vector pMDC32Co2

The vector backbone contains the kanamycin resistance gene, the origin of replication for *E. coli* (pBR322 origin), and the minimal replicon (pVS1) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), the putative promoter of *pCo2*, the Gateway® recombination sequences (attR1 and attR2), the chloramphenicol resistance gene (CMr), the *ccdB* gene for negative selection of the plasmid, the nopaline synthase (*Nos*) terminator, the cauliflower mosaic virus 35S promoter (*CaMV35S*), the hygromycin resistance gene, the cauliflower mosaic virus 35S terminator (*A35S*) and the left border sequence (LB).
Supplementary Figure 9 Map of the destination vector pMDC32-ProOs10g0552200

The vector backbone contains the kanamycin resistance gene, the origin of replication for E. coli (pBR322 origin), and the minimal replicon (pVS1) for stable maintenance in E. coli. The T-DNA cassette contains the right border sequence (RB), the putative promoter of Os10g0552200, the Gateway® recombination sequences (attR1 and attR2), the chloramphenicol resistance gene (CMr), the ccdB gene for negative selection of the plasmid, the nopaline synthase (Nos) terminator, the cauliflower mosaic virus 35S promoter (CaMV35S), the hygromycin resistance gene, the cauliflower mosaic virus 35S terminator (A35S) and the left border sequence (LB).
Supplementary Figure 10 Map of the destination vector pMDC32-ProOs06g0250600

The vector backbone contains the kanamycin resistance gene, the origin of replication for *E. coli* (pBR322 origin), and the minimal replicon (pVS1) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), the putative promoter of *Os06g0250600*, the Gateway® recombination sequences (*attR1* and *attR2*), the chloramphenicol resistance gene (CMr), the *ccdB* gene for negative selection of the plasmid, the nopaline synthase (*Nos*) terminator, the cauliflower mosaic virus 35S promoter (*CaMV35S*), the hygromycin resistance gene, the cauliflower mosaic virus 35S terminator (*A35S*) and the left border sequence (LB).
Supplementary Figure 11 Map of the destination vector pMDC32OsRCC3

The vector backbone contains the kanamycin resistance gene, the origin of replication for *E. coli* (pBR322 origin) and the minimal replicon (pVS1) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), the putative promoter of *OsRCC3*, the Gateway® recombination sequences (*attR*1 and *attR*2), the chloramphenicol resistance gene (CMr), the *ccdB* gene for negative selection of the plasmid, the nopaline synthase *Nos* terminator, the cauliflower mosaic virus *35S* promoter (*CaMV35S*), the hygromycin resistance gene, the cauliflower mosaic virus *35S* terminator (*A35S*) and the left border sequence (LB).
Transgenic line | Salt treatment | Root dry weight (g) | Shoot dry weight (g) | Total dry weight (g) | Shoot Na⁺ (mM) | Shoot K⁺ (mM) | Root Na⁺ (mM) | Root K⁺ (mM)
--- | --- | --- | --- | --- | --- | --- | --- | ---
R52-1 | 0 mM NaCl | 0.05 | 0.22 | 0.28 | 2.85 | 269.77 | 0.96 | 132.53
R52-2 | 0 mM NaCl | 0.10 | 0.38 | 0.48 | 3.07 | 330.35 | 0.64 | 113.04
R52-3 | 0 mM NaCl | 0.11 | 0.37 | 0.48 | 12.77 | 346.47 | 0.74 | 116.19
R52-4 | 0 mM NaCl | 0.10 | 0.35 | 0.45 | 6.23 | 310.77 | 0.62 | 113.57
R52-5 | 0 mM NaCl | 0.07 | 0.28 | 0.35 | 5.61 | 312.22 | 0.75 | 130.48
R52-6 | 0 mM NaCl | 0.12 | 0.39 | 0.51 | 11.53 | 387.29 | 0.68 | 112.18
R52-7 | 0 mM NaCl | 0.12 | 0.43 | 0.55 | 3.03 | 309.65 | 0.62 | 112.55
R52-8 | 0 mM NaCl | 0.10 | 0.38 | 0.48 | 6.82 | 294.94 | 0.59 | 113.98
R52-9 | 0 mM NaCl | 0.14 | 0.51 | 0.65 | 4.45 | 308.80 | 0.55 | 126.93
R52-10 | 0 mM NaCl | 0.15 | 0.48 | 0.62 | 6.35 | 324.32 | 0.61 | 134.32
R52-11 | 0 mM NaCl | 0.07 | 0.30 | 0.37 | 3.93 | 324.50 | 1.34 | 121.91
R52-12 | 0 mM NaCl | 0.07 | 0.31 | 0.38 | 4.63 | 344.25 | 0.69 | 118.28
R52-13 | 0 mM NaCl | 0.09 | 0.31 | 0.40 | 5.37 | 323.87 | 0.61 | 119.67
R52-14 | 0 mM NaCl | 0.11 | 0.42 | 0.53 | 3.91 | 320.63 | 0.83 | 123.73
R52-15 | 0 mM NaCl | 0.10 | 0.37 | 0.47 | 2.54 | 320.21 | 0.63 | 122.18
R52-16 | 0 mM NaCl | 0.11 | 0.39 | 0.50 | 2.22 | 296.70 | 0.60 | 113.89
R52-17 | 0 mM NaCl | 0.13 | 0.46 | 0.59 | 7.75 | 296.12 | 0.62 | 133.88
R52-18 | 0 mM NaCl | 0.10 | 0.32 | 0.41 | 4.41 | 322.93 | 0.68 | 119.08
R52-19 | 0 mM NaCl | 0.14 | 0.50 | 0.64 | 6.12 | 317.66 | 0.72 | 133.08
R52-20 | 0 mM NaCl | 0.08 | 0.28 | 0.36 | 1.73 | 339.48 | 0.87 | 126.21
R57-1 | 0 mM NaCl | 0.09 | 0.34 | 0.43 | 1.89 | 248.64 | 0.72 | 115.75
R57-2 | 0 mM NaCl | 0.09 | 0.36 | 0.45 | 1.29 | 220.91 | 0.61 | 116.27
R57-3 | 0 mM NaCl | 0.11 | 0.37 | 0.48 | 2.93 | 313.63 | 0.67 | 115.49
R57-4 | 0 mM NaCl | 0.13 | 0.44 | 0.57 | 7.17 | 299.44 | 0.55 | 105.46
R57-5 | 0 mM NaCl | 0.07 | 0.31 | 0.38 | 3.77 | 311.99 | 0.71 | 118.67
R57-6 | 0 mM NaCl | 0.18 | 0.57 | 0.75 | 9.82 | 354.48 | 0.58 | 120.48
R57-7 | 0 mM NaCl | 0.08 | 0.31 | 0.40 | 1.74 | 309.59 | 0.67 | 120.36
R57-8 | 0 mM NaCl | 0.09 | 0.33 | 0.43 | 3.10 | 299.29 | 0.56 | 110.30
R57-9 | 0 mM NaCl | 0.09 | 0.34 | 0.43 | 2.33 | 302.55 | 0.61 | 119.21
R57-10 | 0 mM NaCl | 0.11 | 0.44 | 0.56 | 4.74 | 298.45 | 0.55 | 109.44
R57-11 | 0 mM NaCl | 0.12 | 0.42 | 0.53 | 4.39 | 299.35 | 0.69 | 113.25
R57-12 | 0 mM NaCl | 0.06 | 0.28 | 0.35 | 3.10 | 282.80 | 0.77 | 130.59
R57-13 | 0 mM NaCl | 0.10 | 0.41 | 0.51 | 7.08 | 312.55 | 0.61 | 110.63
R57-14 | 0 mM NaCl | 0.15 | 0.55 | 0.70 | 4.26 | 284.95 | 0.61 | 128.43
R57-15 | 0 mM NaCl | 0.08 | 0.32 | 0.39 | 5.40 | 301.98 | 0.64 | 113.25
R57-16 | 0 mM NaCl | 0.17 | 0.57 | 0.74 | 7.05 | 325.93 | 0.58 | 118.02
R57-17 | 0 mM NaCl | 0.09 | 0.32 | 0.41 | 2.65 | 325.13 | 0.64 | 118.92
R57-18 | 0 mM NaCl | 0.10 | 0.33 | 0.43 | 5.00 | 277.37 | 0.57 | 112.80
R57-19 | 0 mM NaCl | 0.17 | 0.57 | 0.73 | 5.13 | 316.82 | 0.64 | 124.38
R57-20 | 0 mM NaCl | 0.07 | 0.26 | 0.33 | 3.83 | 292.77 | 0.62 | 122.68

Supplementary Table 1 Phenotypic data of transgenic T1 Fatmawati transformed with pOs10g0552200:AtAVP1 (R52) and pOsRCC3:OsOVP4 (R57) under 0 mM and 70 mM NaCl treatment

The rice plants were grown in hydroponics with salt treatment imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. Rice plants were harvested 10 d after the 70 mM NaCl treatment had been reached.
Transgenic line & Salt treatment & Root dry weight (g) & Shoot dry weight (g) & Total dry weight (g) & Shoot Na\(^+\) (mM) & Shoot K\(^+\) (mM) & Root Na\(^+\) (mM) & Root K\(^+\) (mM) \\
--- & --- & --- & --- & --- & --- & --- & --- & --- \\
R52-1 & 70 mM NaCl & 0.07 & 0.27 & 0.34 & 14.69 & 243.54 & 93.05 & 81.72 \\
R52-2 & 70 mM NaCl & 0.07 & 0.23 & 0.30 & 15.48 & 236.45 & 92.27 & 77.50 \\
R52-3 & 70 mM NaCl & 0.08 & 0.27 & 0.35 & 25.77 & 235.26 & 89.19 & 75.73 \\
R52-4 & 70 mM NaCl & 0.06 & 0.23 & 0.29 & 36.93 & 255.10 & 102.11 & 75.30 \\
R52-5 & 70 mM NaCl & 0.08 & 0.30 & 0.38 & 59.64 & 237.19 & 102.04 & 75.89 \\
R52-6 & 70 mM NaCl & 0.09 & 0.29 & 0.38 & 32.18 & 200.41 & 97.09 & 84.10 \\
R52-7 & 70 mM NaCl & 0.10 & 0.29 & 0.40 & 30.88 & 212.38 & 95.38 & 82.95 \\
R52-8 & 70 mM NaCl & 0.05 & 0.20 & 0.25 & 25.47 & 246.14 & 99.90 & 79.12 \\
R52-9 & 70 mM NaCl & 0.08 & 0.30 & 0.38 & 58.12 & 321.58 & 91.63 & 66.10 \\
R52-10 & 70 mM NaCl & 0.07 & 0.22 & 0.29 & 57.29 & 251.18 & 101.21 & 69.21 \\
R52-11 & 70 mM NaCl & 0.08 & 0.27 & 0.35 & 16.61 & 186.40 & 101.68 & 85.49 \\
R52-12 & 70 mM NaCl & 0.07 & 0.23 & 0.30 & 14.68 & 164.86 & 90.16 & 79.40 \\
R52-13 & 70 mM NaCl & 0.09 & 0.31 & 0.40 & 32.57 & 231.90 & 93.50 & 84.32 \\
R52-14 & 70 mM NaCl & 0.08 & 0.27 & 0.35 & 24.72 & 231.79 & 91.79 & 77.79 \\
R52-15 & 70 mM NaCl & 0.08 & 0.27 & 0.35 & 27.88 & 247.10 & 92.54 & 77.66 \\
R52-16 & 70 mM NaCl & 0.12 & 0.40 & 0.52 & 63.84 & 211.27 & 85.86 & 71.46 \\
R52-17 & 70 mM NaCl & 0.08 & 0.28 & 0.36 & 58.18 & 263.28 & 97.22 & 76.02 \\
R52-18 & 70 mM NaCl & 0.07 & 0.24 & 0.31 & 31.61 & 288.55 & 91.13 & 76.79 \\
R52-19 & 70 mM NaCl & 0.13 & 0.42 & 0.55 & 49.74 & 215.35 & 88.06 & 71.46 \\
R52-20 & 70 mM NaCl & 0.08 & 0.27 & 0.36 & 11.72 & 117.16 & 96.82 & 81.35 \\
R57-1 & 70 mM NaCl & 0.08 & 0.27 & 0.35 & 27.96 & 227.83 & 97.86 & 83.61 \\
R57-2 & 70 mM NaCl & 0.10 & 0.31 & 0.40 & 35.29 & 219.27 & 92.39 & 76.66 \\
R57-3 & 70 mM NaCl & 0.09 & 0.27 & 0.36 & 29.22 & 230.55 & 93.45 & 83.67 \\
R57-4 & 70 mM NaCl & 0.10 & 0.30 & 0.40 & 59.00 & 209.20 & 89.49 & 65.89 \\
R57-5 & 70 mM NaCl & 0.06 & 0.19 & 0.25 & 18.93 & 246.29 & 92.90 & 78.43 \\
R57-6 & 70 mM NaCl & 0.14 & 0.39 & 0.53 & 44.54 & 221.84 & 85.63 & 72.54 \\
R57-7 & 70 mM NaCl & 0.10 & 0.30 & 0.40 & 31.81 & 218.11 & 98.44 & 85.59 \\
R57-8 & 70 mM NaCl & 0.08 & 0.23 & 0.31 & 27.93 & 270.51 & 95.13 & 85.40 \\
R57-9 & 70 mM NaCl & 0.08 & 0.26 & 0.34 & 23.66 & 241.31 & 89.86 & 81.11 \\
R57-10 & 70 mM NaCl & 0.10 & 0.27 & 0.37 & 38.11 & 236.95 & 90.93 & 76.90 \\
R57-11 & 70 mM NaCl & 0.09 & 0.27 & 0.36 & 30.67 & 225.95 & 97.71 & 79.13 \\
R57-12 & 70 mM NaCl & 0.06 & 0.19 & 0.25 & 13.11 & 227.78 & 93.48 & 82.91 \\
R57-13 & 70 mM NaCl & 0.08 & 0.28 & 0.36 & 28.97 & 216.37 & 87.71 & 78.22 \\
R57-14 & 70 mM NaCl & 0.12 & 0.37 & 0.49 & 51.17 & 211.67 & 90.76 & 74.02 \\
R57-15 & 70 mM NaCl & 0.07 & 0.25 & 0.32 & 24.01 & 246.28 & 94.81 & 79.56 \\
R57-16 & 70 mM NaCl & 0.12 & 0.35 & 0.47 & 44.70 & 215.44 & 87.84 & 74.84 \\
R57-17 & 70 mM NaCl & 0.07 & 0.22 & 0.29 & 20.88 & 202.81 & 91.20 & 82.38 \\
R57-18 & 70 mM NaCl & 0.09 & 0.30 & 0.39 & 39.34 & 229.75 & 86.30 & 77.35 \\
R57-19 & 70 mM NaCl & 0.09 & 0.30 & 0.40 & 44.47 & 235.35 & 95.07 & 76.51 \\
R57-20 & 70 mM NaCl & 0.09 & 0.27 & 0.36 & 28.14 & 240.11 & 94.90 & 79.92

Supplementary Table 1 (continued) Phenotypic data of transgenic T1 Fatmawati transformed with pOs10g0552200::AtAVP1 (R52) and pOsRCC3::OsOVP4 (R57) under 0 mM and 70 mM NaCl treatment

The rice plants were grown in hydroponics with salt treatment imposed in 25 mM increments over 3 d period (20 mM on the last day) starting when the 4th leaf was fully expanded (17 d after germination). The youngest, fully expanded leaves and the total roots were used for the measurement of ion concentration. Rice plants were harvested 10 d after the 70 mM NaCl treatment had been reached.
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