SALINITY TOLERANCE AND TRANSCRIPTOMICS IN RICE

by

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ABSTRACT

Climate change-induced events are causing salinization of many rice growing areas, requiring the development of tolerant varieties to meet up with the demand for food of ever incrasing human population. A wide natural variation of rice genotypes including the genotypes from the sub-species *Indica*, *Japonica* and wild relatives were studied morpho-physiologically to identify the level and mechanism of salt tolerance.

In general, *Indica* varieties performed better than both *Japonica* and wild species. The existence of qualitatively different mechanisms of salt tolerance across the genotypes is identified. For example Pokkali, a salt tolerant *Indica* variety, displayed both 'Na exclusion' and 'ion balance' mechanisms whereas PSBRc50 and IR58 showed only 'Na exclusion' and the *Japonica* genotypes, Banikat and Nipponbare showed only 'ion balance'. The results demonstrated that the tolerance is dependent on the level of stress as Nipponbare appeared as moderately tolerant at 40mM NaCl but as susceptible at 80mM. Multivariate analyses was used to simplify the complex salinity tolerance picture by categorizing the gnoeytpes according to the level of tolerance exhibited.

The gene expression response of eight rice genotypes was interrogated by the weighted continuous morpho-physiological trait responses using a modified version of the 'Significance Analysis of Microarrays' (SAM) to comprehensively elucidate the trait specific expression of genome wide transcripts. More genes were found to be differentially expressed for the traits under salt stress compared to normal conditions. Strikingly, for 'biomass', a contrasting number of positive and negative genes were expressed under unstressed and stressed conditions, respectively. Around sixty genes were identified as to be

involved in Na^+, K^+ and anion homeostasis, transport and transmembrane activity under stressed condition. Gene ontology enrichment analysis identified the genes involved in the major global molecular functions such as signal transduction (>150 genes), transcription factor (81 genes) and translation factor activity (62 genes), protein phosphatase, transferase, hydrolase activity and oxidoreductase activity. The gene network analysis demonstrated that the transcription factors and translation initiation factors form the major gene networks and are mostly active in nucleus, cytoplasm and mitochondria whereas the membrane and vesicle bound proteins form the second network active in plasma membrane and vacuoles.

The differential expression of four tolerant and two susceptible *Indica* genotypes under stress was further analysed using Gene-spring software. More genes were found to be differentially regulated in susceptible than tolerant genotypes with a substantial commonality between these genotypes. The genes under important biological processes and molecular functions are identified and discussed. Highly induced stimulus responsive gene Os01g0159600 (Embryonic abundant protein 1 (OsLEA1a) and Os05g0382200 (Na^+/H^+ exchanging protein-like) can be mentioned for instance. The genes that is located within the salt stress related QTL were identified, for example the transcription factor gene Os01g0303600 (Zinc finger C3HC4 type (RING finger) located within the QTL Saltol and qSKC1 QTLs in chromosome 1. The transcriptomics data also used to predict the salinity tolerance status of the genotypes with unknown tolerance by validating against the OSC filtered PLS-DA model created using the combined differentially expressed significant genes in the known tolerant and known susceptible genotypes. The combined physiological and transcriptomic approach of this study gives a complementary whole organism assessment of plants responses to salt stress.

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All the 12 genotypes were subjected to normalization as described in section 2.12.2 and the differentially expressed genes for the above mentioned varieties were identified by comparing the expression of treated samples vs. control samples.

LIST OF ABBREVIATION

2S- Two known susceptible genotypes
4T- Four known tolerant genotypes
AAP- Amino Acid Transporter
ANOVA- Analysis of Variance
aRNA – amplified RNA
BPH- Brown planthopper
BR/BRR- BRRI dhan29
BRRI- Bangladesh Rice Research Institute
C- Control
cDNA – Complementary DNA
cRNA- Complementary RNA
DMSO - Dimethyl sulfoxide
EST – Expressed Sequence Tag
ET- Ethylene
FAO- Food and Agriculture Organisation
FL/FL4- FL478
GO Analysis – Gene Ontology Analysis
h- Hour
HS/Has- Hassawi
IRRI- International Rice Research Institute
JA- Jasmonic Acid
LRR- leucine-rich repeat
Lt/Ola- *O. latifolia*
LV- Latent variable
MIAME Minimum Information about a Microarray experiment
Min- Minute
mRNA – Messenger RNA
Nb/Non- Nonabokra
NB-LRR- Nucleotide-binding leucine-rich-repeat
NCBI – National Center for Biotechnology Information
Np/Nip- Nipponbare
Nt – Single stranded unit of DNA/RNA
OSC- Orthogonal Signal Correction
PC- Principal Component
PCA- Principal Component Analysis
PCR- Polymerase Chain Reaction
PK/Pok- Pokkali
PLSDA- Partial Least Square Discriminant Analysis
PS- Pooled susceptible genotypes
Ps/PSB- PSBRc50
BN/Ban- Banikat

PT- Pooled Tolerant genotypes
QTL- Quantitative Trait Loci
Rf/Oru- *O. rufipogon*
RIN – RNA Integrity Number
s- Second
SA- Salicylic Acid
Sus – Susceptible
T- Treated
Tol – Tolerant
Unk- Unknown
UTP - uridine 5'-triphosphate

Chapter 1. GENERAL INTRODUCTION

1.1 The experimental plant

1.1.1 Rice: Taxonomy, botany and the genome

Rice, a member of the family Gramineae or Poaceae ("true grass"), belongs to the genus *Oryza* which includes species of which 22 are wild and only two are cultivated (Vaughan *et al*, 2003). Rice can be found in the tropical and subtropical humid parts of Asia, Africa, Australia, Central and South America (Chang 1985). The cultivated species of rice are semi aquatic plants, which can grow up to 5 meters in deep water while some genotypes grow on dry land. Generally, rice is an annual crop with around 3 to 6 months of life cycle (variation depends on varieties) but sometimes produces new tillers (ratoon) after harvesting. The mature plant has a main stem and several tillers which would bear a terminal flowering head and panicle. The morphological development of rice has two phases namely, vegetative phase that includes germination, establishment of seedling, tillering and stem elongation and reproductive phase that includes panicle initiation, booting and grain filing stages (Maclean *et al.* 2002). Rice is also classified as a natural inbred crop which can give much advantage in plant breeding programmes (Blair *et al*, 2002).

O. sativa, an important model species for the monocots and cereals (e.g., barley, wheat, sorghum and maize), possesses 12 pairs of chromosomes and a relatively compact genome of approx. 500 Mbase (n=12) than the multi-gigabase genomes of barley, wheat and maize. *O. sativa* has two main subspecies namely, *Indica* and *Japonica* and are mostly diploid (2n=2x=24) with genome AA (Li *et al.* 1997; Samuel, 2001; Chang, 2003). However, the wild species under *Oryza* genus contains both diploid (2n=2x=24) and tetraploid (2n=4x=48)

forms and represents ten types of genomes: AA, BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ and HHKK (Vaughan *et al.* 2003). Thus the wild rice is considered to be a useful source for genes in rice improvement programmes especially for biotic and abiotic stress resistance (Ratnayaka, 1999). The limitation however appears due to differences in homology between the genome of *O. sativa* and its wild species. This can make the process of gene transfer difficult due to limitation of low crossability and recombination of the chromosomes (Samuel, 2001).

1.1.2 Rice: Wild relatives, domestication and loss of resistance

The wild species provides richness in terms of genetic variation and can be exploited for crop improvement. Rice, with its two domesticated types namely, *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice) is very rich in terms of wild relatives having unique domestication histories that goes back to ~9,000 y ago (Molina *et al*. 2011). The 21 wild relatives of the domesticated rice under the genus Oryza falls under four species group namely, *O. sativa*, *O. officialis*, *O. granulate* and *O. ridelyi* (Sweeney and McCouch 2007). Phenotypically, *O. sativa* differs greatly from its wild relatives in many ways, with long awns and severe shattering being predominant in wild relatives in contrast to its domesticated counterparts (Xiong*et al.* 1999; Bres-Patry *et al.* 2001; Thomson *et al.* 2003; Li *et al.* 2006a). The present day rice *O. sativa* is considered to be domesticated from the wild species *O.rufipogon* and *O.nivara* (Khush 1997; Kovach *et al*. 2007; Sweeney and McCouch 2007). The *O. sativa* again has two genetically distinct subspecies groups namely, *Indica* (cultivated mainly in Asia) and *Japonica* (cultivated in temperate regions of world) that are divergent morphologically, physiologically and are also partially isolated by a post-zygotic barrier (Li *et al.* 1997; Chang, 2003; Kovach *et al*. 2007). Previously these two subspecies were believed to

be domesticated by two independent geographical events in Asia (Vitte *et al*. 2004; Londo & Chiang 2006; Gao and Innan 2008) as shown in Figure 1.1. However, a recent study by Molina *et al.* (2011) suggested a single evolutionary origin of domesticated rice that happened in China.

Crop domestication, the ongoing dynamic process since the prehistoric human civilization, involves selection for desired traits (which are beneficial to human) that makes a wild species more adaptable for cultivation and human consumption (Kovach *et al*. 2007). However, it reduces the fitness of a crop to survive in the wild as human selection for desired traits is often intense (He *et al*. 2011). As a result of the domestication process the extreme genetic diversity of a species is left behind and a very strong genetic bottle neck is generated which caused many genes to be lost from the gene pool. The genes for the desired traits were thus lost in the process of domestication (Wright *et.al*, 2005). For example, the submergence tolerance gene *Sub1* is believed to be originated in wild species of rice (Fukao *et al*. 2009); waxy gene for the glutinous rice is altered in the process of domestication (Olsen and Purugganan 2002) and rice seed shattering genes *sh4 and qSH1* are pertinent examples of crop domestication (Konishi *et al*. 2006).

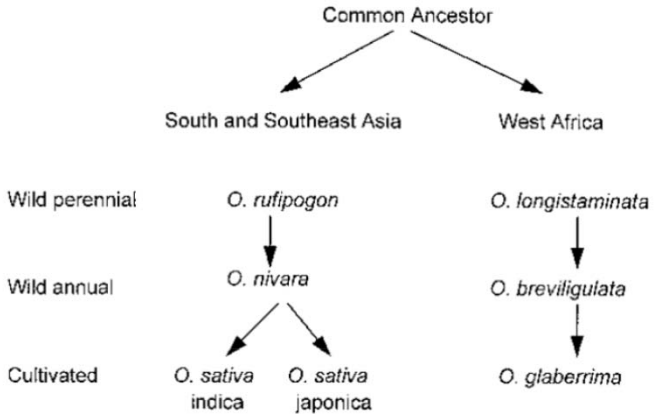


Figure 1.1 The simplified evolutionary pathway of the origin of *O. sativa* ssp*. Indica* and *O. sativa* ssp*. Japonica* (source: Khush 1997).

1.1.3 Rice: Food security, economic impact and the challenges in cultivation

Rice is said to be the oldest domesticated grain crop (around 10000 years) and the most consumed cereal grain grown on the planet. Rice is grown in 9% of the world's arable land which accounts for third highest worldwide production of food, after maize and wheat (FAOSTAT, 2010). In terms of global human nutrition and calorific intake, 21% of per capita energy and 15% of per capital protein is provided by rice (with the percentage in developing countries being 27% and 20%, respectively), ranking it as the most important among the crops that feed the world (Khush, 2001). Growing rice is the important livelihood activity of millions of households around the world and forms the major source of revenue and foreign exchange for several Asian and African countries. The climate changed induced natural catastrophes; along with the environmental (biotic and abiotic) stresses pose a great threat to the food security and the economical development of world's 60% population. Realizing this fact the United Nation has set 'reducing hunger and poverty' as one of the 'Millennium Development Goals' and declared 2004 as the International Year of Rice.

The food demand of the growing population was met by the 1970s and 1980s green revolution in rice production, which however, tend to diminish in recent years causing a deficit in the supply. In 2008, more than 120 countries had grown more than 685 million tons of rice from approximately 159 million hectares of production area (FAOSTAT, 2010). Over the next 50 years, the population of the world is predicted to increase by about 50% while the scarcity of land and water resources tends to grow. In an attempt to estimate the food supplies for the growing human population, the International Rice Research Institute predicted that 800 million tons of rice (with a 40% increase in consumption) will be required in 2025 (Purevdorj and Kubo, 2000). Thus, an increased production of rice will be crucial to achieve long-term global food security but before that the challenges in rice cultivation need to be addressed. Besides the dwindling land and water resources and oversimplified crop management system, the environmental threats posed by biotic (e.g., insects, diseases and pests etc.) and abiotic stresses (e.g., drought, salinity, cold etc.) are the major challenges of rice cultivation. This study, however, focuses specifically on the abiotic stress- soil salinity which is responsible for a major loss of global rice production.

1.2 Soil Salinity

A soil becomes saline when it contains high concentrations of NaCl along with the soluble compounds of other mineral salts such as Ca, Mg, K, Fe, B, $SO4^{2-}$, $CO3^{2-}$ and $CHO3^{-}$ (Szabolcs, 1989). Soil salinity is often measured in terms of electric conductivity (EC) and expressed as mS m^{-1} for low salinity or dS m^{-1} for high salinity, based on the principle that high salt solution causes more electricity to move through it. It is also measured in terms of exchangeable Na percentage (ESP) or Na absorption ratio (SAR) and pH of saturated soil

paste extract. The USDA Salinity Laboratory defines a soil as saline when it have an electrical conductivity (EC) of 4 dS m^{-1} or more equivalent to 40 mM NaCl, ESP <15 % and a pH below 8.5 (USSL, 2005). The threshold values that exert the harmful effects can however depend on the climatic condition, soil water regime and type of plant (Maas, 1986). A salinity of 4-8 dS m^{-1} is considered as moderately saline soil which causes serious damage to most agricultural crop plants and a salinity of 8-16 dS m^{-1} is considered as high salinity which causes only highly tolerant plants to survive.

1.2.1 Causes of soil salinity

Soil salinity, excess accumulation of salts typically at surface soil, in a particular location can be due to primary (naturally induced) or secondary salinization (human induced).The slow weathering of the earth's minerals over eons contributed to the release and deposition of significant amounts of inorganic compounds (e.g., sodium, calcium, magnesium, chloride, sulphate and carbonate etc.) that ultimately deposited into the oceans. The intrusion of sea water into rivers and aquifers along with tidal overflow, cyclones and tsunamis makes the area in close vicinity vulnerable to salinity (Flowers, 1999; Taiz & Zeiger, 2002). Primary salinity is also found throughout the arid and semi-arid regions in the form of ancient salt deposits, fossil salts, and saline groundwater where salts are raised by capillary action to the surface of the soil (Carter, 1975; Flowers, 1999). Soil in a particular area may also become saline because of some secondary sources, such as irrigation. When the plants use the water, the salts are left behind in the soil and evaporation brings them to the surface where they accumulate and damage crops (Carter, 1975). Long term irrigation merely increases the amount of salt in the soil and the situation can be aggravated by poor drainage (Epstein *et al.* 1980; Serrano *et al.* 1999b; Flowers *et al.* 2000). The other sources for secondary

salinization are residual salts from water and soil amendments, animal wastes, chemical fertilizers, applied sewage sludge, the disposal of gas and oil field brines etc.

1.2.2 Global distribution of soil salinity

Soil salinity is more prevalent in arid and semi-arid regions (Ghassami *et al.* 1995, Mashali 1999) with different degree being reported in Australia, America, Africa, Asia, Middle East and India (Prakash and Chandha, 1983, Schofield and Ruprecht, 1989, Richter and Kreitler, 1993, Funakawa *et al.* 2000 and Marie and Vengosh, 2001). FAO Land and Plant Nutrition Management Service reported that salinity and/or sodicity affected about 6.5% of the world's land (831 m ha) either salinity or sodicity extending over all the continents (FAO, AGL 2000; Martinez-Beltran and Manzur, 2005 and Rengasamy 2006). The Environment Program of United Nations estimated that 9-34% of world's irrigated land with a world average of 20% is adversely affected by soil salinity (Ghassemi *et al.* 1995; Szabolcs, 1992; Flowers, 1999). Approximately 60 million ha of irrigated land (Zhang *et. al*., 2001; Tester and Davenport, 2003) and 32 million ha of dry land (2.1%) are affected by varying degrees of soil salinity by human-induced processes (FAO, AGL 2000). In the deltaic country Bangladesh, over 30% of the net cultivable area lies in the coastal zone of Bay of Bengal, of which approximately 53% is affected by salinity where the land use is roughly 50% of the country's average (Petersen & Shireen, 2001; Haque, 2006). Over the last three decades the salinity affected area in this country has increased from 0.83 million ha in 1966-75 to 3.1 million ha. According to the Intergovernmental Panel on Climate Change, Bangladesh is stated to lose the largest amount of cultivated land due to rising sea levels globally. It is predicted that 45cm rise in sea levels would inundate 10% and 1m rise will inundate over 21% of the country's land area (Rahman *et al.* 2007). The other concern is that the area under irrigation

is increasing worldwide day-by-day leaving more area to be affected by salinity stress. A FAO estimation showed that 0.25-0.50 million ha of irrigated lands are becoming unsuitable for production due to salts build-up every year (Martinez-Beltran & Manzur, 2005).

1.2.3 Impact of salt stress on global agricultural production

Salinization of soil, one of the oldest environmental phenomena, is considered as one of the important processes of land degradation (Kassas, 1987; Thomas and Middleton, 1993). The problem of soil salinization is prevalent at a varying degree in all continents of the world (Tanji, 1990), referring to it in some regions as 'Silent Killer' of natural production since it usually kills plants and soil organisms in the affected areas or as 'White Death' since it conjures up white images of lifeless shining lands studded with dead trees. The effect of salt stress on agriculture can range from agricultural production being slightly affected to completely reduced (in extreme cases). Irrigated land constitutes 17% of world's cropland and provides 30% of the overall agricultural production (Hillel, 2000). Among the world's irrigated land, 20% is affected by varying degrees of salinity causing a substantial loss in global agricultural productivity.

It is estimated that the total cost of soil salinity to agriculture per year is approximately $US 12 billion which is expected to increase in future (Dregne *et al.* 1991; Ghassemi *et al.* 1995; Pitman *et al.* 2007). On a global scale, this may appear small, but can have substantial impact in regions where production of staple crops using irrigation is affected by soil salinity. Global food production is estimated to be increased by 38% by 2025 and by 57% by 2050 to meet up the demand of the growing world population (Wild, 2003). With little possibility of increase in irrigated land in some part of the world, the food demand can be met by

increasing yield and by bringing the problem soils back under production by developing suitable crop varieties.

1.3 Harmful effects of salt stress on plants

A soil is considered saline if it contains soluble salts in sufficient quantities that can interfere with the growth of most crop species (Elphick *et al.* 2001; Werner and Finkelstein, 1995). The harmful effects of salts in inhibiting plant growth can broadly be discussed in two categories namely, osmotic stress and ionic toxicity (Figure 1.2). Due to the presence of high salt under salt stress, the osmotic pressure in the soil solution exceeds that of plant cells reducing the plant's ability to take up water and essential minerals nutrients like K^+ and Ca^{2+}(Glenn, Brown & Khan, 1997; Munns, James & Läuchli, 2006). In extreme cases, the soil solution may become hyper-osmotic causing the root cells to lose water instead of absorbing it. Water scarcity disrupts maintenance of cell turgor and cell elongation causing wilting and ultimately death of a plant. This effect of salt stress can thus be described as the drought effect of salt stress (Campbell, 1993; Zhang *et al.* 2001; Apse and Blumwald, 2002; Munns *et al.* 2002).

The sodium ion (Na^+), if present in the cytosol at a concentration higher than the adequate level can be very harmful for most plants (Tuteja 2007). On the other hand, potassium (K^+) is an essential macronutrient and should be abundant in the cell for efficient metabolic functioning as it is involved in osmo-regulation, maintenance of membrane potential and turgor and enzyme activation (K^+ is the co-factor for more than 50 enzymes) (Fox and Guerinot, 1998; Maathius and Amtman, 1999; Mäser *et al.* 2002a; Cuin *et al.* 2003). Since Na^+ and K^+ are physicochemically similar monovalent cations when fully hydrated, they

compete in saline conditions for uptake through shared transport systems (Schachtman and Liu, 1999). Moreover, at a high concentration, Ca^{2+}can be displaced from the plasma membrane by Na^{+}which negatively impacts intracellular K^{+} influx in the cell and changes the homeostasis of other ions such as NO^{3-} (Cramer, Epstein & Lauchli, 1998). Thus excessive uptake of Na^{+} alters (mainly elevates) Na^{+}/K^{+} ratios and exerts metabolic toxicity as Na^{+} and K^{+} competes for the binding sites of many enzymes and disrupts many crucial processes in which K^{+} is involved (Bhandal & Malik, 1988; Zhang *et al.* 2001; Tester & Dabenport, 2003; Munns, James &Läuchli, 2006). High Na^{+} on the other hand can dissipate the membrane potential and facilitate the uptake and build up of Cl^{-} in the cytosol and exert a direct toxic effect on cell membranes and on metabolic activities in the cytosol (Greenway an Munss, 1980; Hasegawa *et al.* 2000; Zhu, 2001; Tuteja 2007). Consequences of these primary effects cause some secondary effects like reduced cell expansion and membrane function, assimilate production, photosynthesis as well as decreased cytosolic metabolism and production of reactive oxygen intermediates (ROSs) which ultimately causes growth inhibition and eventual death of plants.

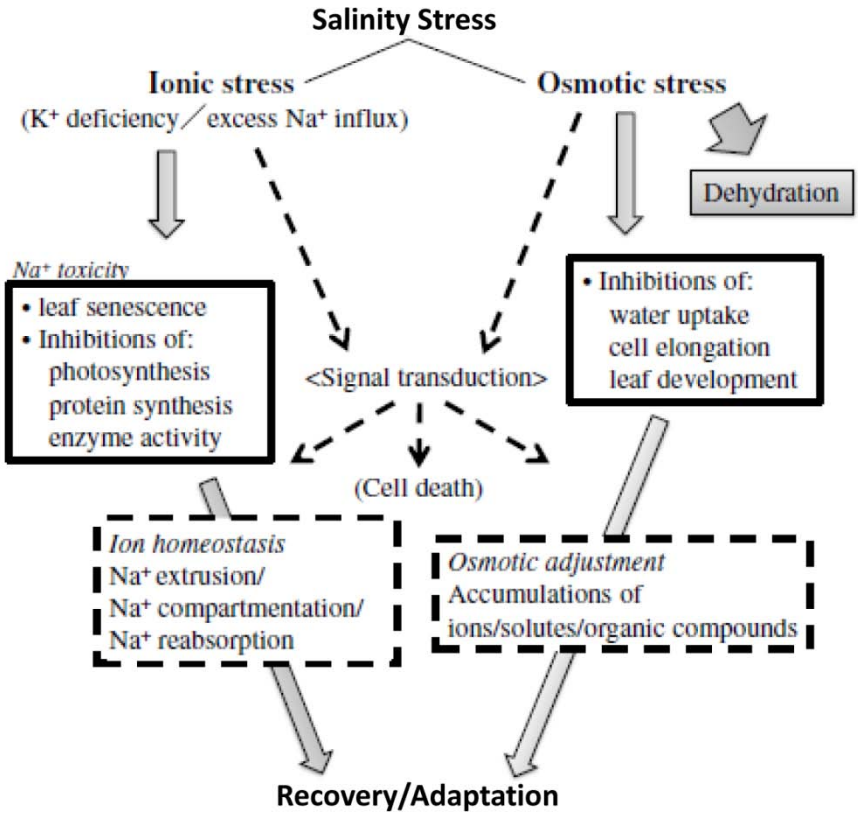


Figure 1.2 Schematic representation of the effect of salt stress on the plant along with corresponding adaptive response used by plants; adapted from Horie *et al.* (2012).

Bold arrows and boxes with bold line indicates the harmful effects of salt stress; dash arrows and boxes with dash line indicates the physiological approaches adapted by plants to overcome the harmful effect of stress

1.4 Mechanisms of Na^+ uptake in plants

In saline soil, epidermal cells of root tips including root hairs are the primary sites for the uptake of inorganic ions through the plasma membrane (Golldack *et al.* 2003; Horie *et al.* 2012). The uptake of salts into roots and translocation into shoots can primarily be attributed to the transpirational flux of the plants (Amtmann and Sanders 1998; Yeo 1998; Blumwald 2000). The H^+-ATPases of plasma membrane acts as the primary pump and generates a proton motive force which drives the transport of Na^+ and K^+ along with other solutes (Braun *et al.* 1986; Craig Plett and Møller 2010; Kronzucker and Britto 2011). The

transport proteins spanning the lipid bilayer of the plasma membrane which is usually impermeable to solutes facilitate the movement of solutes in and out of the cytosol.

Sodium (Na^+), a positive ion mainly enters the cytosol passively as the potential in plant cells is negative inside the plasma membrane (Blumwald 2000; Hasegawa 2013; Yamaguchi *et al.* 2013; Adams and Shin 2014). Once it enters epidermal cells or cortical cells, Na^+ may follow a symplastic (connected by plasmodesmata) or apoplastic pathway (bypass flow) before it encounters the Casparian bands in endodermis layer (Yeo, Yeo & Flowers 1987; Yadav, Flowers & Yeo 1996; Ochiai & Matoh 2002; Gong, Randall & Flowers 2006; Krishnamurthy *et al.* 2009). Apoplastic enzymes shows more tolerance to salts than cytoplasmic enzymes, in both halophytes and glycophytes, indicating the ability of apoplast to withstand relatively high concentrations of Na^+ (Thiyagarajah *et al.* 1996; Adams and Shin 2014).

There exists considerable ambiguity in the mechanism of radial movement of Na^+ across the Casparian bands to the xylem (Kronzucker and Britto 2011; Krishnamurthy *et al.* 2011). It is believed that the Casparian bands in the endodermis layer prevents the symplastic movement of Na^+ and in case of rice, further radial movement of Na^+ occurs through the so-called 'apoplastic bypass', where Na^+ move through the apoplast by solvent drag (Ranathunge *et al.* 2005; Ochiai and Matoh, 2002; Gong *et al.* 2006; Craig Plett and Moller 2010) and through the leakage opened up by the lateral roots (Hasegawa 2013; Yamaguchi *et al.* 2013; Wang *et al.* 2006). The presence of Casparian bands is the strategic mechanism adapted by plants to control the entry of Na^+ to the xylem stream which, in turns, controls salt build up in shoots (Ranathunge *et al.* 2005; Ochiai & Matoh 2002; Krishnamurthy *et al.* 2011). But the structural barriers such as suberin lamellae and deposition of silicon in rice

roots and the presence of phi cell layers in *Brassica* restrict apoplastic bypass of Na^+ (Miyamoto *et al.* 2001; Karahara *et al.* 2004; Gong *et al.* 2006; Fernandez-Garcia *et al.* 2009; Shi *et al.* 2013). There exists variability in the bypass flow and Na^+ uptake in rice roots which can be attributed to the chemical composition of these structural barriers (Yeo and Flowers, 1983 ; Cruz *et al.* 1992; Stasovsky and Perterson, 1993; Schreiber *et al.* 2005).

Besides, the continuity of such barriers to bypass flow may be interrupted during the emergence of lateral roots which originates from the pericycle of seminal and crown roots and protrudes through the cortex before appearing on the surface and provide increased absorptive area along with anchorage of the plant in the soil (Banoc *et al.* 2000; Ma *et al.* 2001; Bailey *et al.* 2002; Want *et al.* 2006). This causes the leakage of solutes into the main root (Hao & Ichii 1999; Ranathunge *et al.* 2005a, b) and is thought to contribute to the major share of the salt (Na^+) uptake from external solution (Hasegawa 2013; Yamaguchi *et al.* 2013; Wang *et al.* 2006). The existence of this phenomenon is, however, debated with skepticism by Krishnamurthy *et al.* 2011 and Faiyue *et al.* 2010a. Moreover, it is also suggested that bypass flow can occur in the lateral roots themselves along with its original location in the area of lateral roots emergence (North & Nobel 1996; Enstone & Peterson 1998; Soukup, Votrubova & Cizkova 2002).

The relative contribution of symplastic and apoplastic pathways to the net Na^+ influx is, however, yet to be well established (Amtmann and Sanders 1998; Kronzucker and Britto 2011; Hasegawa 2013), which is believe to depend on plant species (Essah 2003). In Arabidopsis, the contribution of the bypass flow is believed to be insignificant (Essah *et al.* 2003) which is significant in case of rice (Yeo *et al.* 1999; Shi *et al.* 2013). Although it is

generally established that the passive flow accounts for only a small percentage (usually 1-5%) of the transpirational flow (Yeo *et al.* 1987), it can be vital in transporting ions at high external concentrations and at high transpiration rates (Yeo 1992; Garcia *et al.* 1997; Anil *et al.* 2005; Flowers & Flowers 2005). Thus, membrane transport of root epidermal and cortex cells are the decisive tools for the uptake or rejection of toxic ions like Na^+ from the environment.

Afterwards, Na^+ enters into the xylem through xylem loading and follows the long distance xylem transport. The loading and transfer of Na^+ into the xylem is important to achieve salt tolerance as it is a crucial step in controlling the distribution of excess Na^+ within the plant, the mechanisms are, however, yet to be fully understood (Craig Plett and Møller 2010). This xylem loading of Na^+ is believed to be passive under high salinity and active under mild salinity (Shi *et al.* 2002; Apse and Blumwald 2007; Craig Plett and Moller 2010).

Finally, Na^+ reaches to all cells including metabolically active mesophyll cells following xylem unloading. For nutrients, leaf mesophyll cells exert another membrane transport to enter into the other long-distance pathway in plants, the phloem (Figure 1.4) and thus, Na^+ may also be recirculated in different cells and/or tissues (Sondergaard, Schulz & Palmgren, 2004). Recirculation of Na^+ from to the roots through phloem was initially believed to be negligible (Tester & Devenport, 2003), but recent studies in maize (Qing *et al.* 2009), sweet pepper (Blom-Zandstra *et al.* 1998) and lupin (Munss *et al.* 1988) suggested the possibility of significant recirculation (Craig Plett and Møller 2010). This recirculation is an important strategy adopted by plants to tolerate excess salts as is observed in *Lycopersicon* (Perez-Alfocea *et al.* 2000) and *Phragmites* (Matsushita & Matoh 1991). The Na^+ was found to be

accumulated at a high concentration in older leaves of a number of monocot and dicot species (Jeschke, Pate & Atkins 1987; Gorham 1990; Ashraf & O'Leary 1994; Ghanem *et al.* 2009) which plant tend to shed as an adaptive strategy to get rid of excess Na^+ (Yeo & Flowers 1982). This adaptive strategy also exists in rice as Yeo & Flowers (1982) observed that the accumulation of Na^+ in younger leaves of rice was not due to dilution of Na^+ by rapid growth.

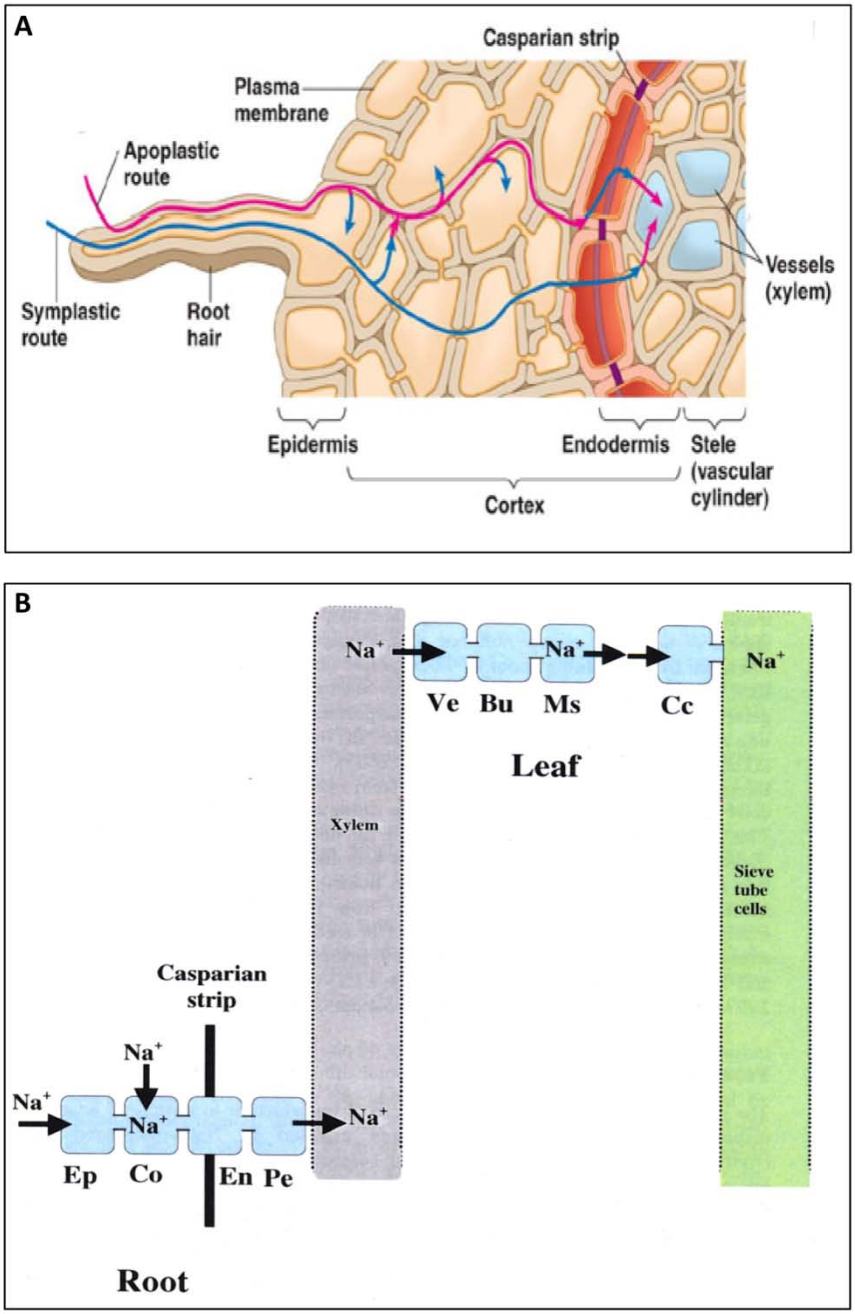


Figure 1.3 Diagrammatic representation of the apoplastic and symplastic pathways of nutrient & water movement from external solution to stele of plants (A; source: <www.bio.miami.edu/dana/226/226F09_10.htm) and the route of Na^+ transport root to leaf; Bu- bundle sheath cell, Co- cortex, Cc- companion cell, Ep- epidermis, En- endodermis, Ms- mesophyll cell, Pe- pericycle, Ve- vessel-associated cell (B; adapted from Sondergaard, Schulz &Palmgren, 2004).

1.5 Mechanism of salinity tolerance

Salinity tolerance depends on a range of anatomical, physiological, biochemical and molecular adaptations of the plant to survive under the stress. Salinity has different effects on plants and thus, there are many mechanisms to tolerate it as well which can be grouped into three main categories namely, osmotic tolerance, ion exclusion and tissue tolerance (Flowers *et al.* 1977; Tuteja 2007; Munns and Tester 2008; Deinlein *et al.* 2014; Roy *et al.* 2014).

Osmotic tolerance mechanisms includes the first adaptive responses of plants immediately after sensing the rise in salt stress outside the roots (Apse and Blumwald, 2002; Munns *et al.* 2006; Flowers and Colmer 2008; Munns and Tester 2008). Very little is known about osmotic tolerance mechanism which is believed to be regulated by rapid, long-distance signalling processes such as ROS waves, long distance electrical signalling, Ca^{2+} signalling etc. that reduce shoot growth and is triggered before shoot Na^{+} accumulation (Munns & Tester 2008; Maischak *et al.* 2010; Mittler *et al.* 2011; Hasegawa 2013). The differences in the initial perception of stress, in the long distance signalling processes and in the responses to the signals cause the differences in osmotic tolerance (Roy *et al.* 2014). The signalling cascade that turns on after salt stress imposition is the current focus of the scientific community. At this point, however, it is believed that the signal of stress is first perceived at membranes by receptors like Ca^{2+} ions, inositol polyphosphates, cyclic nucleotide monophosphates, nitric oxide, G-protein-coupled receptors, kinases, or histidine kinase and other small molecules etc. followed by transducing it to the nucleus which induces or represses some stress

responsive genes leading ultimately to plant adaptation to stress tolerance (Tuteja 2007; Reddy *et al.* 2011).

The mechanism of ion exclusion, on the other hand, is well understood as it is relatively easy to phenotype the traits involved in Na^+ and Cl^- transport in the roots. Tolerance to salt through this mechanism is mainly achieved by restricting the accumulation of Na^+ and Cl^- in the leaves below toxic levels which is attributable to the traits such as compartmentation of ions in the vacuoles of cortical cells, retrieval of Na^+ from the xylem and efflux of ions back to the soil (Munns and Tester 2008; Zhang and Shi 2013; Adolf *et al.* 2013; Deinlein *et al.* 2014).

If the ion exclusion mechanisms fail, the concentrations of Na^+ and Cl^- in leaves may rise to a level which is toxic to normal metabolic functioning. Plants can cope with the toxic level of Na^+ and Cl^- in the leaves by exerting the tissue tolerance mechanism which mainly involves compartmentaion of excess ions at cellular and intracellular level (mainly in vacuole). This process requires higher level controls to coordinate transport and biochemical processes which usually involves ion transporters, proton pumps and synthesis of compatible solutes etc. (Roy *et al.* 2014; Ahmad *et al.* 2013; Horie *et al.* 2012; Petronia Carillo 2011; Munns & Tester 2008; Tuteja 2007).

Mechanisms of salt tolerance take place at whole plant, cellular and molecular levels of organisation (Munns *et al.* 2002). At whole plant level, salt tolerance involves the selective uptake of salts by root cells (especially at the epidermis and endodermis layer), loading of the xylem (the cells of the stele can preferentially load K^+ instead of Na^+), xylem unloading (salt is removed to the upper part of roots, stem, and leaf sheaths from the xylem), loading of the phloem (tolerant plants loads less Na^+ and Cl^- in the phloem) and finally the excretion

of excess salt through salt glands or bladders. The prevalence of well-developed mechanisms allow halophytes (plants that grow well in high salinity) to tolerate salts by controlling the uptake and transport of ions and excrete the excess salt. Glycophytes (plants whose growth is affected by salinity), on the other hand are not equipped with such sophisticated anatomical and physiological tolerance mechanisms but still can employ the first three mechanisms to tolerate the stress to a certain degree (Munns *et al.* 2002; Flowers and Colmer 2008). In general, higher ratio of shoot: root and higher growth rates along with the absence of apoplastic bypass are the contributory features that reduces the entry of salt in the transpiration stream and eventually restricts accumulation of salts in leaves (Pitman, 1984 and Garcia *et al.* 1997).

At cellular and organelle level, ion compartmentation (sequestering the ions in the vacuole of the cell to keep them out of cytoplasm) is the main mechanism of salt tolerance. In spite of considerable influx of Na^+, both halophytes and glycophytes can maintain non-toxic level of Na^+ by compartmentalising the excess cytosolic Na^+ (and Cl^-) into the vacuoles. This not only averts the deleterious effects of Na^+ in the cytosol but also allows the plants to use NaCl as an osmoticum, maintaining an osmotic potential that drives water into the cells (Blumworld 2010). The compartmentation of excess cytosolic Na^+ (and Cl^-) into the vacuoles is evident as leaves of some species were found to function normally even at concentrations well over 200 mM, when the enzyme activity was supposed to be suppressed completely (Munns *et al.* 1983). Generally, at 100 mM Na^+ most enzymes are inhibited and similar concentration of Cl^- is also believed to be toxic. Tolerant plants can sequester the excess toxic ions into the vacuole of the cell followed by accumulation of organic solutes such as proline, glycine betaine, sugar alcohols, polyamines and proteins etc. in the cytoplasm to

balance the osmotic pressure (Munns and Tester 2008; Hasegawa *et al.* 2000). The regulatory networks and the metabolic rearrangements that ultimately controls the level of osmolyte are important in conferring salinity tolerance; an area which is yet to be fully understood (Deinlein *et al.* 2014).

At the molecular level, ion transporters (reviewed in next section) play vital roles in regulating ion homeostasis. Na^+ enters into the cell by competing with other cations, mainly K^+ through high affinity K^+ carriers and/or non-selective low affinity cation channels that are strongly influenced by Ca^{2+}(Amtmann and Sanders 1999; Hasegawa 2013; Munns 2002). Na^+ can be excluded out of the cell from the cytoplasm or intracellularly compartmentalized into the vacuole through Na^+/H^+ antiporters which is regulated by pH gradient across the plasmalemma and tonoplast, respectively (Blumwald, 2000). The transporters regulating ion homeostasis in chloroplasts and mitochondria are, however, yet to be known (Hasegawa 2013; Adams and Shin 2014; Maathuis 2014).

Among the mechanisms reviewed above, a particular plant can adapt a combination of mechanisms to tolerate salinity. To date, there is no such evidence that these mechanisms are mutually exclusive (e.g., tolerance to osmotic phase of salt toxicity isn't prevented by ion exclusion) or that plants can only employ one strategy at a time (Roy *et al.* 2014). But depending on the circumstances, it may be possible that some tolerance mechanisms are more effective than others. For example, at moderate salt stress, 'osmotic tolerance' may be more effective compared to Na^+ exclusion which may be more effective at high salt stress (Munns *et al.* 2012). The interactions with other abiotic stresses such as drought or low water availability and high/low temperature etc. can of importance in this regard. The

existence of all these various mechanisms makes salinity tolerance a complex trait which evidently involves many genes making the development of tolerance variety difficult using traditional breeding methods (Parida and Das 2005; Chinnusamy *et al.* 2005; Tuteja 2007; Munns and Tester 2008; Hasegawa 2013; Deinlein *et al.* 2014; Roy *et al.* 2014).

1.6 Transporters involved in Na^+ homeostasis

The molecular physiological and genetic studies over the last decades have increasingly gained knowledge on the membrane proteins such as water channels and ion transporters in regulating ion homeostasis under saline condition (Blumwald 2000; Pardo *et al.* 2006; Munns and Tester 2008; Maurel *et al.* 2008; Ward *et al.* 2009; Horie *et al.* 2012).

Plants have evolved a number of K^+ transporters such as the KT/HAK/KUP transporter family (Gierth and Mäser 2007; Ward *et al.* 2009) as high concentration of K^+ (~10-200 mM) is required for efficient metabolic functioning in the cytosol. Sodium, being physico-chemically similar to potassium, exploits some of these K^+ transporters to enter the cytosol (Maathuis *et al.* 1997; Maser *et al.* 2001, Maser, Gicrth & Schroeder, 2002; Very & Sentenac, 2002, 2003). In many plant species, voltage-independent (or weakly voltage-dependent) non-selective cation channels (VIC/NSCCs) are believed to be the dominant way of toxic Na^+ influx (Kader and Lindberg 2005; Davenport & Tester 2002 & 2000; Demidchik & Tester 2002; Amtmann *et al.* 1997). Besides, the cyclic nucleotide-gated channels and ionotropic glutamate receptor-like channels are also suggested to be involved in Na^+ influx (Demidchik & Tester 2002). The molecular identity of these NSCCs is, however, yet to be known (Senadheera *et al.* 2009; Roy and Chakraborty 2014).

High-affinity potassium transporters (HKTs) were also suggested to have an important role in regulating Na^+-influx in some species (Ali *et al.* 2010; Hauser *et al.* 2010; Horie *et al.* 2010 & 2009; Byrt *et al.* 2007; Davenport *et al.* 2007; Platten *et al.* 2006; Uozumi *et al.* 2000; Horie *et al.* 2001; Golldack *et al.* 2002; Maser *et al.* 2002). In rice, nine *HKT* homologues (*OsHKT* l to 9) have been identified (Garcia *et al.* 2003). The *HKT* gene family in rice is thus suggested to play a vital role in ion homeostasis despite some of the members being shown to transport Na^+.

Among the transporters of *HKT* group, *HKT1* is believed to have the greatest potentiality in improving salt tolerance, because it frequently appeared as the most likely candidate while studying the QTLs for Na^+ exclusion (Mian *et al.* 2011; James *et al.* 2006; Ren *et al.* 2005). In durum wheat, the salinity tolerance was improved by successfully incorporating novel *HKT* alleles from Triticum monococcum using marker assisted selection approach (James *et al.* 2012; Munns *et al.* 2012). Besides, *HKT2* was also reported to increase level of tolerance to salt, which however, didn't use the Na^+ exclusion technique (James *et al.* 2012).

In rice, Horie *et al.* (2001) suggested that *OsHKT1* and *OsHKT2* encode a Na^+ and a Na^+/K^+-coupled transporter, respectively, while Garciadeblas *et al.* (2003) showed *OsHKT4* as a low affinity Na^+-transporter. *OsHKT5* another Na^+ transporter is believed to increase salt tolerance by maintaining shoot K^+ homeostasis under salt stress (Ren *et al.* 2005; Rus, Bressan & Hasegawa, 2005). However, later it was observed that the *AtHKT1;1*-mediated Na^+ re-absorption in *Arabidopsis* is actually similar to that of *OsHKT1;5* mediated salt tolerance in rice (Hauser and Horie 2010; Horie *et al.* 2009).

Besides, salt overly sensitive (SOS) pathway was also found to play a vital role in regulating Na^+ transport in plants (Qiu *et al.* 2002; Mahajan *et al.* 2008; Yang *et al.* 2009; Ji *et al.* 2009). In Arabidopsis, three independent SOS mutant loci were identified (Zhu *et al.* 1998) and the Na^+/H^+ antiporter (*NHX*) that sequesters the Na^+ in the vacuole was found to be encoded by SOS1 gene (Apse *et al.* 1999; Shi *et al.* 2000; Zhang and Blumwald 2001; Leidi *et al.* 2010; Barragan *et al.* 2012). Other transport proteins involved in Na^+ homeostasis are inward low-affinity cation transporters and rectifying potassium channels (Schachtman *et al.* 1997; Amtmann & Sanders, 1999; Golldack *et al.* 2003).

Altered accumulation of shoot Na^+ has already been achieved by manipulating the expression of these genes which suggests that these genes are to be targeted for improving salinity tolerance in crops (Roy *et al.* 2014). Even though the success is limited (as can be measured by the apparent lack of successful commercial varieties), better understanding of the cell type specific expression under stress may hold the clue for potential improvement in developing successful salt tolerant crop varieties (Møller *et al.* 2009).

1.7 Crop species with varying degrees of salt tolerance

Salinity is considered as a powerful force that leads to adaptation and speciation (Edelist *et al.* 2009, Lowry *et al.* 2009 and Flowers *et al.* 2010). Depending on the ability to grow in saline environments plants can be grouped as halophytes and glycophytes. Halophytes are native to saline environments and are able to complete life cycle in a range of saline conditions starting from 200mM to seawater concentration (Flowers *et al.* 1986; Colmer *et al.* 2006; Munns and Tester, 2008; Flowers *et al.* 2010). Notable examples of halophytes are saltbushes (*Atriplex* spp.), samphires (*Halosarcia* spp.), cordgrasses (*Spartina alterniflora*, *S.*

patens), saltgrass (*Distichlis spicata*), alkali grass (*Puccinellia phryganodes*) and shoregrass (*Monanthochloe littoralis*) etc. Glycophytes or non-halophytes, on the other hand, are relatively salt intolerant whose biomass accumulation and growth can be greatly inhibited under high salt concentration and many can't survive at a concentration of 200mM NaCl (Greenway and Munns, 1980; Flowers and Colmer, 2008; Munns and Tester, 2008). *Arabidopsis thaliana* is often considered as the model glycophytic plant to study salinity tolerance as many physiological mechanisms of salt tolerance of halophytes were found in this species (Hasegawa *et al.* 2013 & 2000; Zhu 2002, 2003; Flowers and Colmer 2008; Munns and Tester 2008). Most of the agricultural crops are glycophytes, although some of them like sugar beet, barley, wheat, canola, cotton, soybean and olives etc. are considered as salt tolerant while tomato, carrots, potato and cabbage are considered as moderately tolerant crops. Salinity is the major nutritional constraint on growth of wetland rice (Yeo and Flowers, 1986; Asch *et al.* 2000; Lin and Kao, 2001) and is considered to be the most sensitive plants to salinity with the critical tolerance level of 40 mM NaCl (Glenn *et al.* 1997).

1.8 Rice genetics: Natural allelic variation and QTLs for salinity tolerance

There exists considerable natural allelic variation among rice germplasm for salinity tolerance that have been exploited to reach to QTLs responsible for salinity tolerance (Lynch and Walsh 1997; Kearsey and Pooni 1998; Collard *et al*. 2005; Yu *et al*. 2012; Ashraf and Foolad 2013; Shahbaz and Ashraf 2013). The natural variation of a species represents the entire recombination events that have taken place over the historic lifetime of that species which resulted in genome wide polymorphisms that facilitates the fine mapping of the QTLs and Genome Wide Association Studies (GWAS) for a particular trait (Mackay *et al*. 2009; Hall

et al. 2010; Assmann 2013). Over the last decades, a number of QTLs were identified in numerous populations of various crop species for various abiotic stresses such as salinity tolerance (Koyama *et al*. 2001; Lindsay *et al*. 2004; Lin *et al*. 2004; Ren *et al*. 2005; Byrt *et al*. 2007; Genc *et al*. 2010; Xue *et al*. 2009; Thomson *et al*. 2010; Ul Haq *et al*. 2010); drought resistance (Price *et al*. 2002b; Price *et al*. 2002a; Quarrie *et al*. 2006; Mathews *et al*. 2008; Von *et al*. 2008; Peleg *et al*. 2009; Chen *et al*. 2010); heat resistance (Yang *et al*. 2002; Mohammadi *et al*. 2008; Mason *et al*. 2010) and cold tolerance (Andaya *et al*. 2006; Baga *et al*. 2007; Kurok *et al*. 2007; Lou *et al*. 2007) etc. A more comprehensive coverage on the abiotic stress related QTLs in crop species can be found in the reviews of Flowers *et al*. 2000; Asins 2002; Langridge *et al*. 2006; Price 2006; Collins *et al*. 2008; Alonso-Blanco *et al*. 2009; Fleury *et al*. 2010; Genc *et al*. 2010; Assmann 2013; Ashraf and Foolad 2013; Bansal *et al*. 2014. The accuracy of QTLs is already well established (Price 2006) and has lead to the identification of a number of candidate genes (Hao and Lin 2010; Roy *et al*. 2011a; Negrão *et al*. 2011; Ashraf and Foolad 2013; Bansal *et al*. 2014).

Salinity tolerance is a cumulative effect of a number of component physiological traits such as Na^+ and K^+ concentrations in root and shoot; xylem loading of Na Na^+, retrieval of Na^+ from shoot, Na^+ exclusion, ion balance, vacuolar sequestration and tissue tolerance etc. Attempts have been made to identify QTLs linked with these traits. For Na^+ or K^+ concentration in shoot and root, Lin *et al*. (2004) eight identified 8 QTLs from F2 and F3 populations from a cross between Nonabokra (highly tolerant *Indica* variety) and Koshihikari (susceptible *Japonica* variety). One of these is QTL *SKC1*, located on chromosome 1 which accounts for 40.1% of phenotypic variance for shoot K^+ concentration as revealed by fine mapping approach using fixed recombinant progeny testing (Ren *et al*. 2005; Jena and

Mackill, 2008). The *SKC1* QTL encodes an HKT family ionic antiporter *OsHKT1;5* in rice that mediates Na^+ reabsorption (Gao and Lin 2005; Rus *et al.* 2006). A similar antiporter, *AtHKT1* was found in Arabidopsis that reduces the concentration of Na^+ in the shoot by transferring excess Na^+ to sieve tubes of the phloem (Sunarpi *et al.* 2005; Horie *et al.* 2009; Hauser and Horie 2010). The ortholog of the *OsHKT1;5* genes in bread wheat is *TaHKT1;5-D* (Byrt *et al.* 2007), candidate for a *Kna1* gene on chromosome arm 4DL and *TmHKT1;5-A* (HKT8), candidate for a *Nax2* on chromosome arm 5AL and *TmHKT7-A2*, candidate for *Nax1* on chromosome arm 2AL (Huang *et al.* 2006). Genome wide association studies in Arabidopsis also confirmed that *HKT1* is associated with shoot Na^+ content and thereby with enhanced tolerance to salt stress (Baxter *et al*. 2010; Atwell *et al*. 2010). Elevated level of tolerance was observed by incorporating vacuolar Na^+/H^+ antiporter genes into rice varieties. For example, Na^+/H^+ antiporter gene *AgNHX1* from *Atriplex gmelini* increased seedling survival (Ohta *et al.* 2002), *PgNHX1* from *Pennisetum glaucum* (L.) enhanced shoot and root lengths (Verma *et al.* 2007) and Na^+/H^+ antiporter *SOD2* gene from yeast lowered Na^+ uptake in the shoots of rice cultivars (Zhao *et al.* 2006).

Other major QTLs identified in rice are *Saltol* (chromosome 1) for ion uptake in salt tolerant cultivar Pokkali that accounted for 64-80% of the phenotypic variation under salt stress and has been reported in other rice varieties as well (Bonilla *et al*. 2002, Gregorio *et al*. 2002; Takehisa *et al.* 2004), *QNa* (chromosome 1) for Na^+ uptake (Flowers *et al*. 2000), *QNa:K* (chromosome 4) for Na^+/K^+ ratio (Singh *et al*. 2001) etc.

For root Na^+/K^+ ratio, Ming-zhe *et al*. (2005) identified two QTLs on chromosomes 2 and 6, Sabouri and Sabouri (2008) identified several QTLs on all but chromosome 9 in rice. For root

and shoot Na^+, K^+ and K^+/Na^+ ratio, Yao *et al*. (2006) identified two QTLs and Ahmadi and Fotokian (2011) detected 14 QTLs on different rice chromosomes; of which the QTL *QKr1.2* for root K^+ content seemed to be most promising as it accounted for 30% variation. Furthermore, Lang *et al*. (2001) identified four QTLs for tissue Na^+/K^+ ratio and one QTL each for Na^+ and K^+ uptake on different chromosomes of rice and Sabouri and Sabouri (2008) identified three QTLs for ion exchange on chromosomes 3 and 10. Of the 13 QTLs identified by Wang *et al*. (2007) on chomomosomes 1, 2, 5, 6, 7 and 12, the QTL *qSC1b* accounted for 45% of the total phenotypic variability. On chromosome 4, Koyama *et al*. (2001) detected 10 QTLs and Lin *et al*. (2004) identified 3 QTLs for seedling survival days under elevated salt stress.

QTL associated with salinity tolerance have been identified in other crop species as well. For example, Xue *et al*. (2010) identified a number QTLs for various traits such as shoot Na^+, K^+ and Na^+/K^+ ratio etc. Lexer *et al*. (2003) detected 10 QTLS for ion uptake and later several candidate genes were found to be linked with those QTLs as revealed by studies based on EST and SNP mapping strategy (Lexer *et al*. 2004, Lai *et al*. 2005). For example, the Ca^{2+} and K^+ transporters genes were co-localized with the QTL for survival and ion uptake. QTL studies with hexaploid bread wheat (Triticum aestivum) identified the locus *Kna1* to control root to shoot transport of Na^+ and K^+ in a way to maintain high K^+/Na^+ ratio in shoot (Dubcovsky *et al*. 1996; Luo *et al*. 1996). In durum wheat (Triticum turgidum L.), however, Na^+ exclusion was reported to be linked to *Nax1* (a locus that promotes Na retention in leaf sheath) and *Nax2* loci, that harbors the Na+ transporters *HKT7* and *HKT8*, respectively (Platt en *et al*. 2006; James *et al*. 2006; Rodrıguez-Navarro and Rubio, 2006; Huang *et al*. 2006, 2008; Byrt *et al*. 2007).

The reactive oxygen species (ROS) such as H_2O_2, superoxide (O_2^-) and hydroxyl radical (^-OH) that are produced under stressed condition can impair normal metabolic functioning (Mittler 2002; Mittler *et al*. 2011). Plants can detoxify these ROS by producing different enzymatic and non-enzymatic antioxidants, a phenomenon that is believed to be associated with salinity tolerance mechanism (Shalata *et al*. 2001; Zhu *et al*. 2005; Zhou *et al*. 2011). Frary *et al*. (2010) identified a number of stress inducible QTLs for the production of various antioxidants in *Solanum pennelli* which may hold value for improving salinity tolerance in crops. Similarly QTLs for the production of various osmo-protectants such as proline were identified in chromosome 2 & 4 of barley under salt stress (Siahsar and Narouei 2010). The transfer of *S. pennellii* early responsive-to- dehydration gene (*SpERD15*) to tobacco (Ziaf *et al*. 2011) and bacterial mannitol 1-phosphate dehydrogenase (*mtlD*) gene to *Solanum tuberosum* L. (Rahnama *et al*. 2011) showed increased production of osmo-protectants and enhanced salinity tolerance. The QTLs can be growth stage and stress condition dependant (as reviewed by Ming-zhe *et al*. 2005; Collins *et al*. 2008; Roy *et al*. 2011; Negrão *et al*. 2011; Assmann 2013; Ashraf and Foolad 2013) which *Indica*tes the complexity of the genetics of salinity tolerance across plant species.

Identification of QTLs and corresponding tightly linked markers along with efficient breeding and biotechnological tools are crucial for success in developing abiotic stress tolerant varieties. Identifying the alleles that expresses differentially in stressed condition is a challenge as abiotic stress is complex and polygenic in nature. Recent advances in genome sequencing has led to the development of several high-throughput technologies such as single nucleotide polymorphisms (SNPs), differential display of reverse transcriptase (DDRT), suppression subtractive hybridization (SSH), serial analysis of gene expression (SAGE),

massive parallel sequence signature (MPSS) and microarray that offers powerful alternatives to identify the candidate genes for various stress conditions.

1.9 Transcriptomics: The high throughput study of whole genome expression

A transcriptome is the complete set of transcripts representing all messenger RNA molecules in a given cell at a particular time point. Transcriptomics, also termed as expression profiling, generally involves a systematic and comprehensive study of all the RNA transcripts that captures gene expression (spatial and temporal expression) of a cell, tissue of an organism under a given biological context (Thompson and Goggin 2006; Duque, Almeida *et al*. 2013). Microarrays, also commonly known as DNA chip, gene chip, or biochip is a high-throughput collection of microscopic spots containing short oligonucleotides (probes) that represent a segment of genes attached to a solid surface. Microarrays quickly became the standard tool in molecular biology, providing a powerful approach for the analysis of genome wide transcriptional response by studying the expression of all the expressed genes in a single experiment.

1.9.1 Brief history of microarrays

Over the past decade, microarrays have been widely used across biological disciplines (Yauk and Berndt 2007). The concept was inspired by the Southern blotting technique where a DNA fragment is attached to a substrate which is then probed with a known gene (Maskos and Southern 1992).

The inherent phenomena of mRNA strand to bind to its complementary DNA sequence is the basic principle of a cDNA microarray. Augenlicht and Kobrin (1982) first used the technique to study the expression of genes in tumour and normal tissue by 378 arrayed lysed bacterial

colonies each with a unique sequence. The major expansion came when more than 4000 human sequences were quantitatively analysed using digital scanning and image processing. Then Kulesh *et al.* (1987) described the use of a collection of distinct DNAs by spotting the cDNAs onto filter paper as arrays for expression profiling. Schena *et al.* (1995) used the miniaturized microarrays for the first time followed by Lashkari, DeRisi *et al.* (1997) who studied the gene expression pattern of the complete genome of *Saccharomyces cerevisiae* on a microarray. The technique is now routinely used for human, microorganisms, animals and plants. Serial analysis of gene expression (SAGE), representation different analysis (RDA) and massively parallel sequence signature (MPSS) are the other methods or technologies that can also be used in transcriptomics analysis.

1.9.2 The underlying principles of microarray

The inherent ability of a single stranded DNA molecule to bind to its complementary strand is the underlying principle of microarray technology. Microarrays are essentially microscope slides (platforms) built by depositing thousands of known DNA fragments (usually of short oligonucleotides) each corresponding to a specific gene at predefined positions (Dunwell, Moya-Leon *et al*. 2001). Gene expression profiling with microarrays involves isolating mRNA from the tissues of interest and converting into a single stranded cDNA with simultenous incorporation of fluorescent labels commonly known as targets. These fluorescently labelled target sequences are then hybridized with the probes deposited on the microarray slides under controlled conditions followed by washing off of weakly bonded sequences that leaves only the strongly bonded strands to remain hybridized.

The target sequences that are now fluorescently labelled bind to a pre-set probe generating a signal whose strength depend on the conditions of hybridization, the number of paired bases of target-probe hybridization and the washing after hybridization. The levels of expression at this stage are detected and quantified by scanning the level of fluorescence of all the spots on the array. Previously, northern blot or reverse transcription polymerase chain reaction (RT-PCR) were used to measure the levels of RNA but the technology was limited to analyze only a few genes at one time (Mitchell, 2008). The microarrays, on the other hand, allow the detection of thousands of genes simultaneously essentially covering the whole genome of a particular species in one single run which makes it a powerful approach and increasingly important in many genomic studies.

1.9.3 The techniques involved in microarray gene expression analysis

Microarrays are manufactured in different ways based on whether the probes are spatially arranged on a silicon chip (e.g., Affymetrix chip), on glass slide (e.g., Agilent slide) or on microscopic beads (e.g., Illumina bead array). The probes used on the microarray platform can be of different types, the cDNA or oligonucleotide arrays being the most common. The PCR products of 500 to 5,000 bp generated from clones of cDNA libraries were the first probes used on a cDNA microarray. The technique, however, was labour intensive and prone to errors as its construction involved many steps including the generation of cDNA libraries, culturing thousands of clones, amplifying these clones and spotting them on a suitable surface (Suhaimi, 2009). The limitations were overcome with the oligonucleotide arrays where probes of shorter target sequence (25 to 60 bp) were synthetically produced on the slides, based on the availability of sequence information in genome databases. This technique is commonly preferred for commercial production as it provides better capacity,

accuracy and reproducibility than cDNA arrays (Chu, Fink *et al*. 2007). The number of probes in an array can be vary extensively (10 probes to up to 2 million micrometre-scale probes) and the length of probe sequences can be shorter (25-mer probes used by Affymetrix) or longer (60-mer probes produced by Agilent). More number of shorter probes can be densely deposited on a single array (cheaper option) whereas longer probes provide more specificity to the target genes. Microarrays can be manufactured by photolithography, printing with fine-pointed pins or ink-jet printing onto glass slides, or using electrochemistry on microelectrode arrays.

Microarray experiments can be conducted by using either one-colour/dye/channel or two-colour/dye/channel techniques depending on the specific applications and needs of the researcher. Cyanine-3 (Cy3) and cyanine-5 (Cy5) having a fluorescence emission wavelength of 570 nm (green) and 670 nm (red), respectively are the most common fluorescent dyes. In two- dye procedure as shown in Figure 1.4, the two samples in question (control and treated or samples from two cell types) are labelled with two fluorescent-dye, hybridised together on a single microarray and scanned to visualise the intensity of the fluorescence. The intensity of a particular target is the cumulative result of the mixture of green and red dye which is then used to identify differentially up and down-regulated probes based on ratio-based analysis. The advantage of this approach is that half as many arrays are required for each experiment compared to one-colour approach. Eppendorf's Dual Chip platform and Agilent's two colour platforms are the example of two-bye based approach. Unlike the two-colour technique, both the control and treated samples are labelled with the same fluorescent dye (Cy3 or Cy5) in the one-colour procedure. The differentially regulated probes are then identified by comparing the intensities of both samples. Gene Chip (Affymetrix),

Bead Chip (Illumina) and Agilent single-channel arrays are popular among the commercially available one-dye based techniques. The relative advantages of one-colour arrays are that the data produced are the absolute intensity values facilitating comparisons across microarrays of other experiments and that one faulty sample can't affect the raw data from another sample and also the experimental design becomes simple and flexible when a large number of samples involved. However, it has been shown that the choice between these two techniques is not critical in influencing the end results as both the techniques provide approximately equivalent levels of biological insight (Patterson, Lobenhofer *et al*. 2006; Paul and Amundson 2008). The choice of platforms depend on probe design, probe content, specificity, sensitivity and reproducibility of the labelling and hybridizing protocols along with the cost involved, time required and ease in data acquisition. Considering the relative advantages of different platforms and techniques, Agilent oligonucleotide arrays with one-colour experimental design has been for opted for this project.

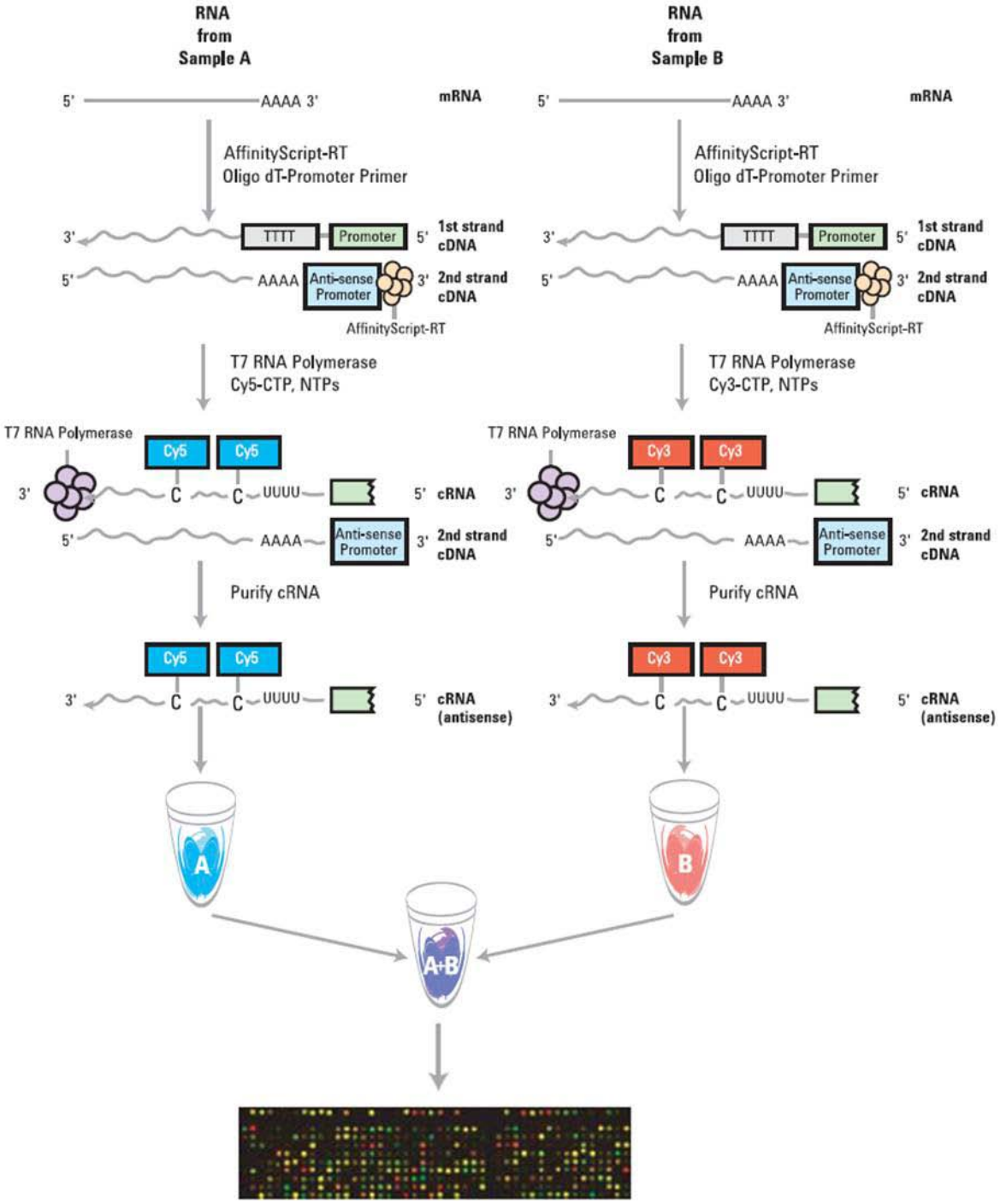


Figure 1.4 Schematic of a two-colour/channel microarray experimental workflow; taken from Agilent's 'Low Input Quick Amp Labeling Protocol for One-Color Microarray-Based Gene Expression Analysis'. Generation of cRNA for a two-colour microarray experiment is shown, for a one-colour microarray experiment, only the Cy3-labeled "B" sample is produced and hybridized.

Over the past decade, the technology has gone through rapid improvement in many aspects, from the vast range of microarray equipment, chemicals and reagents, measurements (statistical analysis software) to bioinformatic analysis. As an example, the increase in the numbers of probes (from 22K to 44k and very recently 60K) printed on the oligunucleotide array produced by the Agilent Technology company can be mentioned.

The rapid progress gained in the field of bioinformatics due to enriched genomic information including genome sequences, gene annotations and functions allowed the development of a number of computational tools facilitating the use of complex statistical models, different types of normalization, and Bayesian statistics besides the initial examination of two fold differences in expression has improved the efficacy of microarray application to gain clearer biological insights (Edwards and Batley 2004).

1.9.4 The application of microarrays

The ever-increasing rate of genome sequencing immediately raises the massive task of identifying the functional roles of genes along with the interacting networks and the cellular processes in which they are involved. DNA is the carrier of genetic information and RNA acts as a messenger (mRNA), passing the actual information from the nucleus into the cytoplasm of the cell where protein is synthesised (Morange, 2009). This process can be directly associated with gene regulation and measured by capturing the entire transcript level at a given condition (Yang and Speed 2002). Microarrays has got the relative advantage of revealing novel genes for biotic and abiotic stress resistance and/or genes of interests to evolutionary biologists and ecologists, since it can investigate thousands of probes in a single

experiment. An elevated (up-regulated) or repressed (down-regulated) expression of a particular mRNA will *Indica*te whether the corresponding gene is active or passive (non-active) under the conditions tested. The actual biological processes, however, are much more complex as biological processes are usually polygenic. However, by looking at the up or down-regulated gene sets from a bio-informatical point of view, we can detect the biological processes along with the genes and pathways responsible that are turned 'on' or 'off' which can enhance our biological understanding at a given point. The microarray can also be used to compare the gene expression patterns between different tissues of an organism, in the same tissue on different organisms or the same tissue but under different conditions which will further enhance our biological understanding. In addition, genome-wide polymorphism can also be surveyed by using this technology which will allow the identification of mutations (Jung, An *et al*. 2008). This technology is thus being extensively used to address a wide range of biological questions in medical and biological research (Yang and Speed 2002).

In addition, the microarrays have gained popularity in evolutionary studies of stress and can be used to detect DNA or RNA that may or may not be translated into proteins, to genotype or re-sequence mutant genomes, to detect gene duplication (Riehle, Bennett *et al*. 2001), sentinel species detection (Letowski, Brousseau *et al*. 2003), fine mapping of genes, polymorphism detection and gene association (Borevitz and Chory 2004). With the high sensitivity, specificity, throughput and cost-efficiency and as the accuracy of microarrays, in describing the transcriptome, is confirmed by other widely accepted assays such as qRT-PCR, microarrays have become the predominant platform for molecular profiling (Deyholos 2010).

1.9.5 Rice genome and platform options for microarrays

With the rapid improvement in the sequencing technologies, the sequencing of more and more crop plant genomes is being completed (Mardis 2008; Lister, Gregory *et al*. 2009). Being a model for monocots and having a small genome size among cereals, rice genome was the first cereal that was completely sequenced (Jiang and Ramachandran 2010). Over 95% of the *Japonica* genome (372.1 Mb) was covered by the map-based rice genome sequence assembly (Matsumoto, Wu *et al*. 2005) and about 56,278 genes (loci) were listed in the Institute for Genomic Research (TIGR) which is released as Rice Genome Annotation database (release 5) in 2007 (Ouyang, Zhu *et al*. 2007). Excluding the transposable elements and alternate splicing sequences, the total non-TE-related genes in rice slightly over forty one thousands (Jung, An *et al*. 2008). These resources along with the information of the Rice Annotation Project database, RAP-DB linked with the European Molecular Biology Laboratory (EMBL), the DNA Data database of Japan (DDBJ) and the NCBI map viewer (Ohyanagi, Tanaka *et al*. 2006). Despite using different criteria and different pseudomolecules to predict genes, there is around 33,315 common genes (loci) that share equivalent loci in TIGR/ MSU and RAP-DB (Wu and Watanabe 2005; Jiang 2010).

Microarray platforms have been developed for many species ranging from microorganisms, plants, animals to human species. For the two subspecies of rice namely *Japonica* and *Indica* several microarray platforms have been developed. Based on draft *Indica* and *Japonica* sequences, Beijing Genomics Institute (BGI) designed the *Oryza sativa* Genome Oligo Set (Version 1.0; 61K). The NSF (National Science Foundation) Rice 45k (45,116) Oligonucleotide Array based on 61,419 gene model predictions from TIGR's osa1 version 3.0 release was designed by The University of California, Davis, USA. Affymetrix designed the GeneChip rice

genome array containing approximately 48,564 transcripts and 1,260 transcripts from the *Japonica* and *Indica* cultivars, respectively. The rice 44K microarray designed by Agilent based on the manually curated annotation from the Rice Annotation Project Database (RAP-DB) consisted of 60-mer oligo-sequences corresponding to RAP loci with transcripts based on 32,325 rice full-length cDNA (representative cDNA), 6,943 EST support, as well as 2,612 *ab initio* gene prediction. There is also provision for custom array designing where researchers can design their own array based on their specific interest. New tools and databases are continually being developed to facilitate the direct comparison between the datasets generated by different platforms. There is limitation in identifying the alternatively spliced transcripts as these are present in eukaryotic genome in large numbers. The unique example of this is the rice gene locus Os03g47610 having eight alternatively spliced transcripts, all encoding a putative thiamine biosynthesis protein. The manufacturer, however, are developing new methods to overcome this limitation. The NSF45k array includes 6,544 oligos corresponding to 15,003 transcripts; the BGI array incorporated 8,320 oligos representing 19,815 transcripts and the Affymetrix Gene Chip designed 9,550 probes representing 19,660 transcripts (Jung, An *et al*. 2008). The Agilent 44k array, which is used in this study, includes 12,544 oligos that were designed computationally to match 17,447 multiple or alternatively spliced transcripts.

1.10 Transcriptomics and abiotic stress resistance gene discovery

Abiotic stresses such as drought, high temperature, cold, submergence and high soil salinity are environmental threats that reduce plant growth and yield. Plants respond to adapt in order to survive upon exposure to these stresses and the adaptive mechanisms can start by

activating or de-activating a series of genes immediately after they sense the stress. The capturing of the whole transcriptome response is a powerful and sensitive detector of stress and screening the complete transcriptome at a given time point allow us to detect any stress-inducible genes which can suggest the specific biological processes and/or the regulation of transcriptional and translational machineries that are induced (Gracey and Cossins 2003).

The application of high throughput genomics-type technologies has greatly enhanced our understanding of plant responses to external factors (Feder and Walser 2005) and revolutionized genome-wide profiling of gene expression by allowing it to be studied in a single experiment (Jiao, Jia *et al*. 2005). In plants, such as *Arabidopsis,* rice, wheat, maize, sorghum, soybean, tomato, petunia, strawberry, ice plants and lima bean, EST based cDNA arrays and oligonucleotide microarrays have been used to understand the underlying biological meaning by studying and comparing the global gene expression patterns (Sreenivasulu, Sopory *et al*. 2007; Oktem, Eyidogan *et al*. 2008). In the recent past, stress-inducible transcripts was identified array based technologies in different plant species, for example *Arabidopsis* (Liang, Zhang *et al*. 2011; Mao, Zhang *et al*. 2012; Proietti, Bertini *et al*. 2013; Rasmussen, Barah *et al*. 2013) and rice (Kawasaki, Borchert *et al*. 2001; Rabbani, Maruyama *et al*. 2003; Ueda, Kathiresan *et al*. 2004; Shiozaki, Yamada *et al*. 2005; Walia, Wilson *et al*. 2007; Kumari, Sabharwal *et al*. 2009; Senadheera, Singh *et al*. 2009; Walia, Wilson *et al*. 2009; Cotsaftis, Plett *et al*. 2011; Lisa, Elias *et al*. 2011).

Bohnert *et al* (2001) identified elevated level of transcripts related to defence, transport, cell rescue and metabolism from the analysis of ESTs generated from cDNA libraries of salinity

stressed rice. Shiozaki *et al* (2005) identified 384 salt stress-inducible ESTs and half of those were involved in the biological processes like stress response, detoxification, growth and development. In an experiment with *Arabidopsis* cDNA microarrays to profile gene expression under cold, drought and high-salinity stress conditions, 53, 277 and 194 genes from 7000 cDNAs were observed to be induced, respectively after cold, drought and high-salinity treatments (Seki, Narusaka *et al*. 2002). Houde *et al* (2006) reported a number of unique sequences enriched in stress-regulated genes, such as those coding for transport, signalling cascades, cryo-protection and transcription factors in a large-scale EST experiment with wheat. In addition, 14 unique ESTs were identified as up-regulated in foxtail millet Sreenivasulu *et al.* (2004) under salt stress and a large number of ESTs related to abiotic stress have been identified in rice (Babu, Sekhar *et al*. 2002; Sahi, Agarwal *et al*. 2003; Sahi, Singh *et al*. 2006). In *Populus euphratica* tree that can tolerant upto 450 mM NaCl, transcripts were identified that were involved in ionic and osmotic homeostasis, elements such as a syntaxin-like protein, magnesium transporter-like protein, metabolism regulators like cytochrome P450, cleavage factor and amino transferase, zinc finger protein, seed imbibition protein and plasma membrane intrinsic protein, photorespiration-related glycolate oxidase and the photosynthesis-activating enzyme Rubisco activase (Gu, Fonseca *et al*. 2004). A few microarray experiments have been reported with rice involving different abiotic stresses (Walia, Wilson *et al*. 2005; Walia, Wilson *et al*. 2007; Senadheera and Maathuis 2009; Diédhiou 2010; Li, Liu *et al*. 2010; Cotsaftis, Plett *et al*. 2011; Ding, Chen *et al*. 2011). The study conducted by Kawasaki *et al.* (2001) was one of the pioneer experiments in rice under salt stress and identified many genes encoding water channel protein isoforms,

subtilisin inhibitor, ABA and stress-induced proteins, glutathione S-transferase, glycine-rich proteins, metallothionein-like proteins, ascorbate peroxidase, tyrosine inhibitor etc.

Transcription factors (TFs) that regulate plants' adaptive responses to stresses are important genes for survival (Oktem, Eyidogan *et al*. 2008) and transcriptomics analysis of whole genomes identified TFs of NAC, WRKY, DREB, MYB/MYC, NAM, ABF/AREB, ATAF1,2, CUC family with their corresponding *cis*-acting elements DRE, MYBRS/MYCRS, ABRE, NACRS (Agarwal and Jha 2010). Transcriptomic studies in cereal plants allowed the identification of short and long-term abiotic stress responsive genes that fall under the category of signal transduction, oxidative stress protection, ion transport, modifications of structural components of cell walls and membranes, stress proteins, aquaporins, protease inhibitorsand antioxidant components (Sahi, Singh *et al*. 2006; Sreenivasulu, Sopory *et al*. 2007).

Besides serial analysis of gene expression (SAGE), representation different analysis (RDA) and massively parallel sequence signature (MPSS) and microarray, the expanded availability of the sequence based technologies like high throughput RNA-Seq and digital gene expression (DGE) profiling is promising further improvements in gene expression profiling (Jain 2012; Strickler, Bombarely *et al*. 2012) with the added advantage of studying SNPs, epigenetic modifications and alternative splicing (Lister, Gregory *et al*. 2009; Jiang and Ramachandran 2010). However, due to the costs involved, microarray based techniques are still a reasonably practical option (Duque, Almeida *et al*. 2013).

The huge datasets generated by genome wide transcriptional analysis in a range of crop species under different biotic and abiotic stresses demanded the development of web based

large public data repositories over the past decade, notably gene expression omnibus (http://www.ncbi.nlm.nih.gov/geo/), (http://www.ebi.ac.uk/arrayexpress/) ArrayExpress and Rice Expression Database (Vij and Tyagi 2007). With the rapid progress in sequencing of new species genomes and advances in transcription profiling technologies along with the bioinformatic utilization of these data, such resources will prove vital in our understanding and manipulation of plant stress tolerance.

1.11 Transcriptomics for salinity tolerance in rice

Plants response to environmental stresses in terms of underlying genetic mechanism can comprehensively be understood by whole genome expression profiling (Mizuno, Kawahara *et al*. 2010). Salt tolerance in rice is advantageous in this regard as rice is particularly sensitive at seedling and reproductive phase and few QTLs having large effects is known to control the trait (Leung 2008). The traits, however, have low heritability and are usually inherited quantitatively (Cuartero, Bolarin *et al*. 2006). The measurements of these traits in segregating populations are not always easy which demands careful coordination of environmental conditions over locations and seasons. Nonetheless, a number of rice varieties with significant yield advantage were released in Philippines, India and Bangladesh using the conventional breeding (Ismail *et al*. 2007). The progress made by conventional breeding coupled with some basic biotechnological techniques such as *in vitro* selection is not enough for future environmental challenges (Ashraf and Harris 2004; Flowers 2004; Yamaguchi and Blumwald 2005). Even though transgenic approached showed some prospect, Cuartero *et al.* (2010) argued to avoid excessive optimism in developing true halotolerant varieties.

It is well documented that the mechanisms involved in salinity tolerance is diverse making it a complex and polygenic trait (Bohnert, Ayoubi *et al*. 2001; Tuteja 2007; Munns and Tester 2008). Introducing a single gene is least likely to improve the tolerance dramatically. Instead, multiple genes involved in the principal mechanism of the processes such as signalling, ion homeostasis, osmotic adjustment, vacuoler compartmentalisation of ions, restoration of enzymatic activity, oxygen free radical scavenging and photorespiration may be necessary (Bohnert, Ayoubi *et al*. 2001). The transcription factors having a cascade effect that can regulate many other downstream genes may also prove vital in this regard. The main starting challenge in this regards is that it is not yet clear what are the genes that is needed to be studied & transferred (Cuartero, Bolarin *et al*. 2010).

The ability to measure the expression levels of entire genome in a single experiment by array based technologies allows biologists to see what are the genes induced or repressed under specific environmental stresses. The limitation is that besides the actual genes that controls the stress response, it detects enormous number of related genes which might be involved in secondary or irrelevant downstream functions (Cuartero, Bolarin *et al*. 2006). Despite the challenge of identifying the relevant target genes the transcriptomic approach provides an efficient tool of identifying the gene(s) involved in specific stress tolerance mechanism.

1.12 Overall aim of the project

This project is aimed at studying the salinity tolerance in wide genetic background of rice which encompasses genotypes from three groups of rice germplasm namely *Indica*, *Japonica* and wild species under moderate and high salt stress. The level and mechanism of salinity tolerance is set to be determined by using multivariate analytical approach based on

combined multiple growth, qualitative and physiological assessment with the aim to categorize the genotypes according to their level of tolerance to salt (see chapter 3). The whole genome transcriptome is set to be profiled using the Agilent 4x44K rice microarray slide. This transcriptomic data is primarily set to be analyzed simultaneously along with the morpho-physiological data with the aim to identify the significantly expressed trait specific genes in the entire wide genetic background of rice represented by the genotypes used in this study (see chapter 4). The main objective of the transcriptomic profiling is however to identify the genotype specific differentially expressed induced genes with the aim to build an *Indica* model of gene expression in rice under salt stress based on the significant induced genes of the four tolerant and two susceptible *Indica* genotypes (see chapter 5). These induced genes in the *Indica* genotypes can then be used to build a model with the aim to identify the transcriptomic fingerprints of tolerance which can be used to predict the tolerance status of the genotypes whose status of tolerance is unknown (see chapter 6). The other objective was to enrich the significant genes in terms of Gene Ontology in a way to identify the biological processes and molecular functions that are operational. The morpho-physiological assessment coupled with in depth transcriptomic profiling of a range of rice genotypes can generate knowledge which can be helpful in enhancing our understanding of the holistic salinity tolerance mechanisms in the wide genetic backgrounds of rice.

Chapter 2. MATERIALS AND METHODS

2.1 Plant materials

Eight genotypes consisting four *Indica*, two *Japonica* and two wild rice accessions were used for the morpho-physiological characterization study. Four more *Indica* genotypes with known response to salt stress including three tolerant and one susceptible genotype were used for gene the expression study (Table 2.1). These genotypes consisted of landraces, cultivars, commercial varieties and wild species and were chosen based on their diverse origins and were obtained from the International Rice Gene Bank Centre (IRGC) of the International Rice Research Institute (IRRI), Philippines.

2.2 Plant culture conditions

The research was conducted in the plant growth room of the School of Biosciences, the University of Birmingham, United Kingdom. Plants were grown twice, firstly for morpho-physiological characterization and secondly for gene-expression studies maintaining identically controlled environmental conditions. Seeds were first heat treated at 48^{o}c for 5 d in a convection oven to break the dormancy. Heat treated seeds were then sterilized with 1% Na-hypochlorite and two drops of tween20 and placed on a presoaked filter paper in a sterile petridish and were incubated at 20^{o}c for 48 h in a plant growth room for germination. Germinated seedlings were then transferred to small test tubes (25 ml) filled with Yoshida nutrient medium for seedling establishment for 7 d (solution was renewed on a daily basis). It helped to attain sufficient seedling growth to transfer individual seedlings to a conical flask (250 ml) filled with Yoshida nutrient solution (Yoshida *et al.* 1976) shown in Appendix I.

Table 2.1 List of plant materials used for morpho-physiological characterization (a) and for the transcriptomic profiling (a & b).

Genotype	Germplasm Group	Accession Number	Viability (%)	Origin
(a) *For morpho-physiological characterization*				
Pokkali	*Indica* (landrace)	IRGC 108921	99	India
PSBRc50	*Indica* (variety)	IRGC 99706	100	Philippines
IR 58	*Indica* (variety)	IRGC 63492	99	Philippines
BRRI dhan 29	*Indica* (HYV)	IRTP 15241	99	Bangladesh
Banikat	*Japonica* (cultivar)	IRGC 67720	99	India
Nipponbare	*Japonica* (cultivar)	IRGC 117274	97	Japan
O. latifolia	Wild species	IRGC 100965	95	Costa Rica
O. rufipogon	Wild species	IRGC 105390	100	Thailand
(b) *Four more genotypes were included for gene expression study*				
IR29	*Indica* (variety)	IRGC 30412	99	IRRI
FL478	*Indica* (variety)	IRGC 66946-3R-178-1-1	100	IRRI
Hassawi	*Indica* (landrace)	IRGC 16817	99	Middle East
Nonabokra	*Indica* (landrace)	IRTP 01231	96	India

Genotypes of list a were morpho-physiologically characterized (chapter 3) and the genotypes in list 'b' were chosen based on prior reputation of salt tolerance e.g., IR29 is a salt sensitive (Thomson *et al.* 2010) and FL478 (Cotsaftis *et al.* 2011), Hassawi (Zhang *et al.* 2012) and Nonabokra (Cotsaftis *et al.* 2011) are salt tolerant genotypes.

Plants were grown twice maintaining same environmental condition in growth room. From the first growing, the morpho-physiological data were collected from the first eight genotypes (list a). From the second growing, the gene expression data were collected from the all twelve genotypes (list a & b)

Chapter 3 uses the morpho-physiological data of eight genotypes (list a) to characterize their salinity tolerance status.

Chapter 4 (modified SAM) uses the morpho-physiological data (obtained from chapter 3) and the gene expression data of the eight genotypes of list a.

Chapter 5 (Differential Expression: the *Indica* model) uses the gene expression data of Pokkali, FL478, Hassawi, Nonabokra (tolerant) and BRRI dhan29, IR29 (susceptible) genotypes.

Chapter 6 (Prediction of salinity tolerance) uses the gene expression data of Pokkali, FL478, Hassawi, Nonabokra (tolerant) and BRRI dhan29, IR29, *O. latifolia, O. latifolia* (susceptible) genotypes.

HYV. High yielding variety, IRGC. International Rice Germplasm Center, IRTP. International Rice Testing Program.

Wrapping with aluminum foil prevented light reaching the nutrient solution and thus prevented unwanted algal growth. Each individual seedling was held in place using a sponge bung in each flask and the roots were suspended in the Yoshida solution. This hydroponic

culture provided direct control over nutrient management whilst also preventing the occurrence of soil borne pests and allowed straight forward access to the roots. Flasks were maintained in a growth room with 16/8 h photoperiod, 28/20°C day/night temperature, 70-75% relative humidity and an average 290 µmol m^{-2} s^{-1} photosynthetic photon flux. The experimental design was set up with both non-saline and salinized nutrient solutions in a randomized complete block design (RCBD) with three replicates.

2.3 Salinity treatments

Seedlings in the flasks were challenged with 40 and 80mM NaCl stress representing moderate and high salt stress, respectively for the morpho-physiological characterization study and with 120mM NaCl stress for gene-expression studies. The salt stress regimes i.e., the strengths and the duration of stress were chosen based on the results of preliminary experiments (data not shown) that were conducted with the objectives to determine a stress regime which will allow the most sensitive plants to remain green allowing the morpho-physiological assessment and the transcriptomic profiling being carried out on all living plants. Along with the NaCl, additional $CaCl_2$ was added to the Yoshida nutrient solution at a molar concentration ratio of 6:1 which helped to maintain constant activity of Ca^{2+} in the growth solution and additional silicon in the form of sodium meta-silicate 9-hydrate (4.5mg/L of culture solution) was added which is essential to avoid lodging of plants and enabling them to withstand the disturbance due to the daily pH monitoring and nutrient renewal. For each of the above mentioned treatments, half strength stress was applied at 13 d after emergence (DAE) initially and the full strength treatment was applied after another 24 h i.e., at 14 DAE. The nutrition solution without any added salt consisted of the

unstressed (control) condition. The pH of the treatment and control solution was maintained at 5.0 using 5M NaOH / HCl on a daily basis using a portable pH meter (Hanna instruments) and the solution was renewed on every alternate days.

2.4 Plant Measurements

2.4.1 Measurements of growth parameters

Leaf elongation rate (LER), root elongation rate (RER) and shoot elongation rate (RER) were measured by measuring the length of fully expanded 3rd leaf (joint of leaf sheath & leaf blade to leaf tip), root (longest measurement of seminal root tip from seed) and shoot (seed to tip of the longest leaf) at 3-6 d after saline treatment (DAS) and total elongation (mm) were calculated over this period and presented as elongation per day i.e., as mm d^{-1}. Coefficient of shoot elongation (CSE) was calculated according to the following formula (Devitt *et al.* 1984).

$$\text{CSE} = \frac{\text{Rate of shoot elongation in stressed plants (cm/d)}}{\text{Rate of shoot elongation in unstressed plants (cm/d)}}$$

Leaf area (cm^2) was measured at 7 DAS using the 'Length- width method' described by Yoshida (1976). The length and maximum width of each individual leaf were measured and LA was calculated using the following equations:

LA for individual leaf = K * Length * Max width of individual leaf and

LA for individual plant = ∑ LA for all individual leaf of a plant

Where, K = 0.67 is the adjustment factor for seedling stage (IRRI, 1972).

Transpiration rate (g DW $^{-1}$ h^{-1}) was measured by estimating the reduction in mass per h per unit (g) biomass at 7 DAS (24 hours cycle including one light and one dark period) using an electric balance. Any water loss other than transpiration was prevented by sealing the individual plant into the conical flask. Plant height (cm) and root length (cm) were recorded at 7 DAS. Plants were harvested at 7 DAS and after blotting the excess solution, plants were washed with de-ionized water several times to remove any surface salt, separated into roots and shoots and dried in a unitherm Dryer at 80^{0}C for 48 h until weights became constant. The shoot and root biomass were then measured using an electric balance.

2.4.2 Measurements of qualitative parameters

Leaf (fully expanded 3rd leaf) rolling score (LRS) was recorded on 3rd day (after 8 h in light) of salt treatment (DAS) on a scale of 1-5, with '1' being fully expanded and '5' fully rolled (Figure 2.1). The modified standard evaluation score (SES) was recorded every day between 3-6 DAS following the standard system of IRRI (Gregorio *et al.* 1997) given in Table 2.2.

Table 2.2 Modified Standard Evaluation Score (SES)

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	HT
3	Nearly normal growth, but leaf tips of few leaves whitish and rolled.	T
5	Growth severely retarded, most leaves rolled, only a few are elongating.	MT
7	Complete cessation of growth, most leaves dry some plants dying	S
9	Almost all plants dead or dying	HS

HT. Highly tolerant, T. Tolerant, MT. Moderately tolerant, S. Susceptible, HS Highly Susceptible

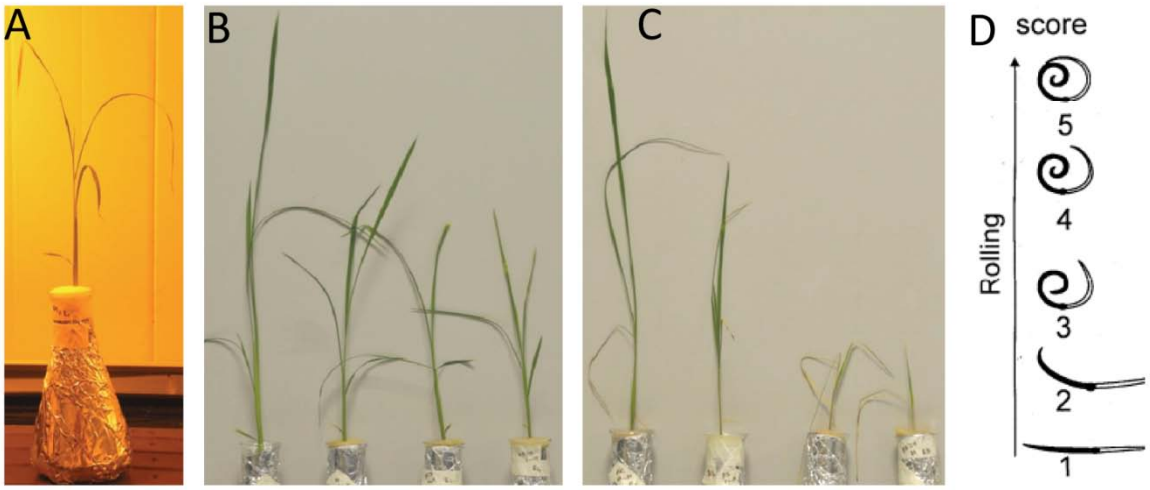


Figure 2.1 Hydroponically grown individual rice seedling (A), seedlings of Pokkali, PSBRc50, IR58 and BRRI dhan29 (L-R) under control (B) and 80 mM NaCl stress (C) condition at 6 DAS (20 DAE) and schematic representation of leaf rolling scores (D)- adapted from O'Toole and Cruz (1980).

2.4.3 Measurements of tissue ion parameters

Oven dried and weighed shoot and root tissues were placed into 1.5ml microfuge tubes and were ground to a fine powder. Powdered tissues were then digested for 5 h with 1M HNO3 at 80°C (1:20 dilution i.e., 1g tissue digested in 20ml 1M HNO3) and centrifuged at full speed for 15 min. The resulting supernatant (extracted sample) was then pipetted out and frozen at -20°C awaiting ion analysis.

Ion chromatography was used to quantify the concentration of cation within the extracted tissue samples using the Dionex® DX500 ion chromatograph (Dionex Corporation, California, USA). Samples were diluted 20 times (1:20) with cation eluent (methane sulfonic acid). A series (0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100 and 150ppm) of cation standard solutions were prepared using NaCl, KCl, $CaCl_2$, $MgCl_2.6H_2O$ and NH_4Cl. The standards and samples were placed into auto-sampling cartridges and placed in the auto-sampler (Dioned AS40 automated sampler). Using the auto-sampler each sample took roughly 22.5 min to run

through the column. The sample was passed at high pressure through the ion exchange column where cations were separated based on their relative affinity to the column. The concentration of Na^+, K^+, Ca^{2+}, Mg^{2+} and NH_4^+ were determined by comparing to the known concentrations of cation standards using the accompanying software (Peaknet® version 5.11).

2.5 Statistical analysis of morpho-physiological data

Where appropriate, all results were tested statistically for differences or correlations. Analysis of variance was performed on all measurements and a student t-test was performed when needed to find statistically significant changes in treated vs. control plants. Significant differences between the means were determined using Duncan's Multiple Range Test (DMRT). Pearson's correlation coefficient between the variables was computed to measure the degree of linear relationship between two variables. Principal component analysis was used to detect underlying sources of morpho-physiological variability, to complement cluster analysis and to investigate patterns of genetic diversity. Traits that were found to be more contributing to the total variation among the rice genotypes by principal component analysis were used for cluster analysis. Cluster analysis was done using Ward's complete linkage method and squared Euclidean distance and the data standardization option were used to convert all variables to a common scale by subtracting the means and dividing by the standard deviation before the distance matrix is calculated to minimize the effect of scale differences. Number of clusters to be formed was determined by finding the best incision point (discriminant analysis) where the similarity and distance values change abruptly. The relationships among the clusters were assessed by measuring the inter-cluster

distances based on cluster centroids. All analyses were done using the Minitab for Windows (version 15) except DMRT which was performed using SAS statistical package (version 9.2).

2.6 Minimum Information about a Microarray Experiment (MIAME)

MIAME is the Minimum Information about a microarray experiment which is essential for appropriate interpretation and potential reproduction of microarray experimental results. The initiative was taken by the Microarray Gene Expression Data (MGED) Society (http://www.mged.org), which requires the minimum information needed to be provided along with the results. The main objectives of the guidelines is to establish a public database making sure that the data submitted can be easily understood and accessed. Among the important contents in MIAME documents are sample and array design description, control elements, experimental design, hybridization procedures and measurements (Brazma *et al.* 2001). A simple outline structure of the current study is given in Appendix II as a MIAME form.

2.7 Tissue sampling and total RNA isolation

The fresh samples (whole seedling including root and shoot) were harvested after 48 h of stressed period (i.e., at 16 DAE) and immediately proceeded to the isolation of total RNA. RNA extractions were carried out using the QIAGEN RNeasy Plant Mini Kit and every step was carried out quickly to avoid the degradation of RNA due to time delay. Each individual seedling represented one treatment and one replicate. The individual seedlings weighing more than the recommended amount of starting material (100 mg) were reduced by removing the sections of root, stem and leaves using sterile scissors for each individual

samples. Care was taken so that the tip, middle and basal portion of roots, stems and leaves were kept in the 100 mg starting material to appropriately represent the tissues from the whole seedling. The tissue samples were then placed in liquid nitrogen and thoroughly ground with pre-chilled mortar and pestle to a fine powder immediately. The tissue powder was decanted into liquid nitrogen-cooled RNase free 2ml micro-centrifuge tubes. Buffer RLT (450 µL) was added to the tube and was vortexed vigorously. The lysate was transferred into a QIA-shredder spin column (lilac) placed in a 2 ml collection tube and centrifuged for 2 m at full speed. The supernatant was then transferred carefully to fresh 2 ml tubes and 0.5 volume of ethanol (96–100%) was added and mixed by pipetting in order to clear the lysate. The samples (usually 650 µL) were then transferred to an RNeasy spin column (pink) placed in a 2 ml collection tube prior to centrifuge at 8000 x g (10000 rpm) for 15 seconds. The spin column membrane was then washed with 700 uL of buffer RW1 by centrifuging at 8000 x g (10000 rpm) for 15 s. The flow through was discarded again and the membrane was further washed with 500 µL of RPE buffer twice at 8000 x g (10000 rpm) for 15 s for the first time and for 2 m for the second time. After discarding the flow through the column was placed in fresh 1.5ml tube and 30µl of RNase free water was added to the column and was kept at room temperature for two minutes. It was then centrifuged at full speed for 1 m to elute the total RNA. The extracted RNA was then aliquoted and immediately stored in a freezer at -70 ^{0}C for further use.

2.8 Quality assessment of the isolated RNA

The integrity of isolated RNA was assessed using the following two methods to ensure the recommended quality and quantity for microarray analysis.

2.8.1 Quantification of RNA using Nanodrop

A Nanodrop ND-1000 VIS spectrophotometer (v. 3.2.1) was used for the quantification of total RNA extracted from the seedlings of rice genotypes. The sample loading area (the receptacle laser cell) was cleaned with RNase free water followed by the initialization of the Nanodrop software with the nucleic acid measurement tab selected. The instrument was initialized and RNA-40 was selected as sample type in the user interface of the software after loading 1 uL of RNase free water (same RNase free water used for RNA extraction) on the receptacle laser cell. On clicking the measure tab, the concentration of RNA (ng/uL), 260/280 ratio and 260/230 ratio is recorded. The 260/280 ratio corresponds to any protein, phenol or alcohol contamination whereas 260/230 ratio *Indica*tes presence of genomic DNA. Only the samples having the 260/280 and 260/230 ratios around 2.0 were selected for further analysis.

2.8.2 Quality assessment of RNA using Bioanalyzer

RNA template quality was assessed before proceeding to further downstream experimental steps. The integrity inspection of RNA sample to be used is important in order to determine that RNA is not degraded during the extraction process. The RNA integrity was detected by the Agilent 2100 bioanalyser using the RNA 6000 Nano Labchip kit. The chip was prepared with the samples and the ladder as per the manufacturer's guidelines and loaded onto the 2100 bioanalyser. The eukaryote total RNA Nano assay was run. The electrograms were generated for each sample showing two distinct peaks representing 18s & 28s ribosomal RNA along with RIN (RNA integrity number) value. The samples showing RIN > 7.0 were selected for the labelling of the RNA samples for subsequent steps.

2.9 The rice genome microarray

The Agilent 4x44K rice microarray (Agilent product number- G2519F; design ID: 15241) was used in this project. The platform consists of 45151 well-characterized probes deposited in each spots, including 1283 spots representing negative controls and 43,724 oligonecleotides that correspond to the transcripts and gene models based on manual annotation of rice genome sequence as described in the Rice Annotation Project Database (RAP-DB). The array is believed to represent about 43,803 rice genes with one 60-mer oligonucleotide probe representing each and consists of 32,325 probes corresponding to RAP loci with full-length representative cDNA, 6,943 probes to RAP loci with EST support, 2,612 probes to gene predicted loci and control probes including false positives and non-rice sequences etc. Among the 28,840 protein-coding genes, 22,532 have single sequence probes, 4,619 have two sequence probes each, 1,235 have three sequence probes each, 330 have four sequences each, 92 have five sequences each, 16 have six sequences each, 11 have seven sequences each, three have eight sequences each, and two have nine sequences each.

2.10 Experimental design of microarray

The gene expression analysis was based on a one colour microarray experiment. The probes that are regulated differentially upon salt stress imposition on each individual genotype was identified by comparing the treated vs. control samples. All the 72 samples (12 genotypes, 2 treatments each with three replicates) were distributed randomly across the 18 slides positioning the replicates of each genotype on separate slides and the control and treated samples of each genotype on the same slide to minimize the experimental variation across slides. A simplified layout of the sample distribution is shown in Figure 2.2.

Replication-1		Replication-2		Replication-3	
Slide 1	PK - T_0	Slide 7	PK - T_0	Slide 13	PK - T_0
	PK - T_1		PK - T_1		PK - T_1
	PS - T_0		PS - T_0		PS - T_0
	PS - T_1		PS - T_1		PS - T_1
Slide 2	IR58 - T_0	Slide 8	IR58 - T_0	Slide 14	IR58 - T_0
	IR58 - T_1		IR58 - T_1		IR58 - T_1
	BR29 - T_0		BR29 - T_0		BR29 - T_0
	BR29 - T_1		BR29 - T_1		BR29 - T_1
Slide 3	BN - T_0	Slide 9	BN - T_0	Slide 15	BN - T_0
	BN - T_1		BN - T_1		BN - T_1
	NP - T_0		NP - T_0		NP - T_0
	NP - T_1		NP - T_1		NP - T_1
Slide 4	LT - T_0	Slide 10	LT - T_0	Slide 16	LT - T_0
	LT - T_1		LT - T_1		LT - T_1
	RF - T_0		RF - T_0		RF - T_0
	RF - T_1		RF - T_1		RF - T_1
Slide 5	FL - T_0	Slide 11	FL - T_0	Slide 17	FL - T_0
	FL - T_1		FL - T_1		FL - T_1
	HS - T_0		HS - T_0		HS - T_0
	HS - T_1		HS - T_1		HS - T_1
Slide 6	NB - T_0	Slide 12	NB - T_0	Slide 18	NB - T_0
	NB - T_1		NB - T_1		NB - T_1
	IR29 - T_0		IR29 - T_0		IR29 - T_0
	IR29 - T_1		IR29 - T_1		IR29 - T_1

Figure 2.2 Simplified layout showing the distribution of 72 samples (12 rice genotypes, 2 treatments and 3 replicates per treatment per genotype) each across 18 Agilent 4x44K microarray slides. (PK) Pokkali, (PS) PSBRc50, (BR29) BRRI dhan29, (BN) Banikat, (NP) Nipponbare, (LT) *O. latifolia*, (RF) *O. latifolia*, (FL) FL478, (HS) Hassawi, (NB) Nonabokra, (T_0) 0mM Nacl-control and (T_1) 120mM Nacl-treated

2.11 One colour Microarray based Gene expression Analysis

This section describes the recommended procedures for sample preparation, labelling with Cy3 dye, hybridization, washing, scanning and feature extraction of Agilent's 60-mer oligonucleotide microarray for Agilent one colour Microarray based gene expression experiment. Details of the procedure can be found in 'Low Input Quick Amp Labeling Protocol v.6.5, May 2010' (available from www.agilent.com/chem/dnamanuals-protocols). The total procedure of microarray has been shown diagrammatically in Figure 2.3.

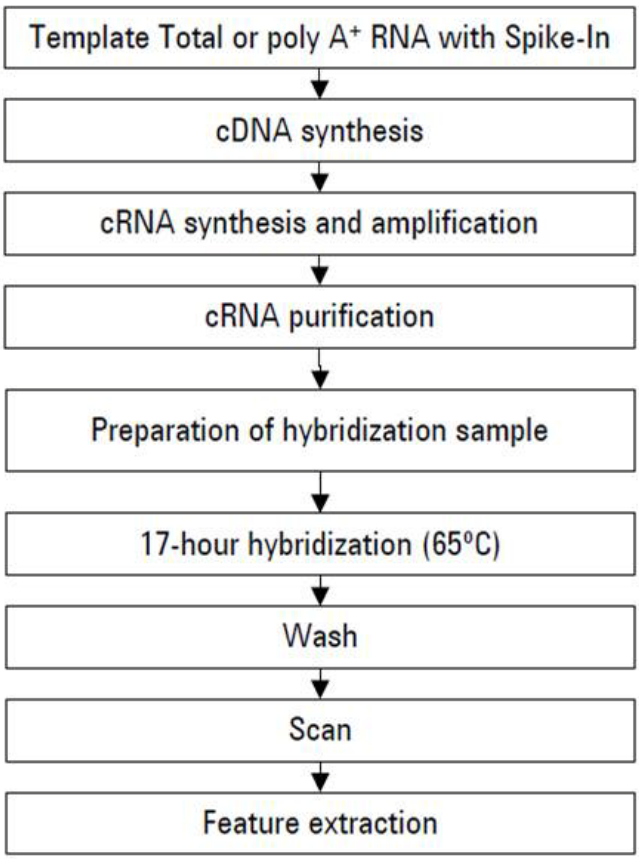


Figure 2.3 Workflow for Agilent one colour Microarray based gene expression experiment (taken from Agilent's Low Input Quick Amp Labeling Protocol v.6.5, May 2010)

2.11.1 Preparation of sample

The quality assured RNA samples were prepared using Agilent's one colour Quick Amp labelling kit (v.6.5, May 2010). It generates fluorescent cRNA (complementary RNA) from the total RNA to be analysed, using T7 RNA polymerase which amplifies (generally 100-fold) the target cRNA incorporating Cy3 labelled CTP. The step consists of four sub-steps namely, preparation of One-Colour Spike-Mix, preparation of labeling reaction, purification of the labeled/amplified RNA and finally quantification of the cRNA generated from the total RNA.

2.11.2 Preparation of labeling reaction

Exactly 300 ng of total RNA were added to the labeling reaction mix containing T7 promoter primer (1.2 µL), spike mix (3.0 µL), and RNase free water to a final volume of 11.5 µL. Both primer and template RNA were then denatured in an incubator for 10 min at a temperature of 65^0C before being placed on ice for another 5 min. The cDNA Master Mix (8.5 µL) containing 5 x buffer (4 µL), DTT (2 µL), dNTPs (1 µL), mmLV-RT (1 µL), RNase out (0.5 µL) was added to the reaction mix prepared for all samples and were mixed by flicking and spinning down. These samples were then incubated again for 2 h at 40^0C in a circulating water bath before placing on ice for 5 min to stop the reaction. The transcription master mix (60 µL) containing RNase free water (15.3 µL), 4x buffers (20.0 µL), DTT (6.0 µL), NTPs (8.0 µL), preheated 50% PEG (6.4 µL), RNase out (0.5 µL), inorganic pyrophosphatase (0.6 µL), T7 RNA polymerase (0.8 µL), and Cy3 dye (2.4 µL) was added to each sample. The samples were again mixed by flicking and spinning down before incubating at 40^0 C for another 2 h. The cRNA was thus synthesised and samples were labelled with Cy3 dye. The samples were then cleaned using RNeasy cleanup procedure. Finally the cRNA was quantified using the

Nanodrop utilizing microarray measurement tab to quantify the yield of cRNA and Cy3 incorporation in each sample.

2.11.3 Hybridization and microarray wash

This is a two day long procedure. On the first day, the blocking reagent was prepared by adding the *Indica*ted amount of RNase free water followed by gentle vortexing and spinning down to mix well. In a further step, the hybridization sample was prepared using a mixture of cRNA (1.65 µg), 10 x blocks (11.0 µL) and fragmentation buffer (2.2 µL) and RNase free water to make up the final volume to 55ul. The samples were incubated at 60^0 C for exactly 30 min to allow the fragmentation of RNA followed by mixing 55 µL 2 x GE hybridization buffer by gentle pipetting to avoid introducing bubbles to stop the fragmentation reaction. A clean gasket slide was placed in assembly with the Agilent label facing up and aligned with the rectangular section of the chamber base. Exactly 100 µL of the sample were loaded onto each gasket well in a 'drag and dispense' manner avoiding the introduction of bubbles. The microarray "active side" was then slowly placed down onto the gasket slide with loaded samples making sure that the "Agilent"-labeled barcode is facing down and the numeric barcode is facing up and that the sandwich-pair is properly aligned. The chamber cover was then placed onto the sandwiched slides and the clamp assembly was hand-tightened. The assembled chamber was then rotated vertically to wet the gasket and assess the mobility of the bubbles before being incubated at 65^0C for 17 h in an Agilent rotisserie. The staining vessels and the wash buffer 2 were prepared and kept at 37^0C overnight for next day washing.

Day two started with preparing two staining troughs with wash buffer 1 at room temperature; the first trough to disassemble the slide and the other one on the stirrer to wash the slide. All the slides in the assembled chamber were disassembled under wash buffer 1 and were placed in a rack in the stirring wash buffer 1. Once all slides were opened, they were stirred for 1 min in wash buffer 1. Wash buffer 2 was then poured into the pre-warmed staining trough and stirred and the slides were washed in wash Buffer 2 for 1 min. Slides were then removed and washed in 100% acetonitrile for 10 s followed by another wash in a stabilising and drying solution for 30 s. The slides were removed slowly from the stabilising and drying solution to allow the reagent to dry off without leaving "water marks" on the slide and placed in a slide box for subsequent use.

2.11.4 Scanning and feature extraction

The slides were assembled into an appropriate slide holder with the numeric barcode visible and were placed into the scanner carousel. The scan setting was set at 'Profile AgilentG3_GX_1Color' for one colour scan. When the scanner was ready, Slot m-n on the scan control main window (m = the Start slot where the first slide is located and n = the end slot where the last slide is located) was set and then the scanning was conducted. Data were acquired by using Agilent feature extraction software version 9.5.3. After the successful completion of extraction, the QC report for each extraction set was critically evaluated and finally, the resulting text files (the raw data files) were saved for further analysis.

2.12 Statistical analysis of microarray data

Gene expression data of all 12 genotypes were analyzed in two different ways namely, the significance analysis of microarrays (SAM) and the GeneSpring which are described in the next sections.

2.12.1 Modified significance analysis of microarrays (SAM)

The gene expression data of the first 8 genotypes were analyzed along with the morpho-physiological data using modified significance analysis of microarrays (SAM). The SAM procedure was first used and described by Tusher *et al.* (2001) where the gene expression data of control and treated samples were analyzed in a single run by denoting the control and treated samples as 1 & 2, respectively. The basic principle of SAM includes scoring each of the genes based on the changes in gene expression which is relative to the standard deviation of repeated measurements. For the genes that have scores greater than an adjustable threshold, the permutations of the repeated measurements are used by SAM to estimate the percentage of genes identified by chance (false discovery rate, FDR). The same SAM procedure is used but two modifications were made to suit the analysis with the experimental design of this project.

Firstly, the control and the treated data were analyzed separately. Secondly, instead of denoting all the 24 control samples (8 genotypes each with 3 replicates) as '1', these were denoted by their corresponding weighted ranks (0-1) which were derived from the actual values of the trait in question. For each of the 14 traits (e.g., for the biomass), the highest value is denoted as '1' and the lowest value as '0' and all the intermediates were weighted accordingly. The same was done for the treated samples as well.

For one particular trait (e.g., for the biomass), the pre-processed (quantile normalized and log2 transformed) gene expression data of the 24 control samples were compiled in one spreadsheet where the samples were denoted by their corresponding weighted ranks (under control condition) for that particular trait. The SAM program is run using the response type as 'qualitative' based on 100 permutations using SAM version 4.0 software. The significant genes were selected based on the criteria of≤5% FDR and ≥2 fold score. A single run of the SAM program (see Tusher *et al.* 2001 for details) generated the output with significant (positive and negative) genes for the trait in question. Thirteen separate run of the SAM program generated the output with significant genes for the remaining thirteen traits under control condition (see figure 4.1 A).

Similarly, the pre-processed (quantile normalized and log2 transformed) gene expression data of the 24 treated samples were compiled in another spreadsheet where the samples were denoted by their corresponding weighted ranks (under treated condition) for each of the fourteen traits. The fourteen separate run of the SAM program generated the significant (positive and negative) genes for each of the fourteen traits (see figure 4.1 B).

These modifications were necessary as unlike the experiment of Tusher *et al.* 2001 where only two human lymphoblastoid cell lines were studied for one single trait (response to ionizing radiation), this project studies fourteen different traits in eight rice genotypes that accounts for a wide range of variation in terms of taxonomy, origin and salt sensitivity. It was thus sensible to analyze the control and treated data separately to identify the trait specific significant genes under control and treated conditions, respectively.

2.12.2 GeneSpring analysis

The GeneSpring analysis was carried out using the transcriptomics data of the four tolerant and two susceptible *Indica* genotypes. Altogether there were 35 samples as the RNA sample of unstressed BRRI dhan29 (replication 2) failed to meet the quality criteria for labelling and hybridization due to likely RNA contamination or degradation during preparation. The raw data files were analysed using Agilent's GeneSpring GX software version 12.5. The quantile method of normalization was used to normalize the data across all arrays setting the threshold raw signal at '1' and setting the baseline transformation to 'Median of all samples'. The experimental grouping was done by setting parameters to the samples. The data were then filtered sequentially by expression, by flags, by data and finally by error (CV <20%). One-way ANOVA was conducted with probability value set at <0.05 and with Benjamini-Hochberg set as multiple testing correction. Differentially expressed significant probes were selected by comparing the treated vs. control samples for each individual genotype based on fold expression differences with a minimum value of 2.0 and a significance value of at least 0.05 using Volcano Plot analysis. The significant probes were further evaluated to answer the specific biological question by comparing between the tolerant and susceptible genotypes.

2.13 Gene Ontology (GO) enrichment analysis

With the advent of technologies, researcher these days can generate high throughput data at whole genome level. However, the challenge is to decipher the underlined biological meaning from this huge data set generated. With the progress in Gene Ontology (GO) term enrichment and the GO databases getting richer day by day, functional annotation enrichment analysis using the Gene Ontology controlled vocabulary can be the promising

strategy to address these issues. Singular Enrichment Analysis (SEA) is one of the three classes of gene ontology enrichment tools described by Huang *et al.* (2009) which can extract the major biological meaning behind large gene lists efficiently. In this SEA analysis, the GO term enrichment of a given set of genes is computed one by one in a linear model by comparing it to a standard or customized annotated reference list. The enriched P-value computed, can identify the genes from the whole differentially expressed gene set that hit a given biological class as compared to pure random chance, allowing the investigator to identify the possible biological processes and molecular functions that are enriched by the identified genes. In this study, the significant candidate genes from the list of comparisons between the individual genotypes and between the tolerant groups were used for GO analysis using the AgriGO web-based tool (Du *et al.* 2010), available from http://bioinfo.cau.edu.cn/agriGO/index.php. The Singular Enrichment Analysis (SEA) was done setting 'Rice TIGR genemodel' as reference and 'Hypergeometric' as statistical test method, 'Hochberg (FDR)' as multi-test adjustment method, 0.05 as p-value cut-off and '5' as minimum number of mapping entries. However, when the number of genes was too low, a Fisher statistical test was conducted setting '1' as the minimum number of mapping entries. The genes under each list were then annotated and defined according to the GO terms directly under the three main categories namely, biological process, molecular function and cellular component. When appropriate, further analysis was focused on the set of genes under important biological processes.

2.14 Mapping of the genes on chromosome and the gene regulatory network

The significant genes were mapped in the twelve chromosomes of rice using the Chromosome Map Tool (http://viewer.shigen.info/oryzavw/maptool/MapTool.do) of GRAMENE genome browser database that provides valuable information through a user-friendly web interface. The list of locus IDs (genes) e.g., RAP ID- Os01g0281000 were provided as input in the 'Chromosome Map Tool' which in turn came up with the chromosomal map *Indica*ting the position of the genes.

The interactions between the significant genes were determined using the 'The Rice Interactions Viewer' web based tool version Interactome 2.0 (http://bar.utoronto.ca/interactions/cgi-bin/rice_interactions_viewer.cgi) developed by the Bio-Analytic Resource- the BAR (http://bar.utoronto.ca/welcome.htm). Prior to using the 'Rice Interactions Viewer', the RAP-DB gene Ids (e.g., Os06g0699400) were converted into MSU (TIGR) ID (e.g., LOC_Os06g48590) using the RiceXPro: Global gene expression profile (http://ricexpro.dna.affrc.go.jp/category-select.php) web based tool. The lists are then provided to the web tool and the output was set to be filtered to include LOC IDs in input set and to remove duplicate interactions.

2.15 Mapping of the genes within the salt stress related QTLs

Mapping of the genes within the salt stress related QTLs was done by two ways. Firstly, using the web based tool 'QlicRice' (http://nabg.iasri.res.in:8080/qlic-rice/qtlbrowser.html) that directly mapped the gene IDs (MSU-Locus IDs) within the corresponding QTL (Smita *et al.* 2011).

Secondly, the genes that are located within the flanking marker region of the QTLs for Shoot Na^+ and K^+ and their ratio located in chromosome 1 (9817791- 13880613 bp) were identified using another web based tool called 'Rice Gene Thresher' (http://rice.kps.ku.ac.th:8080/Web2/) (Thongjuea *et al.* 2009). The detailed manual on how the above two procedure were done can be found in respective websites.

2.16 Building of OSC-PLSDA model and Prediction of salinity tolerance of unknown genotypes

The normalized datasets of the 71 samples obtained by Gene-Spring software as described in the section 2.12.2 were used as the starting datasets. From this datasets, two reduced data matrix namely, Reduced Matrix 1 & 2 were created. The Reduced Matrix 1 was created by combining the differentially expressed significant genes of four known tolerance genotypes (4T) viz., Pokkali, FL478, Hassawi and Nonabokra and two known susceptible genotypes (2S) viz., BRRI dhan29 and IR29 (as shown in Supplementary Table 5.1) comprising 3137 unique genes and 71 samples. The Reduced Matrix 2 was created by combining the differentially expressed significant genes of same four known tolerant genotypes (as of Reduced Matrix 1) and four known susceptible genotypes (4S) viz., BRRI dhan29, IR29, Nipponbare and *O. rufipogon* (as shown in Supplementary Table 6.1) comprising 6303 unique genes and 71 samples. The reduced matrices were then transposed and imported into Matlab and the PLS Toolbox (Eigenvector). Sample and class names were abbreviated for simpler plotting, and initially six classes were defined (Sus-C, Sus-T, Tol-C, Tol-T, Unk-C, and Unk-T where Sus = susceptible, Tol = tolerance, Unk = unknown, C = control and T = tolerant. After the first PCA, only three groups remained defined, these are Sus, Tol, and Unk and the clear separation in PC1 by control versus treated, and in PC2 by susceptible versus tolerant prompted the

pooling of all susceptible and all tolerant samples into one class each. After reclassification into only three classes (susceptible, tolerant, unknown), the two known classes were used to create a PLS-DA model. An in-house PLS-DA script (plsda_auto_v18.m) was used to test and optimize the model. Classification error being minimal at two latent variables (LVs), and the model was tested using the Venetian blinds internal cross validation with 1000 repetitions. LV1 and LV2 weightings were merged into one Variable Importance vector, for which the minimal number of genes was calculated to result in an optimized model (using forward selection method) of only 109 genes for Reduced Matrix 1 and only 585 genes for Reduced Matrix 2. The same dataset of "known" samples (109 and 585 forward selected data for Reduced Matrix 1 & 2, respectively) was used, but preprocessed applying orthogonal signal correction (OSC (#components = 1, #iterations = 0, tolerance = 99.9%), Mean Center) in order to filter out components that are not predictive of class separation. This procedure resulted in a very good model for prediction of the same samples used for model building (using internal cross validation). Based on this model, the PLS Toolbox predicts into which class a sample falls and the samples with unknown tolerance status were added as "validation" dataset without changing the model.

Chapter 3. MORPHO-PHYSIOLOGICAL CHARACTERIZATION OF RICE GENOTYPES FOR SALINITY TOLERANCE

ABSTRACT

Climate change-induced events are causing salinization of many rice growing areas, requiring the identification of new sources of genetic variation for salt tolerance in plant genetic resources since commonly grown cultivars are salt sensitive. To identify the level and mechanism of salt tolerance across a wide range of genotypes we used a novel multivariate screening method using multiple growth and physiological traits simultaneously. Four *Indica*, two *Japonica* and two wild rice genotypes were grown hydroponically under 40 and 80mM NaCl stresses. Fourteen different growth, qualitative and physiological traits e.g., plant height, biomass, root and shoot elongation rates and tissue ion accumulation etc. were recorded. In general, *Indica* varieties performed better than both *Japonica* and wild species. Our approach identified the existence of qualitatively different mechanisms of salt tolerance across the genotypes. For example Pokkali, a salt tolerant *Indica* variety, displayed both 'Na exclusion' and 'ion balance' mechanisms whereas PSBRc50 and IR58 showed only 'Na exclusion' and the *Japonica* genotypes, Banikat and Nipponbare showed only 'ion balance'. The results demonstrated that the tolerance is dependent on the level of stress and that this varies between genotypes; Nipponbare is moderately tolerant to 40mM NaCl but not to 80mM. We also suggest that the use of multivariate analyses can simplify the complex salinity tolerance picture and can effectively reveal the genetic determinant of salinity tolerance from a wide range of germplasm. The results reported here identify different physiological mechanisms of tolerance across the genotypes and provide a sound basis for future studies examining their underlying molecular mechanisms.

3.1 INTRODUCTION AND AIM

Soil salinity, a major factor limiting agricultural productivity worldwide, affects 6.5% of the world's total land (Metternicht and Zinck 2003) and 10-50% of the world's irrigated land (Ghassemi *et al.* 1995; FAO 2002) and causes 0.25-0.50 m ha of irrigated land to become non-productive each year. Salinity can be due to salt build up due to increased irrigation (Quesada *et al.* 2002; Martinez and Manzur 2005) or by seawater incursions (Abrol 2004). Salinity can be exacerbated by rising sea levels due to global warming (Mainuddin *et al.* 2011) or by increased use of irrigation (Rengasamy 2006).

Rice is a staple food for half of the world's population and as a glycophyte is sensitive to soil salinity, suffering from severe yield reduction even at moderate salt levels (Zeng *et al.* 2002). Seedling and flowering stages are the most susceptible to salt stress (Lutts *et al.* 1996). Salinity exerts three main effects on plants; drought stress, direct ion toxicity and nutritional imbalance (Munns *et al.* 2002; Flowers and Colmer 2008). Elevated osmotic pressure in the soil solution reduces soil water potential affecting turgor maintenance and potentially causing wilting, growth reduction and in severe cases death of plants (Munns *et al.* 2006). Disruption of ion uptake can affect ion homeostasis and lead to imbalances of K^+ and other ions increasing the Na^+:K^+ ratio (Apse and Blumwald 2002; Horie *et al.* 2012). Na^+ and Cl^- ions enter into the cells and have direct toxic effects on enzyme activity and cytosolic metabolism (Maser *et al.* 2002; Taiz and Zeiger 2002).

Some rice plants can tolerate salt stress by reducing the absorption of toxic ions, reducing cytosolic Na^+ load thus maintaining a low cytosolic Na^+:K^+ ratio. Alternatively or additionally toxic ions can be compartmentalized into less sensitive organelles like the vacuole

(Blumwald 2000; Munns and Tester 2008). Abiotic stresses are also shown to rapidly elevate Ca^{2+} in cytosol which acts as an important signalling molecule that elicits defence responses (DeFalco *et al.* 2010).

However, it is known from field studies that salt sensitivity in rice can vary between cultivars and this variation could be exploited to discover novel genes and proteins that confer tolerance to salt (Thomson *et al.* 2010; MacGill *et al.* 2012). While some efforts are being made to identify further tolerant genotypes from existing germplasm with *Indica* rice receiving more attention compared to *Japonica* and wild rice, only a few comparative salinity evaluations have been conducted with *Indica* and *Japonica* rice (Lee *et al.* 2003) and with *Indica* and wild rice species (Nakamura *et al.* 2002; Nakamura *et al.* 2004; Awala *et al.* 2010). Most critically, few tolerant and moderately tolerant *Japonica* genotypes (Lee *et al.* 2003) or wild species (Awala *et al.* 2010) have been identified. Indeed, a comparative study involving the salinity response of *Indica, Japonica* and wild species of rice has never been reported until now. Further lack of knowledge exists for genotype screening which has been based on scores for a single growth or physiological trait, but continuous variation in such traits makes it difficult to clearly quantify genotypic differences (Zeng 2005). Qualitative evaluation scores alone based on visual vegetative damage (Gregorio *et al.* 1997) are useful for mass screening at the seedling stage but not effective at lower levels of stress because at low stress the symptoms of damage such as leaf rolling and chlorosis are not always obvious (Zeng *et al.* 2002). The lack of an effective screening method is therefore a major barrier to the effective identification of tolerant germplasm that could be used in breeding for different types of rice.

Our novel approach is to use statistical analyses of multiple variables which can be used to screen and identify genotypes responding in a similar manner to salt stress, subsequently facilitating appropriate selection of material to feed directly into breeding programmes, or the easier identification of genes that may be responsible for salt tolerance. Cluster analysis was effective in categorizing rice genotypes under salt stress using growth parameters alone (Zeng *et al.* 2002) or using both growth and ion accumulation parameters separately (Zeng 2005). The latter study reported that the classification based on ion accumulation parameters correlates with growth performance. These studies establish that rice genotypes can be categorized according to the level of tolerance using both growth and physiological traits simultaneously. However, neither study compared diverse genetic material ranging from *Indica* to *Japonica* and wild species genotypes.

Our study has therefore aimed at the broad comparative evaluation of the level and mechanism of salinity tolerance in three groups of rice germplasm namely *Indica*, *Japonica* and wild species under moderate and high stress levels using multivariate analysis on combined multiple growth, qualitative and physiological assessment as a novel screening approach.

3.2 RESULTS

Seedlings of eight rice genotypes (Table 3.1) were characterized in terms of their growth, physiological and qualitative performances under 0, 40 and 80 mM NaCl stresses, representing control, moderate and high salt stress using *in vivo* hydroponic rice seedling culture system (see section 2.1 to 2.5 in chapter 2) and this section describes the variation (Figure 3.1) that is observed in the level and mechanism of salt tolerance of these eight rice genotypes.

3.2.1 Variability within and between genotypes, treatments and their interaction

Seedlings of rice genotypes varied both inter- and intra-genotypically following exposure to moderate (40mM NaCl) and high (80mM NaCl) salt stress. Statistically significant differences were observed between the genotypes and between the interactions of genotypes and treatments for all traits except for transpiration rate and between the treatments except for shoot K^+ (Table 3.2).

3.2.2 Growth characterization

Leaf, root and shoot elongation rates were decreased at increased external salt in all genotypes; either Pokkali or PSBRc50 showed the least reduction and BRRI dhan29 showed the highest reduction at both salt levels. Banikat had the smallest reduction in root elongation rate at the higher salt level (Figure 3.2 and Table 3.3). Co-efficient of shoot elongation (see section 2.4.1) was found to be the highest in PSB Rc50 (0.77) followed by Nipponbare (0.66) at moderate salt level, which however, declined drastically at high salt level *Indica*ting the inability of these two genotypes to maintain shoot growth at high salt level (Table 3.3). *Indica* land race Pokkali and *Japonica* cultivar Banikat, on the other hand,

were able to maintain shoot growth at both moderate and high salt levels while the shoot growth of BRRI dhan29 was severely affected at both salt levels. The rate of transpiration (g DW^{-1} h^{-1}) varied significantly between treatments showing a gradual decrease in Pokkali and PSBRc50 with the increase in salt stress (Table 3.3). Interestingly, TR was found to be increased in BRRI dhan29 and Nipponbare at moderate salt level, which decreased again at high salt level.

Leaf area, plant height, root length and total biomass decreased with increased external NaCl in all genotypes except *O. latifolia* at both salt levels and Nipponbare at high salt level showed slight increase in root length. Pokkali showed the least reduction in all these parameters except PSBRc50 in leaf area at moderate salt level and Nipponbare in root length at high salt level. *O. latifolia* showed the highest reduction in all these parameters except *O. rufipogon* in plant height at moderate salt level and Nipponbare in leaf area at high salt level (Table 3.2).

Table 3.1 Salinity reactions of eight rice genotypes in terms of Leaf Rolling Score (LRS) and Standard Evaluation Score (SES) under moderate (40mM NaCl) and high (80mM NaCl) stress levels.

Genotype	Germplasm group	Accession	Origin	LRS		SES	
				40mM	80mM	40mM	80mM
Pokkali	*Indica* landrace	IRGC108921	India	2.0^{a}	2.7^{a}	1.7^{a}	3.0^{a}
PSBRc50	*Indica* variety	IRGC99706	Philippines	2.3^{a}	4.3^{a}	2.3^{ab}	5.0^{b}
IR58	*Indica* variety	IRGC63492	Philippines	2.7^{a}	3.7^{a}	5.0^{d}	7.7^{d}
BRRI dhan29	*Indica* HYV	IRTP15241	Bangladesh	5.0^{b}	5.0^{b}	7.7^{c}	9.0^{c}
Banikat	*Japonica* cultivar	IRGC67720	India	3.3^{ab}	4.0^{ab}	3.7^{bc}	7.7^{c}
Nipponbare	*Japonica* cultivar	IRGC117274	Japan	4.7^{b}	5.0^{b}	3.7^{bc}	9.0^{d}
O. latifolia	Wild species	IRGC100965	Costa Rica	4.7^{b}	5.0^{b}	7.0^{d}	9.0^{d}
O. rufipogon	Wild species	IRGC105390	Thailand	3.7^{ab}	4.7^{ab}	4.3^{c}	9.0^{d}

LRS was recorded based on the fully expanded 3rd leaf on 3 days after salt treatment (DAS) on a scale of 1-5, 1= fully expanded and 5 = fully rolled. SES was recorded on 6 DAS on a scale of 1-9, 1= highly tolerant and 9 = highly susceptible. The LRS and SES score for each of the genotypes were '1' under control (0mM NaCl) condition. Data presented as the mean (n=3) and different letters in a column *Indica*te significant differences at $P \leq 0.05$ by Duncan's Multiple Range Test (DMRT); (HYV) High yielding variety, (IRGC) International Rice Germplasm Center, (IRTP) International Rice Testing Program.

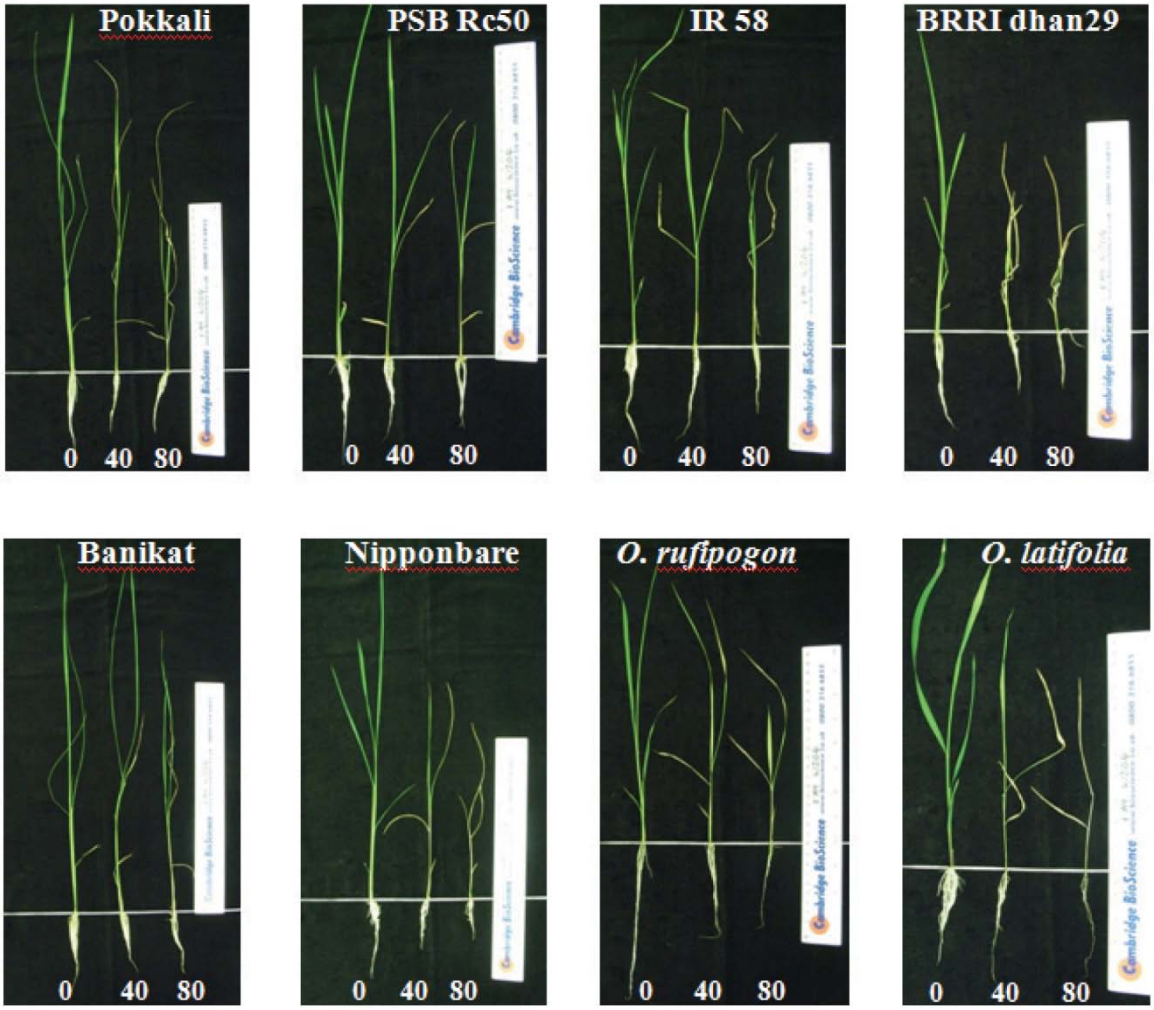


Figure 3.1 Seedlings of eight rice genotypes of 0, 40 & 80 mM NaCl stress showing the evidence of decreased growth at 7 days after stress application.

Table 3.2 Mean squares and F-tests (Two-way ANOVA) of genotypes, treatments and their interactions for growth and physiological traits

Sources (df)	LER	RER	SER	PH	RL	LA x 10^2	TR	Bio mass x10^4	Shoot Na^+ x10^4	Shoot K^+ x10^4	Shoot Na/K	Root Na^+ x10^4	Root K^+ x10^4	Root Na/K	Shoot Cl^- x10^4	Root Cl^- x10^4
G (7)	0.5 ***	0.2 ***	11.2 ***	64.0 ***	16.5 ***	68.3 ***	2.3	28.3 ***	11.4 ***	27.0 ***	0.5 ***	2.3 ***	1.3 ***	1.5 ***	8.8 ***	2.3 *
T (2)	1.5 ***	3.5 ***	157 ***	180 ***	30.1 ***	493 ***	24.0 ***	168 ***	138 ***	6.4	4.0 ***	58.7 ***	8.4 ***	39.1 ***	222 ***	42.5 ***
GxT (14)	0.1 ***	0.1 **	5.9 **	4.0 **	2.2 *	6.9 *	0.6	4.6 ***	4.2 ***	10.0 *	0.1 ***	1.5 ***	0.5 ***	0.5 **	3.9 ***	2.7 ***
Error (48)	0.02	0.02	1.9	1.3	1.1	30.6	1.3	0.6	0.6	4.6	0.1	0.1	0.1	0.2	0.9	0.8

*, ** and *** *Indica*te significant at p≤0.05, P≤0.01 and P≤.001, respectively.

(df) degree of freedom, (G) genotype, (T) treatment, (GxT) genotype-treatment interaction, (LER) leaf elongation rate, (RER) root elongation rate, (SER) shoot elongation rate, (PH) plant height, (RL) root length, (LA) leaf area, (TR) transpiration rate.

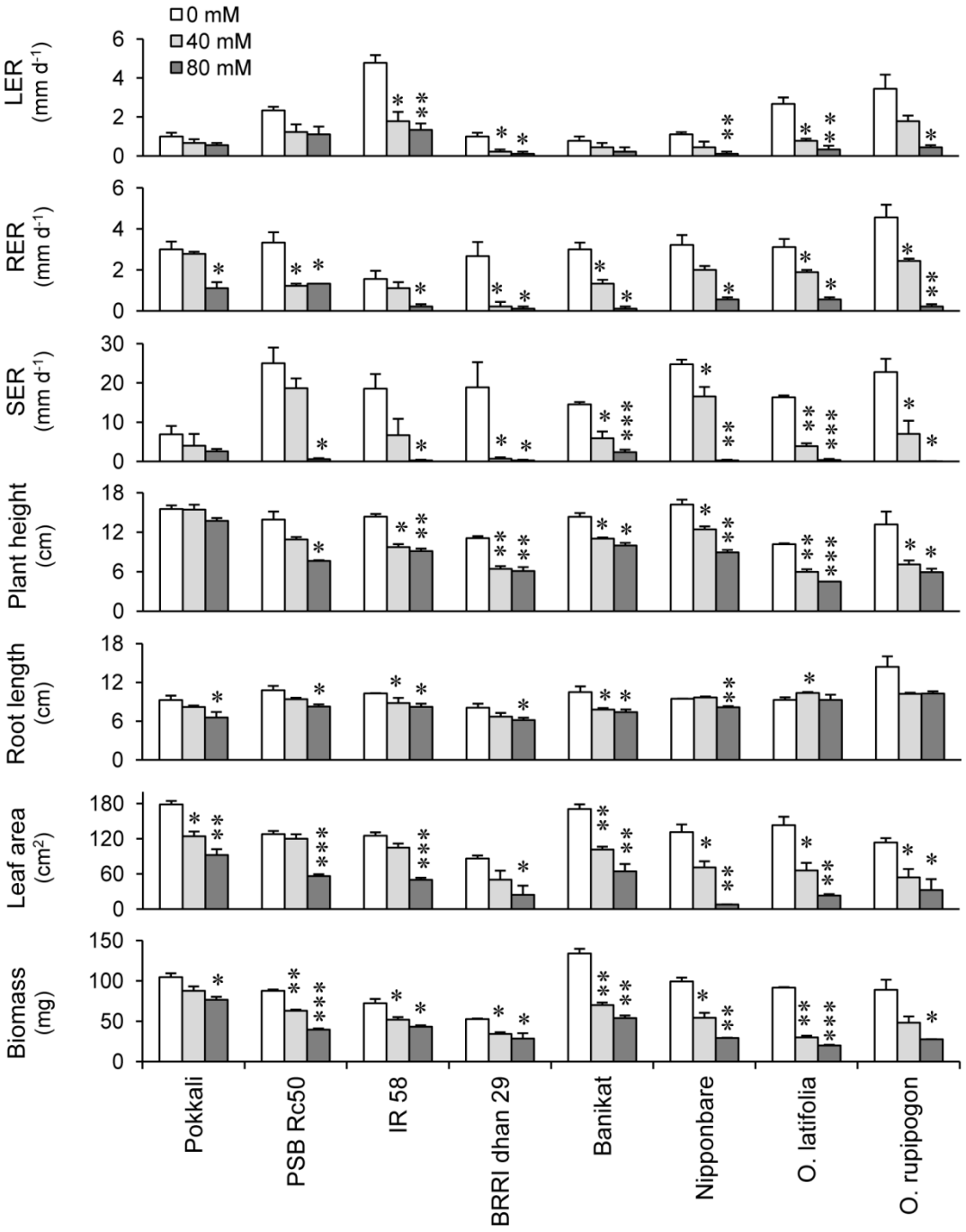


Figure 3.2 Differential growth responses of four *Indica*, two *Japonica* and two wild rice genotypes under 0, 40 and 80mM NaCl stress.

Plants were grown at 28/20°C day/night temperature and 16/8 h light/dark period and were challenged with NaCl stress at 14 DAE. The elongation in the leaf, root and shoot were measured over a period of 3 day (3 – 6 DAS) and the rates are presented as elongation per day i.e., as mm d^{-1}; and the rest of the traits were recorded at 7 DAS. Data presented as mean ± SE (n=3). Significant differences with control response at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ are *Indica*ted by *, ** and ***, respectively by student's t-test. (DAE) days after emergence, (DAS) days after salt treatment, (LER) leaf elongation rate, (RER) root elongation rate, (SER) shoot elongation rate.

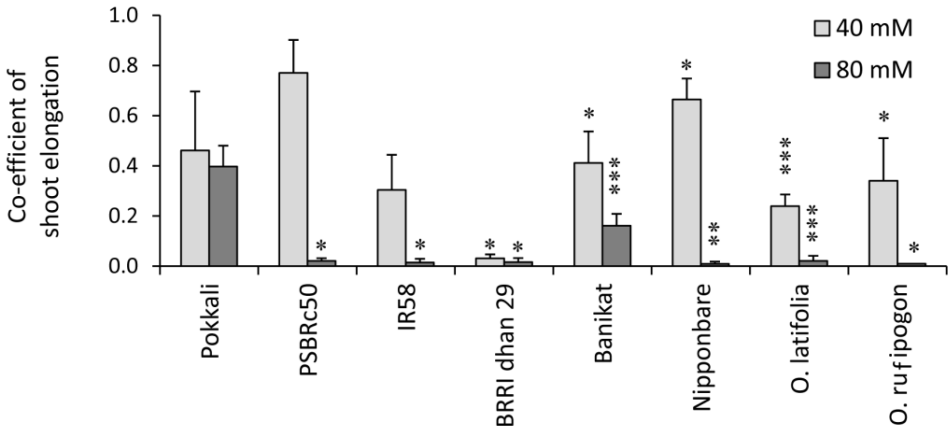


Figure 3.3 The effect of salt stress on coefficient of shoot elongation (ratio of shoot elongation rate in treated to control plants) of the seedlings of eight rice genotypes.

Plants were grown at 28°C (16/8 h light/dark). Data is presented as means ± S.E (n=3). The statistically significant differences determined by t-test using the actual rate of shoot elongation data (not shown) are *Indica*ted by * (P<0.05), ** (P<0.01) and *** (P<0.001).

3.2.3 Qualitative assessment

The visual evaluation scores, Standard Evaluation Score, SES and Leaf Rolling Score, LRS showed significant variation in response to salt stress among the rice genotypes. Both SES and LRS increased with the increase in stress level *Indica*ting greater susceptibility at higher stress level (Table 3.1). Under moderate and high salt, the *Indica* cultivar Pokkali showed a higher degree of tolerance (SES = 1.67 & 3, respectively) followed by PSBRc50 (SES = 2.33 & 5, respectively). The *Indica* variety BRRI dhan29 at both salt levels and the wild species at the high salt level were found to be the most susceptible. BRRI dhan29, Nipponbare and *O. latifolia* showed the highest LRS at high salt stress while Pokkali showed the least at both the salt stresses (2.0 and 2.7 at 40 & 80mM NaCl, respectively) followed by PSBRc50 (2.3) at moderate and IR58 (3.7) at high salt stress.

Table 3.3 Genotypic variation in percent reduction of growth traits in rice seedlings under 40 (A) and 80 mM (B) NaCl stress

Genotypes	% redution							
	LER	**RER**	**SER**	**Leaf Area**	**TR**	**Plant height**	**Root length**	**Bio mass**
(A) Under 40 mM NaCl stress								
Pokkali	36.1^{a}	25.0^{a}	53.9^{abc}	19.9^{bcd}	12.0^{a}	6.0^{c}	10.2^{abc}	15.7^{c}
PSBRc540	42.8 ^{a}	62.5^{ab}	23.0^{c}	6.2^{d}	4.1^{a}	20.9^{bc}	12.2^{ab}	28.1^{bc}
IR58	63.9^{a}	26.2^{bc}	69.6^{ab}	15.7^{dc}	-41.1^{a}	32.0^{ab}	14.5^{ab}	26.8^{bc}
BRRI dhan 29	80.6^{a}	90.5^{a}	96.9^{a}	43.8^{abc}	-23.5^{a}	42.2^{a}	16.0^{ab}	34.7^{bc}
Banikat	55.5^{a}	52.9^{b}	58.9^{abc}	39.9^{abc}	-1.8^{a}	22.8^{abc}	25.1^{a}	47.4^{ab}
Nipponbare	63.9^{a}	33.3^{bc}	33.6^{bc}	44.3^{abc}	2.5^{a}	23.0^{abc}	-2.1^{bc}	45.3^{ab}
O. latifolia	68.5^{ab}	36.3^{bc}	76.1^{ab}	54.9^{a}	-34.3^{a}	41.2^{a}	-11.9^{c}	67.3^{a}
O. rufipogon	45.9^{a}	44.4^{b}	66.0^{abc}	51.1^{ab}	2.3^{a}	42.3^{a}	27.3^{a}	40.2^{abc}
(B) Under 80 mM NaCl stress								
Pokkali	44.4^{b}	63.2^{bc}	60.3^{c}	47.8^{b}	-2.7^{a}	17.0^{d}	27.5^{a}	26.7^{e}
PSBRc540	54.6^{ab}	57.7^{c}	97.9^{a}	56.0^{b}	1.1^{a}	44.2^{ab}	23.1^{ab}	54.7^{bcd}
IR58	70.4^{ab}	80.6^{ab}	98.5^{a}	60.0^{ab}	-76.6^{a}	36.3^{bc}	20.1^{ab}	39.8^{ed}
BRRI dhan 29	91.7^{a}	93.3^{a}	98.4^{a}	69.3^{ab}	11.9^{a}	44.7^{ab}	23.5^{ab}	45.8^{cd}
Banikat	77.8^{ab}	96.7^{a}	88.9^{b}	62.8^{ab}	-17.4^{a}	30.4^{c}	28.9^{a}	59.6^{bc}
Nipponbare	88.9^{a}	81.0^{ab}	99.1^{a}	93.8^{a}	33.9^{a}	44.8^{ab}	13.7^{ab}	70.3^{ab}
O. latifolia	85.2^{ab}	81.0^{ab}	97.9^{a}	83.2^{ab}	-4.3^{a}	55.7^{a}	-1.1^{b}	78.2^{a}
O. rufipogon	83.6^{ab}	94.4^{a}	99.8^{a}	69.9^{ab}	-10.5^{a}	53.7^{a}	27.2^{a}	67.6^{ab}

Data presented as the mean (n=3) and different letters in a column *Indica*te significant differences at *P* ≤0.05 by Duncan's Multiple Range Test (DMRT); (LER) leaf elongation rate, (RER) root elongation rate, (SER) shoot elongation rate, (PH) plant height, (RL) root length, (TR) transpiration rate.

3.2.4 Physiological characterization: tissue ion analysis

Na^+ concentration and the Na^+/K^+ ratio in the shoot increased significantly at higher stress levels in the majority of genotypes (Figure 3.4). At both moderate and high salt levels, Pokkali accumulated least Na^+ (200 ± 6.1 and 201 ± 5 mmol kg^{-1}DW, respectively) and maintained lowest Na^+/K^+ (0.21 and 0.23, respectively) in the shoot followed by PSB Rc50 (241 ± 9 mmol kg^{-1}DW) at moderate, and IR58 (318 ± 11 mmol kg^{-1}DW) at high salt levels. Wild species *O. rufipogon* (548 ± 12 mmol kg^{-1}DW) followed by *O. latifolia* (494 ± 9 mmol kg^{-1}DW) accumulated the highest Na^+ in the shoot at moderate salt level; at high salt level, however, Nipponbare (860 ± 13 mmol kg^{-1}DW) followed by *O. rufipogon* (715 ± 13 mmol kg^{-1}DW) showed the highest accumulation. Shoot Na^+/K^+ was highest in the wild species and BRRI dhan29 at both salt levels. Shoot K^+ decreased in PSBRc50, IR58 and the wild species and increased in Pokkali, Banikat and Nipponbare with the increase in stress level. However, only the increase in Pokkali and the decrease in PSBRc50 at high salt level were significant (Figure 3.4).

Root Na^+ and Na^+/K^+ increased significantly with the increase in salt stress in all genotypes (Figure 3.5). *O. rufipogon* accumulated the least root Na^+ (153 ± 9 mmol kg^{-1}DW), however, Nipponbare maintained least Na^+/K^+ (0.87) in the root at moderate salt level. At high salt level, PSBRc50 (247 ± 12 mmol kg^{-1}DW) followed by Pokkali (257 ± 6 mmol kg^{-1}DW) accumulated the least root Na^+ and also maintained least Na^+/K^+ (1.84 and 2.14, respectively). BRRI dhan29 (347 ± 11 mmol kg^{-1}DW) and Banikat (595 ± 13 mmol kg^{-1}DW) accumulated the highest Na^+ in the root at moderate and high salt levels, respectively. Like shoot Na^+/K^+, root Na^+/K^+ was also high in the wild species and BRRI dhan29 at both salt levels (Figure 3.5). Pokkali was a moderate accumulator of root Cl^- but translocated least Cl^-

to the shoot compared to other genotypes (Figure 3.4 & Figure 3.5). The situation was the reverse in *O. rufipogon* which accumulated least Cl^- in the root but translocated highest Cl^- in the shoot. The concentration of Ca^{2+} was found to be increased in both root and shoot of all genotypes with the increase in stress levels while no such clear trend was observed for Mg^{2+} and NH_4^+ (Figure 3.6). Along with the Cl^-, the concentrations of anions: PO_4^{3-}, NO_3^- and SO_4^{2-} were also measured as the anion chromatographic column detects these anions automatically. These data however were not critically evaluated but shown in Figure 3.7 for any future application.

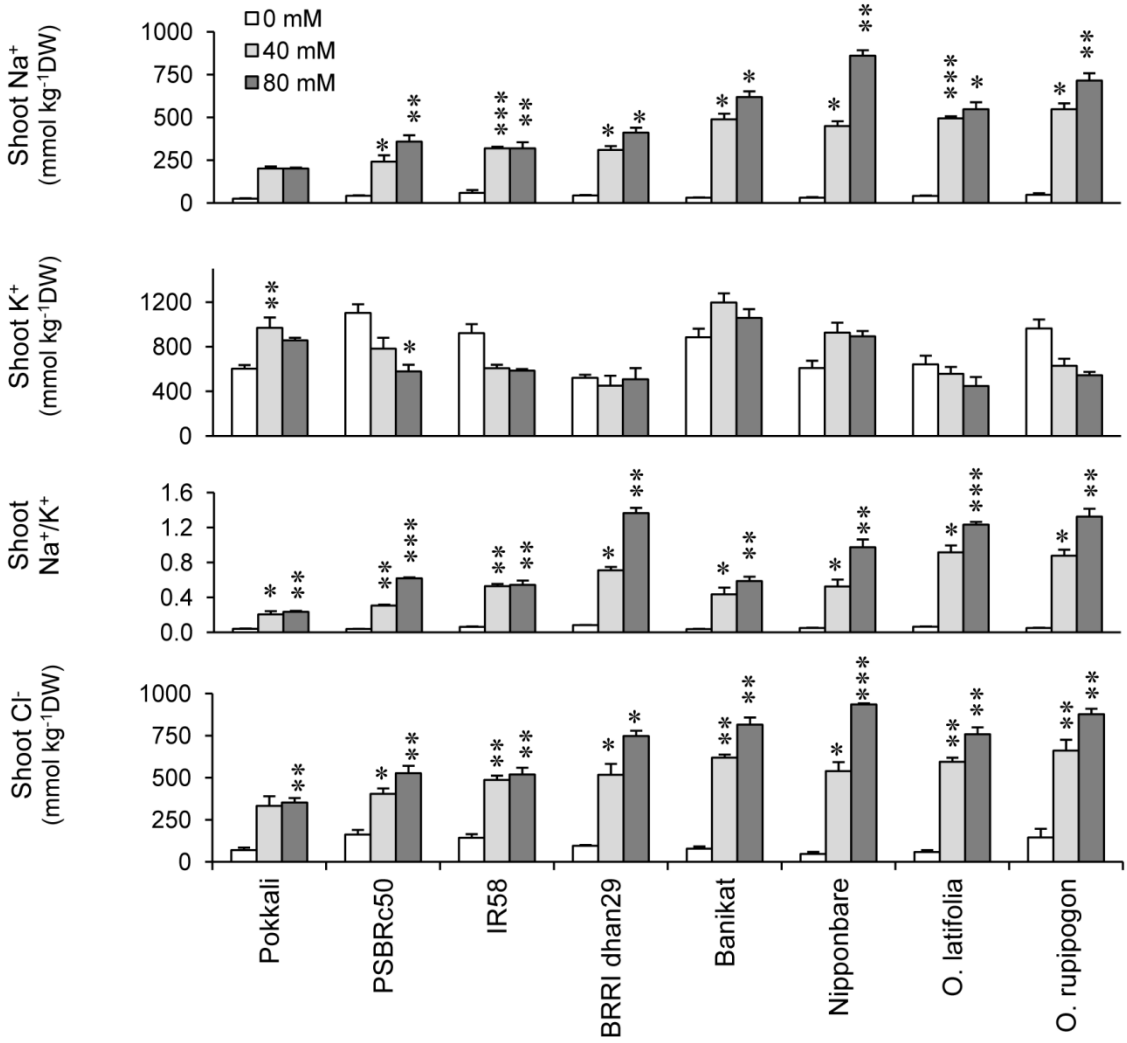


Figure 3.4 Genotypic differences in the concentrations of shoot Na^+, K^+, Cl^- and Na^+/K^+ of 0, 40 and 80mM NaCl stressed rice seedlings.

Plants were grown at 28/20°C day/night temperature and 16/8 h light/dark period and were challenged with NaCl stress at 14 DAE. Shoot tissue was harvested at 7 DAS and the chromatographic analysis was carried out using Dionex® DX500 system. Data presented as mean ± SE (n=3). Significant differences with control at *P* ≤0.05, *P* ≤0.01 and *P* ≤0.001 are *Indica*ted by *, ** and ***, respectively by student's t-test. (DAE) days after emergence, (DAS) days after salt treatment.

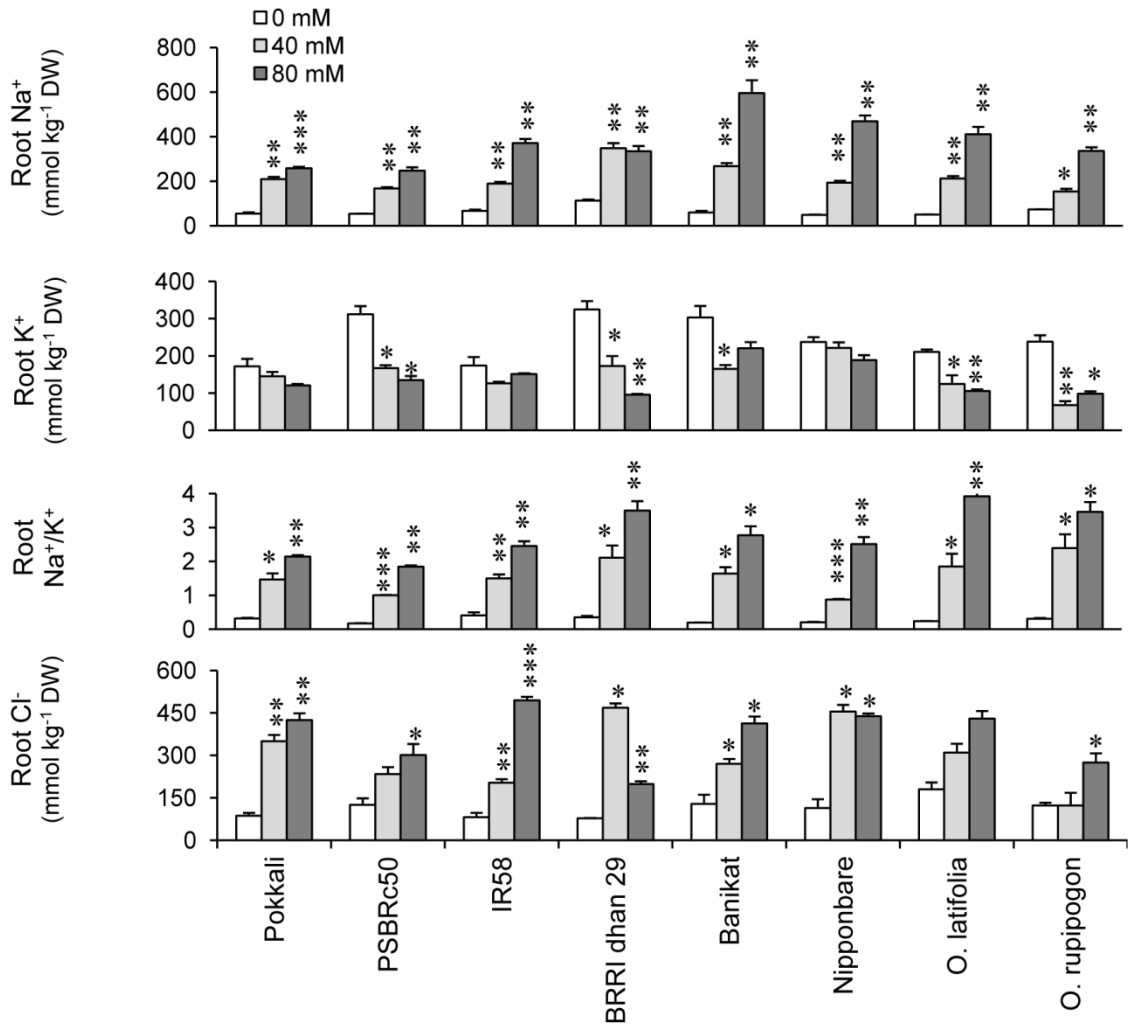


Figure 3.5 Genotypic differences in the concentrations of root Na^+, K^+, Cl^- and Na^+/K^+ of 0, 40 and 80mM NaCl stressed rice seedlings.

Plants were grown at 28/20°C day/night temperature and 16/8 h light/dark period and were challenged with NaCl stress at 14 DAE. Root tissue was harvested at 7 DAS and the chromatographic analysis was carried out using Dionex® DX500 system. Data presented as mean ± SE (n=3). Significant differences with control at *P* ≤0.05, *P* ≤0.01 and *P* ≤0.001 are *Indica*ted by *, ** and ***, respectively by student's t-test. (DAE) days after emergence, (DAS) days after salt treatment.

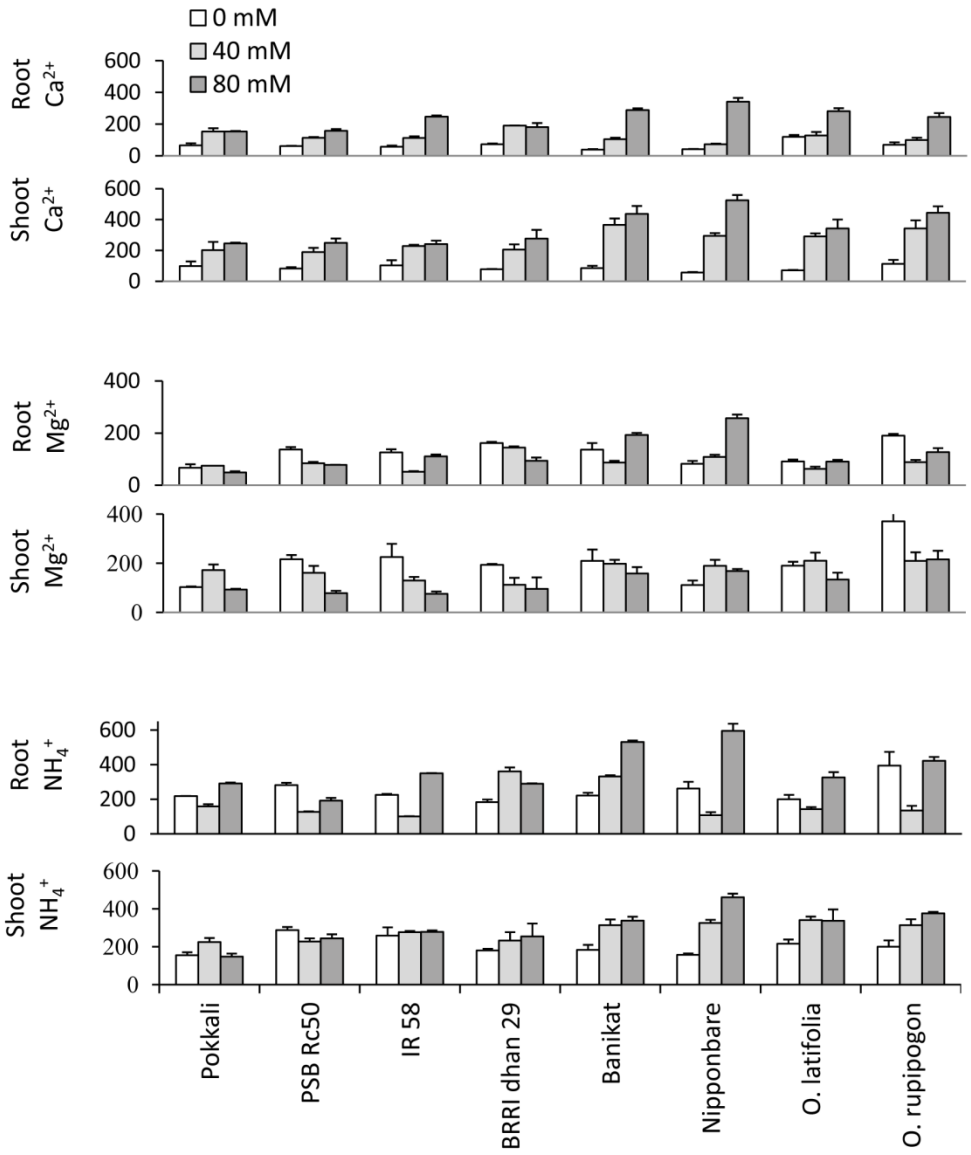


Figure 3.6 Genotypic variation in the concentrations of Ca^{2+}, Mg^{2+} and NH_4^+ (mmol kg^{-1} DW) in roots and shoots of 0, 40 and 80 mM NaCl stressed seedlings of eight rice genotypes.

Plants were grown at 28/20°C day/night temperature and 16/8 h light/dark period and were challenged with NaCl stress at 14 DAE. Shoot tissue was harvested at 7 DAS and ion analysis was carried out using Dionex® DX500 system. Data is presented as means ± S.E (n=3); (DAE) days after emergence, (DAS) days after salt treatment.

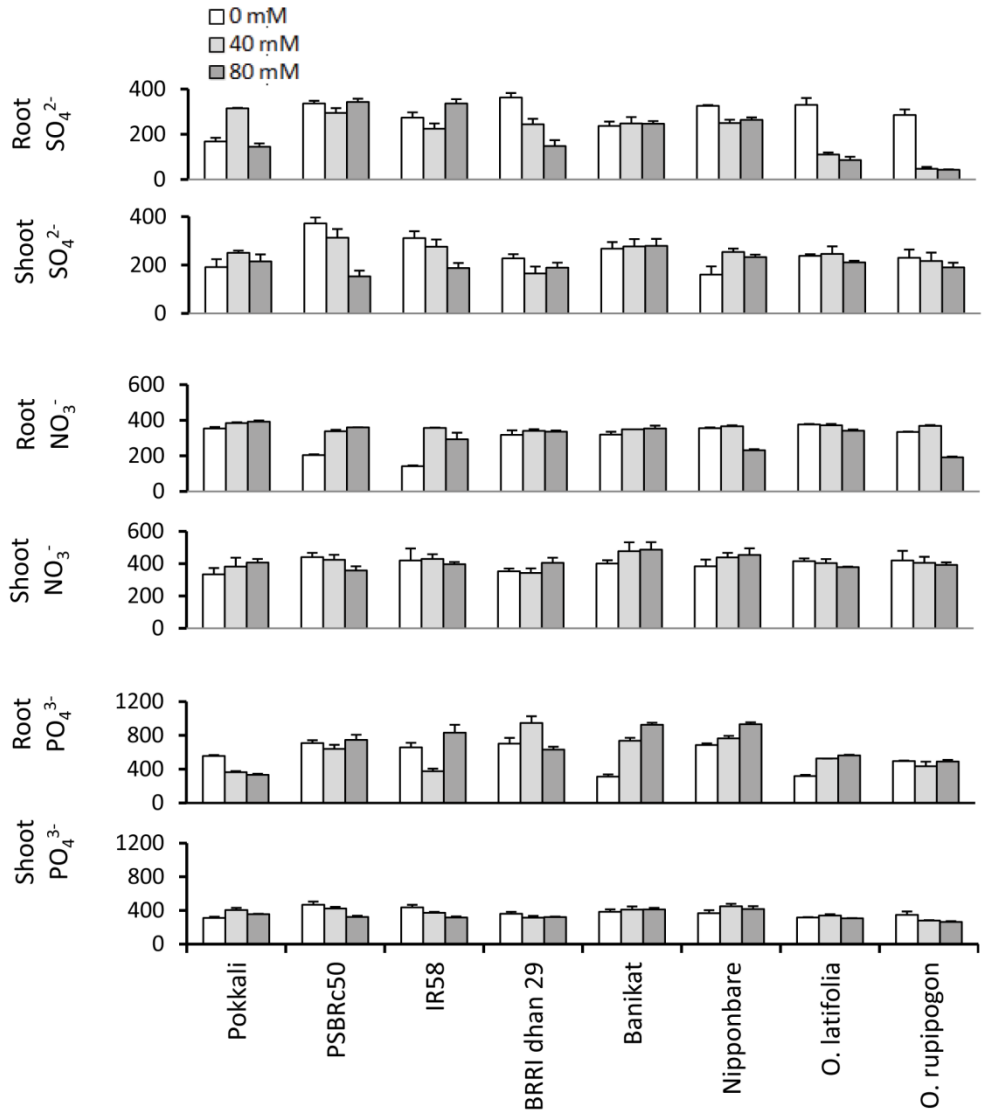


Figure 3.7 Genotypic variation in the concentrations of PO_4^{3-}, NO_3^- and SO_4^{2-} (mmol kg^{-1} DW) in roots and shoots of 0, 40 and 80 mM NaCl stressed seedlings of eight rice genotypes.

Plants were grown at 28/20°C day/night temperature and 16/8 h light/dark period and were challenged with NaCl stress at 14 DAE. Shoot tissue was harvested at 7 DAS and ion analysis was carried out using Dionex® DX500 system. Data is presented as means ± S.E (n=3); (DAE) days after emergence, (DAS) days after salt treatment.

3.2.5 Multivariate assessment of genotypic variability

3.2.5.1 Pearson's Correlation Analysis

Significant correlation was observed among the majority of the traits except for root length at both salt stresses and leaf elongation rate and root Cl^- at high salt stress. However, at 40 mM NaCl stress, leaf elongation rate showed significant negative correlation with root Cl^- and shoot elongation rate showed significant negative correlation with root Na/K . It *Indica*tes that root Na^+ and Cl^- hinder the elongation of leaf and shoot at moderate salt level. However, other growth and physiological parameters were not significantly affected by root Na^+ and Cl^- at moderate salt level. The tolerance parameters like shoot Na/K, standard evaluation score and leaf rolling score (which are significantly positively correlated within themselves) showed significant negative correlation with the growth parameters like plant height, leaf area and total biomass at both salt stresses. It clearly *Indica*tes that shoot Na/K can be considered as the single most important physiological parameter to study salinity tolerance in rice as it evidently restricts the plant growth.

Table 3.4 Correlation matrix of the studied parameters under 40 mM and 80 mM NaCl stressed seedlings of eight rice genotypes

Parameters	LER	RER	SER	Plant Height	Root Length	Biomass	Leaf Area	TR	SES	LRS	Shoot Na^+/K^+	Shoot Cl^-	Root Na^+/K^+
RER	0.22 *(0.37)*												
SER	0.18 *(-0.12)*	0.11 *(0.24)*											
PH	-0.17 *(0.17)*	0.43 *(0.33)*	0.38 *(0.78*)*										
RL	0.53 *(0.17)*	0.58 *(-0.08)*	0.46 *(-0.52)*	-0.15 *(-0.54)*									
Biomass	-0.04 *(0.22)*	0.45 *(0.37)*	0.22 *(0.88**)*	0.91*** *(0.94***)*	-0.21 *(-0.55)*								
LA	0.09 *(0.40)*	0.26 *(0.42)*	0.22 *(0.82*)*	0.80** *(0.75*)*	-0.15 *(-0.39)*	0.86** *(0.93***)*							
TR	-0.26 *(0.04)*	-0.74* *(-0.81*)*	-0.36 *(-0.41)*	-0.81* *(-0.59)*	-0.19 *(0.23)*	-0.85** *(-0.49)*	-0.68 *(-0.34)*						
SES	-0.16 *(-0.44)*	-0.55 *(-0.78*)*	-0.55 *(-0.67)*	-0.87** *(-0.73*)*	-0.13 *(0.41)*	-0.91** *(-0.84**)*	-0.78* *(-0.88**)*	0.61 *(0.68)*					
LRS	-0.46 *(-0.45)*	-0.30 *(-0.36)*	-0.21 *(-0.74*)*	-0.63 *(-0.86**)*	0.03 *(0.38)*	-0.80* *(-0.95***)*	-0.90** *(-0.93***)*	0.43 *(0.39)*	0.78* *(0.83*)*				
Sht Na^+/K^+	0.16 *(-0.56)*	-0.08 *(-0.49)*	-0.37 *(-0.72*)*	-0.90** *(-0.88**)*	0.37 *(0.38)*	-0.88** *(-0.88**)*	-0.89** *(-0.84**)*	0.65 *(0.49)*	0. 80* *(0.83*)*	0.74* *(0.88**)*			
Sht Cl^-	0.09 *(-0.67)*	-0.05 *(-0.61)*	-0.19 *(-0.42)*	-0.65 *(-0.55)*	0.29 *(0.40)*	-0.57 *(-0.69*)*	-0.77* *(-0.78*)*	0.51 *(0.37)*	0.51 *(0.86**)*	0.62 *(0.8*)*	0.78* *(0.73*)*		
Rt Na^+/K^+	0.18 *(-0.55)*	-0.05 *(-0.63)*	-0.77* *(-0.39)*	-0.70 *(-0.74*)*	-0.11 *(0.31)*	-0.44 *(-0.65)*	-0.56 *(-0.58)*	0.37 *(0.61)*	0.57 *(0.75*)*	0.32 *(0.61)*	0.69 *(0.84**)*	0.54 *(0.58)*	
Rt Cl^-	-0.87** *(0.27)*	-0.26 *(0.07)*	-0.09 *(0.29)*	0.16 *(0.46)*	-0.47 *(0.09)*	-0.11 *(0.33)*	-0.17 *(0.22)*	0.25 *(-0.21)*	0.25 *(-0.16)*	0.52 *(-0.42)*	-0.13 *(-0.57)*	-0.25 *(-o.21)*	-0.28 *(-0.29)*

The values *Indica*te the Pearson's correlation co-efficient (analysed using the Minitab software v.15).
The Pearson's correlation co-efficient values of 40 mM NaCl stress is shown as normal font and that of 80 mM stress is shown in parenthesis and in *Italic* font.
*, ** and *** *Indica*te significant correlation at p<0.05, P<0.01 and P<0.001, respectively.
(LER, RER, SER) leaf, root & shoot elongation rate, respectively, (TR) transpiration rate, (SES) standard evaluation score, (LRS) leaf rolling score.

3.2.5.2 Principal Component Analysis

There were significant differences between genotypes in their responses to salt over a range of parameters. To determine the parameters that contributed most towards the overall genotypic variability, principal component analysis (PCA) was performed on the whole data set. Six principal components with eigenvalues higher than unity were extracted and explained 99.1% and 97% of total variation among the eight rice genotypes at moderate and high salt levels, respectively. The first and second principal components accounted for 49% and 21.7% of total variation, respectively at moderate salt level and 53.6% and 17.6%, respectively at high salt (Table 3.5). A scatter plot of the first and second principal components shows the distribution of the eight rice genotypes (Figure 3.8A1 & A2). The pattern of growth, qualitative and physiological responses of 40 and 80mM NaCl stressed rice seedlings are shown in the scree plot and loading plot of principal components (Figure 3.8B1, B2, C1 & C2). Under both levels of stress, the traits biomass, standard evaluation score, plant height, leaf area, shoot Na^+/K^+, LRS, shoot Cl^- and root Na^+/K^+ contributed most in decreasing order towards the overall variability on PC1 (Table 3.5).

Table 3.5 Component loadings of growth, qualitative and physiological traits at 40 mM (A) and 80 mM (B) NaCl stress and percentage of total variance derived from the principal component analysis

Variables	PC1	PC2	PC3	PC4	PC5	PC6
(A) at 40 mM NaCl stress						
Biomass	0.28	-0.03	-0.07	0.16	-0.15	0.03
Plant height	0.27	-0.08	0.11	0.09	-0.05	-0.12
Leaf area	0.27	-0.05	-0.15	0	0.22	0.16
Root elongation rate	0.13	0.26	0.11	0.37	0.09	-0.37
Shoot elongation rate	0.12	0.09	0.46	-0.33	-0.03	0.2
Leaf elongation rate	0.02	0.37	-0.18	-0.27	0.04	0.15
Root length	-0.02	0.35	0.31	0.02	0.3	0
Root Cl-	-0.03	-0.35	0.23	0.04	0.04	-0.43
Root Na+/K+	-0.19	0.11	-0.39	0.2	-0.22	-0.12
Shoot Cl-	-0.21	0.15	0.14	0.26	-0.28	0.29
Leaf rolling score	-0.25	-0.1	0.27	0.09	-0.08	-0.19
Transpiration rate	-0.26	-0.15	-0.01	-0.07	0.11	0.27
Shoot Na+/K+	-0.27	0.16	0.02	0.09	0.03	-0.09
Standard evaluation score	-0.28	-0.09	-0.09	-0.03	0.12	-0.04
Eigenvalue	11.77	5.22	2.35	1.91	1.43	1.1
% of Total variance	49	21.7	9.8	8	6	4.6
% Cumulative variance	49	70.8	80.6	88.5	94.5	99.1
(B) at 80 mM NaCl stress						
Biomass	0.26	0.12	-0.06	0.14	-0.01	-0.08
Plant height	0.24	0.19	-0.05	0.03	-0.07	0.17
Leaf area	0.26	0.01	-0.04	0.11	-0.01	-0.35
Root elongation rate	0.16	-0.17	0.49	-0.11	0.07	0.13
Shoot elongation rate	0.21	0.24	0.05	0.24	0.16	-0.25
Leaf elongation rate	0.13	-0.26	-0.15	-0.46	-0.19	-0.14
Root length	-0.14	-0.07	0.19	-0.39	-0.17	-0.46
Root Cl-	0.1	0.21	-0.09	-0.51	0.34	0.01
Root Na+/K+	-0.22	-0.02	-0.12	0.23	0.33	-0.28
Shoot Cl-	-0.24	0.23	0.07	0.03	-0.17	0.02
Leaf rolling score	-0.26	-0.02	0.13	-0.01	-0.02	0.16
Transpiration rate	-0.16	-0.04	-0.47	-0.03	-0.05	-0.38
Shoot Na+/K+	-0.26	-0.09	0.02	0.19	0.01	0.02
Standard evaluation score	-0.26	0.09	-0.19	-0.06	-0.01	0.04
Eigenvalue	12.87	4.22	2.07	1.77	1.27	1.07
% of Total variance	53.6	17.6	8.6	7.4	5.3	4.4
% Cumulative variance	53.6	71.2	79.8	87.2	92.5	97

(PC) principal component

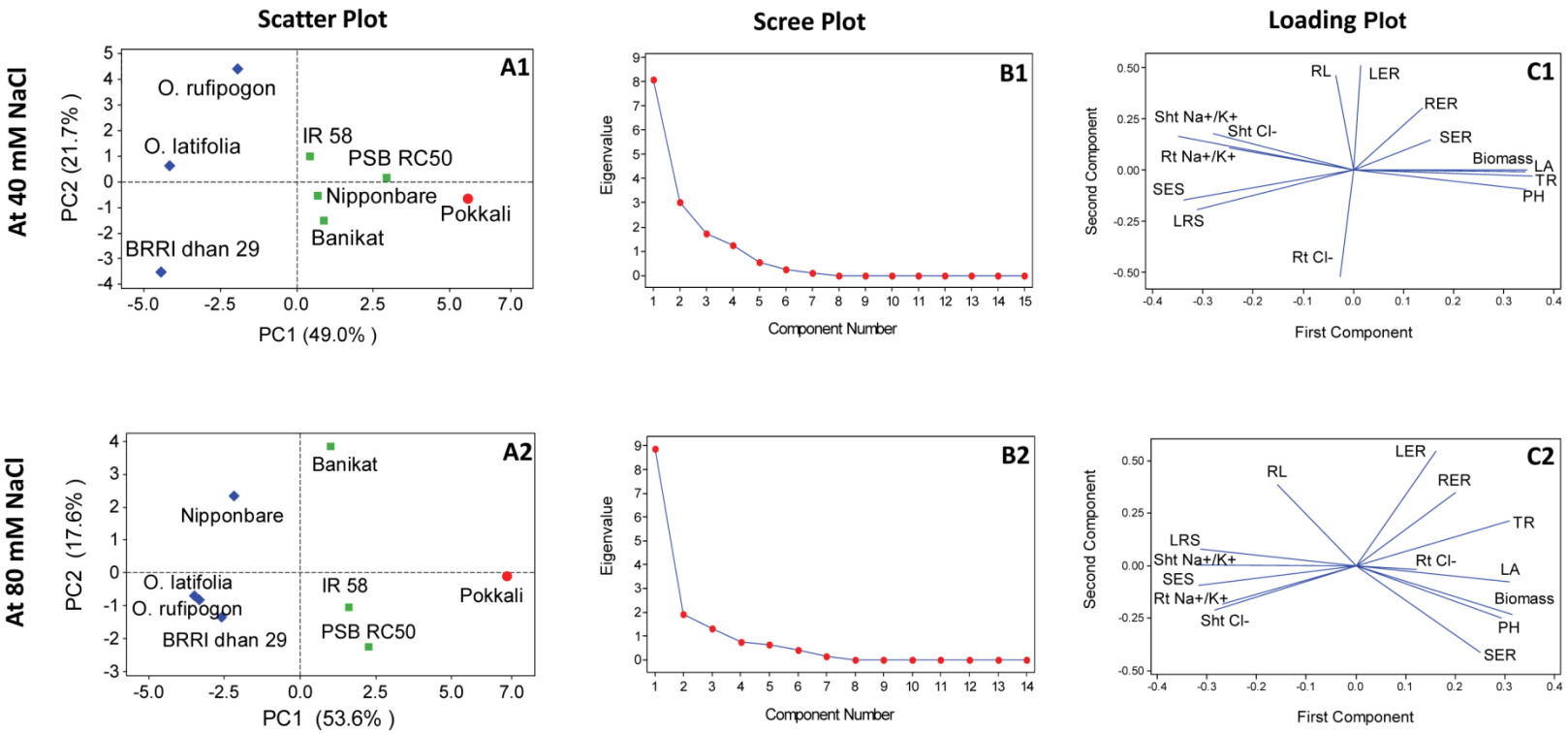


Figure 3.8 Principal component analysis (PCA): scatter plots (A1 & A2) showing the relationships among four *Indica*, two *Japonica* and two wild rice genotypes; scree plots (B1 & B2) showing eigenvalues of principal components and loading plots (C1 & C2) showing the loadings of fourteen growth, qualitative and physiological traits under 40 (A1, B1 & C1) and 80mM (A2, B2 & C2) NaCl stress.

PCA was performed with standardized data (mean subtracted from the variable and then divided by the standard deviation). (RL) root length, (LER) leaf elongation rate, (SER) shoot elongation rate, (RER) root elongation rate, (LA) lear area, (PH) plant height, (RL) root length, (TR) transpiration rate, (Sht) shoot, (Rt) root, (SES) standard evaluation score, (LRS) leaf rolling score.

3.2.5.3 Cluster Analysis

The PCA results were further corroborated by cluster analysis which was done using the traits contributing most to the variation accounted for on PC1 and PC2. The clustering algorithm grouped the eight rice cultivars into three distinct groups at both moderate and high salt level (Figure 3.9). The average inter-cluster distance based on cluster centroids are shown in Table 3.6. The known salt tolerant cultivar Pokkali emerged as the most distinct genotype, forming a distinct cluster at both moderate and high salt levels. The *Indica* genotypes PSBRc50 and IR58 and the *Japonica* genotype Banikat were found together in the same cluster at both moderate and high salt levels and can be categorized as moderately tolerant cultivars. Interestingly, at moderate salt level the *Japonica* cultivar Nipponbare was located within the moderately tolerant group, but at high salt level it emerged as a highly susceptible genotype which *Indica*tes that Nipponbare is tolerant only at moderate salt level. The *Indica* genotype BRRI dhan29 and the wild species *O. latifolia* and *O. rufipogon* were always classified as susceptible genotypes.

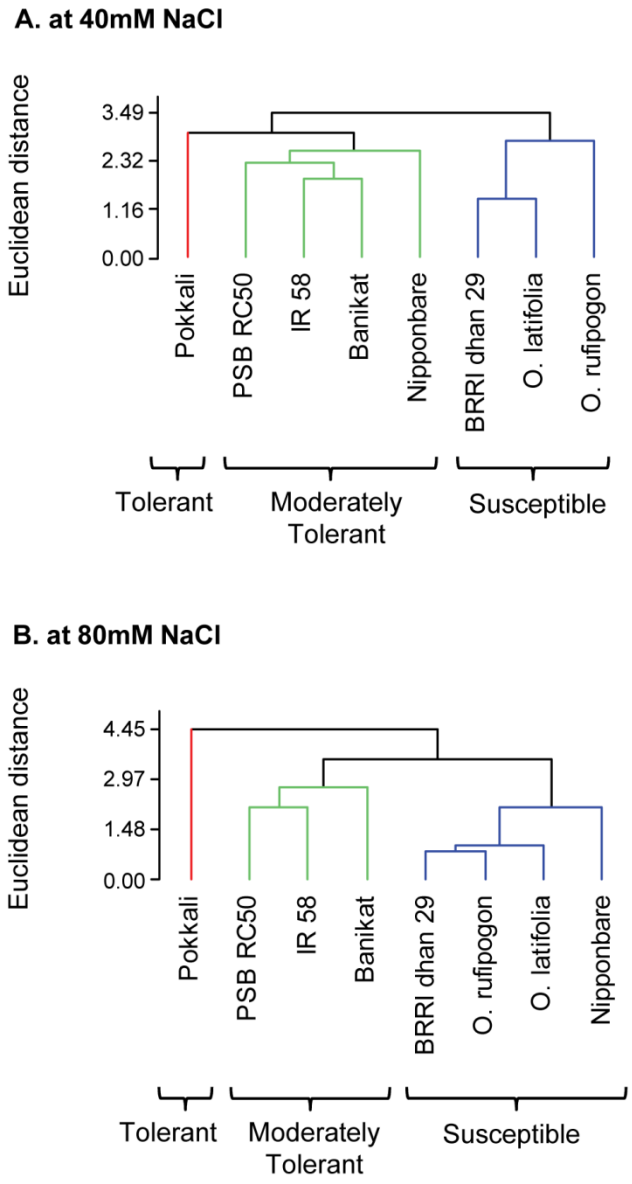


Figure 3.9 Cluster diagram showing the relationships among four *Indica*, two *Japonica* and two wild rice genotypes under 40 (A) and 80mM (B) NaCl stress.

Eight traits, namely biomass, standard evaluation score, plant height, leaf area, shoot Na^+/K^+, LRS, shoot Cl^- and root Na^+/K^+ were identified as the most contributing towards the overall variability of the rice genotypes in PCA and were used for cluster analysis using squared Euclidean distance and Ward's complete linkage method.

Table 3.6 Average inter cluster distance based on cluster centroids of the three clusters of eight rice genotypes under 40 mM (A) and 80 mM (B) Nacl stress.

Average inter cluster distance between the clusters					
(A) at 40 mM NaCl stress			*(B) at 80 mM NaCl stress*		
Cluster	**I**	**II**	Cluster	**I**	**II**
II	4.12		**II**	4.25	
III	7.60	3.91	**III**	7.69	3.68

3.3 Discussion

Unlike previous studies, this study evaluated the comparative salinity tolerance of four *Indica*, two *Japonica* and two wild species of rice using growth, qualitative and physiological traits. Use of multivariate analysis allowed these parameters to categorize the genotypes based on their levels of tolerance and point the way towards a more informative and effective approach to genetically broad-based screening of rice genetic resources. Additionally the approach was able to identify some unexpected responses of some genotypes to salt stress.

Contrasting genotypic differences were observed in response to salt stress. According to SES and LRS, the *Indica* genotypes Pokkali and PSBRc50 showed more tolerance than the two *Japonica* genotypes, and BRRI dhan29 followed by the wild species were most susceptible. Similar trends were observed for percent reduction in leaf, root and shoot elongation rates. At the higher salt level all the genotypes except Pokkali and PSBRc50 showed over 80% reductions in leaf, root and shoot elongation rates. Since growth of rice

has been reported to adapt to the initial osmotic stress within 24 hours of salt stress imposition (Yeo *et al.* 1991), the reduction in growth rate at 3-6 DAS can be attributed to the toxic effects of salt build-up. On this basis Pokkali and PSBRc50 showed greater resistance, a conclusion further strengthened since Pokkali, and PSBRc50 along with the other *Indica* genotype IR58 had the lowest reduction in leaf area, plant height and total biomass.

The concentrations of shoot Na^+, K^+ and their ratio are important physiological traits for studying salinity tolerance (Lee *et al.* 2003; Kader and Lindberg 2005; Zeng 2005). Shoot Na^+ was increased at the higher stress level and varied between the genotypes. In general, *Indica* genotypes accumulated less Na^+ in the shoot, followed by wild species and *Japonica* genotypes. Among the *Indica* genotypes, Pokkali took up less Na^+ and more K^+ in the shoot and hence maintained a low Na^+/K^+ ratio, consistent with greater tolerance. In Pokkali, the concentrations of Na^+ in the root and shoot were similar but the concentration of K^+ was higher in the shoot than in the root, suggesting that increased translocation of K^+ to the shoot maintained a low Na^+/K^+ ratio (Figure 3.4 & Figure 3.5). This observation is consistent with the suggestion that at these salt concentrations Pokkali employs two tolerance mechanisms simultaneously: 'Na^+ exclusion' by restricting the uptake of Na^+ and 'ion balance' by uptaking more K^+ maintaining a low Na^+/K^+ ratio. The other *Indica* genotypes like PSBRc50 and IR58 did not accumulate more K^+ in the leaf, but maintained low Na^+/K^+ by restricting uptake of Na^+ in the shoot. In contrast to Pokkali, 'Na^+ exclusion' is the only mechanism of salinity tolerance employed by these *Indica* genotopes. *Japonica* genotypes Banikat and Nipponbare showed elevated level of Na^+ in

the shoot compared to *Indica* genotypes, but were able to maintain low Na^+/K^+ ratio by translocating more K^+. Previous studies using different *Japonica* varieties, reported that *Japonica* rice lacked the mechanism for high K^+ uptake and that the trait would need to be transferred from *Indica* varieties to achieve salinity tolerance (Lee *et al.* 2003). The current data, however, identifies elevated level of K^+ occurring within the *Japonica* subspecies suggesting that tolerance mechanisms can vary among varieties even within a particular subspecies. This strongly supports the need for enhanced germplasm screening that is precise and high throughput. Our data *Indica*tes that 'tolerance' does not necessarily need to be transferred via *Indica* x *Japonica* crosses, but can be achieved more easily by crossing within *Japonica*.

Principal component analysis was undertaken to determine the overall variation among the rice varieties and to identify combinations of traits that contributed most towards salinity tolerance. Extracting trait combinations using a multivariate approach can facilitate subsequent investigations integrating whole genome expression patterns under salt stress (Pandit *et al.* 2010; Cotsaftis *et al.* 2011; Baisakh *et al.* 2012; Cuin *et al.* 2012). Pokkali diverged from all other varieties in the PCA and formed a distinct group at both moderate and high salt levels. The separation can be attributed to the low reduction in biomass, plant height, leaf area and low shoot Na^+/K^+ and the variety is categorized as the most tolerant among the eight studied varieties. The *Indica* genotypes PSB Rc50 and IR58 and the *Japonica* variety Banikat were all found to be in the same cluster at both moderate and high salt levels and were categorized as moderately tolerant cultivars. It is noteworthy that, at moderate salt level (40mM NaCl) the *Japonica* cultivar Nipponbare

was located in the moderately tolerant group, but at high salt level (80mM NaCl) it had moved to the susceptible group. This is explained by the greater decrease in percent reduction in shoot elongation rate, leaf area, plant height, biomass and greater increase in shoot Na^+, Na^+/K^+ and shoot Cl^- under high stress level compared to moderate stress. It is not clear whether this represents a qualitative difference in the salt response of this variety or whether the other genotypes studied would show a similar pattern if tested against even higher salt levels. This response to salt stress has not previously been detected in this widely studied variety. The *Indica* genotype BRRI dhan29 and the wild species *O. latifolia* and *O. rufipogon* always grouped in the third cluster and were therefore categorized as susceptible. However, this classification contrasts with the finding of Nakamura *et al.* (2002) where *O. latifolia* (IRGC100965) was classified as tolerant. The difference between the conclusions of the two studies may be due to the difference in the way treatments have been applied; the Nakamura group stressed plants at 68 d compared to 14 d in the present study, consistent with tolerance arising later in development in this wild species. In addition, the Nakamura group determined salt tolerance solely on survival whereas the current study combined multiple growth and tissue ion traits analysed with multivariate analysis. Our classification of *O. latifolia* along with BRRI dhan29 and *O. rufipogon* as susceptible *Indica*tes that they may not be useful germplasm sources for breeding programmes for salt tolerance, and once again *Indica*tes a need for precise and wider screening of genetic resources in order to improve the efficiency of breeding programmes (MacGill *et al.* 2012).

3.4 Conclusion

In conclusion, we have been able to identify different mechanisms of salt tolerance operating amongst diverse germplasm of rice, that varieties do not necessarily express tolerance or susceptibility consistently between different levels of salt stress, and that in this particular case, wild species of *Oryza* do not hold value for improving crops' tolerance to salt stress *Indica*ting wide crossing with *Indica* subspecies varieties or wild species is not always necessary to transfer tolerance into new varieties of *Japonica* subspecies. We propose that to come to such conclusions, new evaluation methods including multivariate approaches need to be employed as demonstrated in this study, and on a wider scale than has previously been the case (Ford-Lloyd *et al.* 2011). We also suggest that as the salinity tolerance picture is clearly complex, for future analysis of the genes that may be interacting to confer salinity tolerance by way of whole genome expression studies (Flowers and Colmer 2008; Mian *et al.* 2009; Deyholos 2010), dissection of that complexity using multivariate analysis techniques will be essential.

Chapter 4. GLOBAL GENE EXPRESSION AND MORPHO-PHYSIOLOGICAL SALINITY RESPONSE OF DIVERSE RICE GENOTYPES UNDER STRESSED AND UNSTRESSED CONDITIONS DETERMINED BY SIGNIFICANCE ANALYSIS OF MICROARRAYS (SAM)

ABSTRACT

Stress responsive gene expression is commonly profiled in a comparative manner involving different treatments or genotypes with contrasting reputation of tolerance/resistance to that stress. In contrast, this piece of research work exploited a range of natural variation which represents a wide variation in terms of taxonomy, origin and salt sensitivity to identify the gene expression under salt stress. The modified 'Significance Analysis of Microarrays' (SAM) was applied to interrogate the gene expression response by the weighted continuous morpho-physiological trait responses of eight rice genotypes to comprehensively elucidate the trait specific expression of genome wide transcripts. More genes were found to be differentially expressed for the traits under salt stress compared to normal conditions. Shoot Na^+, ratio of shoot Na^+ & K^+, root K^+ and shoot Cl^- being most prominent traits. Strikingly, for 'biomass', a contrasting number of positive and negative genes were expressed under unstressed and stressed conditions, respectively. The result indentify around sixty genes as involved in Na^+, K^+ and anion homeostasis, transport and transmembrane activity under stressed conditions. Gene ontology enrichment analysis identified 1.36% (578 genes) of the entire

transcriptome involved in the major global molecular functions such as signal transduction (>150 genes), transcription factor (81 genes) and translation factor activity (62 genes), protein phosphatase, transferase, hydrolase activity and oxidoreductase activity under salt stress. The chromosomal mapping of the genes suggests that majority of the signal transduction; transcription and translation initiation factors are located on chromosomes 1, 2, 3, 6 & 7. The gene network analysis showed that the transcription factors and translation initiation factors form the major gene networks and are mostly active in nucleus, cytoplasm and mitochondria whereas the membrane and vesicle bound proteins form a secondary network active in plasma membrane and vacuoles. The novel genes identified here provide picture of a synergistic salinity response representing the potentially fundamental mechanisms that are active in the wide natural genetic background of rice. They may hold evolutionary components of adaptive phenomena to cope with the unsuited environments.

4.1 INTRODUCTION and AIM

Abiotic stresses such as soil salinity, water scarcity, elevated temperatures, nutrient deficiency and heavy metal toxicity etc. greatly reduce agricultural productivity worldwide. The yield of rice, one of the major food crops that feed the world, can be reduced by up to 50% making it highly sensitive to soil salinity (Zeng *et al.* 2002). Salinity tolerance, a complex trait both physiologically and genetically, requires a wide range of physiological and biochemical responses upon exposure to stress (Cotsaftis *et al.* 2011; Flowers, 2004; Munns and Tester, 2008; Wu *et al.* 2013). However, the naturally occurring genetic variation across rice varieties, cultivars, landraces and wild species provides the advantage to identify factors such as genes, proteins and metabolites which can then be utilized by conventional breeding and genetic engineering technologies for improvement of tolerance levels (Horie *et al.* 2012; Langridge and Fleury, 2011; Roy *et al.* 2011). The advances in the high throughput multi-omics techniques such as transcriptomics, proteomics, metabolomics, RNA-seq and next generation sequencing etc. along with the progress made in the ever spreading arena of bioinformatics, have given rise to the systems biology approach (Duque *et al.* 2013). This allows the investigation of the natural genotypic variation holistically to gain deeper biological insight on how the plant functions as a whole by discovering the putative functions of genes, proteins and metabolites in a specific biological context by dissecting the complex regulatory networks associated with stress adaptation and tolerance (Mochida and Shinozaki, 2011; Shelden and Roessner, 2013). The global gene expression is described as the function of genotypes that can be charted across tissues and cell types upon stress imposition

(Galbraith and Edwards, 2010). This systems biology approach can provide unique advantages to bridge the physiological and phenotypic observations and the stress dependent genome wide information of genes, transcripts, proteins and metabolites that can reveal the evolutionary adaptive diversification of complex phenomena like biotic and abiotic stress tolerance mechanisms (Feder and Walser, 2005).

This chapter focuses on the holistic application of transcriptomics - the microarray based expression of the whole genome using the Agilent 44K oligonucleotide array for rice under salt stress. Recently there has been a substantial improvement in microarray platforms and work reported related to crop species includes *Arabidopsis* (Kumari *et al.* 2008; Ma *et al.* 2005), barley (Close *et al.* 2004; Ozturk *et al.* 2002), maize (Fernandes *et al.* 2002; Wang *et al.* 2003) and wheat (Clarke and Rahman, 2005) etc. In rice, several microarray platforms were used such as tilling arrays (Jiao *et al.* 2005; Lei *et al.* 2005; Li *et al.* 2006; Stolc *et al.* 2005), subtractive cDNA library (Kumari *et al.* 2009; Sahi *et al.* 2003; Shiozaki *et al.* 2005), cDNA microarrays (Chao *et al.* 2005; Kawasaki *et al.* 2001; Rabbani *et al.* 2003; Ueda *et al.* 2006), NSF 45K 70-mer oligo microarrays (Senadheera *et al.* 2009) and Affymetrix gene chips (Cotsaftis *et al.* 2011; Walia *et al.* 2007a; Walia *et al.* 2009; Walia *et al.* 2007b) to study the salinity and/or drought response, and reported stress responsive genes that are involved in various molecular functions and biological processes such as oxidative stress, signalling, transcription, translation, transporter, primary and secondary metabolism etc. To date, however, use of Agilent 44K microarrays to study the salinity response has not been reported. Also most of the above reports studied two to four genotypes with contrasting responses to salinity stress and analysed the morpho-physiological and gene expression data separately.

In contrast this study, considered the approach in a more holistic way analysing the gene expression data of eight rice genotypes (see section 2.1 and Table 2.1 in chapter 2) together with the morpho-physiological observations that allowed the identification of trait specific gene expression patterns in the global genotypic variation of rice genotypes. The eight rice genotypes used in this study span landraces, cultivars and HYVs with a range of sensitivity to salt stress stretching from susceptible to highly tolerant. Taxonomically they belong to three different rice groups namely, *Indica* (four genotypes), *Japonica* (two genotypes) and wild species (two genotypes) with diverse geographical origins. With the ecological diversity, species distribution and range of salt sensitivity, these rice genotypes can be considered as the representative gene pool of wider natural variability of rice genotypes. A modified SAM (Significance Analysis of Microarrays) approach was used that provides a powerful option to analyze the morpho-physiological and gene expression data simultaneously allowing a more comprehensive understanding of the constitutive and salinity induced gene expression patterns in the seedlings of wide range of rice genotypes by identifying the trait specific significant (positive and negative) genes.

4.2 RESULTS AND DISCUSSION

The gene expression data of the first eight genotypes such as Pokkali, PSBRc50, IR58, BRRI dhan29, Banikat, Nipponbare, *O. latifolia* & *O. rufipogon* (see table 2.1) were analyzed along with their morpho-physiological data using modified SAM (significance analysis of microarrays) approach (see section 2.12.1 for details of the analysis procedure). The unstressed and stressed samples were subjected to separate modified SAM analysis to identify the constitutive and stress induced gene expression pattern in the wide genotypic background of rice, respectively for each of the fourteen morpho-physiological traits.

4.2.1 Globally, more genes are expressed under salt stress

In general, larger numbers of genes were found to be significantly expressed, both positively and negatively due to stress x genotype interaction for most of the important traits, such as shoot Na^+, shoot Na^+/K^+, root K^+, shoot Cl^- and leaf elongation rate compared to that due to constitutive genotypic variation only, except for root Na^+, leaf area and root Cl^- (Figure 4.1). For example, 1275 and 2391 probes were positively and negatively expressed, respectively for shoot Na^+ under stressed condition compared to only 4 & 8 probes being positively and negatively expressed, respectively under unstressed conditions. The scenario was similar with shoot Na^+/K^+, root K^+, root Na^+/K^+, shoot Cl^- and leaf elongation rate. This suggests that plants deploy their adaptive mechanism by differentially expressing more genes under stressed conditions. The reason for the exceptionally low number of differentially expressed probes in unstressed conditions for most of the traits is not clear. It might be because of the fact that

constitutively the variation in these traits are less which resulted in the identification f fewer globally expressed genes or the genotypic differences in constitutive gene expression is diluted and more random when counted globally across all the genotypes.

Under stressed conditions, fewer probes are found to be significantly differentially expressed for the traits such as root Na^+, leaf area, root elongation rate and leaf rolling score which probably *Indica*tes their lesser involvement in the salinity tolerance mechanism globally. The complete lists of the significant probes for each of the traits along with the corresponding fold scores (d), q-values and functional annotations under unstressed and stressed conditions can be found in supplementary tables 4.1a-n & 4.2a-n, respectively.

The exceptionally higher number of positively (+12749) and negatively (-6882) expressed probes for root Cl^- under unstressed condition compared to only 35 positive and 2 negative probes under stress conditions is probably an example of aberrant case which can't be explained at the moment. The case is also similar for root Na^+ with 331 probes expressed negatively under unstressed condition compared to only 1 probe being negatively expressed under stress condition. These probes were, therefore, not subjected to further investigation. Instead, emphasis was given on the important morpho-physiological traits (as mentioned above) and the corresponding significant genes due to stress x genotype interaction.

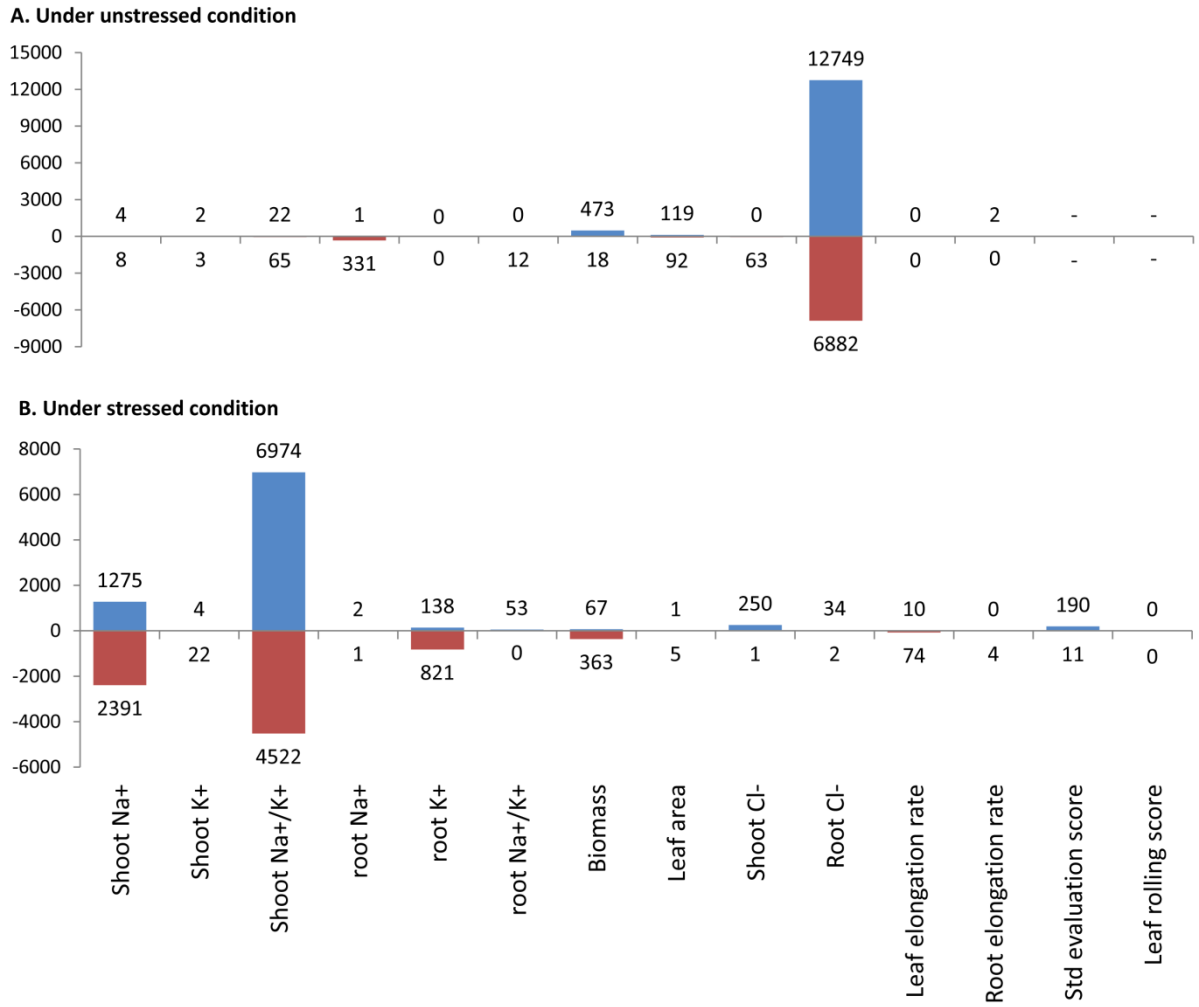


Figure 4.1 Number of significant positively (blue bars) and negatively (red bars) expressed genes for each of the fourteen morpho-physiological traits in the eight rice genotypes representing wide natural variation under unstressed (A) and stressed (B) conditions as determined by the modified SAM approach.

The detailed description of the modified SAM procedure can be found in the section 2.12.1. The analysis for std. evaluation score and leaf rolling score under unstressed conditions wasn't possible as all the genotypes scored the same making weighting of the genotypes impractical. The significant genes were selected based on the criteria of FDR (<5%) and fold score (>2.0). The complete lists of the significant probes along with the corresponding fold score (d), q-value and functional annotation can be found in supplementary table 4.1 & 4.2.

4.2.2 More negatively expressed genes may be responsible for restricting growth (biomass) under salt stress

The genes that are found to be expressed for the trait biomass under both unstressed and stressed conditions were critically evaluated. It is interesting to report that for biomass, one of the most important correlants of salinity tolerance, a larger number of genes (473) were positively expressed (that significantly enriched the GO categories 'Transferase activity' and 'Electron carrier activity') and fewer genes (18) were negatively expressed, under unstressed conditions (Figure 4.1 & Supplementary Table 4.1g). But with the application of salt stress, the scenario was qualitatively changed with a greater number of genes (363) being negatively expressed (that significantly enriched 'Serine hydrolase activity') and fewer (67) being expressed positively (Figure 4.1 & Supplementary Table 4.2g). It *Indica*tes that under stressed condition, genes are expressed negatively in a way to restrict plant growth in wide natural variation of rice genotypes. Only 6 genes being common between the positively expressed and no genes being common between the negatively expressed genes under unstressed and stressed conditions respectively *Indica*ted that globally rice genotypes employ different sets of genes in response to salt stress (Figure 4.2 A1 & A2).

The genes that are expressed significantly with respect to the standard evaluation score (SES), a qualitative assessment trait, showed substantial commonality with the genes that expressed for the trait biomass (Figure 4.2 B1 & B2) *Indica*ting the strength of SAM analysis to identify the corresponding significant genes for the morpho-physiological traits in genetically diverse rice germplasm under salt stress. The lists of the genes along with

corresponding functional annotation and fold scores are shown in supplementary table 4.3.

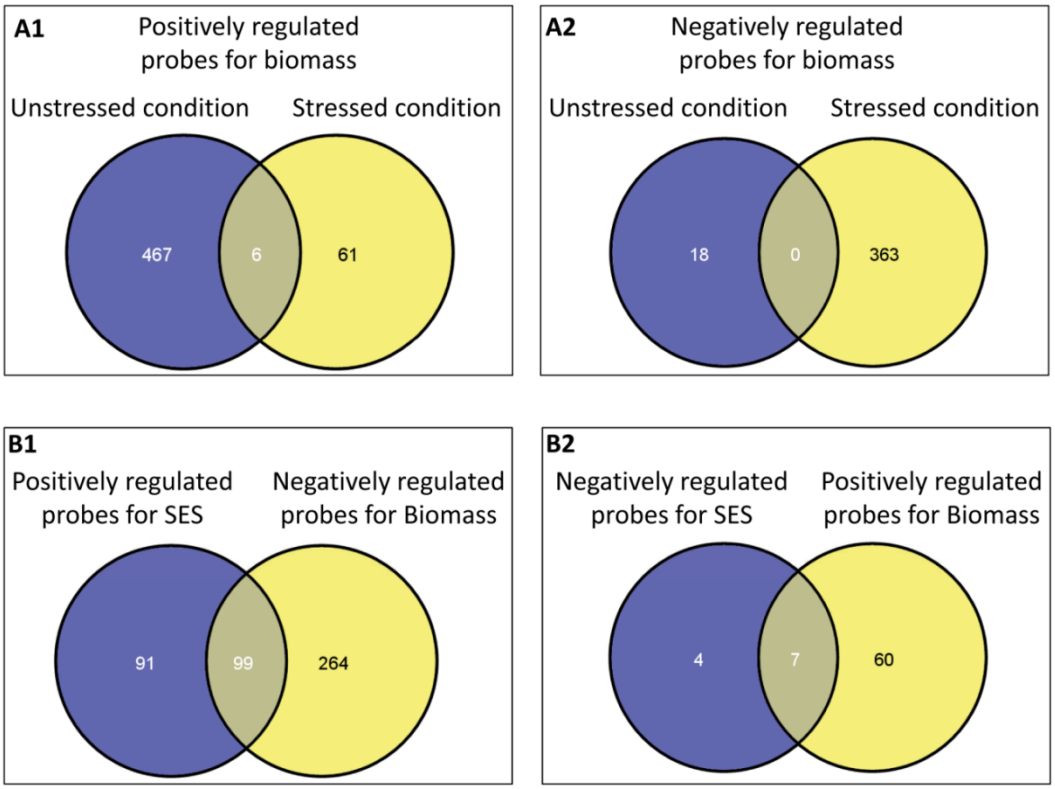


Figure 4.2 The number of positively (A1) and negatively (A2) expressed genes common between stressed and unstressed condition for biomass and showing the positive (B1) and negative (B2) genes that commonly expressed for the traits, standard evaluation score (SES) and Biomass under stressed condition. The lists of the genes along with corresponding functional annotation and fold scores are shown in supplementary table 4.3.

Genes that are differentially expressed for tissue ion concentrations were also compared with the genes that are expressed (both positively and negatively) for biomass to comprehend if there is any commonality. It was observed that 28 common genes along with 17 and 276 unique genes that were positively expressed for shoot Na^+ and Na^+/K^+, respectively were negatively expressed for biomass meaning that these genes may contribute to accumulate toxic Na^+ in shoots which negatively affected the biomass under stressed condition (Figure 4.3A1). The reverse was true also, with 4 common genes along with 3 and 38 unique genes that were found to be negatively expressed for the traits, shoot Na^+ and Na^+/K^+, respectively were found to be positively expressed for biomass (Figure 4.3A2). The 59 genes that were positively expressed for shoot Cl^-, another potentially toxic ion, were negatively expressed for biomass (Figure 4.3C1). The lists of the genes along with corresponding functional annotation are shown in supplementary table 4.4.

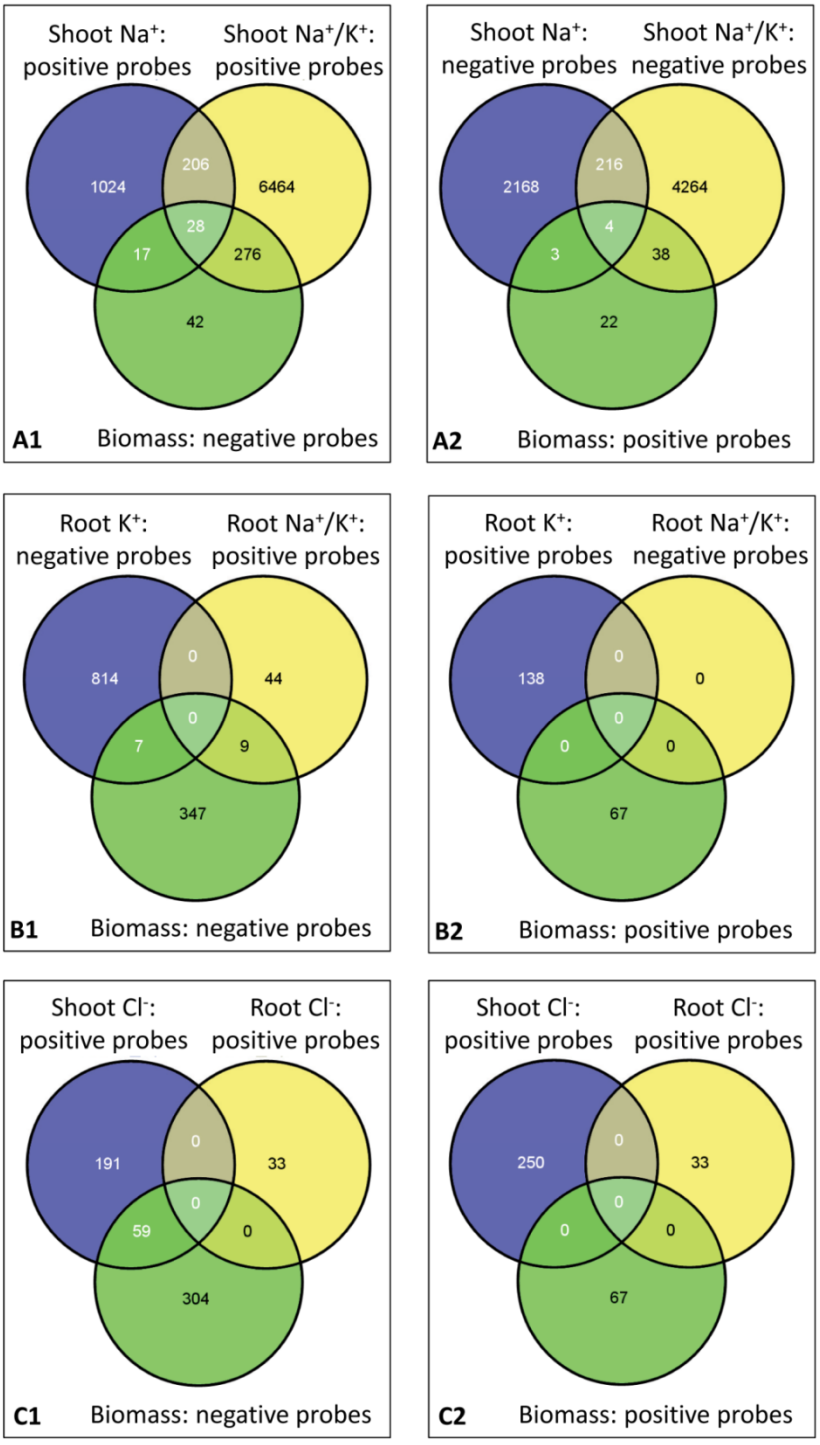


Figure 4.3 Venn diagrams showing the number of positive and negative genes that are commonly expressed for biomass and tissue ions under stress condition in wide natural variation of rice genotypes. The lists of the genes along with corresponding functional annotation are shown in supplementary table 4.4.

4.2.3 Genes involved in ion homeostasis and transport

Before starting the gene ontology analysis, the lists of genes were mined to identify the putative genes that might be involved in ion transport with particular attention being given to the genes that were found to be significant for shoot Na and shoot Na/K in the wide natural variation of rice genotypes by modified SAM analysis (see section 2.12.1). In total, 60 genes were found to be involved in ion homeostasis and transport processes (Table 4.1). The cation transporter family protein (Os06g0701600) and cation/proton exchanger (Os01g0557500) are found to be negatively expressed for shoot Na. Among the genes involved in sodium homeostasis, the notables are Na+/H+ exchangers (Os09g0286400, Os05g0382200, Os11g0648000 and Os12g0641100) and around 10 genes (e.g., Os03g0656500, Os07g0102100, Os03g0337500, Os01g0932500, Os02g0519100, Os03g0575200 and Os04g0682800 etc.) were found to be involved in potassium transport. Genes for other cations such as Ca^{2+}, Mg^{2+} and anions such as ammonium, nitrate, sulphate and phosphate were also significantly expressed in wide natural variation of rice genotypes under salt stress. Several membrane intrinsic and ion channel related genes having putative roles in ion homeostasis were also found to be significantly expressed e.g., aquaporin (Os09g0541000); membrane transporter (Os01g0704100), tonoplast integral protein (Os01g0975900, Os05g0231700); vesicle associated membrane protein (Os12g0639800) and ion channels (Os04g0643600, Os06g0527400, Os02g0255000, Os03g0758300, Os02g0117500 and Os01g0588200).

Among the significant genes for shoot Na, 15 genes were found to be involved in ABC transport (not shown in table). These genes are Os01g0290800, Os01g0356000, Os01g0609200, Os01g0609300, Os01g0966100, Os02g0189800, Os03g0142800, Os03g0332700, Os04g0194500, Os05g0120000, Os07g0522500, Os08g0384500, Os09g0472100, Os09g0572400 and Os10g0205500. The ABC-transporter proteins are believed to transport various substrates such as ions, amino acids, sugars and peptides across cellular membranes besides their role in detoxification, plant growth and developmental processes (Davidson *et al.* 2008; Hall, 2002; Hasegawa *et al.* 2000; Martinoia *et al.* 2002). In yeast, ABC transporters are found to be involved in cation homeostasis (Miyahara *et al.* 1996) but their role in plants is yet to be identified (Kang *et al.* 2010; Rea, 1999). Nine other genes (Os01g0945300, Os03g0576900, Os04g0201500, Os04g0460300, Os04g0674600, Os06g0125400, Os06g0125500, Os04g0597800 and Os03g0719900) were found to be involved in amino acid or peptide transport and 10 genes (Os01g0226600, Os02g0160400, Os02g0574000, Os02g0827200, Os03g0170900, Os03g0197100, Os03g0218400, Os03g0363500, Os04g0678900 and Os06g0523400) were found to be involved in sugar transport.

Table 4.1 Lists of genes (among the significant genes for (a) shoot Na^+ and (b) shoot Na^+/K^+) involved in ion homeostasis and transports under salinity stress in wide natural variation of rice genotypes.

Name	**Annotation**	***(a) among the significant genes for shoot Na^+***		***(b) among the significant genes for shoot Na^+/K^+***	
		Fold score	q-value (%)	Fold score	q-value (%)
Os01g0557500	Cation/proton exchanger 1a.	-2.48	3.03		
Os01g0645200	Bile acid:sodium symporter family protein.	-2.35	3.35		
Os05g0382200	Na+/H+ exchangeing protein-like.			2.02	3.29
Os06g0152200	Salt-tolerance protein.	-2.11	4.88		
Os06g0701600	Cation transporter family protein.	-2.96	1.30		
Os08g0503700	Sodium/sulphate symporter family protein.			-2.26	2.81
Os09g0286400	Sodium/hydrogen exchanger family protein.			2.25	2.34
Os09g0299400	Sodium-and chloride-activated ATP-sensitive potassium channel.			-2.72	1.79
Os09g0484900	Sodium-dicarboxylate cotransporter-like.			-2.01	3.89
Os10g0436900	Sodium/calcium exchanger membrane region domain containing protein.	-2.13	4.88		
Os11g0648000	Sodium/hydrogen exchanger subfamily protein.			2.13	2.81
Os12g0170300	Bile acid:sodium symporter family protein.			2.20	2.34
Os12g0641100	Sodium/hydrogen exchanger family protein.			-2.64	1.79
Os01g0210700	Potassium channel (Fragment).	-2.08	4.88		
Os01g0369300	Potassium transporter 1 (AtPOT1) (AtKUP1) (AtKT1). Splice isoform 2.	-2.11	4.88		
Os01g0648000	Potassium channel.			2.64	1.36
Os01g0696100	K+ channel, two pore family protein.			2.33	1.79
Os01g0932500	K+ potassium transporter family protein.			2.65	1.36
Os02g0519100	K+ potassium transporter family protein.			2.40	1.79
Os02g0612700	K+ channel tetramerisation domain containing protein.	-2.52	2.51		
Os03g0337500	K+ potassium transporter family protein.	-3.57	0.71		
Os03g0575200	K+ potassium transporter family protein.			1.88	3.89
Os03g0656500	K-exchanger-like protein.	-2.24	3.62		
Os04g0401700	Potassium transporter 5 (AtPOT5) (AtHAK1) (AtHAK5).			-2.15	3.29
Os04g0682800	Potassium efflux system protein family protein.			-2.28	2.81
Os06g0625900	Potassium transporter 8 (AtPOT8) (AtHAK8).	-2.94	1.44		
Os06g0671000	Potassium transporter 1 (AtPOT1) (AtKUP1) (AtKT1).			-2.22	2.81

Os07g0102100	K+ potassium transporter family protein.	2.85	1.92		
Os07g0669700	Potassium transporter 4 (AtPOT4) (AtKUP3) (AtKT4).	-3.17	0.93		
Os01g0678500	Two-pore calcium channel.	-2.15	4.88		
Os01g0908500	Mg2+ transporter protein, CorA-like family protein.	-2.13	4.88		
Os02g0138900	Low affinity calcium antiporter CAX2.	-2.54	2.51		
Os02g0720700	Cl- channel, voltage gated family protein.			1.99	3.29
Os04g0605500	Calcium-transporting ATPase 8, plasma membrane-type (EC 3.6.3.8)	-2.93	1.44		
Os04g0653200	Low affinity calcium transporter CAX2 (Fragment).			1.88	3.89
Os05g0594200	Calcium/proton exchanger superfamily protein.	2.65	3.03		
Os03g0150800	High affinity phosphate transporter 2 (Phosphate transporter).			2.91	0.88
Os03g0161200	Sulfate transporter 3.1 (AST12) (AtST1).	-2.95	1.30		
Os03g0195800	High affinity sulphate transporter.			3.62	0.38
Os03g0838400	Ammonium transporter.	-2.99	1.30		
Os04g0185600	Phosphate transporter 6.	-3.65	0.71		
Os05g0477800	High-affinity sulfate transporter HvST1.			2.69	1.09
Os08g0155400	Nitrate transporter (Fragment).	-2.67	2.15		
Os08g0406400	Sulfate transporter (Fragment).	2.67	2.51		
Os09g0240500	Sulfate transporter 4.1, chloroplast precursor (AST82).	-2.26	3.62		
Os10g0444600	Phosphate transporter (Fragment).	-2.50	2.51		
Os01g0588200	Voltage-dependent anion channel.			2.78	1.09
Os01g0704100	Membrane transporter.	-2.52	2.51		
Os01g0975900	Tonoplast membrane integral protein ZmTIP1-2.	3.31	0.80		
Os02g0117500	Glutamate receptor 3.2 precursor (Ligand-gated ion channel 3.2) (AtGluR2).			3.38	0.39
Os02g0255000	Cyclic nucleotide-gated ion channel 1 (AtCNGC1)			2.09	2.81
Os02g0823100	Plasma membrane intrinsic protein (Plasma membrane integral protein ZmPIP1-5	-2.61	2.15		
Os03g0129100	Seven transmembrane protein MLO2.	-2.78	1.92		
Os03g0758300	Cyclic nucleotide-gated ion channel 2 (AtCNGC2)	-2.35	3.35		
Os04g0643600	Cyclic nucleotide-gated channel C (Fragment).	-2.13	4.88		
Os05g0231700	Tonoplast membrane integral protein ZmTIP4-2.	3.35	0.67		
Os06g0527400	Cyclic nucleotide-gated calmodulin-binding ion channel.			1.84	3.89
Os08g0555000	Transmembrane 9 superfamily protein member 2 precursor (p76).	2.83	1.92		
Os09g0541000	Plasma membrane intrinsic protein 2c, (PIP2c, TMP2C, RD28-PIP, WSI-TIP	2.56	3.35		
Os12g0639800	Vesicle-associated membrane protein 722 (AtVAMP722)	-2.70	1.92		

4.2.4 Gene Ontology Enrichment

The Gene Ontology analysis was carried out using Singular Enrichment Analysis (SEA) web based tool (see section 2.13) to identify the biological processes or molecular functions that are significantly enriched by the identified positively or negatively expressed genes for each of the traits. However, it was not possible to carry out successful GO analysis for the traits for which there were only a few positive or negative probes as the number of genes were not sufficient for GO analysis. The detailed results of the GO analysis (lists of significant GO categories of molecular functions and biological processes along with GO terms, p-values, FDR values, GO flash charts and schematic diagrams) are shown in supplementary table 4.5 & 4.6 and the significantly enriched molecular functions (MFs) and biological processes (BPs) along with the numbers of corresponding genes are simplistically compiled in Table 4.2-4.5. The genes mentioned in the Table 4.2–4.5 are described in the following sections.

4.2.5 Global regulation of biological processes (BP) under salt stress

More biological processes (BP) are significantly enriched by the induced genes than by the constitutive genes (Table 4.3 & 4.5). Positively expressed constitutive genes for biomass and leaf area significantly enriched BPs such as developmental process, apoptosis, response to abiotic stimulus and oxidation reduction (Table 4.3), whereas no BPs were enriched by the induced genes for biomass and leaf area (Table 4.5). Under unstressed conditions, no BPs were significantly enriched by the genes that are expressed for shoot Na^+ and shoot Na^+/K^+ (Table 4.3), whereas most of the BPs were enriched by the genes that are expressed for these tissue ion traits under stressed conditions (Table 4.5). This

clearly shows that salt stress expresses genes differentially which enrich different BPs in response to the stress across the range of genotypes. The BPs that are activated under stressed conditions fall under the overall category of Apoptosis, Stress Response, Signalling process, Transport, Metabolic and Catabolic process, Cellular and Developmental processes etc. These were determined by the diagram for significant biological processes generated by SEA analysis as shown in Figure 4.4 as an example and the details (GO terms, p-values, FDR values, GO flash charts, lists of genes under each BP and schematic diagrams) are shown in Supplementary Table 4.6 b1, b2 & c. The role of individual genes is not described in detail in this section, instead is discussed according to the molecular functions activated by these genes in the next section.

Table 4.2 List of significant GO categories of molecular function under unstressed condition for differentially expressed (positive) genes for biomass (determined by SEA analyiss). The details (GO flash charts and schematic diagrams) can be found in supplementary table 4.5a & b.

Molecular Functions: GO category		Positive Genes		
		Biomass (+473)		
GO term	***Description***	***Number of genes***	***p-value***	***FDR***
GO:0016765	Transferase activity, transferring alkyl or aryl (other than methyl) groups	5	0.0011	0.047
GO:0009055	Electron carrier activity	10	0.0016	0.047

Table 4.3 List of significant GO categories of biological process under unstressed condition for differentially expressed (positive and negative) genes for different morpho-physiological traits (determined by SEA analyiss). The details (GO terms, p-values, FDR values, GO flash charts and schematic diagrams) can be found in supplementary table 4.5a & b.

Biological Processes	Positive Genes		Negative Genes
Description	**Biomass** (+473)	**Leaf area** (+119)	**Root Na** (-331)
Biological regulation; including intracellular signaling cascade (7)			45
Developmental process	5		5
Apoptosis (or programmed cell death)	15	7	
Regulation of transcription, DNA-dependent			20
Response to abiotic stimulus	6		
Transport (including protein transport)			35
Oxidation reduction	7		7

Table 4.4 List of significant GO categories of molecular function under stressed condition for differentially expressed (positive and negative) genes for different morpho-physiological traits (determined by SEA analyiss). The details (GO terms, p-values, FDR values, GO flash charts and schematic diagrams) can be found in supplementary table 4.6a & b.

GO category: Molecular Functions		Positive Genes			Negative Genes			
		Shoot Na^+ (+1275)	**Shoot Na^+/K^+** (+6974)	**Root K^+** (+138)	**Shoot Na^-** (-2391)	**Shoot Na^+/K^+** (-4522)	**Root K^+** (-821)	**Biomass** (-363)
Signal transducer activity			107			56		
Binding	Transcription factor activity				81			
	Translation factor activity (nucleic acid binding)		36			26		
	SNAP receptor activity					6		
	Chaperone binding					6		
	Manganese ion binding				18	18		
	Alkali metal ion binding (including potassium ion binding)		8					
	2 iron, 2 sulfur cluster binding					5		
Catalytic activity	Phosphoprotein phosphatase activity (including protein serine/threonine phosphatase activity)				21			
	Protein methyltransferase activity		13					
	Serine O-acyltransferase activity (including serine O-acetyltransferase activity)				5			
	Serine hydrolase activity (including endopeptidase activity)	10	55			25		5
	Metalloexopeptidase activity		16			9		
	Oxidoreductase activity, acting on CH-OH group of donors					39		
	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen					12		
Electron carrier activity		27	125	6	48	109	17	

Table 4.5 List of significant GO categories of biological process under stressed condition for differentially expressed (positive and negative) genes for different morpho-physiological traits (determined by SEA analysis). The details (GO terms, p-values, FDR values, GO flash charts and schematic diagrams) can be found in supplementary table 4.6a & b.

GO Category: Biological Processes		Positive Genes			Negative Genes		
		Shoot Na^+	Shoot Na^+/K^+	Root K^+	Shoot Na^+	Shoot Na^+/K^+	Root K^+
Apoptosis	Programmed cell death			9			
Stress Response	Response to abiotic stimulus		29		12	29	
	Response to chemical stimulus (response to endogenous, organic substance and hormone)		110		19	77	6
	Response to biotic stimulus	8	14			11	
	Cellular response to stimulus		77			46	
Signalling process	Signal transduction, intracellular signalling process; signalling pathway		125				
Trans-port	Transmembrane transport					35	
	Di-, tri-valent inorganic cation transport; and transition metal ion transport				9	13	
Metabolic processes	Regulation of transcription, gene expression				173		
	Negative regulation of gene expression (silencing)					9	
	Translation	45					
	Cellular nitrogen compound biosynthetic process (amine, amino acid biosynthetic process)					61	
	Protein modification by small protein conjugation or removal		31		19	22	
	Protein amino acid dephosphorylation				16		
	Generation of precursor metabolites and energy (including photosynthesis, light harvesting)		26		43	67	
	Small molecule metabolic process		15			208	
	Cellular lipid metabolic process					51	
	Cellulose metabolic process		17				
	Secondary metabolic process		31		11	34	
Catabolic process	Including protein, polysaccharadie catabolic process		126				
Cellular process	Cell cycle		17		7	23	
	DNA conformation change (DNA packaging)	16					
	DNA recombination		32				
	Microtubule cytoskeleton organization		6				
	Cellular macromolecular complex subunit organization	19					
Develop-mental process	Multicellular organismal process	8	88		27	31	10
	Cellular cell wall organization or biogenesis		70		9	13	
	Reproduction		66		18		
	Regulation of anatomical structure size		9				
	Oxidation reduction	29		6	54		12

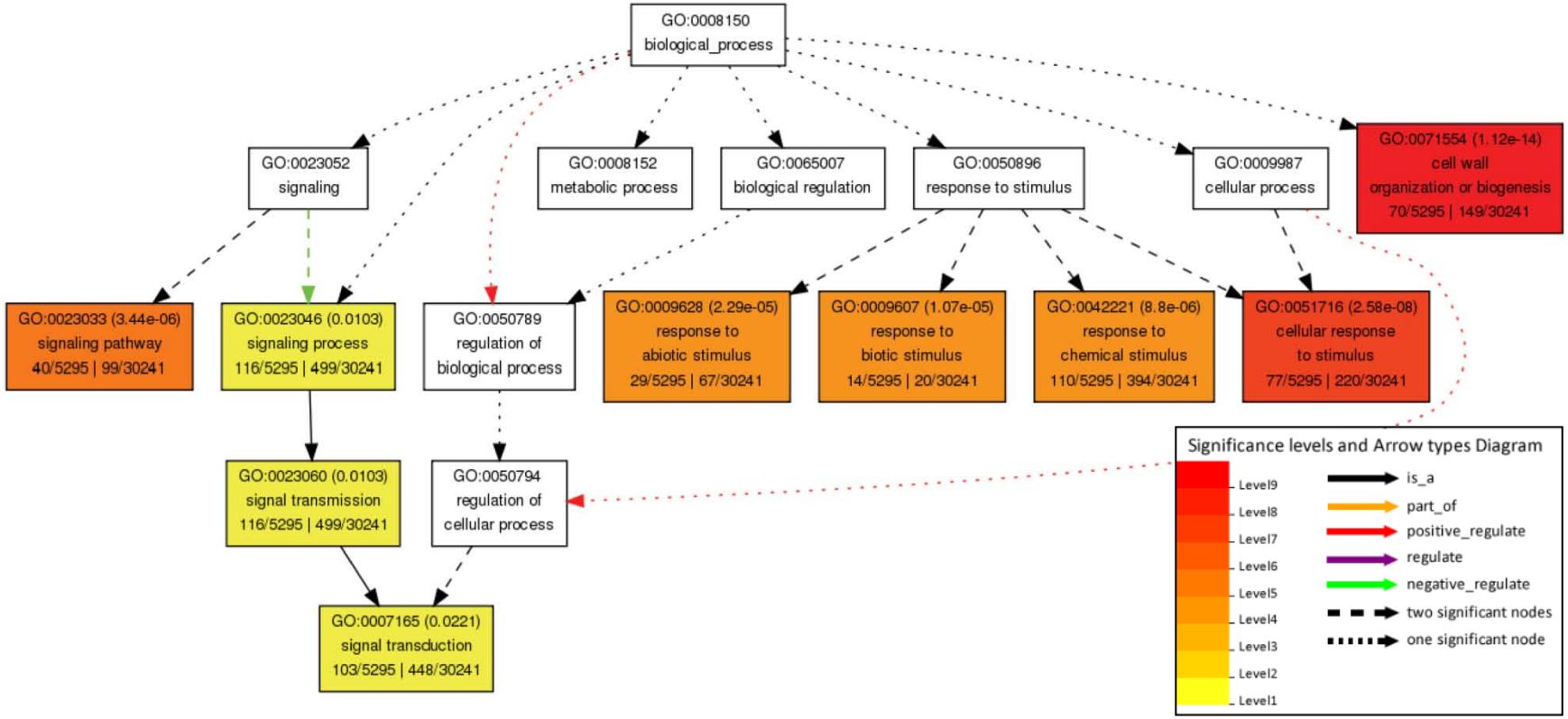


Figure 4.4 A simplified version (full version is shown in supplementary table 4.6c) of the diagram showing only the important gene ontology (GO) terms of the significantly enriched biological processes determined by SEA analysis (see section 2.13) using the significant positive genes for shoot Na^+ in wide natural variation of rice genotypes upon exposure to salt stress. This is shown as an example only. Similar diagrams are generated for the positive and negatively expressed genes mentioned in Figure 4.1. The detailed results of SEA analysis are shown in Supplementary Table 4.6 and are simplistically compiled and represented in Table 4.2-4.5.

4.2.6 Global regulation of molecular functions (MF) under salt stress

Salt stress significantly enriches more molecular functions (MF) in the wide genetic background of rice compared to unstressed conditions. The MFs that are significantly enriched by the positively and negatively expressed genes under stressed conditions fall under the major category of signalling, binding, catalytic activity and electron carrier activity. Only two MFs 'Electron carrier activity' and 'Transferase activity' are enriched under unstressed conditions with a marginal significance level (FDR=0.047 for both MFs, Table 4.2, Supplementary Table 4.5a2); which were also enriched under stressed conditions with more genes being involved in enriching these molecular functions (Table 4.4 & Supplementary Table 4.6a2).

Apart from 'Electron carrier activity', none of the MFs were affected by the genes that positively and negatively expressed root K^+ (Table 4.4). It *Indica*tes that in wide gene pool of rice the genes that are expressed for root K^+ have little role in enriching the molecular functions under stressed conditions. The case is almost similar with the genes that positively expressed for shoot Na^+ except that 10 of the genes significantly enriched 'Serine endopeptidase activity'. But, the transcription factor activity, ion binding, protein phosphatase and serine transferase activity are turned on by the genes that significantly repressed themselves to maintain less Na^+ in shoots suggesting the global importance of these repressive genes in response to salt stress.

However, both the positively and negatively expressed genes for shoot Na^+/K^+ have significantly enriched a number of MFs that includes signal transducer activity, transcription and translation factory activity, serine hydrolase and metalloexopeptidase

activity. The molecular functions that are only activated by the negative genes for shoot Na^+/K^+ are SNAP receptor activity, chaperone binding, manganese, iron and sulphur ion binding and oxidoreductase activity (Table 4.4). The details (GO terms, p-values, FDR values, GO flash charts and schematic diagrams) can be found in Supplementary Table 4.6 a1, a2 & c. The individual genes that enriched the molecular functions mentioned above are further discussed below.

4.2.6.1 Signal transducer activity

Signal transduction is the starting point of the plant's adaptive response towards the environmental stresses. It starts with sensing the stress by the receptors in membranes, which then generates secondary signal messengers like calcium, reactive oxygen species, kinases and phosphates followed by the activation of transcription factor genes that eventually coordinates the plant's adaptive biochemical and physiological responses (Huang *et al.* 2012; Proietti *et al.* 2013; Zhu, 2002). In this experiment, 107 and 54 signalling related transcripts are found to be positively and negatively expressed, respectively in the wide natural rice genotypes (Table 4.6).

Altogether, transcripts of 19 (Os08g0442700, Os07g0134200, Os06g0334300, Os07g0107800, Os05g0155200, Os07g0259100, Os03g0701700, Os04g0169100, Os02g0820900, Os07g0132500, Os01g0239700, Os08g0446400, Os01g0140400, Os01g0836800, Os07g0522600, Os02g0131600, Os02g0117500, Os02g0245100, and Os06g0225300) and 12 (Os10g0346600, Os11g0473000, Os05g0529300, Os06g0680500, Os06g0717200, Os03g0343400, Os01g0176400 and Os01g0114600) receptors are positively and negatively expressed, respectively (Table 4.6). Transcripts of three

receptor like kinases (RLK) such as Os08g0442700, Os07g0134200 and Os06g0334300 are positively expressed. RLKs are known to regulate plant architecture and also play roles in stress defence by sensing the extracellular signals and activating downstream pathways by phosphorylating target proteins (Marshall *et al.* 2012; Tanaka *et al.* 2012). In *Arabidopsis*, a receptor-like protein kinase gene (RPK1) was reported to be induced by several abiotic stresses including salt stress (Hong *et al.* 1997) and very recently, in rice, a putative RLK gene, OsSIK1, with extracellular leucine-rich repeats (Ouyang *et al.* 2010) and a cysteine-rich repeat (CRR) RLK sub-family gene, ARCK1 (Tanaka *et al.* 2012) was reported to be induced by salt and drought stresses.

Transcripts of 5 Ethylene receptor genes (Os05g0155200, Os07g0259100, Os03g0701700, Os04g0169100 and Os02g0820900) were found to be positively expressed under salt stress. The role of ethylene in salt stress response is reported to be equivocal (Achard *et al.* 2006; Pierik *et al.* 2006). In tobacco, an ethylene receptor gene, NTHK1 was reported to promote leaf growth (Cao *et al.* 2006), which demonstrated the significance of these ethylene receptor genes to be studied further in determining their role in salt tolerance.

Among the leucine-rich repeat containing transcripts, three (Os01g0239700, Os08g0446400 and Os01g0140400) were found to be positively expressed and two (Os06g0717200 and Os11g0514500) were found to be negatively regulated in this study. Cheng *et al.* (2009) reported a leucine-rich-repeat type receptor-like protein kinase, OsRPK1 to be induced by multiple stresses in plasma membrane of cortex cells in rice roots and de Lorenzo *et al.* (2009) reported an increase in expression of leucine-rich

gene, Srlk in *Medicago truncatula* roots upon exposure to salt stress. The Srlk gene also had a homologue, Os05g0414700, which was also found to be upregulated in this study.

Protein kinases regulate the phosphorylation and dephosphorylation of other proteins and play a crucial role in stress signal transduction. To date, several plant protein kinases are reported to be activated by osmotic stress (Zhu, 2002) particularly the mitogen-activated protein (MAPK/MPK) kinase (Fujita *et al.* 2006; Sinha *et al.* 2011). In addition, Serine/threonine protein kinases have also been known to be involved in multi-stress tolerance in plants (Zhao *et al.* 2009). At least, 33 and 13 protein kinase domain containing proteins were found to be positively and negatively expressed in the wide natural variation of rice genotypes. Five MAP kinases including MAP kinase 2 (Os06g0699400), 6 (Os06g0154500) and MAPK homolog MMK2 (Os10g0533600) were positively regulated and three MAP kinases (Os06g0708000, Os06g0367900 and Os05g0566400) are negatively expressed in wide natural rice variation (Table 4.6).

Among the serine/threonine kinases, four (Os01g0323000, Os01g0631700, Os10g0136400 and Os07g0537200) was positively expressed and only one (Os06g0496800) was found to be negatively regulated. Among the many other positively expressed transcripts, notable are heat shock protein, mannose binding, extension, histidin kinases, NPH3 domain containing protein and Zn-finger domain containing proteins. Several unknown hypothetical proteins were also found to be differentially expressed in wide natural variation of rice genotypes. The chromosomal distribution of the positively expressed genes is shown in Figure 4.5.

Table 4.6 Lists of positively (a) and negatively (b) expressed transcripts (for shoot Na+/K+) that are involved in the Molecular Function 'Signal transducer activity'

Name	Annotation
(a) Positively expressed transcripts (107)	
Os08g0442700	Receptor-like kinase.
Os07g0134200	Receptor-like protein kinase 3.
Os06g0334300	Receptor-like protein kinase.
Os07g0107800	Phytosulfokine receptor precursor (EC 2.7.1.37) (Phytosulfokine LRR receptor kinase).
Os05g0155200	Ethylene receptor homolog.
Os07g0259100	Ethylene receptor.
Os03g0701700	Ethylene receptor.
Os04g0169100	Ethylene receptor.
Os02g0820900	Ethylene receptor-like protein 2.
Os07g0132500	Lectin-like receptor kinase 7;2.
Os01g0239700	Leucine-rich receptor-like protein kinase.
Os08g0446400	Leucine-rich repeat, plant specific containing protein.
Os01g0140400	Leucine-rich repeat, plant specific containing protein.
Os01g0836800	Lung seven transmembrane receptor family protein.
Os07g0522600	Metabotropic gamma-aminobutyric acid receptor, type B family protein.
Os02g0131600	Mitochondrial import receptor subunit TOM22 homolog (Translocase of outer membrane 22 kDa subunit homolog) (TOM9).
Os02g0117500	Glutamate receptor 3.2 precursor (Ligand-gated ion channel 3.2) (AtGluR2).
Os02g0245100	Peroxisomal targeting signal type 2 receptor.
Os06g0225300	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor (EC 2.7.1.37) (BRI1-associated receptor kinase 1) (Somatic embryogenesis receptor-like kinase 3).
Os01g0665200	Mitogen-activated protein kinase, Blast and wounding induced
Os06g0699400	MAP kinase 2.
Os05g0576800	MAP kinase homolog.
Os06g0154500	Mitogen-activated protein kinase (MAP kinase 6).
Os10g0533600	Mitogen-activated protein kinase homolog MMK2 (EC 2.7.1.37).
Os09g0349800	Protein kinase domain containing protein.
Os09g0349600	Protein kinase domain containing protein.
Os08g0493800	Protein kinase domain containing protein.
Os04g0540900	Protein kinase domain containing protein.
Os02g0111800	Protein kinase domain containing protein.
Os06g0693200	Protein kinase domain containing protein.
Os02g0153200	Protein kinase domain containing protein.
Os04g0658700	Protein kinase domain containing protein.
Os03g0791700	Protein kinase domain containing protein.
Os05g0525600	Protein kinase domain containing protein.
Os08g0203700	Protein kinase domain containing protein.
Os02g0151100	Protein kinase domain containing protein.
Os01g0976900	Protein kinase domain containing protein.
Os10g0155800	Protein kinase domain containing protein.
Os01g0960400	Protein kinase domain containing protein.
Os10g0497600	Protein kinase domain containing protein.
Os01g0664200	Protein kinase domain containing protein.
Os01g0110500	Protein kinase domain containing protein.
Os01g0741200	Protein kinase domain containing protein.
Os02g0218400	Protein kinase domain containing protein.
Os02g0227700	Protein kinase domain containing protein.
Os02g0153100	Protein kinase domain containing protein.
Os03g0148700	Protein kinase domain containing protein.
Os06g0693000	Protein kinase domain containing protein.
Os01g0514700	Protein kinase domain containing protein.

Os01g0114900	Protein kinase domain containing protein.
Os01g0738300	Protein kinase domain containing protein.
Os05g0414700	Protein kinase domain containing protein.
Os06g0654600	Protein kinase domain containing protein.
Os10g0533800	Protein kinase family protein.
Os07g0131100	Protein kinase family protein.
Os03g0772600	Protein kinase family protein.
Os12g0562500	Protein kinase-like protein (Fragment).
Os01g0323000	Ser Thr specific protein kinase-like protein.
Os01g0631700	Ser Thr specific protein kinase-like protein.
Os10g0136400	Serine/threonine kinase.
Os07g0537200	Serine/threonine protein kinase family protein.
Os01g0223900	Curculin-like (mannose-binding) lectin domain containing protein.
Os02g0527900	Curculin-like (mannose-binding) lectin domain containing protein.
Os02g0150800	Cyclin-like F-box domain containing protein.
Os12g0256000	Esterase/lipase/thioesterase domain containing protein.
Os05g0407500	Esterase/lipase/thioesterase domain containing protein.
Os07g0613300	Exportin-t.
Os03g0284100	Expressed protein (Pseudo-response regulator 9) (Timing of CAB expression 1-like protein).
Os03g0637600	Extensin protein-like.
Os08g0332800	F7O18.23 protein (SWP1) (Struwwelpeter 1 protein).
Os08g0230300	Galactose oxidase, central domain containing protein.
Os06g0199800	GPCR, family 2, secretin-like protein.
Os06g0111400	Guanine nucleotide binding protein (G-protein), alpha subunit family protein.
Os06g0163000	Heat shock protein STI (Stress inducible protein) (GmSTI).
Os01g0923700	Histidine kinase-like protein.
Os01g0114700	LRK33.
Os07g0584200	NPH3 domain containing protein.
Os04g0477000	NPH3 domain containing protein.
Os06g0625300	Peptidoglycan-binding LysM domain containing protein.
Os06g0687800	Pincher.
Os07g0130700	Resistance protein candidate (Fragment).
Os08g0376700	Response regulator 1.
Os02g0618200	Response regulator receiver domain containing protein.
Os06g0654300	Response regulator receiver domain containing protein.
Os09g0532400	Response regulator receiver domain containing protein.
Os03g0224200	Response regulator receiver domain containing protein.
Os07g0537900	SRK3 gene.
Os05g0525000	TMK protein precursor.
Os01g0904700	Two-component response regulator ARR1. Splice isoform 2.
Os06g0183100	Two-component response regulator ARR14.
Os06g0574200	UspA domain containing protein.
Os02g0218600	UspA domain containing protein.
Os09g0416700	Vesicle transport v-SNARE family protein.
Os02g0205400	WD40-like domain containing protein.
Os02g0830200	ZmRR2 protein (Response regulator 2).
Os04g0524300	ZmRR2 protein (Response regulator 2).
Os05g0112000	Zn-finger, RING domain containing protein.
Os01g0974400	Zn-finger, RING domain containing protein.
Os03g0275300	Zn-finger, RING domain containing protein.
Os06g0716000	Protein of unknown function DUF668 family protein.
Os04g0433600	Protein of unknown function DUF668 family protein.
Os09g0573200	Conserved hypothetical protein.
Os09g0470900	Conserved hypothetical protein.
Os03g0738800	Hypothetical protein.
Os07g0501800	Hypothetical protein.
Os04g0631900	Hypothetical protein.

Os01g0690900	Hypothetical protein.
(b) Negatively expressed transcripts (54)	
Os10g0346600	BP-80 vacuolar sorting receptor.
Os11g0473000	ER lumen protein retaining receptor (HDEL receptor) (PGP169-12).
Os05g0529300	ER lumen protein retaining receptor (HDEL receptor).
Os06g0680500	Glutamate receptor 3.1 precursor (Ligand-gated ion channel 3.1) (AtGLR2).
Os06g0717200	Leucine-rich repeat/receptor protein kinase precursor.
Os11g0514500	Sorghum bicolor leucine-rich repeat-containing extracellular glycoprotein precursor.
Os03g0343400	Photolyase/blue-light receptor (Photolyase/blue light photoreceptor PHR2).
Os01g0176400	Photoreceptor-interacting protein-like.
Os01g0114600	Receptor-like kinase ARK1AS (Fragment).
Os06g0496800	Serine/threonine kinase receptor precursor.
Os08g0480100	Signal recognition particle receptor protein (Fragment).
Os05g0100700	Somatic embryogenesis receptor kinase-like protein.
Os08g0174700	Somatic embryogenesis receptor-like kinase 2.
Os06g0708000	MAP kinase homolog.
Os06g0367900	Mitogen-activated protein kinase homologue.
Os05g0566400	Mitogen-activated protein kinase. Blast and wounding induced
Os01g0206800	Protein kinase domain containing protein.
Os08g0203400	Protein kinase domain containing protein.
Os05g0588300	Protein kinase domain containing protein.
Os05g0258400	Protein kinase domain containing protein.
Os05g0480400	Protein kinase domain containing protein.
Os02g0228300	Protein kinase domain containing protein.
Os01g0116400	Protein kinase domain containing protein.
Os06g0676600	Protein kinase domain containing protein.
Os02g0821400	Protein kinase domain containing protein.
Os01g0779300	Protein kinase domain containing protein.
Os02g0106900	Protein kinase domain containing protein.
Os11g0678000	Protein kinase family protein.
Os01g0114100	Protein kinase family protein.
Os01g0121100	AR401.
Os01g0958100	Cell division transporter substrate-binding protein FtsY family protein.
Os03g0284100	Expressed protein (Pseudo-response regulator 9) (Timing of CAB expression 1-like protein).
Os03g0637600	Extensin protein-like.
Os07g0535700	F-box protein interaction domain containing protein.
Os06g0111400	Guanine nucleotide binding protein (G-protein), alpha subunit family protein.
Os11g0206700	Guanine nucleotide binding protein (G-protein), alpha subunit family protein.
Os05g0186100	Histidine-containing phosphotransfer protein 4.
Os01g0855600	Hs1pro-1 protein.
Os02g0259100	Hypothetical protein.
Os02g0459600	Legume lectin, beta domain containing protein.
Os11g0102200	NPH1-1.
Os03g0206700	NPH3 domain containing protein.
Os12g0117600	NPH3 domain containing protein.
Os11g0118300	NPH3 domain containing protein.
Os03g0347700	NPH3 domain containing protein.
Os11g0118500	NPH3 domain containing protein.
Os05g0127200	Phosphoinositide-specific phospholipase C (PLC) family protein.
Os07g0694000	Phosphoinositide-specific phospholipase C.
Os07g0695100	Response regulator receiver domain containing protein.
Os02g0729400	Rhodanese-like domain containing protein.
Os12g0117400	RPT2-like protein.
Os11g0143300	Type-A response regulator.
Os01g0560200	Vesicle transport v-SNARE family protein.
Os01g0707300	Vesicle transport v-SNARE family protein.

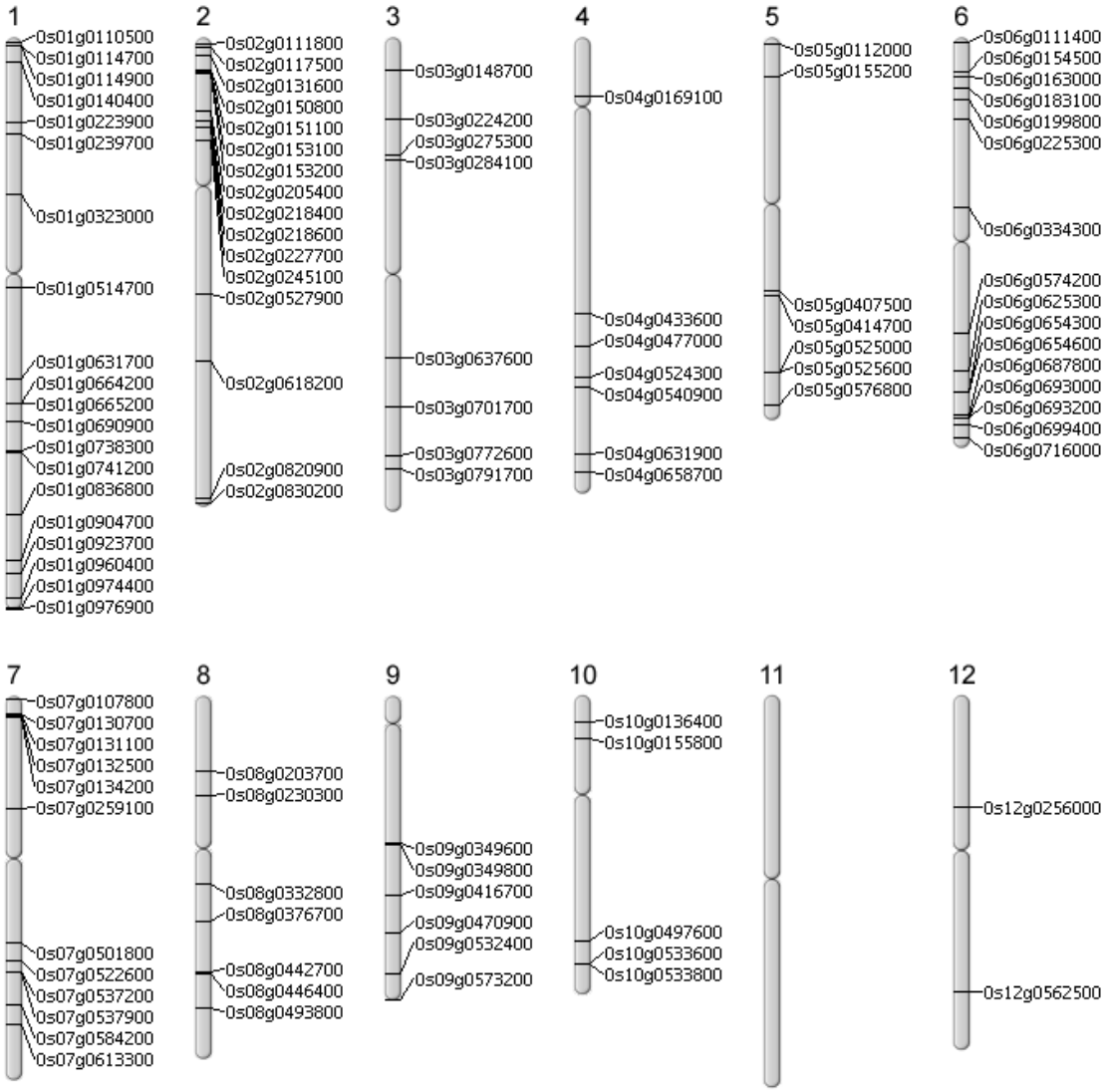


Figure 4.5 The chromosomal distribution of the 107 positively expressed genes across the 12 chromosomes that significantly enriched the 'signal transducer activity'. Chromosome 1, 2, 6 and 7 contain most of the genes while no genes were located in chromosome 11.

The chromosome map is obtained by submitting the list of genes to the web based 'Chromosome Map Tool' http://viewer.shigen.info/oryzavw/maptool/MapTool.do (see section 2.14 for details).

4.2.6.2 Transcription factor (TF) activity

The rice genome is known to have 1772 TFs (http://grassius.org/index.html) and a number of TFs had already been identified with complex patterns of expression under different environmental stresses (Chen and Zhu, 2004). These transcription factors generally fall under the family of WRKY, DREB (dehydration-responsive element-binding), CBF (C-repeat binding factor), MYB, bZIP (basic-leucine zipper), ERF, zinc-finger, helix-loop-helix and NAC (Duque *et al.* 2013; Sreenivasulu *et al.* 2007). In this study, among the transcripts that negatively regulated for shoot Na^+/K^+, 81 transcripts were found to significantly enrich 'Transcription factor activity' (Table 4.7). The four bZip family TFs (Os01g0542700 encoding OsbZIP4, Os03g0770000 encoding OsbZIP32, Os08g0543900 encoding OsbZIP68 and Os11g0154800) that were found to be salt responsive in this study have not been reported before. Some of the identified salt and drought responsive bZIP proteins are OzBZ8 (Mukherjee *et al.* 2006), OsbZIP15 (Zou *et al.* 2008), OsbZIP23 (Xiang *et al.* 2008), OsbZIP46 (Tang *et al.* 2012), OzAREB1 (Jin *et al.* 2010) and OsbZIP16 (Chen *et al.* 2012).

Among the 70 identified WRKY genes in rice and *Arabidopsis* (Dong *et al.* 2003; Goff *et al.* 2002), transcripts of twelve TFs were found to be salt stress responsive in this study (Table 4.7). Of these, OsWRKY6 (Os03g0798500) and OsWRKY42 (Os02g0462800) were found to be low Pi (Chen *et al.* 2009) and low boron (Kasajima *et al.* 2010) responsive in *Arabidopsis* and herbivore responsive in *Nicotiana attenuata* (Skibbe *et al.* 2008); OsWRKY24 (Os01g0826400) stress responsive in *Arabidopsis* (Wei *et al.* 2013); OsWRKY34 (Os01g0665500 and Os07g0583700) cold responsive in *Arabidopsis* (Zou *et al.* 2010);

OsWRKY34 (Os02g0265200) heat responsive in *Arabidopsis* (Li *et al.* 2010). However, the TFs WRKY31 (Os01g0750100), WRKY32 (Os02g0770500), WRKY44 (Os08g0276200), WRKY49 (Os01g0730700), WRKY63 (Os06g0158100) and WRKY71 (Os02g0181300) were not reported earlier (Table 4.7).

Several NAC type transcription factors e.g., SNAc1,6 and ONAC045 were reported to be salt and drought stress responsive in rice (Lata *et al.* 2011); however, none were found in this study. The other important TFs found to be salt responsive in this study include CBF like protein, E2F protein, ethylene responsive TFs, heat shock and MADS-box proteins and AP2 domain containing proteins. Some of these TFs are reported to play a role in abiotic and biotic stress tolerance in rice and other crops; however, a detailed investigation of the previously unidentified TFs will provide valuable information in explaining salinity tolerance mechanisms in rice. The complete lists of the transcription factor genes are shown in Table 4.7 and chromosomal distribution of these TFs are shown in Figure 4.6.

Table 4.7 Lists of transcripts (for shoot Na+/K+) that are involved in transcription factor activity

Name	Annotation
Os01g0952800	Basic helix-loop-helix dimerisation region bHLH domain containing protein.
Os01g0542700	Basic-leucine zipper (bZIP) transcription factor domain containing protein.
Os03g0770000	Basic-leucine zipper (bZIP) transcription factor domain containing protein.
Os08g0543900	BZIP transcription factor RF2b.
Os11g0154800	DNA-binding factor of bZIP class (Fragment).
Os01g0826400	WRKY transcription factor 24.
Os01g0750100	WRKY transcription factor 31.
Os02g0770500	WRKY transcription factor 32.
Os01g0665500	WRKY transcription factor 34.
Os07g0583700	WRKY transcription factor 34.
Os02g0265200	WRKY transcription factor 39.
Os02g0462800	WRKY transcription factor 42 (Transcription factor WRKY02).
Os08g0276200	WRKY transcription factor 44 (WRKY DNA-binding protein 44) (TRANSPARENT TESTA GLABRA 2).
Os01g0730700	WRKY transcription factor 49.
Os03g0798500	WRKY transcription factor 6.
Os06g0158100	WRKY transcription factor 63.
Os02g0181300	WRKY transcription factor 71 (Transcription factor WRKY09).
Os03g0680800	BEL1-related homeotic protein 14 (Fragment).
Os03g0762000	Casein kinase II alpha subunit.
Os06g0127100	CBF-like protein.
Os02g0203000	Concanavalin A-like lectin/glucanase domain containing protein.
Os04g0597300	DNA-binding WRKY domain containing protein.
Os01g0678700	DP protein.
Os01g0165000	DRE binding protein 2.
Os03g0152100	E2F dimerization factor.
Os02g0537500	E2F homolog.
Os04g0669200	Ethylene response factor 3.
Os05g0497200	Ethylene responsive element binding factor 4 (AtERF4).
Os05g0497300	Ethylene responsive element binding factor 5 (AtERF5).
Os06g0194000	Ethylene responsive element binding factor 5 (AtERF5).
Os02g0655200	Ethylene responsive element binding factor3 (OsERF3).
Os01g0934300	Flowering-time protein isoform beta.
Os01g0658900	G-box binding factor 1.
Os03g0640800	HD-Zip protein (Homeodomain transcription factor) (ATHB-14) (Homeodomain-leucine zipper protein 14).
Os03g0745000	Heat shock factor (HSF)-type, DNA-binding domain containing protein.
Os02g0232000	Heat shock transcription factor 29 (Fragment).
Os03g0854500	Heat shock transcription factor 31 (Fragment).
Os06g0603000	Heme oxygenase 1 (Fragment).
Os02g0147800	Homeo protein (Fragment).
Os01g0818400	Homeobox domain containing protein.
Os03g0109400	Homeobox domain containing protein.
Os04g0541700	Homeobox domain containing protein.
Os04g0548700	Homeobox domain containing protein.
Os05g0129700	Homeobox protein rough sheath 1.
Os03g0188900	Homeobox-leucine zipper protein ATHB-6 (Homeodomain transcription factor ATHB-6) (HD-ZIP protein ATHB-6).
Os09g0528200	Homeodomain leucine zipper protein (Fragment).
Os03g0224700	HSP protein (Fragment).
Os03g0673000	Knotted1-type homeobox protein OSH10 (Fragment).

Os03g0727200	Knotted1-type homeobox protein OSH3.
Os01g0201700	MADS box protein.
Os06g0712700	MADS-box protein SPW1.
Os05g0437700	OSE2-like protein (Fragment).
Os02g0797100	Pathogenesis-related transcriptional factor and ERF domain containing protein.
Os04g0547600	Pathogenesis-related transcriptional factor and ERF domain containing protein.
Os04g0610400	Pathogenesis-related transcriptional factor and ERF domain containing protein.
Os05g0549800	Pathogenesis-related transcriptional factor and ERF domain containing protein.
Os06g0691100	Pathogenesis-related transcriptional factor and ERF domain containing protein.
Os07g0227600	Pathogenesis-related transcriptional factor and ERF domain containing protein.
Os08g0408500	Pathogenesis-related transcriptional factor and ERF domain containing protein.
Os08g0521600	Pathogenesis-related transcriptional factor and ERF domain containing protein.
Os11g0129700	Pathogenesis-related transcriptional factor and ERF domain containing protein.
Os01g0883100	PISTILLATA-like MADS box protein.
Os01g0174000	Protein HY5 (AtbZIP56).
Os06g0712600	Protein of unknown function DUF702 family protein.
Os02g0649300	Short highly repeated, interspersed DNA (Fragment).
Os06g0252300	TF-like protein (Fragment).
Os01g0899800	ANT (Ovule development protein aintegumenta).
Os06g0657500	ANT (Ovule development protein aintegumenta).
Os03g0341000	AP2 domain containing protein RAP2.2 (Fragment).
Os03g0191900	AP2 domain family transcription factor homolog (AP2 domain transcription factor) (ABI4:abscisic acid-insensitive 4) (ABI4).
Os09g0369000	AP2 domain transcription factor RAP2.3 (Related to AP2 protein 3) (Cadmium-induced protein AS30).
Os02g0657000	AP2 domain-containing protein Rap211.
Os09g0423800	AP2-1 protein (Fragment).
Os02g0546600	AP2-domain DNA-binding protein.
Os04g0539500	AtGATA-3 protein (GATA transcription factor 3).
Os03g0313100	BABY BOOM.
Os08g0442400	BABY BOOM.
Os08g0472400	Conserved hypothetical protein.
Os09g0456700	Conserved hypothetical protein.
Os01g0200300	Hypothetical protein.
Os03g0231000	Hypothetical protein.

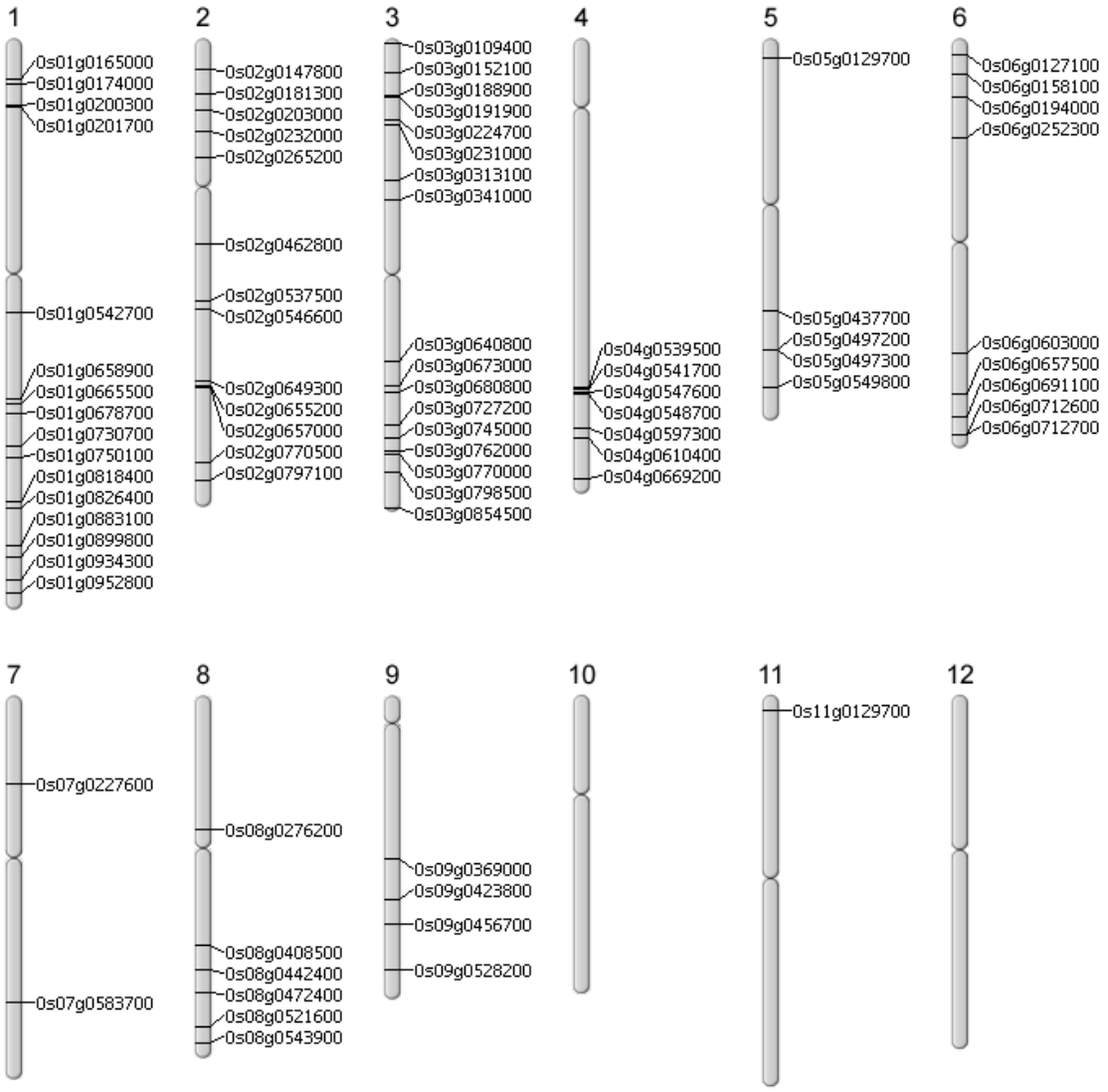


Figure 4.6 The chromosomal distribution of the 81 transcription factors across the 12 chromosomes with no TFs located in chromosome 10 & 12.

The chromosome map is obtained by submitting the list of genes to the web based 'Chromosome Map Tool' http://viewer.shigen.info/oryzavw/maptool/MapTool.do (see section 2.14 for details).

4.2.6.3 Translation factor activity

The regulation of translation, that facilitates the selective synthesis of required proteins, is one of the versatile strategies plants have evolved to cope with the environmental stresses. Generally, in eukaryotes, eukaryotic initiation factor (eIF4E) recognizes the 5'-cap structure of mRNAs to begin the canonical cap-dependent translation. The eIF4G and eIF4A then interacts with eIF4E to form the eIF4F (cap-binding complex) and the factors eIF4B, eIF3, eIF1 and eIF1A are subsequently recruited that ultimately regulates translation (Echevarría-Zomeño *et al.* 2013). Under stresses, the eukaryotic cells usually inhibit this translation initiation that affects the activity of initiation factor eIF2 and eIF4E, a mechanism mainly unknown in plants (Clemens, 2001; Muñoz and Castellano, 2012; Wek *et al.* 2006). In this study, among the positively and negatively expressed genes that are identified significant for shoot Na/K by SAM analysis, it was observed that 36 and 26 genes, respectively significantly enriched the translation factor activity in wide natural rice genotypes (Table 4.8).

Among the positive genes the most noticeable fall under the category of translation initiation factors such as Os02g0146600 (eIF4A), Os03g0566800 (eIF4A-3), Os05g0566500 (eIF-3 zeta), Os07g0124500 (eIF3 p110), Os07g0555200 (eIF4G), Os07g0597000 (eIF-5A), Os03g0758800 & Os12g0507200 (eIF-5A) (eIF-4D), Os05g0592600 (eIF 2 family protein), Os02g0101100 (eIF 3 family protein) and Os02g0557600 & Os05g0498400 (IF SUI1 family protein); transcription elongation factors such as Os03g0196900 (TFIIB), Os11g0166800 (TFS-II), Os03g0441000 & Os01g0846900 (TFIID), Os07g0662500 (EF-1-beta'), Os06g0571400 (EF-1-gamma), Os01g0742200 (EF-2) and Os03g0565500(mEF-G-1) etc.

Several elongation factors e.g., Os03g0177400 & Os03g0178000 (EF-1 α), Os11g0116400 (EF-P), Os12g0541500 (EF-Ts), Os07g0614500 (EF-1-beta) and Os02g0220500 & Os02g0220600 (EF-1-gamma) etc and several translation initiation factors e.g., Os02g0300700 (EIF-1A), Os07g0681000 (eIF-2-beta) (P38), Os01g0120800 (eIF-3 theta), Os07g0167000 (eIF-3 p48), Os01g0970400 (eIF4E-1), Os02g0794400 (IF-3 family protein), Os05g0107700 (TFIIA-gamma) and Os07g0639800 (IF6 family protein) etc. were also found to be negatively expressed. The complete lists of genes are shown in Table 4.8 with their corresponding chromosomal distribution shown in Figure 4.7.

Table 4.8 Lists of positively (a) and negatively (b) expressed transcripts (for shoot Na+/K+) that significantly enriched the translation factor activity in wide natural variation of rice genotypes

Name	**Annotation**
(a) Positively expressed transcripts **(36)**	
Os01g0229100	Conserved hypothetical protein.
Os02g0122300	Conserved hypothetical protein.
Os04g0237300	DNA-directed RNA polymerase alpha chain (EC 2.7.7.6) (PEP) (Plastid- encoded RNA polymerase alpha subunit) (RNA polymerase alpha subunit).
Os03g0851100	Eftu.
Os03g0196900	TFIIB-related protein (Fragment).
Os11g0166800	Transcription elongation factor S-II, N-terminal domain containing protein.
Os03g0441000	Transcription initiation factor TFIID component TAF4 domain containing protein.
Os01g0846900	Transcription initiation factor TFIID domain containing protein.
Os07g0662500	Elongation factor 1-beta' (EF-1-beta').
Os06g0571400	Elongation factor 1-gamma (EF-1-gamma) (eEF-1B gamma).
Os01g0742200	Elongation factor EF-2 (Fragment).
Os03g0565500	Elongation factor G 1, mitochondrial precursor (mEF-G-1).
Os02g0146600	Eukaryotic initiation factor 4A (eIF4A) (eIF-4A).
Os03g0566800	Eukaryotic initiation factor 4A-3 (eIF4A-3) (eIF-4A-3).
Os05g0566500	Eukaryotic translation initiation factor 3 subunit 7 (eIF-3 zeta) (eIF3d) (p66).
Os07g0124500	Eukaryotic translation initiation factor 3 subunit 8 (eIF3 p110) (eIF3c).
Os07g0555200	Eukaryotic translation initiation factor 4G.
Os07g0597000	Eukaryotic translation initiation factor 5A (eIF-5A).
Os03g0758800	Eukaryotic translation initiation factor 5A-2 (eIF-5A) (eIF-4D).
Os12g0507200	Eukaryotic translation initiation factor 5A-2 (eIF-5A) (eIF-4D).
Os05g0592600	Initiation factor 2 family protein.
Os02g0101100	Initiation factor 3 family protein.
Os05g0575300	Translation initiation factor IF-2, chloroplast precursor (PvIF2cp).
Os02g0557600	Translation initiation factor SUI1 family protein.
Os05g0498400	Translation initiation factor SUI1 family protein.
Os02g0456200	G1 to S phase transition protein 1 homolog.
Os01g0528000	Hypothetical protein.

Os01g0652800	Hypothetical protein.
Os01g0655400	Hypothetical protein.
Os06g0688100	Hypothetical protein.
Os02g0812400	Nucleotidyl transferase domain containing protein.
Os05g0277300	Peptide chain release factor 1.
Os07g0503700	Proteasome component region PCI domain containing protein.
Os01g0887200	Winged helix DNA-binding domain containing protein.
Os06g0597400	ZLL/PNH homologous protein.
Os04g0168100	Zn-finger, C2H2 type domain containing protein.
(b) Negatively expressed transcripts (26)	
Os04g0533000	ATP-dependent RNA helicase p54 (Xp54).
Os05g0227700	Conserved hypothetical protein.
Os07g0191700	Conserved hypothetical protein.
Os03g0177400	EF-1 alpha.
Os03g0178000	EF-1 alpha.
Os11g0116400	Elongation factor P (EF-P).
Os12g0541500	Elongation factor Ts (EF-Ts).
Os07g0614500	Elongation factor 1-beta (EF-1-beta).
Os02g0220500	Elongation factor 1-gamma (EF-1-gamma) (eEF-1B gamma).
Os02g0220600	Elongation factor 1-gamma (EF-1-gamma) (eEF-1B gamma).
Os02g0300700	Eukaryotic translation initiation factor 1A (EIF-1A) (EIF-4C).
Os07g0681000	Eukaryotic translation initiation factor 2 beta subunit (eIF-2-beta) (P38).
Os01g0120800	Eukaryotic translation initiation factor 3 subunit 10 (eIF-3 theta) (Eukaryotic translation initiation factor 3 large subunit) (eIF3a) (p114).
Os07g0167000	Eukaryotic translation initiation factor 3 subunit 6 (eIF-3 p48) (eIF3e) (Mammary tumor-associated protein INT-6) (Viral integration site protein INT-6) (MMTV integration site 6).
Os01g0970400	Eukaryotic translation initiation factor 4E-1 (eIF4E-1) (eIF-4E-1) (mRNA cap-binding protein) (eIF-4F 25 kDa subunit) (eIF-4F p26 subunit).
Os12g0607100	Histone-lysine N-methyltransferase, H3 lysine-9 specific (EC 2.1.1.43) (Histone H3-K9 methyltransferase) (H3-K9-HMTase) (Suppressor of variegation protein 3-9).
Os02g0794400	Initiation factor 3 family protein.
Os05g0107700	Transcription initiation factor IIA gamma chain (TFIIA-gamma).
Os07g0639800	Translation initiation factor IF6 family protein.
Os06g0338900	Nucleotidyl transferase domain containing protein.
Os05g0277300	Peptide chain release factor 1.
Os02g0606100	Quinoprotein amine dehydrogenase, beta chain-like domain containing protein.
Os02g0641800	RNA helicase.
Os12g0165700	Transcription factors TFIIS, elongin A, CRSP70, conserved domain containing protein.
Os01g0772200	Winged helix DNA-binding domain containing protein.
Os10g0397200	Winged helix DNA-binding domain containing protein.

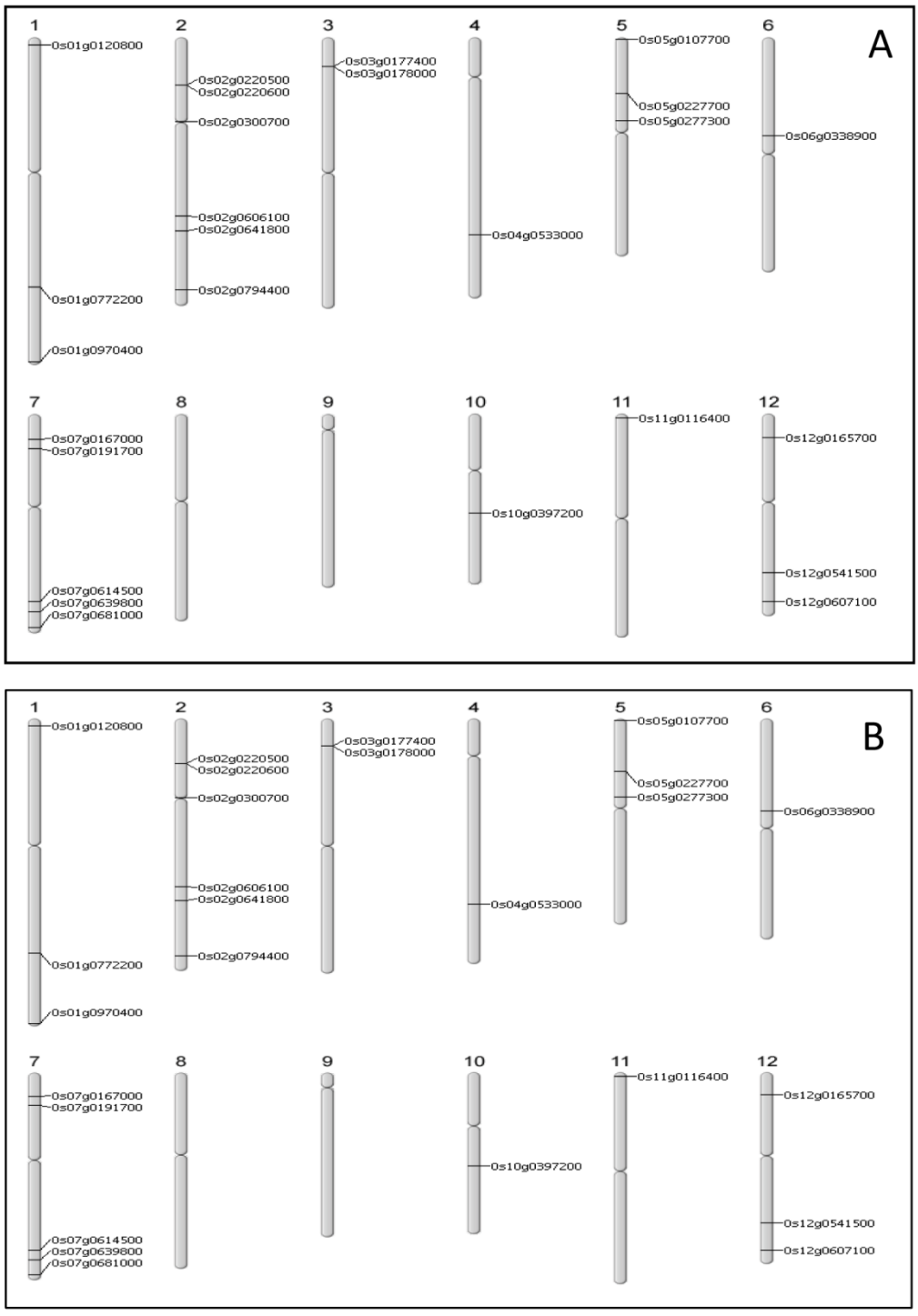


Figure 4.7 The chromosomal distribution of the 36 positively (A) and 26 negatively (B) expressed genes for shoot Na/K that significantly enriched translation factor activity in wide natural rice genotypes under salt stress.

The chromosome map is obtained by submitting the list of genes to the web based 'Chromosome Map Tool' http://viewer.shigen.info/oryzavw/maptool/MapTool.do (see section 2.14 for details).

4.2.6.4 SNAP receptor and Chaperone activity

SNAP receptor activity is regulated by a super family of proteins known as SNAREs [soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors] that act as a marker to identify a membrane and selectively interact with SNAREs on other membrane surfaces to mediate membrane fusion thus providing a continuous flux of membranes via transport vesicles. This vesicle traffic is believed to be involved in cell homeostasis, growth and development of plants (Kim and Brandizzi, 2012; Tyrrell *et al.* 2007). In this study, among the genes that are negatively expressed for shoot Na/K, six genes that significantly enriched the SNAP receptor activity in wide rice genotypes under salt stress were identified. The bet like SNARE- AtBS14a (Os02g0820700 & Os08g0563300) that were found to be significant was reported to control cell growth in *Arabidopsis* (Tai and Banfield, 2001). The syntaxin identified is AtSYP52 (encoded by Os02g0119400) was very recently described to act as t-SNARE when distributed in membrane TGN/PVC and plays a putative inhibitory role when present on the tonoplast in *Arabidopsis* (De Benedictis *et al.* 2013). Another syntexin, OSM1/SYP61, was also reported to be involved in osmotic stress tolerance in Arabidopsis (Zhu *et al.* 2002). However, three other syntexins encoded by Os07g0164300, Os01g0254900 and Os06g0168500 that were found to be significant in this study might be novel syntexin and it would be of interest to know their specific role in future.

Chaperones are proteins involved in non-covalent folding or unfolding of other proteins and are believed to be expressed in response to high temperature and other cellular stresses. Yamada *et al.* (2002) identified a cytosolic chaperonin-containing TCP-1α (CCTα)

homologue that displayed enhanced salt tolerance in the mangrove plant, *Bruguiera sexangula*. In this study, six transcripts that significantly enriched chaperone binding activity under salt stress were identified. These are GrpE type 2 (Os08g0338700), GrpE protein family protein (Os04g0431100 & Os09g0284400), DRF2 (Os12g0456200) and one protein of unknown function (Os12g0456200) and another conserved hypothetical protein. The lists of the genes can be found in Supplementary Table 4.6a2.

4.2.6.5 Ion binding

Under the molecular functional category of binding, genes were identified that significantly enriched the GO categories like Manganese ion binding, Alkali metal ion binding (including potassium ion binding) and iron, sulfur cluster binding. Most of the genes encode for proteins such as phosphatases, Kinases, Germin family protein, CBL kinases etc. Most of the genes were negative genes that are expressed for shoot Na^+ and shoot Na/K, except for only 8 positive genes for shoot Na/K that significantly enriched metal ion binding activity. The complete lists of the genes can be found in Supplementary Table 4.6a2.

4.2.6.6 Catalytic activity

A number of catalytic molecular functional activities determined by the SEA analysis was found to be significantly enriched by the genes that are positively and negatively expressed for shoot Na, shoot Na/K, root K and biomass (Table 4.4). These catalytic

activities mainly include Phosphoprotein phosphatase (including protein serine/threonine) activity, Protein methyltransferase activity, Serine O-acyltransferase, hydrolase and endopeptidase activity, Metalloexopeptidase activity and Oxidoreductase activity etc (Table 4.4 & Supplementary Table 4.6a2)

4.2.7 Interacting network of genes under salt stress

All the significant genes described above (in total 578) were evaluated to see if there is any interaction between the proteins encoded by these genes using the web based tool 'The Rice Interactions Viewer' that queries a database of 37472 predicted and 430 confirmed Rice interacting proteins (see section 2.14). The interactive networks analysis of the lists of genes revealed that there are two networks which are *Indica*ted by circle in Figure 4.8. In the larger network, LOC_Os03g08050 (Os03g0177400) seemed to be the central protein which encodes for 'Protein elongation factor (EF-1 alpha)'. Most of the proteins in this network seem to be localized mainly in nucleus (blue), cytoplasm (pink) and mitochondria (light blue) and encode mainly for the translation factors such as LOC_Os02g56740 (protein translation initiation factor eIF-2B subunit epsilon, LOC_Os05g51500 (protein eukaryotic translation initiation factor 5B), LOC_Os07g44620 (protein eukaryotic translation initiation factor 6), LOC_Os05g41900 (protein translation initiation factor SUI1), LOC_Os02g19770 (protein eukaryotic translation initiation factor 1A), LOC_Os07g36940 (protein eukaryotic translation initiation factor 4G) etc and transcription factors such as LOC_Os06g14190 (protein NF-X1-type zinc finger protein). On the contrary, only a few proteins are located in the chloroplast (green) and plasma membrane (brown) and vacuole (yellow). The proteins localized in the chloroplast (green)

are mainly catalytic proteins such as LOC_Os12g13390 (protein aspartyl aminopeptidase, putative), LOC_Os01g13190 (protein histidinol dehydrogenase, chloroplast precursor), LOC_Os02g10120 (protein lipoxygenase) and LOC_Os07g42180 (protein exportin 1). Proteins expressed in the vacuole includes LOC_Os06g23160 (protein bacterial transferase hexapeptide domain containing protein), LOC_Os01g12870 (protein eukaryotic translation initiation factor 3 subunit E-interacting protein) and LOC_Os02g39350 (protein eukaryotic translation initiation factor 2A) etc.

On the other hand, in the smaller network, all the proteins are SNAREs type proteins (SNAP receptor activity) with three localized in the vacuole encoding protein syntaxin (LOC_Os01g15110), protein vesicle transport v-SNARE protein (LOC_Os01g51120) and protein SNARE domain containing protein (LOC_Os02g02720) and localized in the plasma membrane encoding protein vesicle transport v-SNARE protein (LOC_Os01g37980).

These finding probably points towards the hypothesis that in the wide natural gene pool, transcription and translation factor genes are the main regulators under salt stress and these genes are probably the initial defence response that co-regulates in an interactive manner which ultimately cascades to induce the downstream genes that are essential for adaptation to environmental stresses.

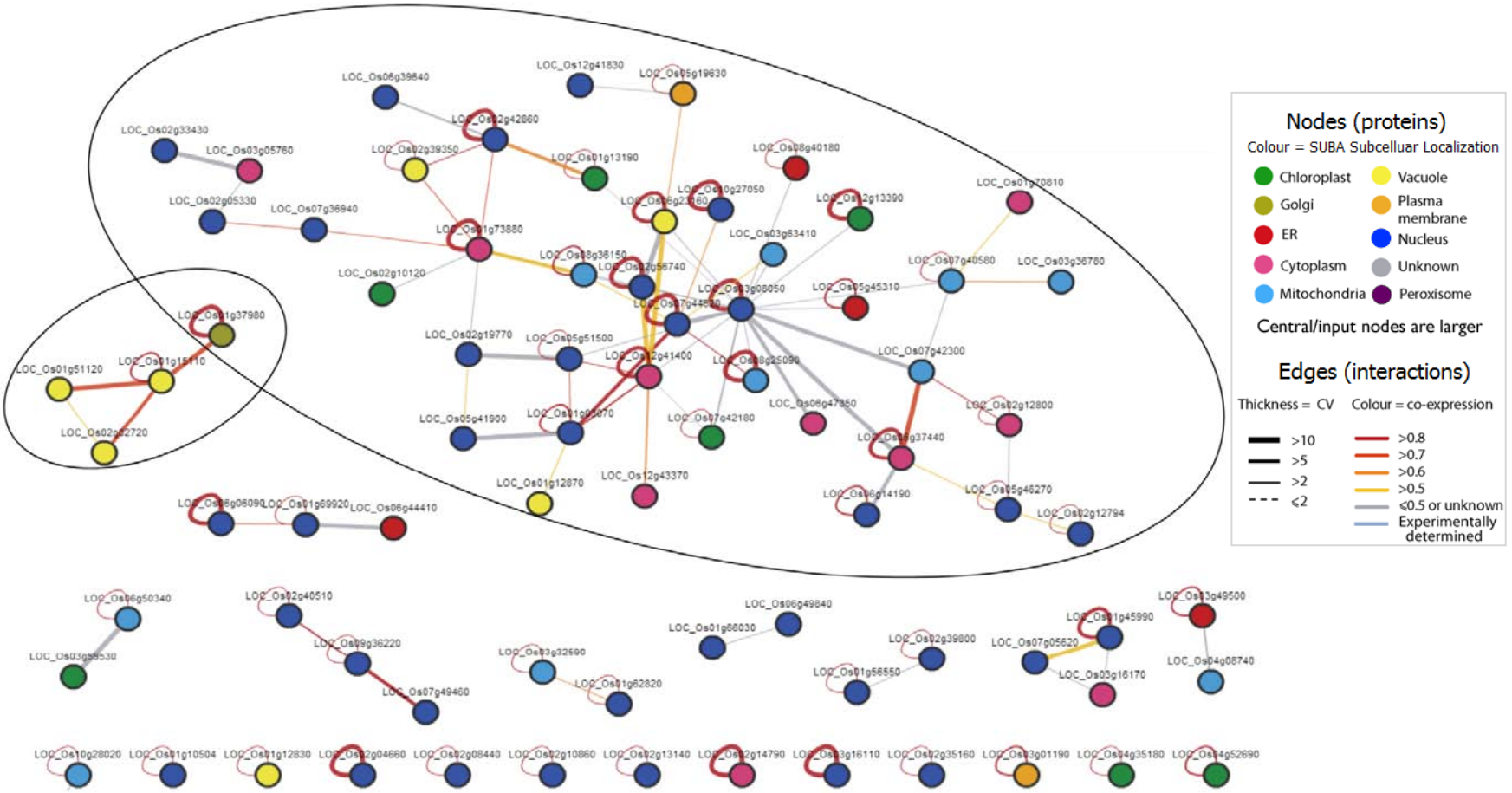


Figure 4.8 Regulatory networks of all the 578 genes that significantly enriched the molecular functional categories under salt stress in wide natural variation of rice genotypes. The web based tool 'Rice Interactions Viewer' (http://bar.utoronto.ca/interactions/cgi-bin/rice_interactions_viewer.cgi) were used to predict the interactions.

4.3 CONCLUSION

This chapter presents the first attempt to explore comprehensive global changes in the pattern of gene expression across wide natural genetic variation of rice genotypes upon exposure to salt stress. Eight rice genotypes representing a gene pool of wide natural variation in terms of taxonomy, origin and salt sensitivity were studied morpho-physiologically along with the whole genome transcriptome analysis using Agilent 4x44K rice microarray slide containing 42478 known and predicted genes. The Significance Analysis of Microarrays (SAM) was applied in a novel way (see section 2.12.1) to decipher the trait specific changes in gene expression by integrating the genome wide expression data with the weighted continuous morpho-physiological trait response of diverse rice genotypes under stressed and normal condition.

Distinctive changes in gene expression were observed for shoot Na, shoot Na/K, root Na and shoot Cl with more genes expressed upon stress imposition. Results suggested a striking difference in the candidates regulating 'biomass', with more genes being regulated negatively under stress conditions, contrasting with normal condition. Mining the data identified 60 genes involved in cation and anion homeostasis, transport and membrane bound activity. Gene ontology enrichment analysis identified 578 global genes (representing 1.36% of the entire transcriptome) involved in the major molecular functions such as signal transduction (>150 genes), transcription factor (81 genes) and translation factor activity (62 genes), SNAP receptor (6 genes) and chaperon binding (6 genes), ion binding activity (41 genes), serine/threonine phosphatase, transferase, hydrolase activity and oxidoreductase activity under salt stress. The mapping of the identified genes in the chromosomes made the global distribution clearer showing that chromosomes 1, 2, 3, 6 & 7 contain the majority

of the genes that significantly enriched signal transduction, transcription and translation initiation factors while chromosomes 10, 11 and 12 seem to hold little value in terms of global salinity response. The regulatory network analysis identified that the transcription factors and translation initiation factors mostly active in nucleus, cytoplasm and mitochondria form the major gene network and the membrane and vesicle bound proteins seem to be regulated interactively in plasma membrane and vacuoles.

The genes that are identified here provide a synergistic global salinity response picture representing the mechanisms that are active in the wide natural variation of rice genotypes, which may not be referred back to individual genotypes at the moment but might be of interest in future to look at their specific role in individual rice genotypes (the issue addressed in chapter 5). Nonetheless, the products of these genes may hold the evolutionary adaptive phenomena to cope with the hostile environments. Several of the identified genes were reported before either in rice or in other crop species, however, the novel genes and the genes with unknown function may enhance our understanding of stress adaptation once the role of these genes are functionally verified.

LIST OF SUPPLEMENTARY TABLES

Supplementary Table 4.1 (a-n). Positively and negatively regulated significant probes for each of the 14 morpho-physiological traits in the eight rice genotypes representing wide natural variation in terms of salinity tolerance and genetic diversity under unstressed condition determined by Significance Analysis of Microarrays (SAM). Significant probes were selected based on the criteria of FDR <5% (q-value) and fold score >2.0 (d) and are presented along with the corresponding Locus ID and annotation.

Supplementary Table 4.2 (a-n). Positively and negatively regulated significant probes for each of the 14 morpho-physiological traits in the eight rice genotypes representing wide natural variation in terms of salinity tolerance and genetic diversity under stressed condition determined by Significance Analysis of Microarrays (SAM). Significant probes were selected based on the criteria of FDR <5% (q-value) and fold score >2.0 (d) and are presented along with the corresponding Locus ID and annotation.

Supplementary Table 4.3 (A1, 2): Lists of probes along with the corresponding functional annotation and fold scores that commonly regulates Biomass under unstressed and stressed conditions (A1) and probes that commonly regulates Biomass and SES under stressed conditions (B1) in wide natural variation of rice genotypes

Supplementary Table 4.4 (A1,2; B1,2 & C1,2) Lists of probes along with corresponding functional annotation that commonly regulate between biomass and tissue ions under stress condition in wide natural variation of rice genotypes.

Supplementary Table 4.5a & b Lists of significant GO categories of molecular functions and biological processes along with GO terms, p-values, FDR values, GO flash charts and schematic diagrams under unstressed condition for differentially regulated (positive and negative) genes for different morpho-physiological traits (determined by SEA analysis).

Supplementary Table 4.6a & b Lists of significant GO categories of molecular functions and biological processes along with GO terms, p-values, FDR values, GO flash charts and schematic diagrams under stressed condition for differentially regulated (positive and negative) genes for different morpho-physiological traits (determined by SEA analysis).

Chapter 5. WHOLE GENOME EXPRESSION PROFILING OF RICE SEEDLINGS WITH CONTRASTING TOLERANCE TO SALT STRESS: THE *INDICA* MODEL OF SALINITY RESPONSE IN RICE

ABSTRACT

Plants respond to salt stress by regulating a series of genes on a holistic basis to cope with the unsuited environment. This project provides a never before attempted scale of profiling the whole genome transcriptional response in the seedlings of four tolerant and two susceptible *Indica* genotypes of rice using Agilent 4x44K rice microarray upon exposure to salt stress. More genes were differentially regulated in susceptible than tolerant genotypes with a substantial commonality between these two contrasting sets of rice genotypes. The differentially expressed transcripts involved in the important biological processes such as response to stimuli, signaling and signaling process and in the important molecular functions such as Transporter activity, Transcription factor, Transcription regulator activity, molecular transducer activity, antioxidant activity and nutrient reservoir activity were further investigated and the putative candidates were suggested. Highly induced stimulus responsive gene Os01g0159600 (Embryonic abundant protein 1 (OsLEA1a) and Os05g0382200 (Na^+/H^+ exchanging protein-like) can be mentioned for instance. Additionally, the genes located within the important salt stress related QTL were identified and discussed. As an example, the transcription factor gene Os01g0303600 (Zinc finger C3HC4 type (RING finger) located within the Saltol and qSKC1 QTLs for shoot K concentration and K:Na ratio in chromosome 1 can be mentioned. Among the datasets of

important identified genes, many were not previously reported and hence, form the set of novel targets which may improve our understanding of salinity tolerance mechanism in rice provided that their role is functionally verified which ultimately will be useful for biotechnological manipulation for rice improvement.

5.1 INTRODUCTION AND AIM

The salt sensitive glycophytic crop plant rice can lose its major share of yield due to abiotic stresses, particularly due to soil salinity worldwide (Chinnusamy *et al.* 2005; Vij and Tyagi, 2007; Zeng *et al.* 2002). Salinity causes accumulation of excess Na^+ and Cl^- in the shoot which is detrimental for plant nutrition and exerts osmotic stress and ionic toxicity that ultimately restricts plant growth (Flowers and Colmer, 2008; Munns, 2002; Munns *et al.* 2006). Plants respond to the stress by activating a series of cellular and metabolic mechanisms which is sensed and transmitted by the signalling and regulatory pathways (Cotsaftis *et al.* 2011; Flowers, 2004; Wu *et al.* 2013). This makes salinity tolerance a complex trait both physiologically and genetically involving a dynamic operation and coordination of a complex network of genes which ultimately leads to the metabolic and physiological adaptation (Bohnert *et al.* 2006; CHAO *et al.* 2005; Duque, 2013; Kim *et al.* 2007; Sreenivasulu *et al.* 2007). This is achieved by the modification of the expression pattern of genes that regulate the synthesis of different categories of proteins, *viz*., transporters, transcription factors, signalling and molecular transducers etc (Hasegawa, 2013; Rasmussen *et al.* 2013; Yamaguchi-Shinozaki and Shinozaki, 2006).

There exists considerable natural genetic variation for salinity tolerance within rice germplasm which means different mechanisms are in operation in different genotypes with different sets of genes being associated, a phenomenon that can potentially be harnessed to better understand and improve the salinity tolerance in rice (Horie *et al.* 2012; Langridge and Fleury, 2011).

Transcriptomics can capture the gene expression pattern in a given biological context by capturing the mRNA abundance of the entire genome. The genetic architecture of the

salinity tolerance mechanism can be better clarified by comparing the transcriptomes across genotypes. In the recent past, transcriptomics was extensively used in different crops including rice to elucidate the pattern of gene expression under diverse biological conditions including biotic and abiotic stresses (Clarke and Rahman, 2005; Close *et al.* 2004; Kumari *et al.* 2008; Shiozaki *et al.* 2005). In rice, the salinity tolerance is studied by various microarray platforms mainly involving two to four genotypes with contrasting salt sensitivities (Cotsaftis *et al.* 2011; Kumari *et al.* 2009; Senadheera and Maathuis, 2009; Ueda *et al.* 2006; Walia *et al.* 2005). The approach has already started to shift towards the use of the high-throughput sequencing techniques such as RNA-seq as the technologies are becoming more affordable day by day (Mutz *et al.* 2013; Shendure, 2008; Wang *et al.* 2009). Yet, microarray based transcriptomics can generate useful biological knowledge and for salinity tolerance in rice, a large scale study of the transcriptome involving multiple susceptible and tolerant check varieties can capture wider gene expression information and may reveal never before identified genes.

The present piece of research work thus aims at finding the changes in the level of expression of the transcripts in four tolerant and two susceptible, *Indica* rice genotypes that can be regarded as the check varieties for salinity tolerance study in rice. The capture of wider transcriptomic responses across these multiple genotypes can provide a wider dataset that can be used to build an *Indica* model of gene expression in rice under salt stress.

5.2 RESULTS AND DISCUSSION

As salinity tolerance has been extensively studied in *Indica* subspecies with most of the tolerant and sensitive check varieties belonging to this subspecies group, in this chapter it is attempted to build a pool of genes that are differentially regulated in the genotypes of this subspecies group with contrasting sensitivity to salt stress. Keeping this objective in mind, the gene expression data of the four *Indica* tolerant genotypes (Pokkali, FL478, Hassawi and Nonabokra) and two *Indica* susceptible genotypes (BRRI dhan29 and IR29), which can respectively be regarded as the tolerant and susceptible set of check varieties to study salt stress response in rice, were analysed using GeneSpring software package (GXv12.5) to identify the differentially expressed probe sets (see section 2.12.2 in chapter 2). This pool of genes can serve as the *Indica* rice model salinity response in tolerant and susceptible genotypes.

5.2.1 Quality control and filtering of gene expression data

After the normalization (quantile) process, the total data set (all the 31 samples) were subjected to Principal component analysis that identified two samples namely, IR29 (unstressed-replicate 3) & Nonabokra (unstressed-replicate 1) as outliers and were thus removed from the data set. The principal component analysis (PCA) and the box plot of normalized intensity values of all six genotypes are shown in Figure 5.1. The subsequent four step filtering process (i.e., filtering by expression, flags, data set and finally by 20% CV) bottlenecked only 21,912 probes which was further reduced to 10,163 probes by the statistical analysis; one-way ANOVA ($p<0.05$).

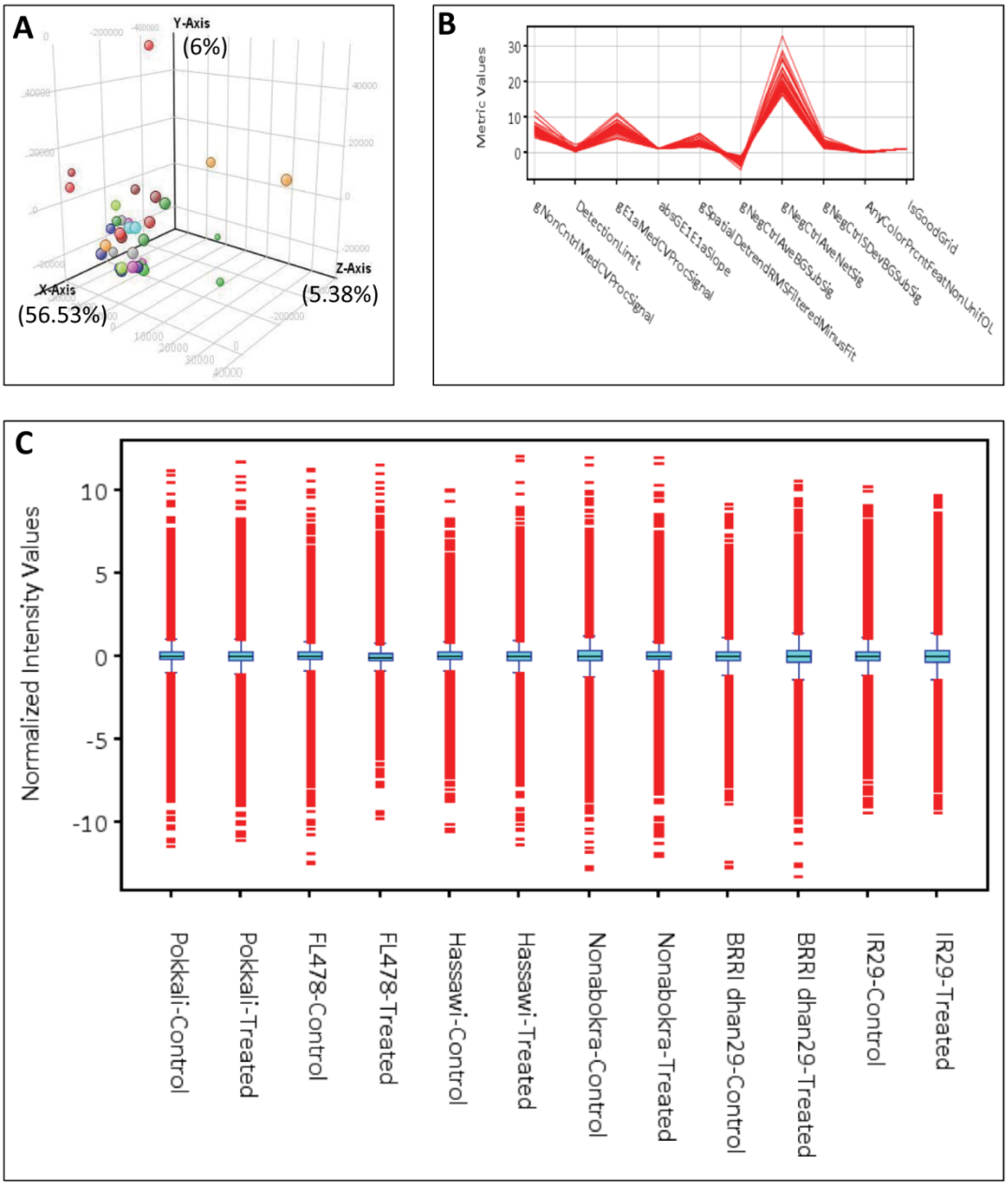


Figure 5.1 Quality control of the samples showing the principal component analysis (A) and QC metrics (B) along with the resultant box plot (C) on 33 samples after two outliers namely, IR29 (unstressed-replicate 3) & Nonabokra (unstressed-replicate 1) were excluded from the Genespring analysis.

5.2.2 Determination of differentially expressed significant genes

From these 10,163 probes, differentially regulated (both up and down-regulated) significant probes were selected by comparing the treated vs. control samples for each individual genotype based on two selection criteria; fold expression difference (≥ 2.0) and a significance value (≤ 0.05) using Volcano Plot analysis. The numbers of differentially expressed probes upon exposure to salt stress in each genotype are shown in Figure 5.2 with the corresponding volcano plot and scatter plot shown in Figure 5.3. The complete lists of up and down regulated probes along with the corresponding p-values, FC values and functional annotations in each individual genotype are shown in supplementary table 5.1a-f. In an attempt to find out the similarity between these genotypes, the numbers of probes that are commonly up or down regulated in these genotypes are shown in Table 5.1.

Among these tolerant and susceptible check genotypes, the differential constitutively expressed probes were also determined by comparing the expression data of each of the tolerant genotypes in unstressed conditions firstly, with the expression data of susceptible genotype BRRI dhan29 and secondly with that of IR29. The probes, however, were not subjected to further analysis in the project but deposited in Supplementary Table 5.2a-h for possible use in future.

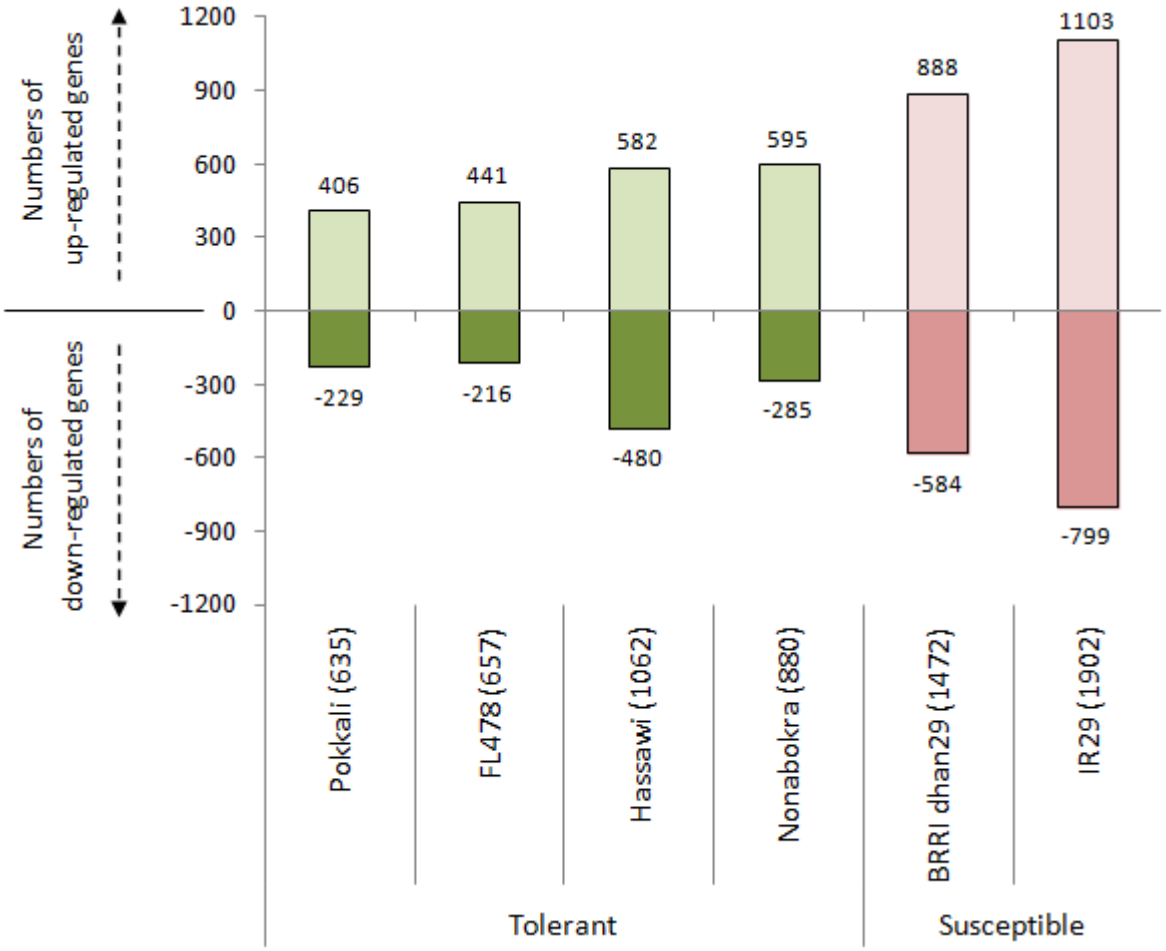


Figure 5.2 Graph showing the numbers of up-regulated (lighter shade, above x-axis) and down-regulated (darker shade, below x-axis) genes under salt stress in four tolerant genotypes namely, Pokkali, FL478, Hassawi and Nonabokra (green bars) and two susceptible genotypes namely, BRRI dhan29 and IR29 (red bars).

The significant genes were selected based on two criteria; p-value ≤0.05 and fold change value ≥2. Lists of up and down-regulated probes along with corresponding p-values, FC values and functional annotation in each individual genotype can be found in supplementary table 5.1a-f.

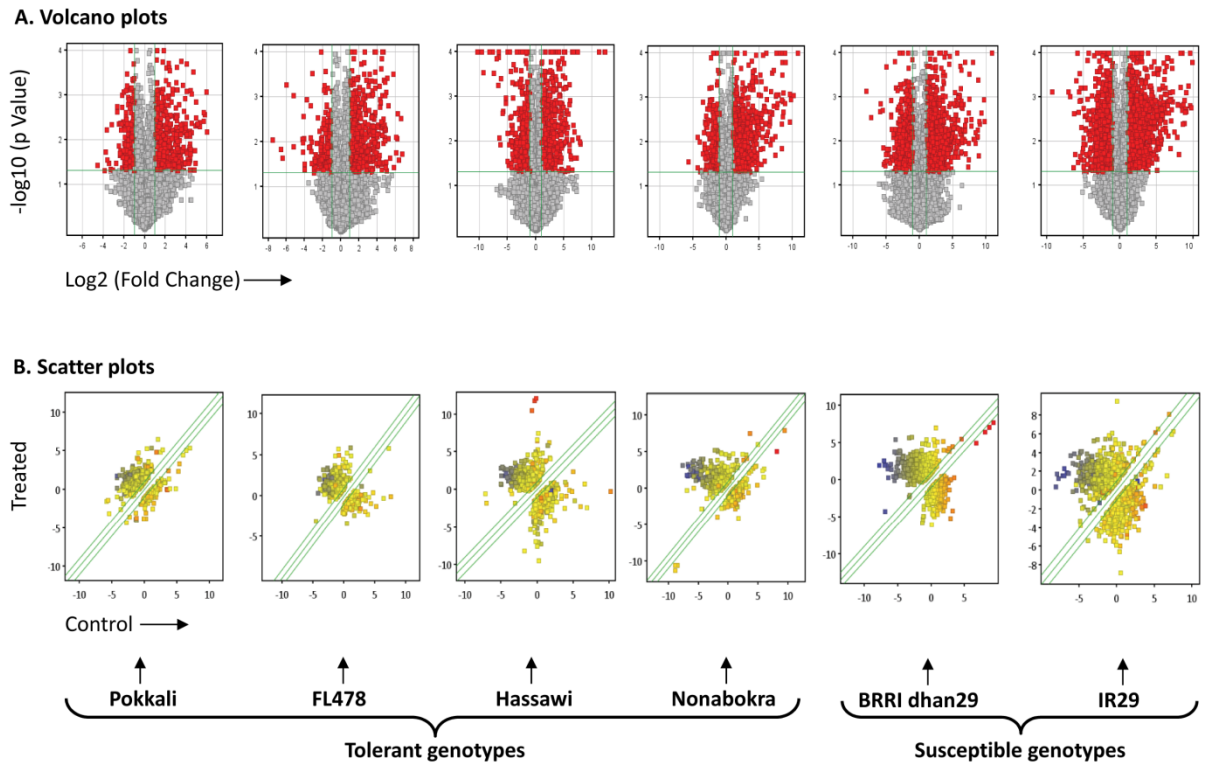


Figure 5.3 Volcano plots (A) and Scatter plots (B) showing the visual representation of the number of differentially expressed significant probes in each genotype under salt stress in four tolerant check genotypes namely, Pokkali, FL478, Hassawi and Nonabokra and two susceptible check genotypes namely, BRRI dhan29 and IR29.

Table 5.1 The number of differentially regulated probes that are common between the genotypes

Genotypes	Pokkali	FL478	Hassawi	Nonabokra	BRRI dhan29	IR29
Pokkali	x	+219 -52	+224 -97	+245 -55	+231 -68	+288 -79
FL478	x	x	+294 -106	+285 -54	+256 -80	+339 -95
Hassawi	x	x	x	+333 -92	+314 -144	+391 -181
Nonabokra	x	x	x	x	+387 -107	+479 -129
BRRI dhan29	x	x	x	x	x	+601 -305
IR29	x	x	x	x	x	X

Numbers followed by '+' and '-' sign *Indica*te the number of commonly up- and down-regulated probes, respectively.

5.2.3 The *Indica* model of salinity response in rice

For each of the four tolerant genotypes, the probes that were found to be significantly differentially regulated (both up and down-regulated) upon exposure to salt stress were pooled together to represent the pool of differentially regulated probes in the tolerant check varieties and are referred to as significant probes in pooled tolerant (PT) genotypes. The same was done with the differentially regulated significant probes of each of the two susceptible genotypes and are referred to as significant probes in pooled susceptible (PS) genotypes. The idea behind this is that the probes that are up or down-regulated in tolerant genotypes will serve as a pool of probes that are differentially expressed in all the four tolerant genotypes and similarly, in two susceptible genotypes. The details of how the pooling was done are shown in Figure 5.4 and Supplementary Table 5.3. This approach provides the advantage of identifying the putative candidate genes by wider comparison of salt responsive transcripts in the pool of tolerant and susceptible genotypes instead of

comparing the individual tolerant and susceptible genotypes as shown in Figure 5.5. The subsequent analysis is thus focused on the lists (a-i) of probes shown in Figure 5.5 and Supplementary Table 5.4 as transcripts in those lists can make the comparison of stress responsive gene expression in tolerant and susceptible genotypes easier.

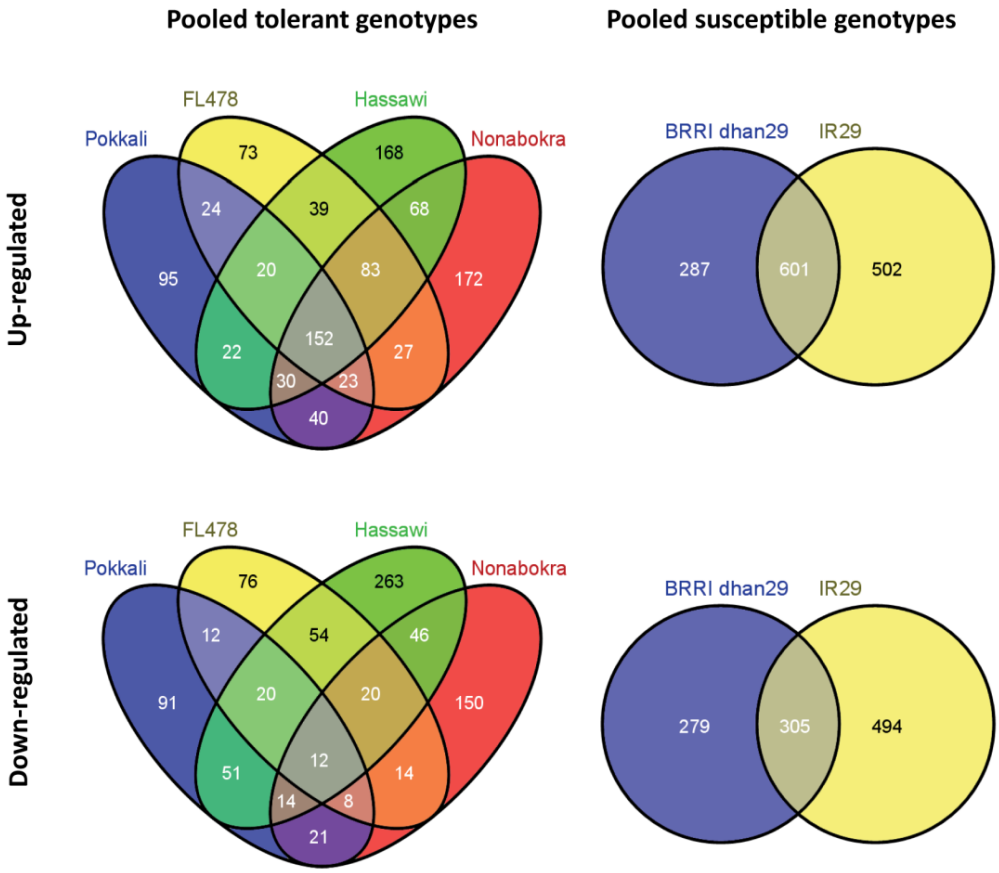


Figure 5.4 Venn diagrams showing the pooling of differentially regulated probe sets in tolerant (Pokkali, FL478, Hassawi and Nonabokra) and susceptible (BRRI dhan29 and IR29) genotypes. Probe sets represented by each of the above numbers are presented as lists in Supplementary Table 5.3.

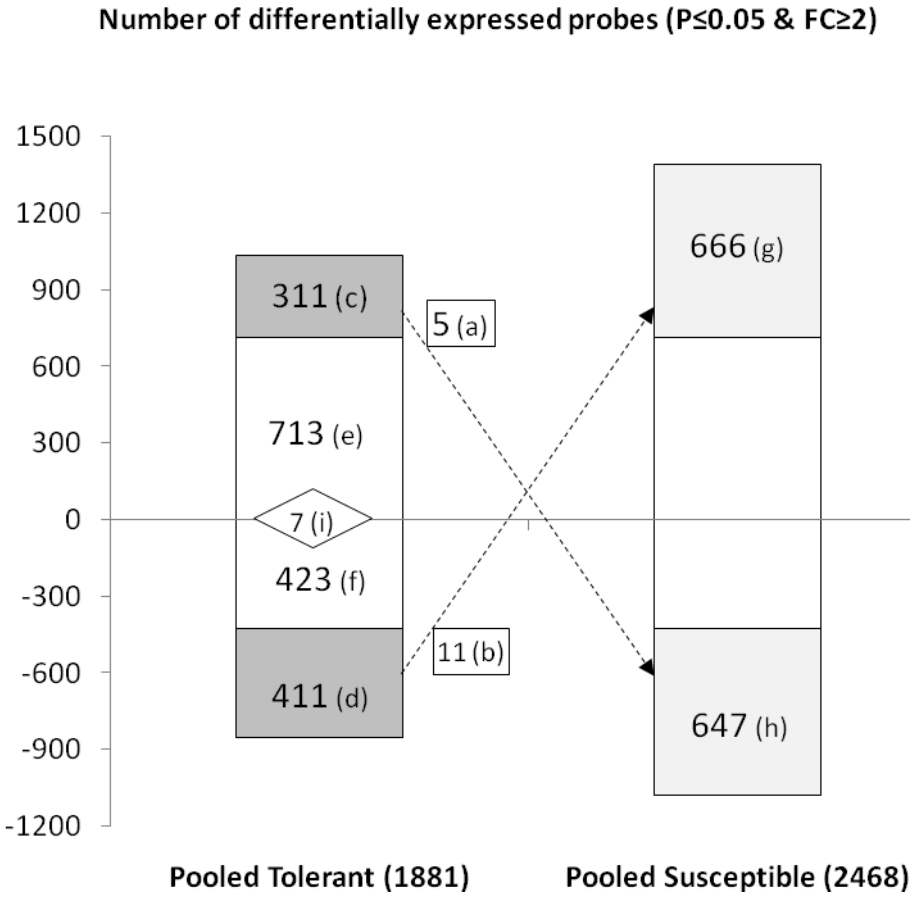


Figure 5.5 Graph showing the numbers of differentially regulated genes in pooled tolerant (PT) *viz*., Pokkali, FL478, Hassawi and Nonabokra and pooled susceptible (PS) *viz*., BRRI dhan29 and IR29 genotypes upon exposure to salt stress.

This graph is derived from figure 5.3 by pooling (shown in figure 5.4) the genes that are commonly up or down-regulated in tolerant and susceptible genotypes. Values in each of the boxes indicate the following-

'a' *Indica*tes that 5 genes are up-regulated in PT genotypes which are also down-regulated in PS genotypes;
'b' *Indica*tes that 11 genes are down-regulated in PT genotypes which are also up-regulated in PS genotypes;
'c' *Indica*tes that 311 genes are only up-regulated in PT genotypes
'd' *Indica*tes that 411 genes are only down-regulated in PT genotypes
'e' *Indica*tes that 713 genes are commonly up-regulated in PT & PS genotypes
'f' *Indica*tes that 423 genes are commonly down-regulated in PT & PS genotypes
'g' *Indica*tes that 666 genes are only up-regulated in PS genotypes
'h' *Indica*tes that 647 genes are only down-regulated in PS genotypes
'i' *Indica*tes that among the 7 genes, some are up-regulated and some are down-regulated in PT genotypes

Genes represented by each of the above numbers (a-i) are presented as lists along with corresponding expression values, p-values, fold change values and functional annotation in Supplementary Table 5.4.

Gene Ontology enrichment analysis

The probes in the lists 'a' to 'i' (see below) were evaluated critically in a way to identify the putative candidates. In addition, the lists 'c' to 'h' were subjected to Singular Enrichment Analysis (SEA), one of the gene ontology enrichment procedures (see section 2.13 in chapter 2) which facilitated the efficient extraction of the major biological meaning behind these large gene lists by focusing on the transcripts under important molecular functions and biological processes. The details of the gene ontology analysis are shown as GO flash charts in Figure 5.6a&b and as lists in supplementary table 5.6. As the number of transcripts in the lists a, b and i were small, it was not possible and necessary to do the GO enrichment to explain these probes.

List a. Probes up-regulated in tolerant genotypes but down-regulated in susceptible genotypes

The transcripts of five genes (Os06g0683700, Os01g0693300, Os11g0586800, Os10g0450000 and Os11g0581900) that were found to be up-regulated in tolerant genotypes but down-regulated in susceptible genotypes by the analytical approach followed in this project should ideally be the strongest candidate genes for salinity tolerance in rice. None of these genes were reported in previous experiments except one of the phylo-genetically related homologous genes Os11g0581900, BIL4 (BRz-insensitive-long hypocotyls 4), reported to mediate plant cell elongation by expressing in many organs of *Arabidopsis* which was suppressed by prolonged application of brassinosteroid-biosynthesis inhibitor, brassinazole (BRz) (Yamagami *et al.* 2009). The significant up-regulation in tolerant genotype Hassawi (FC +2.07) and down-regulation in susceptible genotype IR29 (FC -1.39) of Os11g0581900 may *Indica*te its role in maintaining growth under salt stress.

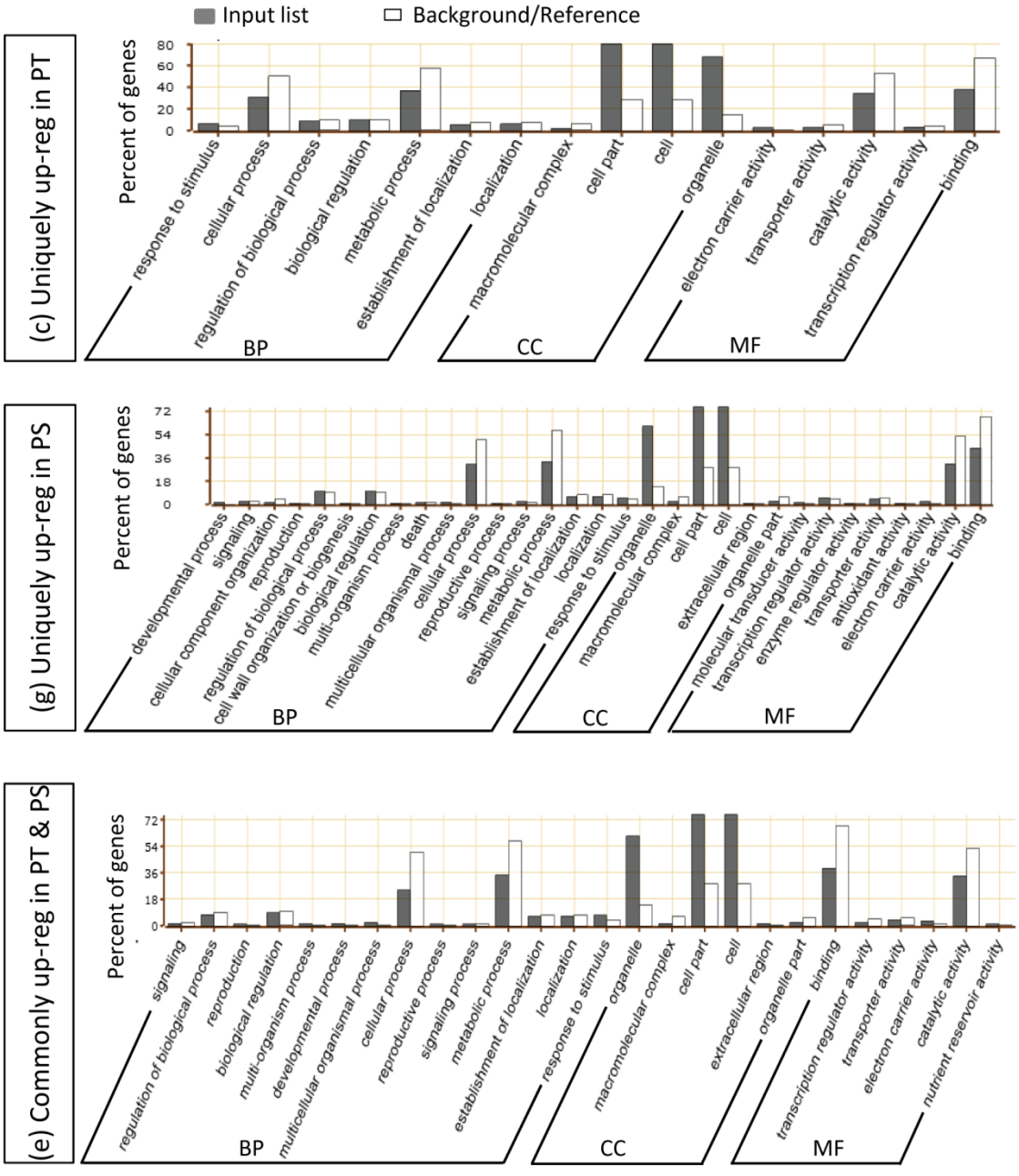
List b. Probes down-regulated in tolerant genotypes but up-regulated in susceptible genotypes

Followed by the five genes in list 'a', the 11 genes in list 'b' should ideally be the next strongest candidate for salinity tolerance in rice. The gene Os11g0701800 encoding a Class III chitinase homologue (OsChib3H-a) was most repressed in tolerant genotype Pokkali and most induced in susceptible genotype BRRI dhan29. This gene was also reported to be induced by JA treatment in susceptible genotype Nipponbare which is a susceptible genotype at high salt stress (Miyamoto *et al.* 2012). Another classs III chitinase, *Oschib1*, was reported to be induced by blast fungus *Magnaporthe grisea* and *Xanthomononas oryzae pv. Oryzae* (Park *et al.* 2004). Chitinases are largely induced by stresses like wounding, pathogen and signalling molecules such as ethylene, JA, ABA, ET, MJ and SA etc. (Jwa *et al.* 2006) and thus may play an important role in salinity tolerance in rice as well.

Another gene, Os02g0467000 encoding cinnamate 4-hydroxylase CYP73 was repressed in tolerant genotype Nonabokra and induced in susceptible genotype BRRI dhan29. A class II cinnamate 4-hydroxylase, CYP73A15 is reported to reduce and delay the lignification in French bean and tobacco (Blee *et al.* 2001). The down-regulation of CYP73 in this study probably suggests its putative role in increasing lignification which might occur in the Casparian strip and thus confer tolerance to salt.

The other genes which were not reported before include Os05g0283600 (Zn-finger, CCHC type domain containing protein), Os11g0307300 (Herbicide safener binding protein), Os01g0217500 (DJ-1 family protein) and genes (Os11g0206100, Os11g0307300, Os06g0722700, Os05g0223300, Os08g0156100, Os02g0300000 and Os04g0601500) of unknown function. As the two reported genes (Os11g0701800 &Os02g0467000) are

evidently playing a role in stress tolerance, these un-reported genes may also be involved in the process and hence, would be of interest to know their function in future.



PT= Pooled tolerant , PS = Pooled susceptible
BP = Biological process, CC = Cellular component, MF = Molecular function

Figure 5.6a Flash charts showing the comparison of overall GO terms between the differentially expressed uniquely and commonly up-regulated probe-sets in pooled tolerant (PT) namely, Pokkali, FL478, Hassawi and Nonabokra and pooled susceptible (PS) namely,

BRRI dhan29 and IR29 rice genotypes under biological process (BP), cellular component (CC) and molecular function (MF) derived from Singular Enrichment Analysis (SEO) using AgriGO web based tool.

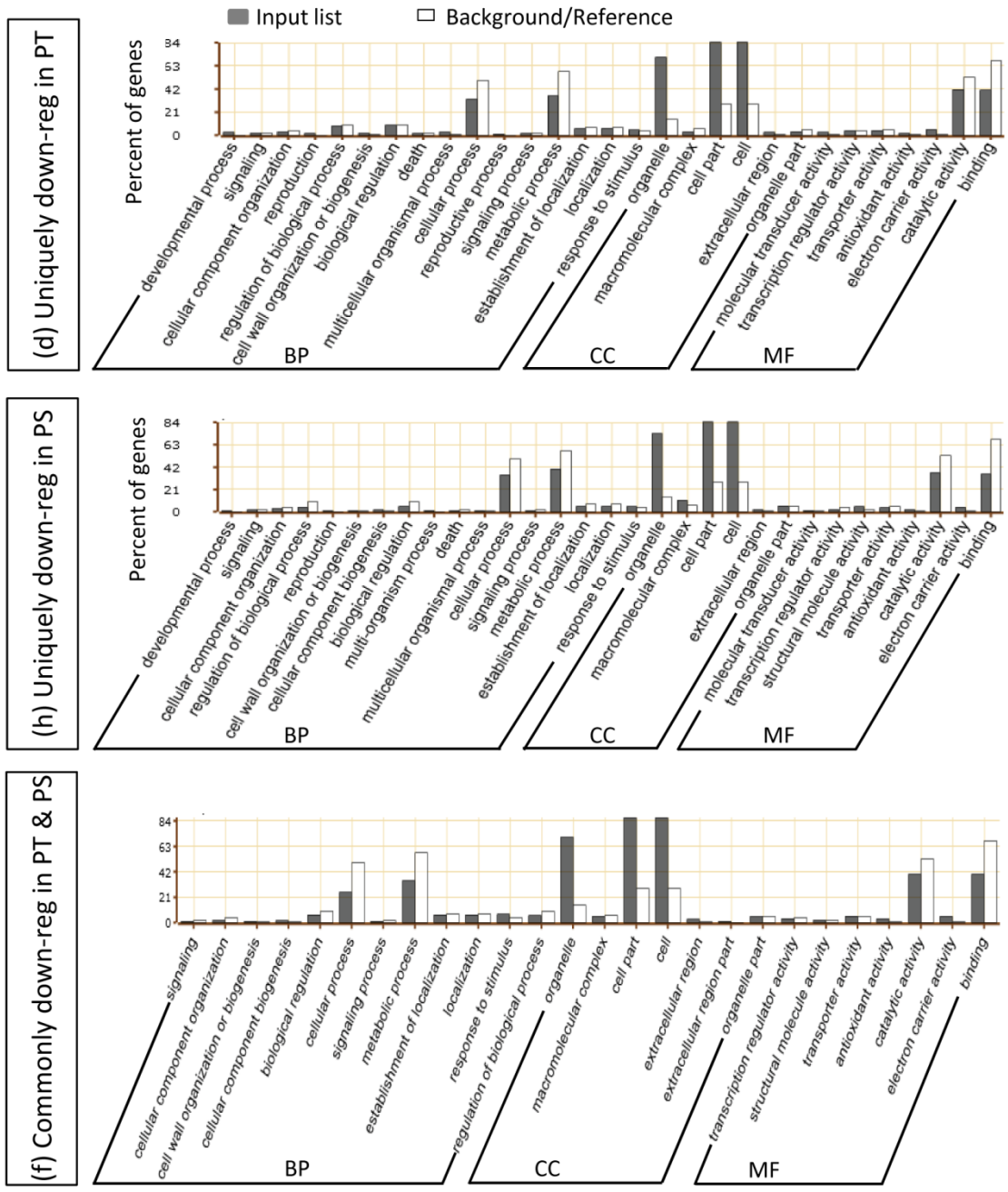


Figure 5.6b Flash charts showing the comparison of overall GO terms between the differentially expressed uniquely and commonly down-regulated probe-sets in pooled tolerant (PT) namely, Pokkali, FL478, Hassawi and Nonabokra and pooled susceptible (PS) namely, BRRI dhan29 and IR29 rice genotypes under biological process (BP), cellular

component (CC) and molecular function (MF) derived from Singular Enrichment Analysis (SEO) using AgriGO web based tool.

List c. Probes uniquely up-regulated in tolerant genotypes

The 311 genes that are uniquely up-regulated in tolerant genotypes but which showed no significant response in susceptible genotypes represents the other strong putative genes for salinity tolerance in rice (List c in Supplementary Table 5.4). Gene Ontology analysis grouped these 311 genes according to several biological processes (BPs) and molecular functions (MFs). Among the BPs, 'response to stimulus' (15 genes) and among the MFs, 'Transporter activity' (7 genes) and 'Transcription regulator activity' (8 genes) are deemed important and discussed further (Table 5.2; Supplementary table 5.5).

Table 5.2 Probes under the selected Biological processes (BP) and molecular functions (MF) derived from the Singular Enrichemnt Analysis (SEA) of the 311 (list c, ST 5.4) uniquely up-regulated probes in pooled tolerant genotypes (Pokkali, FL478, Hassawi and Nonabokra) upon exposure to salt stress.

RAP-DB ID (Locus ID)	**Annotation**	**Fold Change (Log2)**			
		Pokkali	**FL478**	**Hassaw**	**Nonabokra**
(BP) **Response to stimulus (15)**					
Os01g0159600	Embryonic abundant protein 1.			2.69	5.78
Os05g0540100	FEN-1.			3.27	
Os01g0959200	Abscisic stress ripening protein 1.	3.09			1.72
Os11g0229500	Disease resistance protein family protein.	2.41			
Os04g0465100	Haem peroxidase, plant/fungal/bacterial family protein.			2.05	
Os07g0694300	Fungal lignin peroxidase family protein.			1.57	
Os04g0549600	Heat shock protein DnaJ family protein.			1.55	
Os10g0542900	Chitinase (EC 3.2.1.14) (Fragment).	1.88		1.39	
Os03g0655400	Similar to Water stress induced protein.			1.37	
Os03g0130300	Cp-thionin.	2.05		1.29	1.05
Os04g0446200	Conserved hypothetical protein.			1.19	
Os01g0149800	Metallothionein-like protein type 2.	1.11	1.12	1.15	1.73
Os02g0135200	Blast and wounding induced mitogen-activated protein kinase.	1.12			
Os06g0275000	Hd1.				1.77
Os01g0369900	NADH:flavin oxidoreductase/NADH oxidase family protein.		1.01		
(MF) **Transporter activity (7)**					

Os05g0382200	Na+/H+ exchangeing protein-like.	3.46	4.45	4.62	4.57
Os10g0418100	Calcium-transporting ATPase 8, plasma membrane-type (EC 3.6.3.8) (Ca(2+)-ATPase isoform 8).	3.12	1.73	2.01	
Os07g0666900	Na/H antiporter Nhx1.	1.50			
Os12g0638200	Peptide transporter.	1.38			
Os02g0313900	H(+)-transporting ATPase.			2.17	
Os02g0825600	Plasma membrane H+ ATPase (EC 3.6.3.6).			2.09	
Os06g0158900	Multidrug-resistance associated protein 3.			1.08	
(MF) **Transcription regulator activity (8)**					
Os01g0868000	Pathogenesis-related transcriptional factor and ERF domain containing protein.		1.78	1.45	
Os08g0521600	Pathogenesis-related transcriptional factor and ERF domain containing protein.		2.74		2.07
Os05g0549800	Pathogenesis-related transcriptional factor and ERF domain containing protein.	1.26			
Os01g0106400	Isoflavone reductase-like protein.		1.68		
Os10g0376900	Basic helix-loop-helix dimerisation region bHLH domain containing protein.				1.80
Os06g0275000	Hd1.				1.77
Os11g0490900	WRKY transcription factor 72.				1.04
Os05g0439100	Hypothetical protein.		1.17		1.00

Among the 15 genes under BP 'response to stimulus', the most up-regulated gene (>5 fold in Nonabokra and 2.69 fold in Hassawi) is Os01g0159600 which encodes 'Embryonic abundant protein 1 (OsLEA1a)'. LEA proteins play important roles in abiotic stress tolerance such as salt, cold and chilling stress (Bhardwaj *et al.* 2013; Shih *et al.* 2010; Wang *et al.* 2007a). Wang *et al.*(2007a) identified 34 OsLEA genes in rice and reported both constitutive and stress responsive expression under several stress conditions. Using the variety Nipponbare, they found no expression of OsLEA1 under cold and salt stresses; however, it was induced by GA and ABA treatment. The higher unique expression of this gene in tolerant genotype Nonabokra *Indica*tes its putative role in salt tolerance in rice.

The gene Os01g0959200 encoding an 'Abscisic stress ripening protein 1 (*OzAsr1*)' was up-regulated in Pokkali (>3 fold) and Nonabokra (1.72 fold). This gene was reported to be

induced by drought and cold stress (Joo *et al.* 2013; Kim *et al.* 2009a) in rice and by salt stress in tomato (Goldgur *et al.* 2007; Kalifa *et al.* 2004).

The gene Os03g0130300 encoding Cp-thionin was also up-regulated in Pokkali (2.4 fold) and Hassawi (1.29 fold). Plant γ-thionins or defensins as they are commonly known are believed to play important roles in plant defence against pests and pathogens (Thevissen *et al.* 2004) and are reported to be abiotic stress responsive in *Panax ginseng* (Lee *et al.* 2011). The role of the thionins, particularly the Cp-thionin (Os03g0130300) identified in this research would be of interest to elucidate their specific functions in rice abiotic stress tolerance.

The transcript of Os10g0542900 (Chitinase, EC 3.2.1.14, Fragment) was upregulated in Pokkali and Hassawi. It was also reported to be induced in Nipponbare leaf disks upon JA treatment (Miyamoto *et al.* 2012) and in *Oryza sativa* L. cv. Jinheung by rice blast fungus *M. Grisea* (Kim *et al.* 2009b). The mitogen-activated protein kinase (Os02g0135200) is induced in Pokkali which was reported to be expressed in pollen mother cells of Nipponbare *Indica*ting its putative role in reproduction (Tang *et al.* 2010). The gene Os08g0459600 (*OsOPR7*) is involved in the biosynthesis of jasmonic acid (Tani *et al.* 2008) and is induced by the phytotoxic effects of copper in rice (Lin *et al.* 2013). The up-regulated gene Os01g0369900 (*OsOPR10*) is phylogenetically related with *OsOPR7* and thus may have a role in abiotic stress signalling in rice. Among the induced genes, Os03g0655400 that is believed to encode a 'Water stress induced protein' was expressed in the transgenic (OsDREB1B transferred) rice, NERICA1 under drought conditions (Ishizaki *et al.* 2013) and in the roots of iron-deficient rice cultivar Tsukinohikari (Kobayashi *et al.* 2009). The flowering time gene 'Heading date 1 (hd1)' which is encoded by Os06g0275000 is believed to be the key gene in rice yield improvement (Endo-Higashi and Izawa, 2011; Tsuji *et al.* 2011) and was also up-

regulated in Hassawi. It is also involved in 'Transcription regulator activity' but its role in salinity response in rice is not yet clear, however, will be of interest to look at in future.

Among the genes that are involved in response to stimulus, which were not reported elsewhere, the important genes include Os04g0549600 (Heat shock protein), Os11g0229500, Os04g0465100 and Os07g0694300 (pathogen related proteins), Os01g0149800 (Metallothionein-like protein type 2) and Os05g0540100 (FEN-1) with one gene of unknown function (Os04g0446200).

Among the seven genes involved in the MF, 'transporter activity', gene Os05g0382200 (Na+/H+ exchanging protein-like) was highly induced (~4 fold) in all four tolerant genotypes. The Na+/H+ exchanger was reported to confer NaCl tolerance in yeast (Nass *et al.* 1997), compartmentalize sodium in the vacuole of halophyte *Salicornia bigelovii* (Parks *et al.* 2002), regulated by the Salt-Overly-Sensitive (SOS) Pathway in *Arabidopsis thaliana* (Qiu *et al.* 2004) and its role in stress-induced signal transduction is reviewed by (Pedersen, 2006). Senadheera *et al.* (2009) reported the increased expression of one putative cation-proton exchanger, OsCHX11 (Os05g31730) in the roots of rice under salt stress. The fact that the gene Os05g0382200 (Na+/H+ exchanging protein-like) is highly induced in all tolerant genotypes, makes it one of the most important candidate genes for improving salinity tolerance in rice.

The gene Os10g0418100 encoding 'Calcium-transporting ATPase 8' is the other next highly induced gene in all the tolerant genotypes except Nonabokra. This gene showed increased expression in rice roots under drought (Jeong *et al.* 2013; Redillas *et al.* 2012) and copper and cadmium stress (Lin *et al.* 2013).

The Na/H antiporter Nhx1 (Os07g0666900) on the other hand was only found to be induced in Pokkali (>1.50 fold). This gene was well studied in rice and reported be expressed under salt stress and localized in the tonoplast (Fukuda *et al.* 2004; Fukuda *et al.* 1999; Mullan *et al.* 2007; Zhao *et al.* 2006).

In this experiment, the gene Os12g0638200 encoding 'Peptide transporter' was induced in Pokkali. Peptide transporters are membrane transport proteins playing roles in transport of peptides and nitrates (Steiner *et al.* 1994; Tsay *et al.* 2007) and recently, an *Arabidopsis* peptide transporter AtPTR3 was reported to be salt inducible (Karim *et al.* 2005).

Among the induced transporters, Os02g0313900 (H(+)-transporting ATPase), Os02g0825600 (Plasma membrane H+ ATPase) and Os06g0158900 (Multidrug-resistance associated protein 3) were not reported before, hence may hold a clue in improving salt tolerance in rice.

The Isoflavonereductase-like protein, (Os01g0106400, *OsIrl*) is reported to be expressed under sulfur deprivation in rice (Lunde *et al.* 2008), maize (Petrucco *et al.* 1996) and *Arabidopsis* (Hirai and Saito, 2004). The 'Basic helix-loop-helix dimerisation region bHLHdomain containing protein encoding gene Os10g0376900 was induced by jasmonic acid in rice leaf disks and suspension cells (Miyamoto *et al.* 2012). The gene Os11g0490900 encoding 'WRKY transcription factor 72' was induced by low-temperature stress in rice NIL containing a major quantitative trait locus qLTG3-1 controlling low-temperature germinability in rice (Fujino and Matsuda, 2010). The other salt induced genes are Os06g0275000 (Hd1) which is discussed in ‘response to stimulus’ section above, and Os05g0439100 with a unknown function.

List d. Probes uniquely down-regulated in tolerant genotypes

Among the 411 uniquely down-regulated genes in tolerant genotypes that showed no significant response in susceptible genotypes, 17 were involved in the biological process 'response to stimulus' (List d in Supplementary Table 5.4, Figure 5.6b and Supplementary table 5.5d). Of these, the down regulation of *LATERAL ROOT PRIMORDIUM 1* (Os09g0531600) suggests its role in restricting root growth in rice under salt stress. In a comparative proteomic analysis in wheat roots, the protein 'Haem peroxidase' (Os05g0134800) was differentially regulated under salt stress (Guo *et al.* 2012), whereas the other down-regulated genes (Os06g0695300 and Os01g0293900) encoding the same protein were not reported in that experiment. Brunings *et al.* (2009) reported the up-regulation of Os08g0452500 (Auxin responsive SAUR protein family protein) and Os08g0539700 (PibH8 protein) in response to pathogen rice blast fungus *Magnaporthe oryzae*. The auxin efflux carrier gene, Os01g0802700 was up-regulated in young florets of the heat-tolerant rice cultivar 996 upon exposure to 40°C heat treatment (Zhang *et al.* 2012) and Os03g0300400 (Pathogen-related protein) were induced by Blast and Leaf Blight Diseases in rice plants where OsSSI2 was knocked out (Jiang *et al.* 2009). The expression of the rest of the genes involved in 'response to stimulus' was not reported elsewhere. Beside these, 16 'transporter activity', 15 'transcription regulator activity', 9 'molecular transducer activity' and 6 'antioxidant activity' genes were also down-regulated (Supplementary table 5.5d). These genes were not discussed further to focus on the up-regulated genes that are likely to be more important in elucidating salinity tolerance mechanism in rice.

List e. Probes commonly up-regulated in both tolerant and susceptible genotypes

Interestingly, transcripts of 713 genes are found to be up-regulated in both tolerant and susceptible genotypes (List e in Supplementary Table 5.4). These commonly up-regulated genes may not hold the clue for differential salinity tolerance between tolerant and susceptible genotypes unless these genes regulate at different degrees in these two categories of genotypes. Data were thus mined to find out the difference in the degree of up-regulation between tolerant and susceptible genotypes. It was generally observed that fewer genes were up-regulated at a higher level in tolerant genotypes than that of their susceptible counterparts.

The genes that are involved in the BP response to stimulus (41) and signalling (9) and in the MF transporter activity (23) and transcription regulator activity (13) as derived from the Gene Ontology enrichment analysis and are discussed further (Table 5.3; Figure 5.6a and Supplementary Table 5.5e).

Among the genes involved in response to stimulus that are up-regulated to a higher degree in tolerant genotypes, Os01g0959100 encoding 'Abscisic stress ripening protein 1' was also reported to be up-regulated in OsNAC5 transgenic rice seedlings upon cold, drought and salt stress (Jeong *et al.* 2013). The gene Os02g0643800 encoding 'Auxin responsive SAUR protein family protein' was reported to be up-regulated in rice panicles after heat stress imposition (Zhang *et al.* 2012). The dehydrins Os11g0451700 (Dehydrin 9), Os11g0454000 (Dehydrin family protein) and Os01g0702500 (Dehydrin RAB25) were highly up-regulated in Nonabokra whereas Os02g0669100 (Dehydrin COR410, Cold-induced COR410 protein) and Os11g0454200 (Dehydrin RAB 16B) showed slightly more up-regulation in susceptible genotypes (Supplementary Table 5.5).

Table 5.3 Probes under the selected Biological processes (BP) and molecular functions (MF) derived from the Singular Enrichemnt Analysis (SEA) of the 713 (list e, ST 5.4) that are commonly up-regaulated in at least one tolerant and in one susceptible genotypes upon exposure to salt stress.

RAP-DB ID (Locus ID)	Annotation	Fold Change (Log2)					
		Tolerant				Susceptible	
		Pokkali	FL478	Hassawi	Nona bokra	BRRI dhan29	IR29
(BP) Response to stimulus (41)							
Os01g0959100	Abscisic stress ripening protein 1.	4.08	4.03	3.86	3.66		3.35
Os07g0694700	Ascorbate peroxidase (EC 1.11.1.11).				1.21		1.80
Os02g0643800	Auxin responsive SAUR protein family protein.		1.14	2.95			1.12
Os11g0592200	Barwin domain containing protein.	1.42				3.20	2.18
Os11g0592000	Barwin.	2.04				2.95	3.41
Os06g0127100	CBF-like protein.		3.79		4.60	3.43	4.84
Os02g0465900	ChaC-like protein family protein.	1.08	1.22	1.97	1.31	2.38	1.91
Os12g0437800	CI2E.		3.45	1.82		2.24	2.56
Os11g0451700	Dehydrin 9.	4.36			10.13	8.91	7.79
Os02g0669100	Dehydrin COR410 (Cold-induced COR410 protein).		1.29	1.73	2.37	2.60	3.06
Os11g0454300	Dehydrin family protein.	4.37	4.94	6.92	9.62	8.40	6.98
Os11g0454000	Dehydrin family protein.			4.53	6.58		5.87
Os11g0454200	Dehydrin RAB 16B.	4.36	5.04	7.27	9.61	9.31	9.61
Os11g0453900	Dehydrin RAB 16D.				8.52	9.77	8.54
Os01g0702500	Dehydrin RAB25.		4.18	4.59	6.97	5.79	5.54
Os05g0349800	Embryonic abundant protein 1.				4.77	5.77	4.32
Os04g0493400	Endochitinase A precursor (EC 3.2.1.14) (Seed chitinase A).	3.52		2.48	3.24	3.93	3.09
Os05g0407500	Esterase/lipase/thioesterase domain containing protein.			1.11		1.60	1.98
Os01g0327100	Haem peroxidase, plant/fungal/bacterial family protein.	1.52				1.73	1.32
Os03g0745000	Heat shock factor (HSF)-type, DNA-binding domain containing protein.			3.34			5.79
Os09g0482600	Heat shock protein 82.				3.39		4.68
Os06g0553100	Heat shock transcription factor 29 (Fragment).	1.74			3.22	4.07	3.89
Os01g0571300	Heat shock transcription factor 31 (Fragment).	1.91	2.12	2.15	3.94	4.28	4.52
Os12g0147200	Hypothetical protein.	4.84	4.78	5.00	6.92	6.79	6.79
Os10g0345100	Multi antimicrobial extrusion protein MatE family protein.	1.60	1.79		2.40	3.26	2.49

Os03g0188100	Multi antimicrobial extrusion protein MatE family protein.			1.80			3.55
Os06g0216300	Oxo-phytodienoic acid reductase (12-oxophytodienoic acid reductase).	5.12	5.81	4.19			1.15
Os01g0963200	Peroxidase BP 1 precursor.	1.24		1.90			2.05
Os03g0390200	Protein kinase 3.	1.32	1.35	1.60	1.99	2.39	3.09
Os12g0626500	Seed maturation protein domain containing protein.				6.66	4.52	4.16
Os06g0341300	Seed maturation protein domain containing protein.				8.33	7.40	4.86
Os02g0782500	Small heat stress protein class CIII.	3.89	3.22	4.91	4.60	5.46	6.38
Os06g0517700	Thionin Osthi1.				4.67	2.70	4.37
Os01g0511100	Universal stress protein (Usp) family protein.	3.18	3.33	3.64	3.87	5.05	5.18
Os10g0437500	Universal stress protein (Usp) family protein.	2.28		1.74		3.37	
Os02g0707900	Universal stress protein (Usp) family protein.	1.42	1.03	1.64	1.95	1.77	1.28
Os01g0849600	Universal stress protein (Usp) family protein.				2.48		1.84
Os01g0783500	Universal stress protein (Usp) family protein.			1.05	1.39	1.78	1.85
Os03g0180900	ZIM domain containing protein.		1.95		1.88	1.77	
Os06g0612800	Zn-finger, A20-like domain containing protein.				1.03	1.60	1.52
Os01g0256500	Zn-induced protein.		1.28	1.97	1.08	1.45	1.92
(BP) Signaling (9)							
Os09g0418000	Protein kinase domain containing protein.	1.81	1.00			2.08	2.32
Os11g0132900	Protein kinase family protein.	1.33	1.17	2.23	1.92	2.49	2.21
Os03g0390200	Protein kinase 3.	1.32	1.35	1.60	1.99	2.39	3.09
Os12g0130200	Ser/Thr protein kinase (Fragment).	1.28	1.13	2.04	1.81	2.36	2.23
Os05g0407500	Esterase/lipase/thioesterase domain containing protein.			1.11		1.60	1.98
Os07g0550400	Receptor-like protein kinase 5.				1.13	2.03	1.93
Os02g0643800	Auxin responsive SAUR protein family protein.		1.14	2.95			1.12
Os03g0180900	ZIM domain containing protein.		1.95		1.88	1.77	
Os08g0307400	Phosphatidylinositol 3-kinase, root isoform (EC 2.7.1.137) (PI3- kinase) (PtdIns-3-kinase) (PI3K) (SPI3K-5).			1.33		1.71	
(MF) Transporter activity (23)							
Os04g0435100	Amino acid/polyamine transporter I family protein.	1.01					1.31
Os04g0659800	Amino acid/polyamine transporter II family protein.	1.20				1.86	2.27
Os01g0645200	Bile acid:sodium symporter family protein.				2.27	2.22	1.42
Os03g0758300	Cyclic nucleotide-gated ion channel 2 (AtCNGC2) (Cyclic nucleotide-and calmodulin-regulated ion channel 2) (DEFENSE NO DEATH 1).			1.37	1.72		1.49
Os03g0218400	Hexose transporter.	4.03	3.07	2.97	1.01	2.36	2.70
Os03g0167200	IQ calmodulin-binding region domain containing protein.			1.02	1.34	2.23	1.42
Os03g0305600	Mitochondrial import inner membrane translocase, subunit Tim17/22 family protein.	4.25	4.22	6.00	7.72	7.77	7.22
Os01g0303300	Mitochondrial import inner membrane translocase, subunit Tim17/22 family protein.		3.53		7.19	6.93	5.80

Os01g0225000	Mitochondrial substrate carrier family protein.	2.66	2.82	3.17	4.21		3.25
Os10g0345100	Multi antimicrobial extrusion protein MatE family protein.	1.60	1.79		2.40	3.26	2.49
Os03g0188100	Multi antimicrobial extrusion protein MatE family protein.			1.80			3.55
Os05g0196100	Multidrug resistance associated protein 1.		1.52		1.30		1.18
Os01g0142800	Peptide transporter.	1.71	2.00	2.31	1.45	1.09	
Os03g0689300	Plasma membrane H+ ATPase (EC 3.6.3.6) (H-ATPase).			2.89	2.42	2.97	3.64
Os07g0191200	Plasma membrane H+ ATPase (EC 3.6.3.6).		1.69	1.20	1.32		1.32
Os11g0151600	Schizosaccharomyces pombe (Fragment).			1.50	1.33	2.05	1.80
Os03g0170900	Sucrose transporter.		1.33			1.37	
Os10g0360100	Sugar transporter protein.	2.36	2.96	3.48	3.51	3.45	2.52
Os03g0161200	Sulfate transporter 3.1 (AST12) (AtST1).		1.86				2.94
Os02g0612900	Temperature stress-induced lipocalin.	1.82	1.75	1.72	1.81	1.30	
Os05g0410500	TGF-beta receptor, type I/II extracellular region family protein.		2.42		2.29	3.11	3.74
Os06g0264500	TGF-beta receptor, type I/II extracellular region family protein.		1.31	1.48	1.46	2.05	2.56
Os10g0492600	Tonoplast membrane integral protein ZmTIP3-1.			4.63	4.42	3.59	4.03
(MF) Transcription regulator activity (13)							
Os12g0168100	AP2 domain containing protein RAP2.6 (Fragment).		3.01	3.39		3.83	4.40
Os03g0379300	Basic helix-loop-helix dimerisation region bHLH domain containing protein.	1.18			1.88		1.43
Os01g0159800	Basic helix-loop-helix dimerisation region bHLH domain containing protein.			1.08	1.13	1.83	2.09
Os06g0127100	CBF-like protein.		3.79		4.60	3.43	4.84
Os01g0165000	DRE binding protein 2.		1.60	1.51	1.95	2.62	2.40
Os03g0745000	Heat shock factor (HSF)-type, DNA-binding domain containing protein.			3.34			5.79
Os06g0553100	Heat shock transcription factor 29 (Fragment).	1.74			3.22	4.07	3.89
Os01g0571300	Heat shock transcription factor 31 (Fragment).	1.91	2.12	2.15	3.94	4.28	4.52
Os04g0541700	Homeobox domain containing protein.		2.50		3.46		3.17
Os01g0867300	OSE2-like protein (Fragment).	1.40	1.37	1.79	2.46	2.87	2.89
Os02g0649300	Short highly repeated, interspersed DNA (Fragment).		4.69	6.53	7.42	6.75	7.91
Os03g0815900	Transcription elongation factor S-II family protein.				1.15	1.59	1.58
Os01g0734000	WRKY DNA binding protein.				1.04	1.91	
(MF) Nutrient reservoir activity (6)							
Os03g0694000	Oxalate oxidase 1 (EC 1.2.3.4) (Germin).	3.68	2.04	2.35	3.38	4.16	3.69
Os03g0804700	Germin-like protein subfamily T member 1 precursor.	3.62	3.11	2.86		1.56	
Os03g0793700	Globulin 2 (Fragment).	3.18	4.82	4.12	4.33	3.25	6.55
Os09g0552400	RmlC-like cupin family protein.	1.05	3.85	2.96		1.99	
Os09g0552600	RmlC-like cupin family protein.		4.98	3.54	2.25	3.45	2.53

The gene Os06g0216300 encoding 12-oxophytodienoic acid reductase which is commonly known as OsOPR1 was highly up-regulated in all tolerant genotypes except Nonabokra compared to susceptible genotypes. The role of OsOPR1 is reported to be involved in Jasmonic acid biosynthesis and is believed to play an important role under environmental stresses in rice (Jang *et al.* 2009). Seed maturation protein domain containing proteins (Os12g0626500 & Os06g0341300) were also up-regulated more in tolerant genotypes.

Most of the heat shock proteins (Os03g0745000, Os09g0482600, Os06g0553100, Os01g0571300, Os12g0147200, Os10g0345100 and Os03g0188100), Universal stress protein (Usp) family protein (Os01g0511100 and Os10g0437500) and most of the genes involved in signalling such as Os09g0418000 & Os11g0132900 (Protein kinase domain containing protein), Os12g0130200 (Ser/Thr protein kinase), Os07g0550400 (Receptor-like protein kinase 5) and Os08g0307400 (Phosphatidylinositol 3-kinase) are more induced in susceptible genotypes than in tolerant genotypes (Supplementary Table 5.5e).

Among the genes involved in the Molecular Function, fewer genes showed higher expression in tolerant genotypes compared to that of susceptible genotypes. For example, the expression of transporter activity gene Os03g0218400 (Hexose transporter) was almost twice in tolerant genotypes Pokkali and FL478 (FC >4 and >3, respectively) compared to that of susceptible genotypes. This gene commonly known as OsMST4 was found to be expressed in almost all plant parts such as root, shoot, leaf blade, leaf sheath, embryo and endosperm except anther of rice plants (Wang *et al.* 2007b). The 'Tonoplast membrane integral protein ZmTIP3-1' gene Os10g0492600 too was slightly more induced in tolerant genotypes Hassawi and Nonabokra along with the Peptide transporter gene Os01g0142800 which showed more expression in FL478 and Hassawi.

Among the genes that showed higher up-regulation in susceptible genotypes in this project, Os03g0305600 (Mitochondrial import inner membrane translocase, subunit Tim17/22 family protein) showed higher expression within 24 hours of stress in rice (Pegoraro *et al.* 2012) and Os03g0689300 (Plasma membrane H+ ATPase gene) showed down-regulation in anthers of rice (Li *et al.* 2011) and the gene Os05g0410500 (TGF-beta receptor, type I/II extracellular region family protein) were reported to be up-regulated in rice genotype FR13A which is believed to play a role in submergence tolerance and bacterial leaf blight resistance in rice (Kottapalli *et al.* 2007). The gene Os04g0659800 (Amino acid/polyamine transporter II family protein), Os03g0167200 (IQ calmodulin-binding region domain containing protein), Os10g0345100 & Os03g0188100 (Multi antimicrobial extrusion protein MatE family protein), Os11g0151600 (Schizosaccharomyces pombe) and Os03g0161200 (Sulfate transporter 3.1) also showed higher up-regulation in susceptible genotypes which were not previously reported and thus provide a set of genes whose function in stress tolerance is yet to be discovered.

Among the 13 transcription regulator activity genes that are commonly up-regulated in both tolerant and susceptible genotypes, the genes Os03g0745000 (Heat shock factor (HSF)-type, DNA-binding domain containing protein), Os06g0553100 (Heat shock transcription factor 29), Os01g0571300 (Heat shock transcription factor 31), Os04g0541700 (Homeobox domain containing protein), Os02g0649300 (homeobox-leucine zipper protein ATHB-6, Short highly repeated, interspersed DNA), Os03g0815900 (Transcription elongation factor S-II family protein) and Os01g0734000 (WRKY DNA binding protein) showed higher up-regulation in susceptible genotypes and were not reported previously in a salt stress context. The other

genes Os01g0165000 (DRE binding protein 2), Os01g0867300 (OSE2-like protein) were reported to be induced by drought in rice plants (Maruyama *et al.* 2012; Wang *et al.* 2011).

List f. Probes commonly down-regulated in both tolerant and susceptible genotypes

Probes representing the transcripts of 423 genes are commonly down-regulated in both tolerant and susceptible genotypes (List f in Supplementary Table 5.4). GO analysis revealed that among the biological processes 26 genes are involved in 'response to stimulus' and 5 are involved in 'signalling' as shown in Figure 5.6b and supplementary table 5.5f. Among the molecular functions, the genes involved in transporter activity (21) and transcription regulator activity (13) are discussed further. The transporter activity genes that are less down-regulated in tolerant genotypes and have not been reported previously are Os04g0607600 (Cation transporter family protein), Os01g0556700 (Dicarboxylate transporter), Os02g0787600 (Ionotropic glutamate receptor family protein), Os03g0195300 (Low affinity sulphate transporter 3) and Os10g0554200 (Nitrate transporter). The gene Os06g0178900 (Vacuolar H+-pyrophosphatase, OsVHP1;2) was reported to be induced by submersion in rice (Muto *et al.* 2011).

The genes involved in transcription regulator activity that are down-regulated in tolerant genotypes to a lower degree than that of the susceptible genotypes and were not reported previously are Os07g0158500 (CCAAT-binding transcription factor, subunit B family protein), Os06g0140700 (Homeobox-leucine zipper protein HAT14, HD-ZIP protein 14), Os03g0671800 (Basic helix-loop-helix dimerisation region bHLH domain containing protein), Os03g0657400 (WRKY transcription factor 60) and Os06g0552900 (Terminal flower1). Among the previously reported genes, Os12g0263800 (Pinoresinol-lariciresinol reductase

TH1) was down-regulated in melatonin-rich transgenic rice expressing a sheep serotonin N-acetyltransferase compared to the wild type (Byeon *et al.* 2013).

List g. Probes uniquely up-regulated in susceptible genotypes

The genes that are uniquely up regulated in susceptible genotypes (but showed no significant response in tolerant genotypes) were not evaluated critically as these genes may not hold any particular clue for salinity tolerance (Figure 5.6b and supplementary table 5.5g) in rice. Among the 666 uniquely up-regulated genes (List g in Supplementary Table 5.4), 26 are involved in the BPs 'response to stimulus' and 13 in 'signalling'; and 22 are involved in the MF 'transporter activity', 7 in 'molecular transducer activity', 27 in 'transcription regulator activity', 5 in 'enzyme regulator activity' and 5 in 'antioxidant activity' (Figure 5.6a and supplementary table 5.5g).

Among the genes involved in transporter activity, Os09g0563200 (K+ potassium transporter family protein) was highly up-regulated (almost 10 fold) in susceptible genotype IR29. Potassium transporter 4 (AtPOT4) (AtKUP3) (AtKT4) encoded by Os07g0669700 was also upregulated in IR29. It may *Indica*te the fact that under salt stress the susceptible genotype IR29 tries to uptake more K in a way to compensate the uptake of more toxic Na or these transporters may play a role in the uptake of Na itself. The other up-regulated transporters are Os12g0512100 (General substrate transporter family protein), Os06g0250600 (EAG/ELK/ERG potassium channel family protein) and Os01g0130000 (Cation efflux protein family protein). None of the above genes were reported previously and hence may be of interest to look for their functions in future.

List h. Probes uniquely down-regulated in susceptible genotypes

The genes that are uniquely down regulated in susceptible genotypes (but showed no significant response in tolerant genotypes) were not evaluated critically as these genes may not hold any particular clue for salinity tolerance in rice. The details of the genes can be found in Supplementary Table 5.4 (List d), Figure 5.6b and supplementary table 5.5h. Among the highly down-regulated transporters the gene Os01g0945300 encoding an 'Amino acid/polyamine transporter I family protein' (>6 fold in IR29), BRRI dhan29), Os01g0232000 encoding a 'Aquaglyceroporin (Tonoplast intrinsic protein (Tipa)' (>2 fold in both IR29 & BRRI dhan29), Os07g0513000 encoding a 'ATP synthase gamma chain, chloroplast' (>2 fold in BRRI dhan29) and Os07g0448400 encoding a 'Plasma membrane integral protein ZmPIP2-6' (>2 fold in both IR29 & BRRI dhan29) can be mentioned.

5.2.4 Mapping of the genes (in list a-h) within the salt stress related QTL

The genes in the lists a-h are mapped within the salt stress related QTLs by the web based tool ‘Qlic Rice’ and the results are shown in Table 5.2. Also the genes that are located within the important QTL (saltol and qSKC1) for shoot Na and K on chromosome 1 (Table 5.3) were identified by using the web based tool ‘Rice Gene Thresher’ and the results are shown in Table 5.4. Detailed procedures can be found in section 2.15 and the detailed results are shown in Supplementary Table 5.7.

None of the genes that are up-regulated in PT but down regulated in PS (list a) and the vice-versa (list b) is located within the salt stress related QTL.

Table 5.4 Genes (from the list a-h) that fall within QTL related to salt stress (data mined using the web based tool 'Qlic Rice'). The details are shown in Supplementary Table 5.7.

RAP (Os ID)	Start(5')	End(3')	Putative Function	QTL Accession ID(s) & Published Symbol	Fold Change (Log2)					
					PK	FL	HS	NB	BR	IR
a. Up-regulated in PT & down in PS (5)										
None	-	-	-	-						
b. Dow-regulated n in PT & up in PS (11)										
None	-	-	-	-						
c. Up-regulated in PT (311)										
Os01g0601000	23942105	23937907	conserved hypothetical protein	CQl1 (Potassium Concentration)			2.75	2.92		
Os01g0826000	35331713	35332202	heavy metal-associated domain containing protein	AQEM001(Salt-Sensitivity); qSDS-1 AQEM008(Salt-Sensitivity); qRNTQ-1			1.79			
Os08g0412800	19615992	19614835	Protein of unknown function DUF1262 family protein	AQFT004(osmotic adjustment capacity); oa8.1	3.78					
d. Down-regulated in PT (411)										
None	-	-	-	-						
e. Up-regulated in PT&PS (713)										
Os01g0224400	6833922	6837677	mitochondrial carrier protein CGI-69, putative, expressed	AQDX002 (Osmotic adjustment capacity) CQl1 (Potassium Concentration)				1.10	1.03	1.24
Os01g0256500	8543254	8542916	Similar to Ramy1, expressed protein	CQl1 (Potassium Concentration)		1.28	1.97	1.08	1.45	1.92
Os01g0348900	13902983	13902449	SalT gene product (Salt-induced protein). jacalin-like lectin domain containing protein	CQl1 (Potassium Concentration)	1.72	1.88		2.25	3.72	2.98

Os02g0649300	26137542	26138414	homeobox-leucine zipper protein ATHB-6, Short highly repeated, interspersed DNA (Fragment).	AQFT002 (Osmotic Adjustment Capacity); oa2.1 CQH6 (Osmotic Adjustment Capacity); oa2.1		4.69	6.53	7.42	6.75	7.91
Os02g0669100	27160910	27159944	dehydrin, putative, expressed	AQFT002 (Osmotic Adjustment Capacity); oa2.1 CQH6 (Osmotic Adjustment Capacity)		1.29	1.73	2.37	2.60	3.06
Os07g0154100	2869105	2870853	viviparous-14, putative, expressed	AQEM004(Salt sensitivity); qSNC-7 AQEM005(Salt sensitivity); qSNTQ-7 AQEM010(Salt sensitivity); qRKC-7 AQEM011(Salt sensitivity); qRKTQ-7			4.37	4.91		5.84
Os08g0472000	23231744	23237712	bZIP transcription factor, putative	AQDX013 (Osmotic Adjustment Capacity)		1.33	1.31	2.71	1.58	1.79
f. Down-regulated in PT&PS (423)										
None	-	-	-	-						
g. Up-regulated in PS (666)										
Os08g0400000	18937925	18941207	Puromycin-sensitive aminopeptidase (EC 3.4.11.-)	AQFT004(osmotic adjustment capacity)					1.08	
Os08g0408500	19414983	19415825	Pathogenesis-related transcriptional factor and ERF domain containing protein.	AQFT004(osmotic adjustment capacity)						1.29
h. Down-regulated in PS (647)										
Os01g0654500	26535978	26532389	NADP-isocitrate dehydrogenase.	CQI2(Sodium Uptake) CQI3(Sodium to Potassium content Ratio)					-1.13	-1.37

PT. Pooled Tolerant genotypes (PK. Pokkali, FL. FL478, HS. Hassawi, NB. Nonabokra); PS. Pooled Susceptible genotypes (BR. BRRI dhan29, IR. IR29)

Table 5.5 The QTL related to salt tolerance in the chromosome 1 of rice. The markers encompass a chromosomal region of 9817791-13880613bp (as shown in diagram below) within which genes were looked for using the web based tool 'Rice Gene Thresher'.

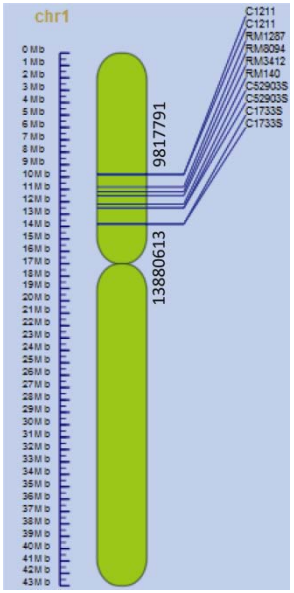
QTL	Trait	Marker(s)	Physical distance (mb)		References
Saltol	K+ : Na+ ratio	RM8094–CP6224	11.23		(Niones, 2004)
Saltol	K+ : Na+ ratio	RM3412–RM140	12.00-12.27		(Niones, 2004)
-	Shoot Na^+ and K^+	RM140–C1733S	12.28-13.82		(Bonilla *et al.* 2002)
Saltol	Shoot Na^+ and K^+	C52903S-C1733S	12.54-13.82		(Bonilla *et al.* 2002)
qSKC1	Shoot K+ conc.	C1211–S2139	9.82->11.28		(Lin *et al.* 2004)
		RM8094-RM10825	-		(Thomson *et al.* 2010)
	Shoot K+ conc.	RM1287-RM10793	-		(Thomson *et al.* 2010)
SKC1	Shoot K+ conc.	-	11.42-11.48		(Ren *et al.* 2005)
qSNK1	Shoot Na–K ratio	RM1287-RM10825	-		(Thomson *et al.* 2010)
qRKC1	Root K+ conc.	RM1287-RM11300	-		(Thomson *et al.* 2010)

Table 5.6 Genes of the list a-h that fall within the QTLs of chromosome 1 (9817791- 13880613bp) that regulate Shoot Na^+ and K^+ and their ratio. The analysis was carried out by the web based tool 'Rice Gene Thresher'. The detailed results can be found in Supplementary Table 5.7.

RAP (Os ID)	Functional Annotation	Start Postion	End Position	Fold Change (Log2)					
				Pokkali	FL478	Hassawi	Nona bokra	BRRI dhan29	IR29
c. Up in PT (311)									
Os01g0303600	Zinc finger C3HC4 type (RING finger) putative	11228884	11231387			1.10			
Os01g0332200	oxidoreductase 2OG-Fe(II) oxygenase family putative	12857959	12865001				2.16		
d. Down in PT (411)									
Os01g0309100	hypothetical protein	11568127	11568773			-1.41	-1.00		
Os01g0293900	Haem peroxidase, plant/fungal/bacterial family protein.	10698384	10699888		-1.53		-1.00		
e. Up in PT&PS (713)									
Os01g0284900	hypothetical protein	10197767	10200138	2.08	1.70	2.85	2.42	2.49	2.88
Os01g0329400	Mitochondrial carrier protein putative	12687209	12692242	1.18		1.10			1.16
Os01g0303300	Mitochondrial import inner membrane translocase, subunit Tim17/22 family protein.	11218610	11220411		3.53		7.19	6.93	5.80
Os01g0306400	hypothetical protein	11375746	11376483		3.40		3.27	4.50	3.13
f. Down in PT&PS (423)									
Os01g0319200	Plant protein of unknown function family protein.	12112177	12115707	-1.18				-3.05	-3.87
Os01g0282800	Tubulin/FtsZ family GTPase domain putative	10096169	10099031		-1.01	-1.04		-1.74	

Os01g0296700	Glycosyl hydrolase family 3 C terminal domain putative	10868228	10871020		-1.59		-2.47	-2.97	
Os01g0284500	Nectarin 1 precursor (EC 1.15.1.1) (Superoxide dismutase [Mn]).	10164907	10165684			-1.46			-1.96
Os01g0294700	Haem peroxidase, plant/fungal/bacterial family protein.	10745361	10746991		-1.27	-1.30	-2.10	-2.03	-2.49
Os01g0287600	Chitinase class I	10338784	10340328			-1.37		-2.28	-2.56
Os01g0297700	Integral membrane protein DUF6 putative	10908252	10911159			-1.82		-2.55	-2.72
g. Up-in PS (666)									
Os01g0347100	Protein of unknown function DUF1399 family protein.	13782751	13787086						1.16
Os01g0315800	dTDP-glucose 4-6-dehydratase-like protein	11888852	11891850						1.09
Os01g0330200	Papain family cysteine protease putative	12728218	12729320					1.68	
Os01g0328300	F-box domain putative	12619893	12622707					1.42	
h. Down-in PS (647)									
Os01g0280400	Tetratricopeptide-like helical domain containing protein.	9959565	9963473						-2.72
Os01g0301900	Protein of unknown function DUF247, plant family protein.	11106957	11110301					-2.79	-2.31
Os01g0306800	hypothetical protein	11409027	11417471						-1.60
Os01g0332100	Plant neutral invertase putative	12852701	12856719						-1.01
Os01g0293200	Protein of unknown function DUF860, plant family protein.	10671946	10673681						-1.01
Os01g0283600	Cinnamoyl-CoA reductase (EC 1.2.1.44).	10141132	10143070					-1.53	

However, **among the 311 genes that are uniquely up-regulated in PT (list c)**, five were located within salt stress related QTL, four of these on chromosome 1 and the rest on Chromosome 8 (Table 5.2 & Table 5.4). Of these genes, the one of particular interest is Os01g0303600 which encodes a transcription factor 'Zinc finger C3HC4 type (RING finger) putative' is located within the chromosome 1 region of the QTL Saltol and qSKC1 for shoot K concentration and K:Na ratio, respectively (Table 5.4). The location of this transcription factor within the important QTL strongly suggests its putative role which might include regulating the other downstream genes that are involved in ion homeostasis and hence provide an immediate candidate gene. The gene Os01g0332200 (oxidoreductase 2OG-Fe(II) oxygenase family putative) was also found to be located within Saltol QTL on chromosome 1. The QTL for Potassium Concentration (CQl1), osmotic adjustment capacity (oa8.1) and salt-sensitivity (qSDS-1 and qRNTQ-1) harboured the genes Os01g0601000 (conserved hypothetical protein), Os08g0412800 (Protein of unknown function DUF1262 family protein) and Os01g0826000 (heavy metal-associated domain containing protein), respectively (Table 5.2). None of the genes were reported previously, hence may be of interest to look for their functional role in salt tolerance.

Among the 411 genes that are uniquely down-regulated in PS (list d), only two genes were located within the salt stress related QTL. The gene Os01g0293900 (Haem peroxidase, plant/fungal/bacterial family protein) which is down-regulated in Hassawi (FC -1.41) and Nonabokra (FC -1.00) is located within the QTL for Shoot K+ concentration (qSKC1) and the hypothetical protein encoding gene Os01g0309100 (down-regulated in FL478 and Nonabokra) is located within the saltol QTL, both on chromosome 1.

Among the commonly up-regulated 713 genes in PT&PS (list e), 11 genes are located within QTL regions, of which 7 are on chromosome 1 (Table 5.3 & Table 5.5).

Two transcription factor genes namely, Os08g0472000 (bZIP transcription factor) and Os02g0649300 (homeobox-leucine zipper protein ATHB-6) fall within the QTL (oa2.1) for Osmotic Adjustment Capacity and might be very important along with the 'Salt-induced protein' encoding gene Os01g0348900 and Os02g0669100 (dehydrin) that falls within the QTL (CQI1) for Potassium Concentration.

Among the commonly down-regulated 423 genes in PT&PS (list f), seven genes are located within the Saltol and qSKC1 QTL region of chromosome 1 (Table 5.5). The transmembrane domain containing protein gene Os01g0297700 may play vital role in ion homeostasis along with the Haem peroxidase, plant/fungal/bacterial family that is involved in response to oxidative stress.

5.2.5 Apoptosis is repressed in tolerant genotypes

Before describing each of the lists (a-i), it was noticed from the GO flash charts (Figure 5.6a&b) that genes involved in apoptosis (death) were only down-regulated (no gene was up-regulated) in PT genotypes whereas apoptosis genes were both up- and down-regulated in PS genotypes (Table 5.5 & Figure 5.6a&b). This suggests that tolerant genotypes may develop an adaptive approach by down-regulating (not up-regulating) the apoptosis genes.

Among the apoptosis genes that are uniquely down –regulated in pooled tolerant genotypes, Os11g0606400 (Disease resistance protein family protein) has been shown to be down-regulated in germinating seedlings of rice variety Hayamasari upon low temperature treatment (Fujino and Matsuda, 2010) and Os08g0539700 (PibH8 protein) was reported to

be up-regulated in pathogen induced 4-5 leaf stage *Japonica* cultivar Monko-to upon inoculation by rice blast fungus *Magnaporthe oryzae* (Brunings *et al.* 2009).

Among the uniquely up-regulated probes in pooled susceptible genotypes, gene Os11g0506800 containing a IQ calmodulin-binding region domain was also reported to be up-regulated and induce programmed cell death in rice under high salt stress (Yang *et al.* 2013) and under heat stress (Sun *et al.* 2012) and yeast and *Arabidopsis* (Kang *et al.* 2006). The other up-regulated gene, Os12g0586000 encoding disease resistance protein 1 (ADP1) which is also known as OsSAPK9, was also reported to be induced in rice by the bacterial leaf streak pathogen (Xu *et al.* 2011) and by *Xanthomonas oryzae* pv.*oryzicola* infection (Xu *et al.* 2013). The rest of the genes such as Os11g0684200, Os08g0396700, Os08g0261000, Os07g0196900 and Os11g0161000 that induce programmed cell death were not reported before and could be of interest in manipulating salt tolerance in rice.

Table 5.7 Probes involved in apoptosis (determined by SEA analysis using the lists d, g & h of Supplementary Table 5.4).

Probes involved in death (apoptosis)		**Fold Change (treated vs control)**					
Locus ID	**Annotation**	**Pokkali**	**FL478**	**Hassawi**	**Nonabokra**	**BRRI dhan29**	**IR29**
Among the uniquely down-regulated probes in pooled tolerant genotypes (ST 5.4 list d)							
Os11g0606400	Disease resistance protein family protein.	-1.37		-1.85			
Os11g0590700	Disease resistance protein family protein.	-1.27					
Os04g0118800	Disease resistance protein family protein.		-1.02				
Os11g0238700	Disease resistance protein family protein.		-1.69				
Os11g0689100	Disease resistance protein family protein.				-1.00		
Os08g0539700	PibH8 protein (Disease resistance protein family protein)				-1.00		
Among the uniquely up-regulated probes in pooled susceptible genotypes(ST 5.4 list g)							
Os11g0506800	IQ calmodulin-binding region domain containing protein.						4.63
Os11g0684200	Disease resistance protein family protein.						1.90
Os08g0396700	DC1 domain containing protein.						1.39
Os08g0261000	Disease resistance protein family protein.						1.07
Os07g0196900	Conserved hypothetical protein.						1.00
Os11g0161000	NB-ARC domain containing protein.					1.71	
Os12g0586000	Disease resistance protein ADR1					1.14	
Among the uniquely down-regulated probes in pooled susceptible genotypes (ST 5.4 list h)							
Os04g0622600	XA1.						-1.48
Os02g0120800	Rop2 small GTP binding protein					-1.31	-1.32
Os02g0312600	Small GTP-binding protein (Fragment).					-1.04	-1.12
Os11g0654800	Hypothetical protein.						-1.03
Os09g0524800	Apoptosis regulator Bcl-2 protein, BAG domain containing protein.					-1.06	
Os08g0246300	Disease resistance protein family protein.					-2.16	
Os10g0131000	Disease resistance protein family protein.					-2.56	

5.2.6 Commonality in the genes obtained by modified SAM and GeneSpring approach

The trait specific significant genes for shoot Na, shoot NaK, Biomass, shoot Cl- that are obtained by using the modified SAM approach (chapter 4) have some commonality with the differentially regulated genes obtained by GeneSpring approach (chapter 5). The detailed lists are shown in supplementary table 5.8. It is noteworthy that these two approaches are different in terms of the dynamics of data involved and the statistical approaches applied. However, both aimed at identifying the genes that are expressed upon stress imposition under different biological context.

5.3 CONCLUSION

The gene expression of four tolerant genotypes and two susceptible genotypes upon exposure to salt stress has been profiled using Agilent 4x44K rice microarray. In general, higher numbers of genes are differentially regulated in response to salt in susceptible genotypes (1472 and 1902 in BRRI dhan29 and IR29, respectively) than in tolerant genotypes (635, 657, 1062 and 880 in Pokkali, FL478, Hassawi and Nonabokra, respectively). The differentially regulated genes in these tolerant and susceptible genotypes are pooled together and are discussed in a comprehensive way by categorising these genes according to their up and down-regulation in the tolerant and susceptible genotypes. The genes that are uniquely up-regulated in tolerant genotypes and the genes that are commonly up-regulated in tolerant and susceptible genotypes (but at a higher rate in tolerant genotypes) provide the strongest candidates for salinity tolerance in rice.

Gene ontology analysis categorized the genes according to the biological processes (BPs) and molecular functions (MFs) of which the genes in the important BPs such as response to

stimulus, signaling and signaling process and the genes with important MFs such as transporter activity, transcription factor, transcription regulator activity, molecular transducer activity, antioxidant activity and nutrient reservoir activity were discussed.

For example, among the uniquely up-regulated genes in tolerant genotypes, 15 genes are involved in response to stimulus and 7 genes are involved in transporter activity and provide strong candidates for salinity tolerance in rice. Among the genes involved in response to stimulus, the most highly induced gene is Os01g0159600 (>5 fold in Nonabokra and >2.5 fold in Hassawi) encoding an 'Embryonic abundant protein 1 (OsLEA1a)' and among the genes involved in transporter activity the most highly induced gene (~4 fold induced in all four tolerant genotypes) Os05g0382200 encoding a Na+/H+ exchanging protein-like can be mentioned.

The genes were also assessed in terms of whether they are located within previously identified salt stress related QTL. Among the uniquely up-regulated 311 genes in pooled tolerant genotypes, five genes are located within QTL, of which the most important one is the transcription factor Os01g0303600 (Zinc finger C3HC4 type (RING finger) which is located within the chromosome (1) region of the QTL Saltol and qSKC1 for shoot K concentration and K:Na ratio can be mentioned here as another example.

Among the identified genes in tolerant and susceptible genotypes, the genes that were not characterized before constitute the most novel target genes and would be of great interest in future to functionally validate the role of these genes which can then be used for biotechnological manipulation to improve the salinity tolerance in rice.

LIST OF SUPPLEMENTARY TABLES

Supplementary Table 5.1a-f: Lists of differentially (both up and down) regulated significant probes upon exposure to salt stress in Tolerant check variety Pokkali (a), FL478 (b), Hassawi (c), Nonabokra (d) and in susceptible check variety BRRI dhan29 (e) and IR29 (f).

From left to right are given the Agilent probename, NCBI Locus ID, p-value, fold change in expression (treated/control; log_2) and the functional annotation. Probe sets are ordered based on fold changes upon exposure to salt stress (most up-regulated to most down-regulated) in Pokkali. Two week old seedlings were subjected to 120mM NaCl stress and RNA was extracted after 48 hours of stress duration.

Supplementary Table 5.2a-g: Lists of constitutive differentially (both up and down) regulated significant probes in Tolerant check variety Pokkali, FL478, Hassawi and Nonabokra when compared to susceptible check variety IR29 (a-d) and BRRI dhan29 (e-h).

Supplementary Table 5.3: Lists of differentially up and down regulated significant probes in pooled tolerant (Pokkali, FL478, Hassawi and Nonabokra) and pooled susceptible (BRRI dhan29 and IR29) genotypes upon exposure to salt stress (as shown in Figure 5.4).

Supplementary Table 5.4: The details (Locus IDs, expression values, p-values and functional annotations) of the differentially regulated probe sets in pooled tolerant (Pokkali, FL478, Hassawi and Nonabokra) and pooled susceptible (BRRI dhan29 and IR29) genotypes upon exposure to salt stress (as shown in Figure 5.5).

Supplementary Table 5.5: The probes under the Biological processes (BP) and molecular functions (MF) derived from the Gene Ontology (Singular Enrichment Analysis, SEA) analysis of the differentially-regulated probes in pooled tolerant (Pokkali, FL478, Hassawi and Nonabokra) and pooled susceptible genotypes (list c-h of figure 5.5) upon exposure to salt stress.

Supplementary Table 5.6: Detailed (raw) results of the Gene Ontology (Singular Enrichment Analysis, SEA) analysis of the the differentially-regulated probes in pooled tolerant (Pokkali, FL478, Hassawi and Nonabokra) and pooled susceptible genotypes (list c-h of figure 5.5) upon exposure to salt stress.

Supplementary Table 5.7 Details of the genes (from the list a-h) that fall within QTLs related to salt stress. Data mined using the web based tool 'Qlic Rice' (a) and 'Rice Gene Thresher' (b).

Supplementary Table 5.8 Lists of genes that is common between modified SAM and GeneSpring approach.

Chapter 6. SALINITY TOLERANCE PREDICTION OF GENOTYPES OF UNKNOWN TOLERANCE: USE OF A OSC-PLSDA MODEL CREATED BY GENE EXPRESSION DATA OF GENOTYPES OF KNOWN TOLERANCE

6.1 ABSTRACT

The salt stress responsive transcriptomic profile of known tolerant and susceptible genotypes was used to predict the salinity tolerance of the genotypes whose tolerance level is unknown. The Orthogonal Signal Correction (OSC) -filtered partial least square discriminant analysis (OSC-PLSDA) was used to classify the transcriptomic fingerprints and predict the salinity tolerance of a range of genetically wide rice genotypes. The model was developed using the classification error as a function of the number of latent variables and was tested using the Venetian Blinds internal cross validation. The minimum number of genes (109 genes) that highly contributed to the classification and model prediction of salinity tolerance status was identified using the Variable Importance vector. Among the genes the notables include 16.9 kDa class I heat shock protein (Os01g0136200), Calcium-binding protein (Os02g0606500), beta-expansin EXPB4 (Os10g0556100) and Expansin Os-EXPA3 (Os05g0276500), Disease resistance protein family protein (Os12g0467300 & Os11g0606400), Late embryogenesis abundant (LEA) group 1 family protein (Os04g0589800) and DREB2C protein (Os08g0521600) etc. The prediction of the salinity tolerance status of the unknown genotypes obtained from this OSC-PLSDA modelling of transcriptomics data is comparable to that obtained by multivariate analysis of the morpho-physiological data. The slight difference between the categorization of these two approaches could be the function of limitation in experimental design and/or insufficient samples for building the OSC-PLSDA

model. However, the results are promising and could be improved if the above two cases are taken care of in future. The whole fingerprints of these transcriptomic profiles could be possible markers for the overall prediction of tolerance or resistance to biotic and abiotic stresses.

6.2 INTRODUCTION AND AIM

Knowing the status of tolerance or resistance of plants to various biotic and abiotic stresses is the essential prerequisite for any crop improvement programmes/acitivities. This is usually done via extensive morpho-physiological studies which require meticulous planning and design, laborious experiments and collection of data for a number of different growth, physiological, metabolic and stress parameters etc. These screening techniques have been successful and have served the scientific community the purpose being to recognize the tolerant or resistant genotypes for different crop species, e.g, Rice landraces Pokkali, Nonbokra, CSR types and *Oryza coarctata* etc are tolerant to salt. These known tolerant and susceptible genotypes and the other resistant and susceptible genotypes for other biotic and abiotic stresses in other crop species were exploited at different levels for crop improvement.

However, scientists are looking for the source of tolerance or resistance in wider genotype groups such as at subspecies level, distant relatives and crop wild relatives. Very recently the submergence tolerance genes sub1A, sub1B and sub1C were identified in some wild species *O. rufipogon* and *O. nivara* and landraces e.g., Kurkaruppan and Goda Heenati (Bailey-Serres *et al.* 2010; Fukao *et al.* 2009; Pucciariello and Perata, 2013; Xu *et al.* 2006) and were successfully utilized for developing submergence tolerant crop varieties such as FR13A and FR43B and breeding lines such as Thalavu, BKNFR76106-16-0-1-0 and IR49830-7-1-2-1-3. Another example is the brown plant hopper resistant gene, bph14 which has originally been derived from the wild rice species, *Oryza officinalis* (du *et al.* 2009). *O. coarctata* T., a wild relative of rice, has long been reputed for its wider adaptability in salt environments and was investigated at molecular level (Das-chatterjee *et al*. 2006; Mahalakshmi *et al*. 2006). Very

recently scientists were successful in introgressing the salt tolerance traits from *O. coarctata* to the modern variety IR56 which opens up the avenue to develop a super salt tolerant rice in future (IRRI, 2013).

Wide hybridization between different species and subspecies provides opportunities to create variation of which some can be of greater benefit to mankind. This will happen if there is the source of tolerance or resistance in either of the parents. It is generally considered that the wild species of Oryza are a useful source of novel genes for resistance to biotic or abiotic stresses and for hybrid development programmes (Ratnayaka, 1999; Samuels, 2001). For example, the trait for tolerance to acid sulphate is found in *O. rufipogon*, tolerance to drought in *O. meridionalis*, resistance to multiple pests in *O. punctata*, trait for high biomass production in *O. grandiglumis* and the traits for resistance to BPH is found in *O. minuta*, *O. eichingeri*, *O. australiensis* and *O. officinalis* (Sundaramoorthi *et al.* 2009; Jena, 2010). Thus looking for this source of tolerance in distant relatives and/or wild relative is crucial.

With the advent of genomic techniques and at the rate the techniques are becoming more affordable opens a new avenue. More and more crop genomes are being sequenced which lead to the development of different sequence based technologies such as microarrays. In the recent years, microarray experiments were conducted extensively which led to the development of different public data repositories. Transcriptomics data from different crop species under different biotic and abiotic stresses at different time points are available there. These data repositories and newly generated transcriptomics data can potentially be exploited to predict the tolerance or resistance status of genotypes of unknown status by

computational model-based comparisons with the genotypes where tolerance or resistance status is known (Gavaghan *et al.* 2002).

This prediction is usually done by multivariate statistical analysis such as unsupervised Principal Component Analysis (PCA) and supervised Orthogonal Signal Correction (OSC) filtered Partial Least Square Discriminant Analysis (OSC-PLSDA) applied on high throughput 'omics' data (Beckwith-Hall *et al.* 2002; Brindle *et al.* 2002; Gavaghan *et al.* 2002; Griffin, 2006). The Orthogonal signal correction (OSC) -filtered partial least square discriminant analysis (OSC-PLSDA) was successfully applied to predict and classify the exposure of rats to organophosphates such as tributyl phosphate (TBP) or triphenyl phosphate (TPP) by characterising the rat urine using 1H Nuclear Magnetic Resonance (NMR) Metabolomic data (Alam *et al.* 2012), to predict the sensory quality ranking of Japanese green tea (Sen-cha) using volatile profiling (Jumtee *et al.* 2011) and to classify the malignant Oral Squamous Cell Carcinoma (SCC) tissues from benign samples and then to predict the unknown samples using the proton HR-MAS NMR spectroscopic data (Srivastava *et al.* 2011).

Despite the potentiality of the transcriptomics and proteomics data to be used for this prediction analysis, so far there is no such report for prediction of the biotic and abiotic stress tolerance of rice genotypes. Rice, being one of the important food crops and susceptible to various biotic and abiotic stresses, has extensively been studied transcriptomically which has enriched the volume of publicly available databases. This opens up a new avenue to screen wide natural germplasm by predicting the tolerance or resistance to biotic and abiotic stresses of these genotypes at different tissues, developmental stages, growth, environmental and stress conditions. This approach can

supplement or can provide an effective alternative to the traditional morpho-physiological assessment based screening of germplasm with an added advantage of predicting the transcriptomic biomarkers/fingerprints of tolerance or resistance. This chapter describes the development of OSC-PLSDA model based on the gene expression data of the genotypes of known tolerance and the prediction of the tolerance status of the genotypes of unknown tolerance coupled with the identification of predictive transcriptomics biomarkers.

6.3 Results and Discussion

Among the twelve genotypes that are studied transcriptomically, eight were morpho-physiologically characterized (Table 2.1) and grouped according to their tolerance status using multivariate analysis as shown in chapter 3. The remaining four genotypes (FL478, Hassawi, Nonabokra and IR29) had not been subjected to morpho-physiological characterization as their salinity tolerance status was well documented (see Table 2.1). The gene expression data (see section 2.11) of the tolerant and susceptible genotypes were used to build the prediction model. The prediction of salt tolerance of genotypes of unknown tolerance status is computationally validated using the orthogonal signal corrected partial least square discriminant analysis (OSC-PLSDA) model. Two OSC-PLSDA models were created using the differentially expressed significant genes in two combinations of genotypes, firstly, using the differentially expressed significant genes of the 4T (Pokkali, FL478, Hassawi and Nonabokra) and 2S (BRRI dhan29 and IR29) genotypes (the reduced matrix 1 of 3137 genes) and secondly, using that of 4T and 4S (BRRI dhan29, IR29, *O. Latifolia* and *O. rufipogon*) genotypes (the reduced matrix 2 of 6306 genes). The details of the analysis procedure are shown in section 2.16 and the results are discussed below.

6.3.1 The reduced matrix 1 (3137 genes)

The *Indica* genotypes Pokkali, FL478, Hassawi and Nonabokra can be regarded as the benchmark of salt tolerant genotypes and BRRI dhan29 and IR29 as the susceptible check varieties. The reduced matrix 1 was created by combining the 3137 unique differentially expressed significant genes of these four known tolerance (4T) and two known susceptible genotypes (2S) as shown in section 2.16.

6.3.1.1 Initial PCA plots of all samples

The initial PCA plots involving all the samples identified some of the samples of *O. latifolia* as outliers as shown in Figure 6.1. This was confirmed in the box-whisker plot of all 12 genotypes created by the Gene-Spring software which also showed the abrupt distribution of *O. latifolia*. This prompted the removal of the outliers for the next PCA. However, it seemed sensible only to use the known samples to see how they are separated in PC1 and PC2 which was done in the next step.

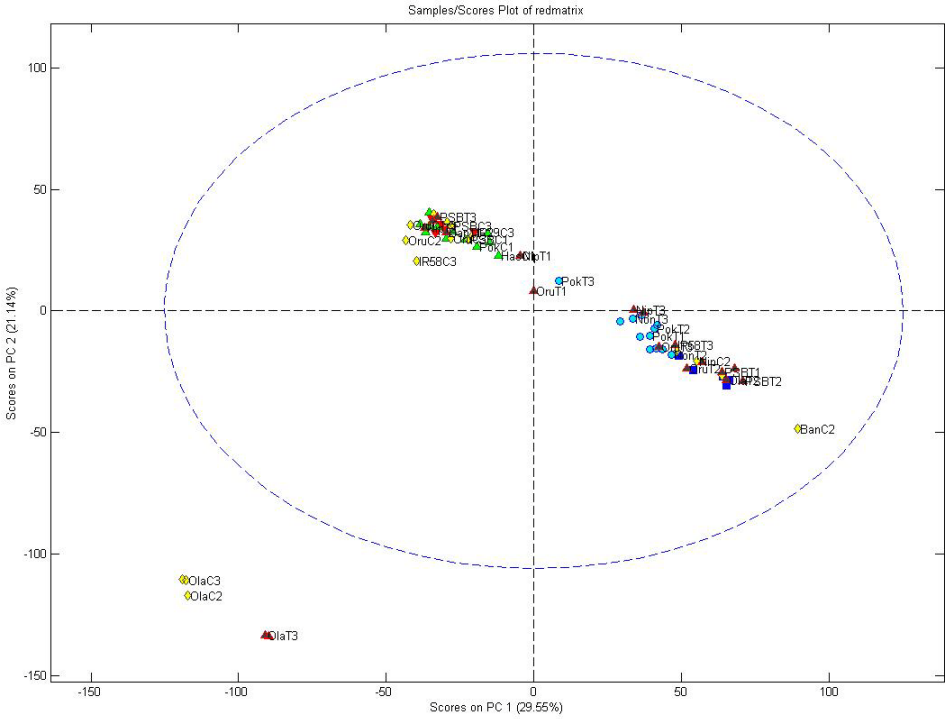


Figure 6.1 The reduced matrix 1 based (3137 genes) initial PCA plots of all samples.

C. Control, T. Treated, Pok. Pokkali, FL4. FL478, Has. Hassawi, Non. Nonabokra, BRR. BRRI dhan29, T1, T2 &T3. Treatment 1, 2 & 3, respectively and C1, C 2 & C 3. Control 1, 2 & 3, respectively.

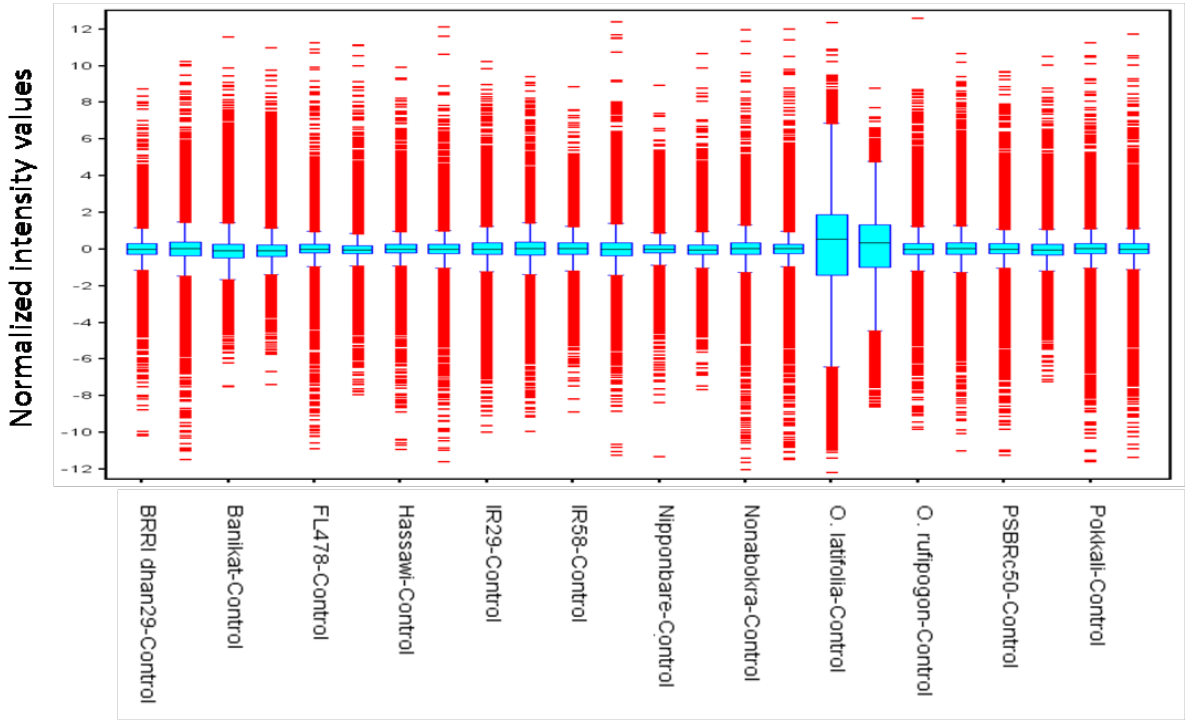


Figure 6.2 Box-whisker plot after normalization of all 12 genotypes created by Gene-Spring (v. 12.58) software showing the aberrant distribution of *O. latifolia* than that of the rest of the genotypes. The details of the differentially expressed genotypes are shown in Supplementary Table 6.1.

6.3.1.2 Initial PCA plots of all known samples

The reduced matrix 1 based (3137 genes) initial PCA plots clearly separated the control versus treated samples in PC1 and susceptible versus tolerant in PC2 which prompted the pooling of all susceptible and all tolerant samples into one class each (Figure 6.3).

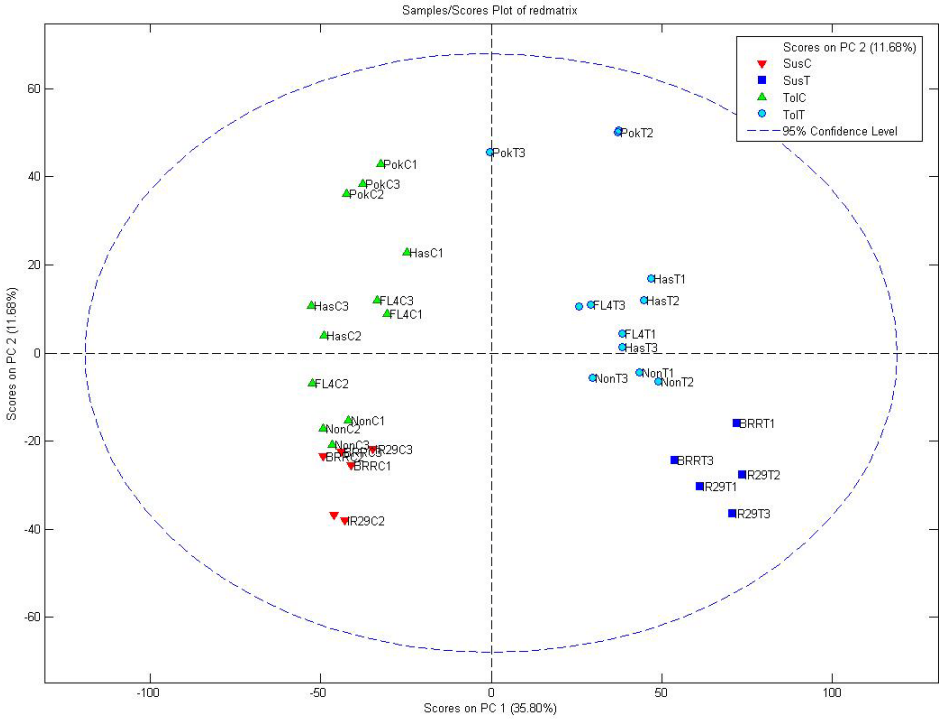


Figure 6.3 The reduced matrix 1 based (3137 genes) initial PCA plots of all known samples.

Sus. Susceptible, Tol. Tolerant, C. Control, T. Treated, Pok. Pokkali, FL4. FL478, Has. Hassawi, Non. Nonabokra, BRR. BRRI dhan29, T1, T2 &T3. Treatment 1, 2 & 3, respectively and C1, C 2 & C 3. Control 1, 2 & 3, respectively.

6.3.1.3 Building of traditional PLS-DA model

After reclassification into only three classes (susceptible, tolerant, unknown), the two known classes were used to create a PLS-DA model (PLS Toolbox plot shown above). An in-house PLS-DA script (plsda_auto_v18.m) was used to test and optimize the model. Classification errors were minimal at two latent variables (LVs), and the model was tested using the Venetian blinds internal cross validation with 1000 repetitions. LV1 and LV2 weightings were

merged into one Variable Importance vector (Figure 6.4), for which the minimal number of genes was calculated to result in an optimized model (using forward selection method) of only 109 genes (Table 6.1).

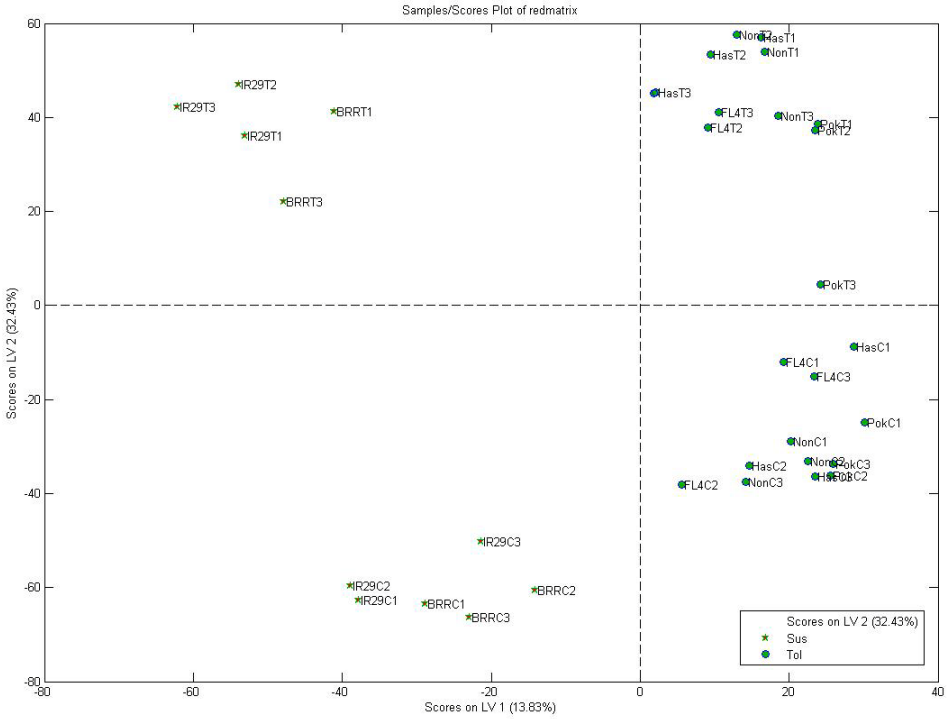


Figure 6.4 Score plots on LV 1 and LV 2 of all known samples based on the reduced matrix 1 (3137 genes) and traditional PLS-DA model.

Sus. Susceptible, Tol. Tolerant, C. Control, T. Treated, Pok. Pokkali, FL4. FL478, Has. Hassawi, Non. Nonabokra, BRR. BRRI dhan29, T1, T2 &T3. Treatment 1, 2 & 3, respectively and C1, C 2 & C 3. Control 1, 2 & 3, respectively and LV. Latent Variable.

Table 6.1 Statistics used to characterise the traditional PLS-DA predictive model

Class	**Classification error rate**	**P value**
Before forward selection		
Susceptible	0.0068561	<0.001
Tolerant	0.0068561	<0.001
After forward selection (optimization resulted in 109 forward selected variables)		
Susceptible	0.0040587	<0.001
Tolerant	0.0040587	<0.001

This procedure resulted in a very good model for prediction of the same samples used for model building (using internal cross validation). However, with such few samples used for model building and internal validation, it is difficult to determine whether this model is over-fit. This model was used for the next (prediction) steps involving the unknown samples.

6.3.1.3.1 Prediction of unknowns using optimized (traditional) PLS-DA model

A simple PLS-DA model based on the forward-selected matrix was created and the "unknown" samples added as a truly independent "validation" dataset. The plot shows that there is still a strong unwanted component separating control and treated samples among the known samples. With the "unknowns" located in the centre between the two "known" classes, this clustering (Figure 6.5) is not strictly by class difference, making the immediate prediction difficult. This prompted to build an OSC (orthogonal signal corrected) PLS-DA model.

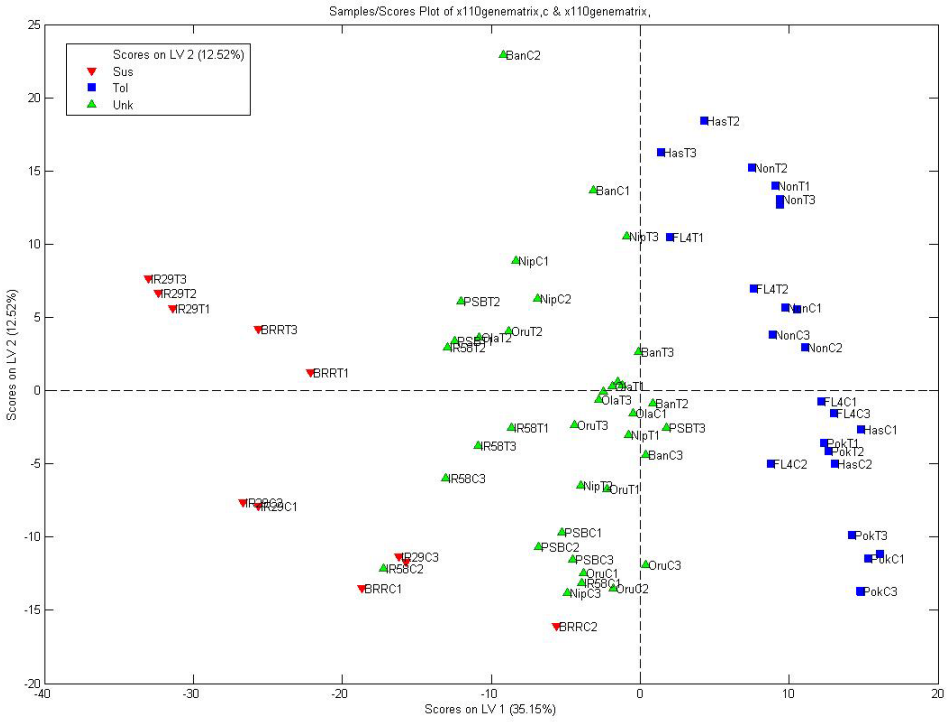


Figure 6.5 Score plots on LV 1 and LV 2 of all samples (known tolerant, known susceptible and unknown) using the traditional optimized PLS-DA model with 109 genes.

The genotypes of unknown salinity tolerance status is located in the centre between the two "known" classes *Indica*ting that these clustering is not strictly by class difference which makes the immediate prediction difficult.

6.3.1.4 Building of an OSC (orthogonal signal corrected) PLS-DA model

The same dataset of "known" samples (109 forward selected data) was used, but pre-processing applied orthogonal signal correction (OSC (#components = 1, #iterations = 0, tolerance = 99.9%, Mean Center) in order to filter out components that are not predictive of class separation. The clear separation of the four tolerant and two susceptible genotypes are shown in Figure 6.6. The detail of the OSC PLS-DA predictive model is shown in Appendix III.

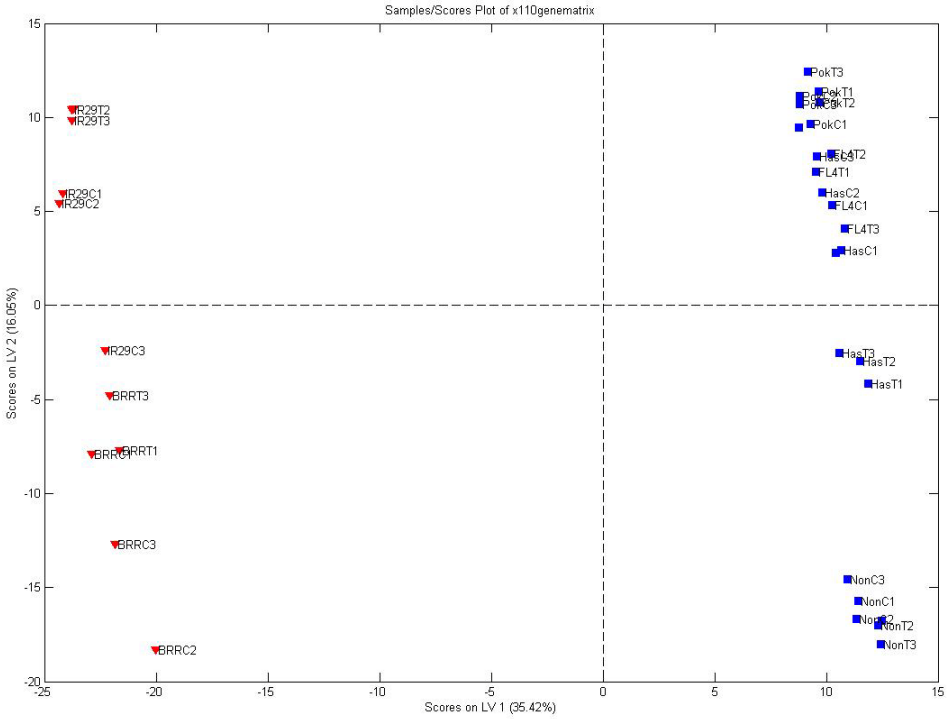


Figure 6.6 Score plots on LV 1 and LV 2 of all known samples based on the reduced matrix 1 (3137 genes) and OSC filtered PLS-DA model.

C. Control, T. Treated, Pok. Pokkali, FL4. FL478, Has. Hassawi, Non. Nonabokra, BRR. BRRI dhan29, T1, T2 &T3. Treatment 1, 2 & 3, respectively and C1, C 2 & C 3. Control 1, 2 & 3, respectively and LV. Latent Variable.

6.3.1.4.1 Prediction of unknowns using OSC filtered PLS-DA model

Again, the "unknowns" were added as "validation" dataset to the OSC filtered PLS-DA model. The classification of "unknowns" (Figure 6.7) was clearer than that based on traditional PLS-DA model (Figure 6.5).

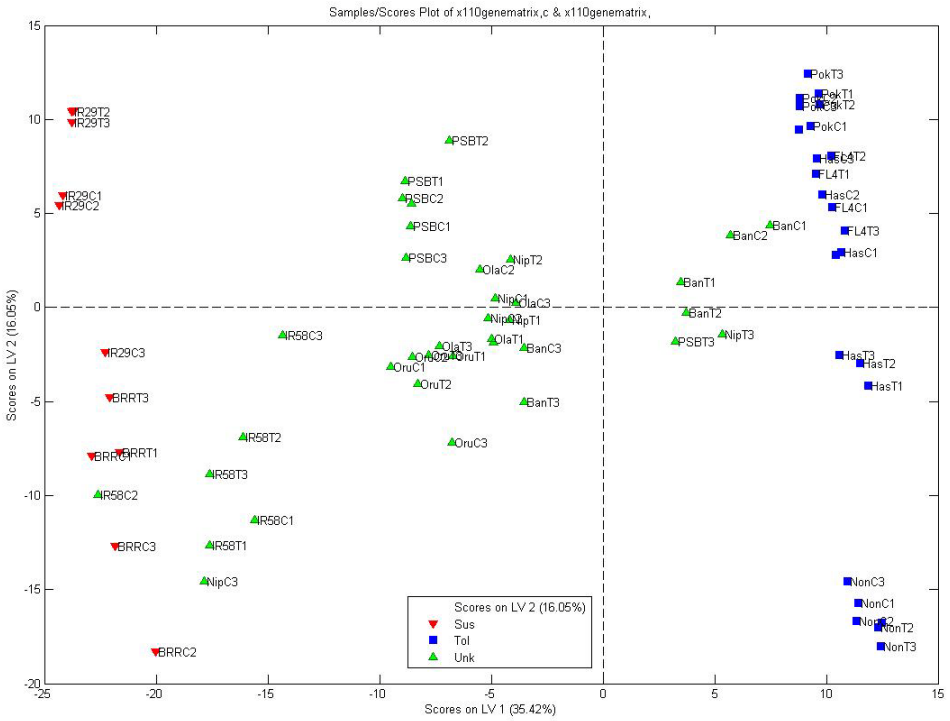


Figure 6.7 Score plots on LV 1 and LV 2 of all samples (known tolerant, known susceptible and unknown) using the using OSC filtered PLS-DA model with 109 genes.

The genotypes of unknown salinity tolerance status is located in the centre between the two "known" classes but the separation is clearer than that of traditional PLS-DA model shown in figure Figure 6.5.

6.3.1.4.2 Prediction of unknown control samples using OSC-PLSDA model

The OSC-PLSDA model thus created using the four known tolerant and two known susceptible genotypes was then used to predict the tolerance status of unknown samples. The prediction results thus obtained are shown in Table 6.2. It was observed that only the prediction of Banikat as tolerant and *O. rufipogon* as susceptible matched with that of the morpho-physiological categorization as shown in Figure 3.9b.

Table 6.2 Prediction of unknown control samples using OSC-PLSDA model with 109 genes.

Genotype	Banikat	IR58	*O. latifolia*	Nipponbare	PSBRc50	*O. rufipogon*
Tolerant	3	0	2	2	0	0
Susceptible	0	3	1	1	3	3

The numbers *Indica*te the number of replicates.

Two of the moderately tolerant genotypes of *Indica* subspecies namely, PSBRc50 and IR58 were predicted as susceptible (Table 6.2) which is a partial mismatch to the morpho-physiological categorization (Figure 3.9b). The wild species *O. latifolia* which is supposed to be a susceptible genotype is predicted as tolerant. The case is also unclear with the *Japonica* genotype Nipponbare which is susceptible or resistant dependent upon level of salt applied.

Considering the morpho-physiological categorization as a yard-stick of salinity tolerance of the studied genotypes, the prediction of the right tolerance status of the unknown control samples was not precise for 66.67% cases. This might be due to the fact that the control samples were validated against the model which is created by using the differentially expressed genes of four known tolerant and two known susceptible genotypes. That is the transcriptomic fingerprint induced by salt stress was used to create the model. But the control samples that are validated against this model simply lacked stress induced

transcriptomic fingerprints and the constitutive markers that existed in the control samples weren't enough for the appropriate prediction of tolerance status. This could be avoided by building the model using the control samples of known tolerant and susceptible genotypes only and then the unknown controls could be validated against that. But with the dataset generated in this project it would not be sensible as there would not be enough control samples to build the model.

The idea behind this approach (i.e., creating the model with the differentially expressed significant genes in control vs. treated samples in the four known tolerant and two known susceptible genotypes and then validating the unknown control samples against this model) was that this model can be used by other researchers to directly predict the tolerance status of unknown genotypes by validating the transcriptomic response of unknown control samples. Now that this approach has not proved fully satisfactory with this data set, it would be of interest to see how the prediction of the unknown treated samples goes with this same model.

6.3.1.4.3 Prediction of unknown treated samples using OSC-PLSDA model

The unknown treated samples were validated against the same OSC-PLSDA model with the forward selected 109 genes. Besides the correct prediction of Banikat and *O. rufipogon* (as in previous approach shown in Table 6.2), *O. latifolia* was also predicted correctly as susceptible in this approach (Table 6.3).

The *Indica* genotype IR58 which was categorized as moderately tolerant by the morpho-physiological assessment is predicted as a susceptible genotype (Figure 3.9b). It could be due the fact that the morpho-physiological assessment was done with 80mM NaCl stress but the

transcriptomic study was conducted with a higher salt stress (120 mM NaCl). It is possible the genotype can no longer tolerate the stress at a higher level (i.e., 120 mM NaCl) and hence, shows a susceptible response. Like the case of Nipponbare which showed some degree of tolerance at 40 mM NaCl stress but appeared as a susceptible genotype at 80 mM NaCl stress (figure 3.9 a & b). The categorization of IR58 as a moderately tolerant genotype was the outcome of multivariate assessment. If only the qualitative trait, standard evaluation score, SES (on a scale of 1-9, 1= highly tolerant and 9 = highly susceptible) is considered, IR58 actually appears to be a susceptible genotype (SES score is 7.7) as shown in Table 3.1.

For the other *Indica* genotype PSBRc50, which too was morpho-physiologically characterized as a moderately tolerant genotype (Figure 3.9b), only one sample (one replicate) was predicted as tolerant (Table 6.3). If all the three replicates were predicted as tolerant, it could be a perfect scenario. However, this is not the case and it is not clear whether or not only one treated replicate showed the predictive transcriptomic markers (keeping in mind that this is a moderately tolerant genotype) that are picked up by the model.

Like the case of PSBRc50, only one sample is predicted as tolerant for the *Japonica* cultivar Nipponbare (Table 6.3). This particular genotype was observed to move to susceptible from moderately tolerant status at higher (80 mM NaCl) salt stress. It could be a possibility that even at higher stress (120 mM NaCl) this particular sample (which is predicted as tolerant) possesses some transcriptomic markers which are picked up by the model even though morpho-physiologically it still appears as a susceptible genotype. It is however, not clear if there is an issue of differences in the genetic base of the model where all genotypes belong

to *Indica* subspecies and this particular genotype which belongs to *Japonica* sub-species, as the other *Japonica* genotype Banikat was predicted quite appropriately.

Table 6.3 Prediction of unknown treated samples using OSC-PLSDA model with 109 genes

Genotype	Banikat	IR58	*O. latifolia*	Nipponbare	PSBRc50	*O. rufipogon*
Tolerant	2	0	0	1	1	0
Susceptible	1	3	3	2	2	3

The numbers *Indica*te the number of replicates.

Even though the unknown treated samples are better predicted than those of unknown control samples, it is still not an exact match to the morpho-physiological outcome. It could be due to the fact that the number of samples used to build the model is insufficient and also that unequal numbers of tolerant (4 genotype) & susceptible (2 genotype) samples is used for building the model. The reason for using the aforementioned 4T and 2S genotypes for building the model is that these genotypes represent the benchmark tolerant and susceptible check genotypes, all belonging to *Indica* sub-species. A model created using these genotypes of the same sub-species group (i.e., *Indica*) with extreme contrast in their response to salt stress may provide the best basis for building the model.

Now that the predictions obtained so far don't exactly match that of morpho-physiological outcome, alternative approaches are sought. One of the alternatives was that adding two more susceptible genotypes would equalize the number of samples between the tolerant and susceptible genotypes used for model building and may improve the prediction. Moreover, by doing so, we are satisfying the general exercise of using the 2/3rd data to build the model and the rest of data sets to validate against the model for any prediction study. It is thus prompting to see how the prediction goes with this alternative approach (model created with the differentially significant genes between treated vs. control samples in in 4T

& 4S genotypes) by adding two more susceptible genotypes namely, *Japonica* cultivar Nipponbare and wild species *O. latifolia*. These genotypes are chosen based on their susceptible ranking at 80mM NaCl stress as obtained by the multivariate analysis based morpho-physiological assessment (Figure 3.9b). The prediction outcome using these four tolerant (Pokkali, FL478, Hassawi & Nonabokra) & four susceptible (IR29, BRRI dhan29, *O. Latifolia*, *O. rufipogon*) genotypes is discussed in the next section.

6.3.1.5 Forward selected 109 genes

The list of 109 forward selected genes used to build the OSC filtered PLS-DA model using the reduced matrix 1 are shown in Table 6.4. Of which, the notables that might have a role in salinity tolerance include 16.9 kDa class I heat shock protein (Os01g0136200), Calcium-binding protein (Os02g0606500), beta-expansin EXPB4 (Os10g0556100) and Expansin Os-EXPA3 (Os05g0276500), Disease resistance protein family protein (Os12g0467300 & Os11g0606400), Heavy metal transport/detoxification protein domain containing protein (Os01g0758000), Late embryogenesis abundant (LEA) group 1 family protein (Os04g0589800), DREB2C protein (Os08g0521600) and Sorbitol transporter (Os12g0514000) etc. No annotations were observed for the genes Os08g0282800, Os04g0258100, Os02g0482200 and Os01g0564000 and 30 genes were hypothetical proteins whose functions in salinity tolerance in rice would be of greater interest.

Table 6.4 List of 109 forward selected genes used to build the OSC filtered PLS-DA model using the reduced matrix 1

Locus ID	Annotation
Os08g0282800	(No Hit)
Os04g0258100	(No Hit)
Os02g0482200	(No Hit)
Os01g0564000	(No Hit)
Os07g0206500	13 kDa prolamin precursor.

Os01g0136200	16.9 kDa class I heat shock protein.
Os08g0417000	2OG-Fe(II) oxygenase domain containing protein.
Os08g0249900	2OG-Fe(II) oxygenase domain containing protein.
Os12g0183300	3'(2'),5'-bisphosphate nucleotidase (EC 3.1.3.7) (3'(2'),5- bisphosphonucleoside 3'(2')-phosphohydrolase) (DPNPase).
Os12g0168000	5-formyltetrahydrofolate cyclo-ligase family protein.
Os10g0556100	beta-expansin EXPB4 [*Oryza sativa* (*Japonica* cultivar-group)].
Os01g0124200	Bowman Birk trypsin inhibitor.
Os02g0606500	Calcium-binding protein.
Os03g0853200	CD9/CD37/CD63 antigen family protein.
Os04g0142400	Conserved hypothetical protein.
Os11g0233400	Conserved hypothetical protein.
Os04g0308500	Conserved hypothetical protein.
Os08g0539200	Conserved hypothetical protein.
Os07g0136400	Conserved hypothetical protein.
Os04g0431300	Conserved hypothetical protein.
Os02g0616100	Conserved hypothetical protein.
Os11g0270000	Conserved hypothetical protein.
Os04g0372400	Conserved hypothetical protein.
Os12g0540000	Conserved hypothetical protein.
Os12g0166700	Conserved hypothetical protein.
Os02g0583700	Conserved hypothetical protein.
Os01g0155800	Conserved hypothetical protein.
Os04g0126900	Conserved hypothetical protein.
Os02g0682700	Conserved hypothetical protein.
Os08g0150600	Conserved hypothetical protein.
Os04g0478000	Conserved hypothetical protein.
Os06g0248300	Conserved hypothetical protein.
Os09g0482300	Conserved hypothetical protein.
Os02g0108000	Conserved hypothetical protein.
Os05g0349400	Conserved hypothetical protein.
Os04g0454600	Conserved hypothetical protein.
Os09g0498200	Conserved hypothetical protein.
Os01g0644200	Conserved hypothetical protein.
Os05g0202800	Conserved hypothetical protein.
Os07g0105000	Cupredoxin domain containing protein.
Os04g0431200	Cyclin-like F-box domain containing protein.
Os06g0113600	Cyclin-like F-box domain containing protein.
Os12g0467300	Disease resistance protein family protein.
Os11g0606400	Disease resistance protein family protein.
Os10g0452900	Eggshell protein family protein.
Os10g0453000	Eggshell protein family protein.
Os05g0276500	Expansin Os-EXPA3.
Os12g0260500	Glucose/ribitol dehydrogenase family protein.
Os04g0390800	Glucose/ribitol dehydrogenase family protein.
Os12g0478200	GRAM domain containing protein.
Os01g0758000	Heavy metal transport/detoxification protein domain containing protein.
Os05g0138300	Hydrophobic protein LTI6B (Low temperature-induced protein 6B).
Os08g0184800	Hypothetical protein.
Os04g0149400	Hypothetical protein.
Os09g0309900	Hypothetical protein.
Os12g0435100	Hypothetical protein.

Os06g0271400	Hypothetical protein.
Os04g0589800	Late embryogenesis abundant (LEA) group 1 family protein.
Os02g0636400	Leucine-rich repeat, cysteine-containing subtype containing protein.
Os10g0392900	Lipolytic enzyme, G-D-S-L family protein.
Os12g0559200	Lipoxygenase (EC 1.13.11.12).
Os10g0361000	Lipoxygenase, LH2 domain containing protein.
Os05g0214300	MtN3 and saliva related transmembrane protein family protein.
Os04g0460900	Non-protein coding transcript, unclassifiable transcript.
Os03g0846200	Peptidase, trypsin-like serine and cysteine domain containing protein.
Os07g0677300	Peroxidase.
Os04g0415800	Plant lipid transfer/seed storage/trypsin-alpha amylase inhibitor domain containing protein.
Os10g0483400	Protein kinase-like domain containing protein.
Os01g0114300	Protein kinase-like domain containing protein.
Os01g0189800	Protein of unknown function DUF1618 domain containing protein.
Os11g0540600	Protein of unknown function DUF247, plant family protein.
Os04g0422600	Protein of unknown function DUF6, transmembrane domain containing protein.
Os04g0494800	Protein of unknown function DUF642 family protein.
Os02g0586000	Quinonprotein alcohol dehydrogenase-like domain containing protein.
Os01g0147700	Region of unknown function, putative Zinc finger, XS and XH domain containing protein.
Os04g0226400	Regulator of chromosome condensation/beta-lactamase-inhibitor protein II domain containing protein.
Os04g0316700	Retrotransposon gag protein family protein.
Os05g0582200	Retrotransposon gag protein family protein.
Os08g0164800	RNA-directed DNA polymerase (Reverse transcriptase) domain containing protein.
Os01g0745400	Sec34-like protein family protein.
Os12g0641000	Similar to Actin 1.
Os03g0643300	Similar to AER123Wp.
Os01g0127600	Similar to Bowman-Birk type proteinase inhibitor D-II precursor (IV).
Os05g0247100	Similar to Chitinase (EC 3.2.1.14) III C00481-rice (EC 3.2.1.14).
Os04g0457000	Similar to Chlorophyll a/b-binding protein CP24, photosystem II (Fragment).
Os06g0564700	Similar to Cysteine synthase (EC 4.2.99.8).
Os08g0521600	Similar to Dehydration responsive element binding protein 2C (DREB2C protein).
Os05g0212300	Similar to Endo-beta-1,4-glucanase precursor (EC 3.2.1.4).
Os03g0830300	Similar to Fw2.2.
Os04g0457500	Similar to Gamma-glutamyltranspeptidase 1 precursor (EC 2.3.2.2) (Gamma-glutamyltransferase 1) (CD224 antigen) [Contains: Gamma- glutamyltranspeptidase 1 heavy chain; Gamma-glutamyltranspeptidase 1 light chain]. Splice isoform 3.
Os07g0468100	Similar to Glutathione S-transferase GST 19 (EC 2.5.1.18).
Os03g0711800	Similar to IRE homolog 1 (Fragment).
Os06g0567900	Similar to L-ascorbate oxidase precursor (EC 1.10.3.3) (Ascorbase) (ASO).
Os10g0409400	Similar to Polygalacturonase isoenzyme 1 beta subunit precursor.
Os04g0561500	Similar to Prolyl endopeptidase (EC 3.4.21.26) (Post-proline cleaving enzyme) (PE).
Os06g0234200	Similar to RAC-like GTP binding protein ARAC8 (GTPase protein ROP10).
Os01g0123000	Similar to Retrofit.
Os12g0514000	Similar to Sorbitol transporter.
Os01g0555100	Similar to TATA-binding protein associated factor 2N (RNA-binding protein 56) (TAFII68) (TAF(II)68).
Os08g0539600	Similar to TGB12K interacting protein 2.
Os12g0630100	Similar to Thaumatin-like protein precursor.
Os12g0630500	Similar to Thaumatin-like protein.
Os03g0144500	Similar to Xyloglucan galactosyltransferase KATAMARI 1 (EC 2.4.1.-) (MURUS3 protein).
Os08g0414600	Soluble quinoprotein glucose dehydrogenase domain containing protein.

Os09g0256100	Sulfotransferase family protein.
Os11g0562100	Terpene synthase family protein.
Os04g0608600	Thioredoxin domain 2 containing protein.
Os05g0213900	Virulence factor, pectin lyase fold family protein.
Os08g0237000	Xyloglucan endotransglycosylase/hydrolase protein 8 precursor (EC 2.4.1.207) (End-xyloglucan transferase) (OsXTH8) (OsXRT5).

6.3.2 The reduced matrix 2 (6306 genes)

The differentially expressed significant genes of four tolerant and four susceptible were combined to create the reduced matrix 2. These represent 2/3rd of the entire dataset and the rest of the samples were validated against the model afterwards. The initial PCA of all the samples are shown in Figure 6.8. Some of the samples of wild species *O. latifolia* appeared to be the outliers and were removed during prediction alaysis. Then a forward selected 585 genes were used to create the optimized OSC-PLSDA model to filter out components that are not predictive of class separation. The PCA of all samples based on 585 forward selected genes are shown in Figure 6.9. The tolerant and susceptible genotypes separated by PC2 as shown in figure 6.9.

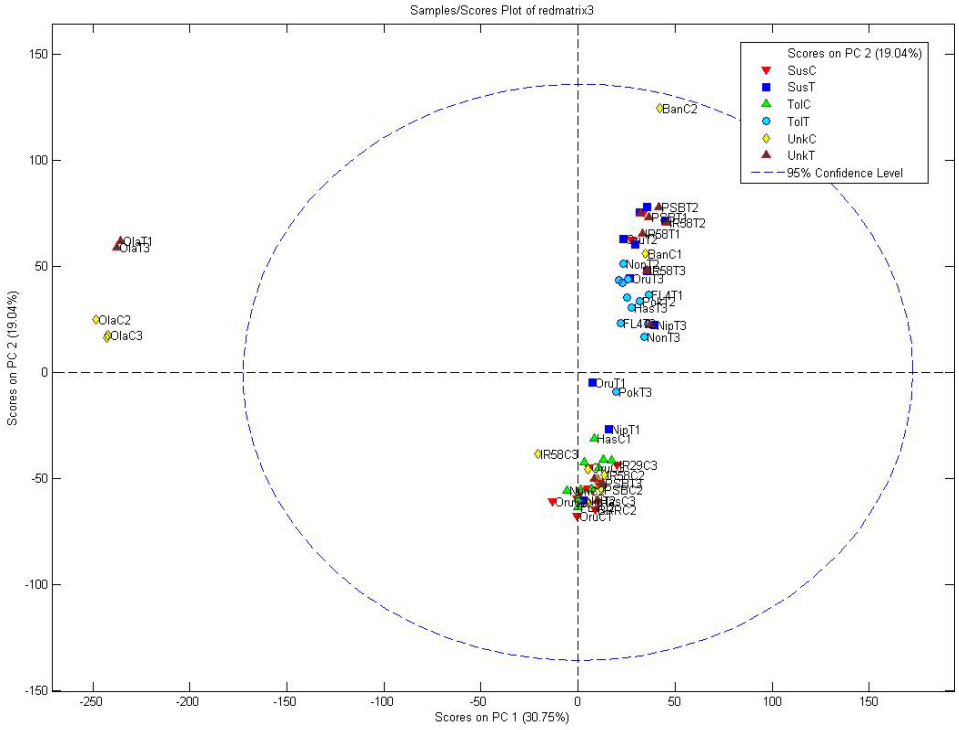


Figure 6.8 The PCA of all the samples based on the reduced data matrix 2 comprising 6306 genes.

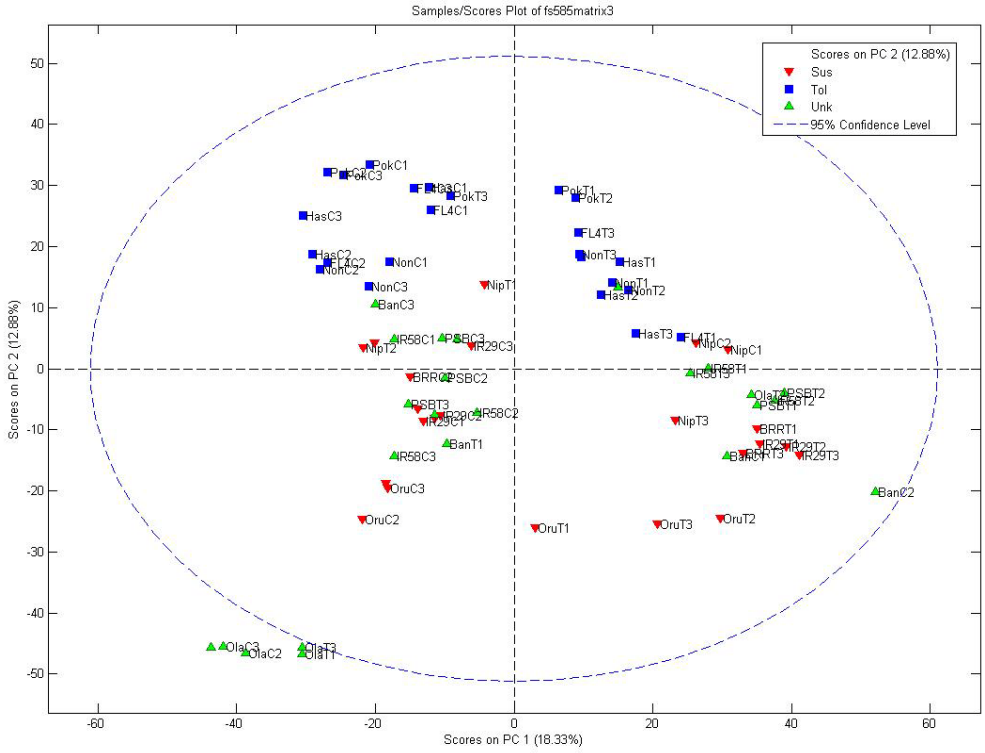


Figure 6.9 The PCA of all the samples based on the reduced data matrix 2 comprising the forward selected 585 genes.

6.3.2.1 Prediction of unknown control & treated samples using OSC-PLSDA model with 585 genes

The unknown control and treated samples of four genotypes which represent 1/3rd of the entire dataset were validated against the reduced matrix 2 (with 6306 genes) and the OSC-PLSDA model (with 585 forward selected genes). Both the control and treated samples of the *Indica* genotypes PSBRc50 and IR58 and the *Japonica* genotype Banikat are predicted as susceptible (Figure 6.9) which is in clear disagreement with the morpho-physiological findings. The prediction of the wild species *O. latifolia* with the reduced matrix 2 and with the OSC-PLSDA model is not the same and not in agreement with the morpho-physiological outcome (Figure 6.9).

Table 6.5 Prediction of unknown control and treated samples using the reduced matrix of 6306 genes and using the OSC-PLSDA model with forward selected 585 genes

Genotype	Banikat	IR58	O. latfolia	PSBRc50	6306 genes
Tolerant			5		
Susceptible	6	6	T2	6	
Genotype	Banikat	IR58	O. latfolia	PSBRc50	585 genes (forward selected)
Tolerant			C1, T1, T3		
Susceptible	6	6	C2, C3, T2	6	

Share

The numbers *Indica*te the number of replicates. C. Control. T. Treatment

Even though there is strong separation by germplasm group as shown in Figure 6.9 which is a proof of a good model building, the prediction is not an exact match to the morpho-physiologically categorized yardstick. But the message obtained from this prediction based on this 4T & 4S approach is that due to the existence of wider diversity in the susceptible group (as it now consists of two *Indica* genotypes, one *Japonica* genotype Nipponbare and one wild species *O. rufipogon*), all further test samples are likely to fall into this group. Having the similar genotype diversity in both groups to build the model creates the chances of

the OSC technique, to select the traits that are looked for. Obviously having fewer types means a higher likelihood that the groups that they are put into might show differences that do not correlate to salt tolerance.

6.4 Conclusion

Looking at all three predictions as shown in Figure 6.2, Figure 6.3 and Figure 6.4, it is now clear that the prediction based on the OSC-PLSDA model with 109 forward selected genes created using the 4T & 2S approach is best so far. However, still the prediction did not match exactly to that of the previous morpho-physiological classification which we considered as the yardstick of tolerance status of the genotypes studied which itself may not be definitive. The reason behind the imperfect prediction in some cases could actually be that the test samples are genuinely intermediate in tolerance status as the PCA clearly shows, and that might be because they have only some, but different combinations of genes that confer tolerance. The predictive model was however, successful in separating the known tolerant, unknown and susceptible, unequivocally. Inclusion of more samples of the similar sub-species group to build the model may improve the prediction of salinity tolerance status of the rice genotypes using the transcriptomics data which then may serve as a genomic database tool to screen the rice genotypes.

LIST OF SUPPLEMENTARY TABLES

Supplementary Table 6.1a-l: Lists of differentially (both up and down) regulated significant probes upon exposure to salt stress in all 12 genotypes namely, *Indica* genotypes Pokkali (a), FL478 (b), Hassawi (c), Nonabokra (d), BRRI dhan29 (e) and IR29 (f), PSBRc50 (g), IR58 (h), *Japonica* genotypes Banikat (i) and Nipponbare (j) and wild relatives *O. latifolia* (k) and *O. rufipogon* (l).

All the 12 genotypes were subjected to normalization as described in section 2.12.2 and the differentially expressed genes for the above mentioned varieties were identified by comparing the expression of treated samples vs. control samples.

Chapter 7. GENERAL DISCUSSION

Soil salinity poses a great environmental threat to world's food security in the face of dwindling agricultural land and increasing human population (Ahuja *et al*. 2010; Tester and Langridge 2010; Mainuddin *et al*. 2011; Bansal *et al*. 2014). Increased salinization of soils either by natural or by human induced processes is forcing plant breeders to look for sources of tolerance, to identify crop traits and the associated genes that confer the tolerance to excess salt either by conventional breeding or by molecular biotechnological manipulations (Martinez and Manzur, 2005; Ashraf and Akram 2009; Ford-Lloyd *et al*. 2011; Rajalakshmi and Parida 2012; Kumar *et al*. 2013). Screening of the germplasm thus became a prerequisite to identify the sources of tolerance.

7.1 Screening of germplasm based on multivariate analytical approach

Eighteen different qualitative, growth and physiological traits of eight genotypes belonging to three different rice sub-species groups namely *Indica*, *Japonica* and wild species were studied simultaneously using multivariate approach to statistically categorize the genotypes according to their level of tolerance (Chapter 3). The use of multivariate approach allowed robust screening of the genotypes, as it takes the variation of different components of the polygenic and complex salinity tolerance features such as root and shoot growth parameters, tissue ions and visual scores etc. into account. Zeng (2005) and Zeng *et al.* (2002) have already demonstrated the effectiveness of using multivariate approach on a limited scale. With the recent rise in plant phenomics and ionomics, the faster and automated collection of data for different phenotypic and physiological parameters from large populations became possible (Houle *et al*. 2010; Furbank and Tester 2011; White *et al*.

2012; Yang *et al*. 2013a) which further opens up the possibility of effective utilization of multivariate approach in broad based screening of genotypes by minimizing the timeframe required for wide screening.

7.2 Comparative morpho-physiological assessment of rice genotypes

The comparative morpho-physiological assessment revealed that qualitatively different mechanisms of salt tolerance are in operation within these germplasm groups (Chapter 3). The 'Na exclusion' and 'ion balance' mechanisms were observed in Pokkali, whereas only the former is prevalent in PSBRc50 and IR58 and only the latter is prevalent in the *Japonica* genotypes, Banikat and Nipponbare. The existence of 'Na exclusion' mechanism and its role is well documented in Pokkali and Hassawi (Cotsaftis *et al*. 2012; Kavitha *et al*. 2012; Platten *et al*. 2013a) and in Nonobokra (Ren *et al*. 2005b) and in other rice genotypes (Asch *et al*. 2000; Ul Haq *et al*. 2010). For the rest of genotypes, further in depth tissue ion analysis would be helpful in confirming the current conclusion of the existence of 'Na exclusion' and/or 'ion balance' mechanism as this conclusion was only reached by comparing the root and shoot ion data. Na exclusion was also found to be in operation in other cereal crop species such as wheat (Munns and James, 2003; Cuin *et al.* 2009, 2010); pearl millet (Krishnamurthy *et al*. 2007) and barley (Garthwaite *et al*. 2005; Shavrukov *et al*. 2010) etc. The genes *HKT1;4* (*Nax1*) and *HKT1;5* (*Nax2*) in wheat (Byrt *et al*. 2007; Huang *et al*. 2006; James *et al*. 2006a; Munns *et al*. 2003), *AtHKT1;1* in Arabidopsis (Davenport *et al*. 2007) and *OsHKT1;5* in rice (Ren *et al*. 2005) is already established to be associated with Na exclusion. The rice genes *OsMapk44* is postulated to be involved in ion balance (Jeong *et al*. 2006) and *OsHKT1;1*, *OsHAK1*, *OsHAK7*, *OsHAK10* and *OsHAK16* are candidates for larger accumulation

of Na in older leaves under salt and alkali stress (Wang *et al*. 2012a; Wang *et al*. 2012b). Once the existence of the mechanisms are confirmed in individual genotypes of rice and other crop species, the specific role of these and novel genes involved in ion balance can be verified which will enhance our understanding on molecular basis of salinity tolerance.

The multivariate analysis based categorization of the genotypes as susceptible and tolerant using allowed comparison of whole genome transcripts which is aimed at identifying the candidate genes that are responsible for salinity tolerance. The whole genome transcriptome of twelve rice genotypes belonging to *Indica*, *Japonica* and wild species having a range of tolerance to salt stress was profiled with the view to capture salt responsive expression of genes from a wide genetic background of rice.

7.3 Modified Significance Analysis of Microarrays: The perspective

Chapter four describes the trait specific gene expression of the entire genetic diversity represented by the rice genotypes of this study. This is done by using the modified Significance Analysis of Microarrays (see section 2.12.1) by analysing both the gene expression data and the weighted morpho-physiological responses simultaneously. Around 60 genes were identified to be involved in ion homeostasis, transport and transmembrane activity at stressed condition along with the genes involved in signal transduction (>150 genes), transcription factors (81 genes) and translation factor activity (62 genes) etc (Supplementary Table 4.6). A regulatory network analysis (see section 2.14) revealed that the transcription factors and translation initiation factors formed the major gene network which is active in nucleus, cytoplasm and mitochondria whereas the membrane and vesicle bound proteins formed another network which is active in plasma membrane and vacuoles

(see section 4.2.7). The genes that are identified here may not be attributed to individual genotypes as the expression of the entire genetic base is analysed simultaneously. However, the results hold importance in terms of identifying the candidate genes which can be helpful in explaining the potentially fundamental mechanisms of salinity tolerance active in the wide natural genetic background of rice. The other fact that can be mentioned here is that the identified genes may also be partly the product of the variation between the genetic block of *Indica*, *Japonica* and wild species as all three species/subspecies groups were analysed simultaneously. This was taken care of when only the tolerant and susceptible *Indica* genotypes were studied to identify the differentially expressed transcripts due to stress imposition which is described in chapter five.

7.4 Differentially expressed transcripts: *Indica* model

The initial plan of this project included the comparison of the physiological and transcriptional response between the *Indica, Japonica* and wild relatives of rice. The genotypes were chosen accordingly based on their prior reputation, so that in each sub-species group there is at least one tolerant and one susceptible genotype. A comparative physiology study involving all the genotypes of all three sub-species groups was needed to verify the tolerance level of the genotypes under study. The results revealed that neither of the two genotypes in the wild relative group is tolerant and that the *Japonica* genotype Nipponbare switched from being moderately tolerant to susceptible at higher salt stresses. Consequently that changed the part of the plan of comparing the transcriptional responses between these subspecies groups. Hence, the focus was subsequently shifted only to the differential expression analysis of the tolerant and susceptible *Indica* genotypes. However,

three more known tolerant and one more known susceptible genotypes were included (see Table 2.1) to broaden the genetic background of *Indica* sub-species and the differentially regulated transcripts were then critically analysed.

More genes were differentially regulated in susceptible genotypes than tolerant genotypes, a notion which was also observed by some of the previous studies (Walia *et al*. 2005; Walia *et al*. 2007; Senadheera *et al*. 2009). This probably suggests that the tolerant genotypes are better prepared to combat the stress in anticipation with a number of stress responsive genes expressed constitutively. The study on Arabidopsis for salinity tolerance (Taji *et al*. 2004a) and with tomato for heat resistance (Bita *et al.* 2011) also reached to the similar conclusion that tolerant plant constitutively express the stress protection related genes even under normal growth condition. The higher number of differentially expressed genes in susceptible genotypes may also be due to the fact that the effect of stress is more felt by susceptible genotypes followed by more genetic adjustment in the network of genes in an attempt (not necessarily successful) to adapt to the stressed condition.

Of the differentially regulated transcripts in four tolerant and two susceptible genotypes under salt stress, 311 were found to be uniquely up-regulated in at least one of the tolerant genotypes and 713 were found to be commonly up-regulated in at least one of the tolerant and one of the susceptible genotypes which can be considered as the most important candidates for salinity tolerance. It is evident from the high throughput genome wide experiments in rice (Walia *et al*. 2005; Walia *et al*. 2007; Senadheera *et al*. 2009; Cotsaftis *et al*. 2011), wheat (Kawaura *et al*. 2006; Mohammadi *et al*. 2008; Aprile *et al*. 2009; Liu *et al*. 2012), barley (Ueda *et al*. 2004a; Ueda *et al*. 2004b; Walia *et al*. 2006; Ueda *et al*. 2006;

Talamè *et al*. 2007), maize (Wang *et al*. 2003; Zheng *et al*. 2010) and Arabidopsis (Nikiforova *et al*. 2003; Taji *et al*. 2004b; Ma *et al*. 2005; Kumari *et al*. 2008; Ghars *et al*. 2008; Rasmussen *et al*. 2013) that numerous genes are involved in abiotic stresses tolerance mechanisms. The discovery of the fact that large number of genes is responsive for stress tolerance has changed the view of the biologists that transferring a gene or two might not be effective in developing successful stress tolerant cultivar (Yamaguchi and Blumwald 2005; Singh *et al*. 2008; Li and Zhang 2013). The apparent lack of successful abiotic stress tolerant commercial cultivar in the market further strengthens this notion (Kole *et al*. 2010; Møller *et al.* 2009; Bhatnagar-Mathur *et al*. 2008). However, that does not necessitate the development of stress tolerant variety by transferring all the stress responsive genes so identified by the high throughput techniques in elite cultivars. Moreover, not all the genes are directly responsible for conferring salinity tolerance as evidence suggested that a lot of genes are simply expressed as a function of adaptation to secondary stress response or stress recovery (Zhu 2001; Xiong and Zhu 2002; Shaik and Ramakrishna 2014).

It is thus important to determine the key genes that are responsible for salinity tolerance. Identifying those genes from hundreds of differentially expressed genes and explaining their specific roles in the mechanism of stress tolerance becomes the next challenge (Edwards and Batley 2004; Vandepoele and Peer 2005; Huang *et al*. 2009; Oh *et al*. 2012; Gillis and Pavlidis 2013; Yang *et al*. 2013b). However, the current development in the field of genomics and bioinformatics allowed several GO analysis to effectively categorize the long list of genes according to the biological processes (BP) such as response to stimulus, signalling etc. and the molecular functions (MF) such as transporter activity and transcription regulator activity etc. they are involved in (Huang *et al*. 2009; Du *et al*. 2010). This study used Singular

Enrichment Analysis (SEA) that facilitated the discussion of the functional roles of the genes to piece together the complete picture of salinity tolerance mechanism. The list of transcripts according to the biological processes and molecular functions they are involved in are given in Table 5.2, Table 5.3 and Supplementary table 5.5 and the individual genes encoded by these transcripts are discussed in section 5.2.3 and this section aims at discussing the results within their wider context.

7.4.1 Transcripts involved in 'response to stimulus'

Under the biological process 'response to stimulus' the notable transcripts that are up-regulated either uniquely in the tolerant genotypes or commonly in both tolerant and susceptible genotypes and involved in 'response to stimulus' (BP) are Embryonic abundant protein 1 (Os01g0159600,Os05g0349800), Abscisic stress ripening protein 1 (Os01g0959200, Os01g0959100), Haem peroxidase, plant/fungal/bacterial family protein(Os04g0465100,Os01g0327100), Heat shock protein (Os04g0549600, Os03g0745000, Os09g0482600, Os06g0553100, Os01g0571300), Universal stress protein (Usp) family protein (Os01g0511100, Os01g0511100, Os02g0707900, Os01g0849600, Os01g0783500) and Dehydrin (Os11g0451700, Os02g0669100, Os11g0454300, Os11g0454000, Os11g0454200, Os11g0453900, Os01g0702500). The transgenic plants generated by transferring either of the genes belonging to the above family have shown increased tolerance to different abiotic stresses. For example, the transfer of an Embryonic abundant protein, *HVA1* from barley to rice has shown increased tolerance to water deficit and salinity stress in rice (Xu *et al*. 1996) and these proteins were also found to be involved in tolerance to desiccation in rice (Shih *et al*. 2010) and freezing in wheat (Sasaki *et al*. 2014). Transfer of Abscisic stress ripening gene, *ASR1* showed increased salt tolerance in tobacco (Kalifa *et al.*

2004b) and its orthologous gene, *LLA23* showed increased tolerance drought and salt in lily (Yang *et al.* 2005). Over-expression of peroxidise gene, *swpa1* in sweet potato (Huh *et al*. 1997) and *TPX2* in tomato (Amaya *et al*. 1999) has shown increased tolerance to salt oxidative and salt stress, respectively. Heat shock proteins have also shown to increase thermo-tolerance to plants (Sun *et al*. 2001; Li *et al*. 2003; Katiyar-Agarwal *et al*. 2003; Wang *et al*. 2005). The Universal stress protein (Usp) family proteins are mostly found to be involved in stress and acid resistance in bacteria (Kvint *et al*. 2003; Seifart Gomes *et al*. 2011) and shown elevated tolerance to drought in cotton (Shamim *et al*. 2013; Maqbook *et al*. 2008 & 2007). Overexpression of multiple dehydrin genes showed increased tolerance to salt and osmotic stress and freezing stress in Arabidopsis (Puhakainen *et al*. 2004; Brini *et al*. 2007; Hanin *et al*. 2011).

7.4.2 Transcripts involved in 'signaling'

Nine transcripts that encode signalling related genes were found to be commonly up-regulated in at least one tolerant and one susceptible genotype. The transcript Os09g0418000 (Protein kinase domain containing protein) encodes for the gene *CIPK5* whose over expression showed increased tolerance to salt in rice (Xiong & Yang 2003) and the ortholog of transcript Os03g0390200 encoding a Protein kinase 3 is *SNRK2* whose over expression showed increased tolerance to drought and salt stress in Arabidopsis (Zhang *et al*. 2010). Overexpression of signalling gene *SAPK4* (Die´dhiou *et al*. 08), *CIPK03*, *OsCIPK12*, and *CIPK15* (Xiang *et al*. 07), MAPK kinase kinase (*MAPKKK/DSM1*), *MAPK44* (Nning *et al*. 2010; Jeong *et al*. 2006) is known to increase tolerance to multiple stress in rice. Role of signalling molecules in activating downstream genes to combat the effect of stress is well documented (Chinnusamy *et al*. 2004; Agarwal and Jha 2010; Huang *et al*. 2012).

7.4.3 Transcripts involved in 'transcription regulator activity'

Among the up-regulated transcripts (21 in total; list c and e in ST 5.5) that encodes transcription regulator activity, the notables are WRKY TF (Os11g0490900, Os01g0734000), Heat shock TF (Os03g0745000, Os06g0553100, Os01g0571300), DREB TF (Os06g0127100, Os01g0165000), bHLH domain containing protein (Os10g0376900, Os03g0379300, Os01g0159800) etc. The transcription factors and their roles in abiotic stress tolerance are well reviewed (Agarwal *et al*. 2006; Ross *et al*. 2007; Agarwal and Jha 2010; Golldack *et al*. 2011; Todaka *et al*. 2012; Kumar *et al*. 2012). Wu *et al*. (2009) reported enhanced tolerance to drought by the overexpression of *Oryza sativa WRKY11* & Heat Shock Protein 101 and (QIU and YU 2009) observed increased tolerance to drought tolerance disease resistance and disease resistance in Arabidopsis by over expressing the gene *OsWRKY45*. The up-regulation of *DREB1A* (Os06g0127100) and *DREB2A* (Os01g0165000) as observed in this study was also found previously to be induced by drought and salt stress (Dubouzet *et al*. 2003). The same study also found the induction of three more DREB TF namely, *OsDREB1B*, *OsDREB1C*, *OsDREB1D*. Wang *et al*. (2008) observed that over expression of *OsDREB1F* enhanced the tolerance to cold, drought, salt stress in both rice and arabidopsis. However, none of the NAC and MYB type TFs was found to be induced in tolerant genotypes whereas Ohnishi *et al*. (2005) reported the up-regulation of *OsNAC6* under salinity, drought, cold, and ABA.

7.4.4 Transcripts involved in 'transporter activity'

Transcripts involved in transporter activity showed specific pattern of up-regulation in tolerant genotypes. No cation/H^+ exchanger or Na^+/H^+ antiporter is up-regulated in susceptible genotypes even though transcripts encoding Plasma membrane H^+ ATPase was found to be up-regulated in both tolerant (Os02g0825600, Os02g0313900) and susceptible

(Os03g0689300, Os07g0191200) genotypes (Table 5.2 & 5.3). Among the rest of the transcripts, notables are Calcium-transporting ATPase 8 (Os10g0418100), Amino acid/polyamine transporter (Os04g0435100, Os04g0659800), Bile acid:sodium symporter (Os01g0645200), Peptide (Os01g0142800) Hexose (Os03g0218400) and sucrose (Os03g0170900, Os10g0360100) transporters etc. Na^+/H^+ exchanger gene is shown to enhance salinity tolerance in rice by *OsNHX5* gene (Bassil *et al*. 2012), in rice and maize by *OsNHX1* gene (Chen *et al*. 2007) and in Arabidopsis by *AtNHX1* gene (Gaxiola *et al*. 2007). *NHX1* is believed to enhance the compartmentalization of Na^+ into the vacuoles (Chen *et al*. 2007) and there is more than 26 report of enhanced salt tolerance by these genes (Agarwal *et al*. 2013). Besides these exchangers or antiporters, the proton pump of the cell membrane acts as a driving force for nutrient uptake (Serrano *et al*. 1999) and the plasma membrane H-ATPase plays major role for this, whose (gene *Avp1*) overexpression has shown to enhance salinity tolerance in Arabidopsis (Gaxiola *et al*. 2007), tobacco (D'yakova *et al*. 2006), maize (Wei *et al* 2008), alfalfa (Bao *et al*. 2009) and cotton (Lv *et al*. 2008). However, no transcript encoding high affinity potassium transporter (HKT) family protein was found to be up-regulated in this study, although rice genome is known to have seven HKT transporter genes (Garciadeblas *et al*. 2003, Platten *et al*. 2006) which have defined roles in Na^+ homeostasis (Yao *et al.* 2010, Horie *et al.* 2007).

7.4.5 Protein of unknown function (PUF)

Among the differentially regulated significant genes thus identified in this study and in the recent studies of similar kind, a large numbers of genes encodes for hypothetical protein whose function is as yet to be known (Garg *et al*. 2012, 2013; Soda *et al*. 2013). But the up-regulation of these genes upon stress imposition signifies the fact that these PUFs may also

have roles in stress tolerance. The lack of similarity with well-characterized sequences (genes and proteins) further increases the interest in these groups of genes, a fact which points towards the possibility of these genes having unique functions and roles in essential pathways (Singh *et al*. 2012). With the progress in genome sequencing and functional annotation, the specific roles of these genes could be unearthed which will may lead to the discovery of novel candidates and new alternate pathways and may further broaden our understanding on stress tolerance (Luhua *et al*. 2008; Pawlowski 2008).

7.5 Identifying candidate genes: current and future context

Microarray is one of the first set of techniques that is used in studying the gene expression at whole genome level in post-genomic era. With the progress in technology and affordability of the whole genome sequencing, more sophisticated techniques is emerging that can be employed to reach to appropriate candidate genes for various biotic and abiotic stresses. Transcript abundance by sequencing the entire set of mRNAs using RNA-seq techniques can provide accurate measurement of gene expression, a fact which already shifted the research paradigm from microarray to RNA-seq approach (Lister *et al*. 2009; Atkinson and Urwin 2012). Moreover, the advances made in pre-genomic era can also be further investigated to identify the candidate genes (Yano *et al*. 2012; Shelden and Roessner 2013; Liu *et al*. 2013; Soda *et al*. 2013; Wang *et al*. 2013; Jain 2012). For example, combining the fine mapping of the QTLs and the expression analysis of the genes that localizes within those QTLs using microarrays can potentially identify the candidate genes within shortest possible time (Krzywinski *et al*. 2009; Deshmukh *et al*. 2010; Pandit *et al*. 010; Cotsaftis *et al*. 2011; Wang *et al*. 2013). In this study, a number of transcripts that is located within the salt

stress related QTL were found to be up-regulated. For example, the up-regulated transcription factor gene 'Zinc finger C3HC4 type RING finger' (Os01g0303600) were located within the QTL *Saltol* and qSKC1 in chromosome 1 can be mentioned.

Moreover, the availability of genome wide high-density molecular markers allows the identification of candidate genes from wide natural variation using Genome Wide Association Studies (GWAS), functional allele mining and SNP value determination (Li and Zhang 2013; Yang *et al*. 2013b; Bansal *et al*. 2014; Bolger *et al*. 2014). Besides, the candidate genes from different crops including the mangroves and wild species (Roy *et al*. 2011b; Rajalakshmi and Parida 2012; Garg *et al*. 2013; Atwell *et al*. 2014) and the advancements in cell or tissue specific expression studies for different biotic and abiotic stresses has already generated huge datasets (Pu and Brady, 2010; Long, 2011; Rogers *et al.* 2012; Ma *et al*. 2013). Because there can be several isoforms of a particular genes which can have similar mechanism of action across species (Platten *et al*. 2013b) and the dynamics of the proteins, metabolic pathways they are involved in along with the possible complexity in epigenetics further poses a daunting challenge to identify the network of genes that can be effectively used in crop improvement programmes (Golldack *et al*. 2011; Oh *et al*. 2012; Duque *et al*. 2013; Garg *et al*. 2013; Cabello *et al*. 2014). A gene network analysis using web based tool was attempted in this study and it was observed that the transcription factors and translation initiation factors formed a network of genes which are mostly active in nucleus, cytoplasm and mitochondria and the membrane and vesicle bound proteins formed another network of genes that are active in plasma membrane and vacuoles (section 4.2.7; Figure 4.8). The recent rise in system biology approach may further allow the comprehensive integration of multi-omics datasets in a way to identify the appropriate biomarker

candidates for enhancing the tolerance to biotic and abiotic stress (Yuan *et al*. 2008; Fukushima *et al*. 2009; Langridge and Fleury 2011; Yang *et al*. 2013b; Cabello *et al*. 2014).

Limitations and future research directions

The constraints in time and resources have limited the scope of this research project. The limitations with their possible solutions and future research directions are listed but are not limited to the following-

1. In the physiology experiment, more genotypes possibly with diverse genetic background especially for the *Japonica* and wild species groups could be used for screening purposes. The multivariate analysis for screening germplasm is often suggested to be more suitable with wider range of genotypes variability.

2. More physiological traits such as chlorophyll content, osmotic pressures and water potentials of the tissue and the nutrient solutions, net photosynthetic rate and dark respiration, CO_2 assimilation rate, osmolytes such as proline, total soluble sugar etc. and the survival rates could be measured. In addition, concentration of ions in xylem sap and in the vacuole and cytosol could be measured that would help in in-depth understanding of ion homeostasis and ion selectivity and in particular better reveal whether plants are performing toxic ion exclusions or ion balance techniques to tolerate excess salts. This could also explain whether the osmotic stress or the ionic toxicity causes the actual harm to plants?

3. The threshold tolerance level of the genotypes could be established and the extent of apoplastic bypass along with the route of ion uptake could be characterized. All these would help us better understand the salinity tolerance process which ultimately would

help us to selectively target the improvement of a few aspects by conventional breeding and genetic manipulation.

4. In addition, it would be better if the physiology study could also be carried under field conditions and if the survival and recovery rates and the yield performances of the genotypes could be measured. Unlike the NaCl based salt stress and the controlled growth condition in laboratory, the field environment is dynamic as many other forms of salts like carbonates and bicarbonates of other mineral nutrients and the macro and micro environmental conditions along with the prevalent soil properties make the condition unique (Yamaguchi and Blumwald 2005; Deyholos, 2010; Mittler and Blumwald, 2010; Leung 2008; Tester and Langridge 2010; Atkinson and Urwin 2012; Cabello *et al*. 2014).

5. In the Significance Analysis of Microarrays chapter, only the first eight genotypes were used. It was not possible to include the four genotypes that were added later as those genotypes were not physiologically characterized. Addition of these genotypes in the SAM analysis would broaden the genetic diversity further while exploring the gene expression in wide natural variation of rice genotypes.

6. Besides analysing the four tolerant and two susceptible genotypes with GeneSpring software, it would be interesting to see the differential gene expression involving two genotypes with contrasting tolerance to salt at a time or to compare every genotype against a single reference susceptible or tolerant genotype. The results could be different as the normalization of the expression signals over a range of genotypes may give the output slightly differently.

7. There could be the effect of background genetic variation in the differential gene expression analysis as diverse genotypes were studied. It could be useful to see the gene expression changes in the recombinant inbred lines (RILs) or near isogenic lines (NILs) with contrasting tolerance to salt that would be helpful to avoid the background genetic variation.

8. The gene expression data derived from the high-throughput microarray experiments are often validated by qRT-PCR technique. The expression of the selected genes could be validated by qRT-PCR experiment.

9. Transcriptome changes at different time points could be studied to capture more transcriptional responses that would probably reveal the expression of genes that are active at different stages of stress period and would possibly provide the better insight into the expression of the significant genes identified in this project at other time points.

10. The effect of few more stresses such as cold, drought, high temperature etc. could be studied. That would probably reveal the additional set of genes involved and also if there is any crosstalk between the stresses along with the possible identification of multi-stress induced genes.

11. It could also be evaluated if there is any correlation between the physiological responses and the expression pattern of selected candidates. That would probably better identify the strongest candidates whose expression is co-related with the changes in the specific physiological responses.

12. Furthermore, the copy number variation and the allelic forms of the selected candidate genes could be identified by using genomic southern blots which might be useful in

explaining the variation in stress adaptation. In addition, the micro-RNAs that play important roles in stress tolerance could be identified.

13. For more precise estimation of gene expression, microarray based techniques can be replaced by sequence based techniques such as RNA-seq. And the selected candidate genes can selectively be sequenced to identify the SNP variations among those genes which possibly help to explain the variation in stress adaptation.

14. The candidate genes are selected based on the abundance of mRNA which may not correlate well with the protein/enzyme activity levels due to possible post-translational modification. The stress responsive role of these selected genes should thus be confirmed by further proteomics and/or metabolomics studies.

15. Microarrays can only suggest genes which are helpful in creating hypotheses. The functions of the selected candidates should be established by using knockouts, T-DNA insertion lines, tilling mutants, siRNA Knock down lines and over expression studies or by using transgenic approaches.

16. Inclusion of more samples of same sub-species group (e.g., *Indica* sub-species) may improve the OSC-PLSDA model further which would ultimately result in better prediction of salinity tolerance status of genotypes whose tolerance status is unknown.

Concluding remarks

The technical advances in multi 'omics' technologies along with the Next Generation Sequence based technologies being affordable day by day, opened the avenue of system biology to better understand the whole biological processes and the molecular networks involved in biotic and abiotic stress tolerance.

Identification of novel network of genes that can sense the stresses, transduce the signals, activate the transcription factors which ultimately can activate the downstream genes that are directly related with stress tolerance such as transporters, exchangers, anitporters etc. will be helpful in designing effective breeding programmes and genetic engineering strategies. The findings of this study will contribute to the ongoing and future investigation of salt stress response in rice and in other cereals for the development of broad spectrum and durable stress tolerant elite cultivars. Development of crop plants with an inherent ability to cope with the unsuited environmental conditions will significantly contribute in achieving global food security.

Chapter 8. REFERENCES

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APPENDICES

Appendix I: Yoshida nutrient medium as adapted from Yoshida *et al.* 1976

Solution	Composition	Amount
A	Amonium nitrate (NH_4NO_3)	91.4 g
	dH_2O, volume to	1000 ml
B	Sodium phoshate 2-hydrate ($NaH_2PO_4\,2H_20$)	40.3 g
	dH_2O, volume to	1000 ml
C	Potassium sulfate (K_2SO_4)	71.4 g
	dH_2O, volume to	1000 ml
D	Calcium chloride ($CaCl_2$)	88.60 g
	dH_2O, volume to	1000 ml
E	Magnesium sulfate 7-hydrate ($MgSO_4{\cdot}7H_2O$)	324 g
	dH_2O, volume to	1000 ml
F	**Micronutrients – (store in dark glass bottle)**	
	Manganous chloride 4-hydrate ($MnCl_2{\cdot}4H_2O$)	1.500 g
	Ammonium molybdate 4-hydrate ($(NH_4)_6Mo_7O_{24}4H_2O$)	0.074 g
	Boric acid (H_3BO_3)	0.934 g
	Zinc sulfate 7-hydrate ($ZnSO_4{\cdot}7H_2O$)	0.035 g
	Cupric sulfate 5-hydrate ($CuSO_4{\cdot}5H_2O$)	0.031 g
	Iron chloride 6-hydrate ($FeCl_3{\cdot}6H_2O$)	7.700 g
	Citric acid monohydrate	11.900 g
	Dissolve each (except $FeCl_3{\cdot}6H_2O$ *which should be dissolved in 100* mL*) separately in 50 ml* dH_2O *in beakers then combine*	
	Add concentrated sulfuric acid (H_2SO_4).	50 ml
	dH_2O, volume to	1000 ml

Amount of stock solution to take per preparation

Stock Solution	Amount (ml)		
	per 4 liter solution	**per 20 liter solution**	**per 60 liter solution**
A	5	25	75
B	5	25	75
C	5	25	75
D	5	25	75
E	5	25	75
F	5	25	75

Add sodium meta-silicate 9-hydrate (4.5mg/L of culture solution)

Adjust pH to 5 (pH 4.5 with nitric acid)

Prepare fresh stock solutions for every two months

Appendix II: MIAME/PLANT frame work

MIAME/PLANT frame work		Experiment information
***I*. Array Design Description**		
Manufacturer	:	Agilent
Array design name	:	4x44K rice one colour microarray slide
Surface & coating specification	:	Glass slide
Platform type	:	*in situ* synthesized (SurePrint inkjet technology)
Type of the reporter	:	Synthetic oligo-nucleotides (60-mer)
Sequences	:	43,803 rice probes represented
Composition	:	Content sourced from the National Institute of Agrobiological Sciences, RefSeq and GenBank 2007
Availability	:	Agilent Product Number - G2519F Design ID - 015241
***II*. Experiment Design**		
1. Plant experimental design		
1) Pooling of experiments		
No. of plants in each pool	:	1
When pooled	:	N/A
Genotype pooled	:	Individual
Grown on	:	3 separate plate
Planted on the same day	:	Yes
2) Experimental Design		
Number of blocks	:	3
Randomized between blocks	:	Yes
2. Plant sample used, extract preparation and labeling		
1) Biosource properties		
Plant strain or lineGenotype	:	Germplasm Accession (12)
Starting material	:	Seed
Development stage	:	seedling stage (16 days old)
Organism part	:	Whole seedling (root + shoot)
2) Biomaterial manipulations		
Growth substrate	:	Yoshida nutrient medium
Growth environment	:	Controlled growth room
Environmental conditions		
Light duration	:	16/8 h (D/N) photoperiod
Light source		Fluorescent lamps
Light intensity		270-300 µmol m^{-2} s^{-1}
Watering conditions		Renewing the nutrient solution daily
Relative Humidity		70-75%
Temperature		28/20°C day/night
Spacing/density of the plant		15cm
Harvesting conditions		As above environmental conditions
Treatment Type		Salt
degree of stress		120 mM NaCl
Stress duration		2 days
Isolation techniques		Whole seedling removed from solution
3) Extraction method		
Quantity extracted		100mg
Extraction source		Fresh sample
Extraction method		Qiagen RNeasy plant mini kit (see chapter 2)
Labelling		As per manufacture's instruction (see chapter 2)

Appendix III: Details of the OSC PLS-DA predictive model

```
X-block: x110genematrix  35 by 110 (sommeru@BIO-T418-
DT1@20130607T171856.51414875 m:20130607175910.399)
Included: [ 7-29 42-47 54-59 ]  [ 1-110 ]
Included (in axis units): [ n/a ]  [ 1-110 ]
Preprocessing: OSC (#components = 1, #iters = 0, tolerance = 99.9%), Mean
Center
Y-block: y  35 by 2 (sommeru@BIO-T418-DT1@20130611T141418.67355725
m:20130611141418.673)
Included: [ 7-29 42-47 54-59 ]  [ 1-2 ]
Preprocessing: Autoscale
Num. LVs: 2

Statistics for each y-block column:
Modeled Class: 1   2
Sensitivity (Cal):  1.000  1.000
Specificity (Cal):  1.000  1.000
Class. Err (Cal):     0     0
RMSEC: 0.0160295 0.0160295
Bias:     0 -1.11022e-016
R^2 Cal: 0.998808 0.998808
```

Percent Variance Captured by Regression Model

	-----X-Block-----		-----Y-Block-----	
Comp	This	Total	This	Total
1	35.42	35.42	99.41	99.41
2	16.05	51.46	0.47	99.88

Appendix IV: Guide on how to use Supplementary Tables

The supplementary tables can be sometimes too large involving hundreds of rows, columns and large diagrams deposited in a single worksheet. The following guidelines may thus be helpful in using these large files.

More than one worksheet in a single excel file:

There may be more than one worksheet in a single excel file, e.g., in supplementary table 4.1 there is 14 worksheets.

Too many rows, columns or diagrams in one worksheet:

In a single worksheet, there may be too many rows, columns and diagrams. Please take your time to browse the entire worksheet. It will be helpful to have a look at the entire worksheet by zooming it out. The entire worksheet may be zoomed in or out by clicking the 'Zoom' in the 'View' menu or by rotating the mouse wheel forward and backward while holding the 'Ctrl' key down.

Some of the columns might be hidden:

In some cases there might be some hidden columns. Whether there is a hidden column or not, can be identified by looking at the top headings of the columns. Missing alphabetic letters in the column heading means that some columns are hidden. To display the hidden columns, please select the columns adjacent to either side of the columns, then right-click a row or column (or a selection of multiple rows or columns), and then click 'unhide'. A column can be make 'hidden' again by right clicking that column and clicking 'hide'.

Data presented as groups of rows in a single worksheet:

The data may be presented as groups of rows separated by several blank rows. Clicking the 'up' or 'down' arrow while holding the 'Ctrl' key down will help to navigate across the groups of data quickly.