Alternative respiratory genes can improve tolerance to abiotic stresses in rice (*Oryza sativa* L.)

Vajira Rupika Wanniarachchi

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Abstract

The non-phosphorylating alternative respiratory pathway (AP) for plants, which consists of two protein groups, alternative dehydrogenases (NDHs) and alternative oxidases (AOXs), has become an area of research interest due to its potential role during stress tolerance. Although the physiological functions of AOXs have been extensively studied those of NDHs are largely unclear and less is known about their responses to abiotic stresses. The main objective of this study was to investigate whether the alterations in AP genes could infer tolerance to abiotic stresses in cereal crops, using rice a model cereal. To achieve this, it was first necessary to identify all candidate genes encoding members of this pathway in rice. To explore the role of AP in stress in rice two approaches were taken; 1. Examine the expression of AP genes in different varieties of rice with varied tolerance to salt and 2. Development of transgenic lines of rice overexpressing AP genes and assess impact of growth under salt stress.

Database screening of the rice genome revealed that both AOX and NDH genes are present in rice. NDH grouped into three distinct families, NDA, NDB and NDC, as in Arabidopsis, but AOX2 type gene was absent. Co-expression of AP genes was observed in response to chemical inhibition of the cytochrome pathway of the mitochondrial electron transport chain, suggesting potential co-regulation. Analysis of promoter regions revealed that these genes share common *cis*-acting regulatory elements, previously found to be functional in Arabidopsis. However, functional analysis of them in rice is yet be investigated.

A number of rice AP genes was co-expressed in response to salinity stress also. A comparative study of two contrasting rice cultivars, Nipponbare and Langi, further revealed the existence of cultivar and tissue-dependent variation in gene expression. As found in other species, AOX proteins exist as homodimers in rice and the reduced (active) form was found to be predominant in mitochondria from salt-stressed tissues

while the oxidized (inactive) form was more abundant in non-stressed tissues, suggesting post-translational regulation of AOX may be more important in rice than protein levels *per se*. A novel polyclonal antibody raised against NDB2 showed a higher abundance of NDB2 in salt-treated shoots than in non-treated shoots, suggesting NDB2 might be important in salinity tolerance responses. However, in contrast to shoots, corresponding bands were not detected in either salt-treated or non-treated roots. This suggests the possibility of having different post-translational regulations in shoots and roots in rice. However, this needs to be further investigated.

A study on 16 different rice cultivars revealed the existence of wide genotypic and tissue-specific variation in gene expression and tolerance mechanisms in response to salinity stress. Expression of *AOX1a* and *NDA2* in leaf tissues showed a strong positive correlation with tissue Na^+ content. But, there was no correlation between AP gene expression and shoot growth. In fact, the expression of *AOX1c* and *NDB2* in roots revealed strong negative correlation with relative growth of roots. Although it is possible that some AP components play a certain role in salinity tolerance in some cultivars, transcriptional data did not reveal any particular trend in AP gene expression in response to salinity stress amongst the cultivars tested, suggesting AP gene expression levels may not be good molecular markers for salt tolerance in rice.

Transgenic rice plants overexpressing salt-responsive *AtAOX1a* or *AtNDB2*, driven by the rice actin-1 promoter were successfully generated via *Agrobacterium*-mediated transformation. Yield performances of segregating transgenic lines under salinity stress were evaluated at panicle initiation stage, and a significant reduction of empty seed production was revealed in some transgenic lines compared to their controls, suggesting a possible increase in tolerance to high salinity. This result suggests that overexpression of heterologous stress-responsive genes could be a potential avenue for improving rice towards a particular stress tolerance, however this needs confirmation when homozygous lines have been selected.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Vajira Rupika Wanniarachchi

Date:

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1 General introduction

1.1 Genetic diversity and domestication of rice

Rice is an annual crop with around 3 to 6 months of life cycle that varies depending on the cultivar and the environment in which it grows. The mature plant has a main stem and several tillers that later bear terminal flowering heads and panicles. Rice completes two distinguishable phases during their life cycle; a vegetative phase and a reproductive phase. The vegetative phase includes germination, early seedling growth and tillering. The reproductive phase is subdivided into the time before and after heading, that is, panicle exsertion and the ripening period (Maclean et al., 2002).

Rice is a member of the Family Poaceae (or Gramineae), belonging to the genus *Oryza*, which consists of more than 20 wild species and two cultivated species. The cultivated species, *O. sativa* (Asian rice) and *O. glaberrima* (African rice) are diploid (2n=24) and have an AA genome (Brar and Khush, 1997; Khush, 1997). Wild species have either 2n=24 or 2n=48 chromosomes representing AA, BB, CC, BBCC, CCDD, EE, FF, GG and HHJJ genomes (Brar and Khush, 1997; Khush, 1997).

The history of rice domestication has long been a subject of debate. It is believed that the cultivated species originated from a common ancestor with an AA genome and became domesticated through two parallel evolutionary pathways (Figure 1.1) (Khush, 1997; Chang, 2003; Purugganan, 2014). The present day rice *O. sativa* is believed to be domesticated from Asian common wild rice, *O. rufipogon* while African rice was domesticated from *O. barthii* (Khush, 1997; Molina et al., 2011; Purugganan, 2014). A number of genetic studies using molecular markers or DNA sequence information have also revealed that *O. sativa* cultivars are more closely related to *O. rufipogon* than to any other *Oryza* species (Lu et al., 2002; Rakshit et al., 2007). It is obvious that during domestication, morphological and physiological traits were altered in response to human selection (Purugganan, 2014).

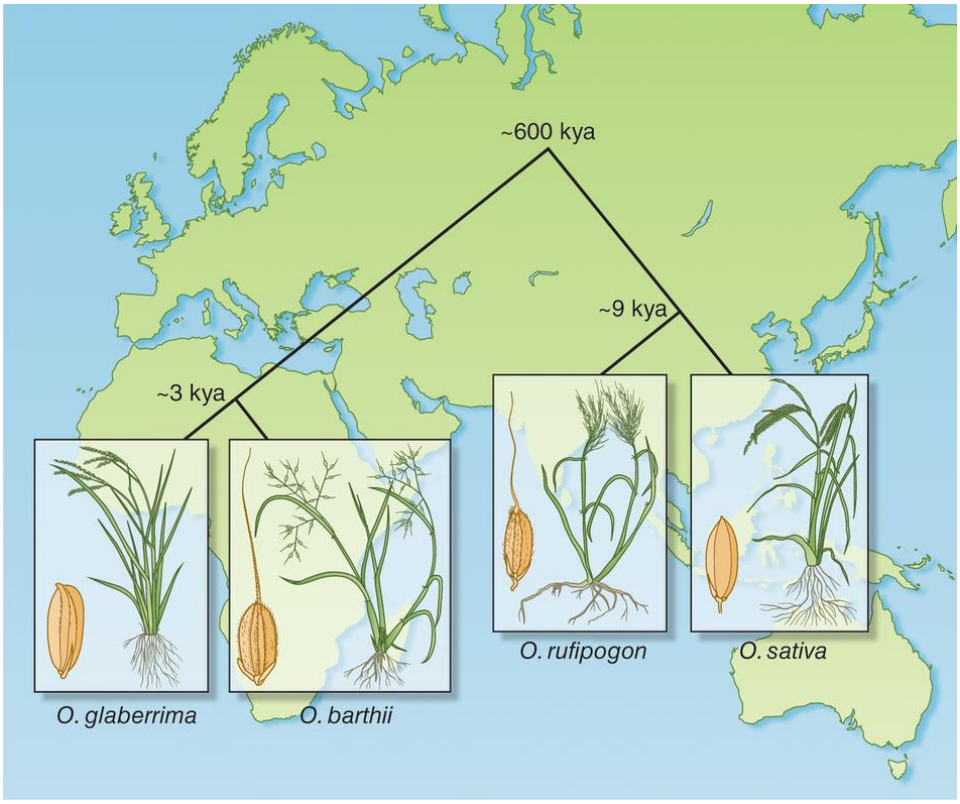


Figure 1.1 Parallel domestication of two rice species (Purugganan, 2014)

O. sativa and *O. glaberrima* represent parallel domestications on two different continents. Around 9,000 years ago, wild *O. rufipogon* was domesticated in Asia and evolved into *O. sativa*. Around 3,000 years ago, *O. barthii* was cultivated in Africa, leading to the evolution of *O. glaberrima*. Kya-thousand years ago (Purugganan, 2014).

Asian rice, *O. sativa*, is one of world's oldest and most important crop species and is believed to have been domesticated ~9,000 years ago (Molina et al., 2011; Purugganan, 2014). It is grown all over the world while *O. glaberrima* is cultivated only in part of Africa. Traditionally, two major sub species, japonica and indica, have been identified in Asian rice based on their morphological characters and responses to chemicals (Oka, 1974; Kovach et al., 2009; Molina et al., 2011). These two groups are different from each other in their adaptation to climatic, ecogeographic and cultural conditions (Chang, 2003). Japonica varieties are cultivated in both lowland and high-elevation upland areas of tropical South East (SE) Asia, and in colder, temperate climates, including north eastern Asia, Europe, Western US, Chile and Australia. Indica varieties are mainly grown in lowland areas throughout South and SE Asia and

China (Zhao et al., 2010). Studies based on genetic variation of Asian rice came up with broad classification system, which categorises Asian rice into five subpopulations; indica, aus, temperate japonica, tropical japonica and aromatic. Of these five subpopulations, indica and aus belong to the indica varietal group whereas tropical japonica, temperate japonica and aromatic are closely related to the japonica varietal group (Garris et al., 2005; Caicedo et al., 2007; Kovach et al., 2007; Zhao et al., 2010) (Figure 1.2).

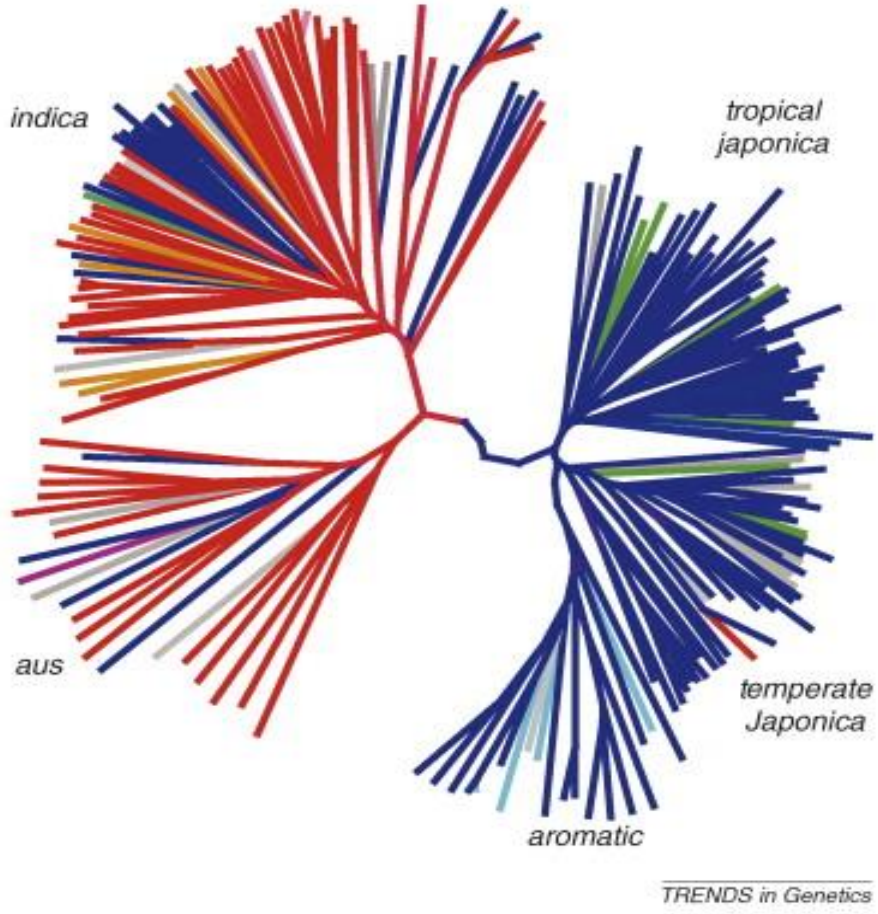


Figure 1.2 Subpopulation structure in Asian rice *O. sativa* (Garris et al., 2005; Kovach et al., 2007)

Characterization is based on the presence of deep genetic differentiation in *O. sativa* using genetic markers or DNA sequence information. The tree clearly illustrates the major division between the two varietal groups (indica and japonica), and their further subdivision into five groups, indica, aus, tropical japonica, temperate japonica and aromatic (Garris et al., 2005; Kovach et al., 2007).

1.2 Rice as a model system for cereals

Rice (*Oryza sativa* L.) is recognized as an ideal model system for genome research in monocots and cereals based on several key features (Delseny et al., 2001; Shimamoto and Kyozuka, 2002). It has the smallest genome (430 Mb approximately) (Arumuganathan and Earle, 1991; Delseny et al., 2001) compared to other major cereals such as sorghum (750 Mb), maize (2400 Mb), barley (4900 Mb) and wheat (16,000 Mb) (Sasaki and Sederoff, 2003; Bennetzen et al., 2005). The rice genome was completely sequenced by the International Rice Genome Sequencing Project in 2004 (IRGSP, 2005) and now is publicly available providing researchers an invaluable resource for genomic comparison studies. Further, the rice genome shows a close relatedness to other major cereals. Comparative mapping studies have revealed that synteny and gene homology are highly conserved between rice and other cultivated cereals. Rice contains 98% of known protein homologs of maize, wheat, and barley (Goff et al., 2002). Once an important gene has been isolated from rice, it can be used as a probe to isolate the corresponding homologue in other economically important cereals like wheat, maize or barley (Delseny et al., 2001; Sorrells et al., 2003). The availability of an efficient transformation system in rice also makes it a good model system for cereals.

1.3 Economic importance of rice

Rice is the most important food staple worldwide. It is grown on approximately 155 million hectares and accounts for one-fifth of the global calorie supply (Pandey et al., 2010). Out of three leading food crops, rice, wheat and maize, in the world, rice records the highest human consumption accounting 85% of total production, compared with 72% for wheat and 19% for maize. Rice provides 21% of global human per capita energy as well as 15% of per capita protein. Rice also provides minerals, vitamins, and fibre (http://www.knowledgebank.irri.org).

More food is needed to feed the rapidly growing human population. The International Rice Research Institute (IRRI, 2000) has predicted that 800 million tons per year more rice will be required in 2025 (Kubo and Purevdorj, 2004). According to the recent Declaration of the World Summit on Food Security, 70% more food is required by 2050 to meet the demand which will predominantly be coming from developing countries (FAO, 2009). To meet this requirement, the current annual average crop production of 32 million metric tons per year needs to be increased up to 44 million metric tons per year (Figure 1.3). This represents a 38% increase in production, which is believed to be sufficient to sustain populations for another 40 years (Tester and Langridge, 2010).

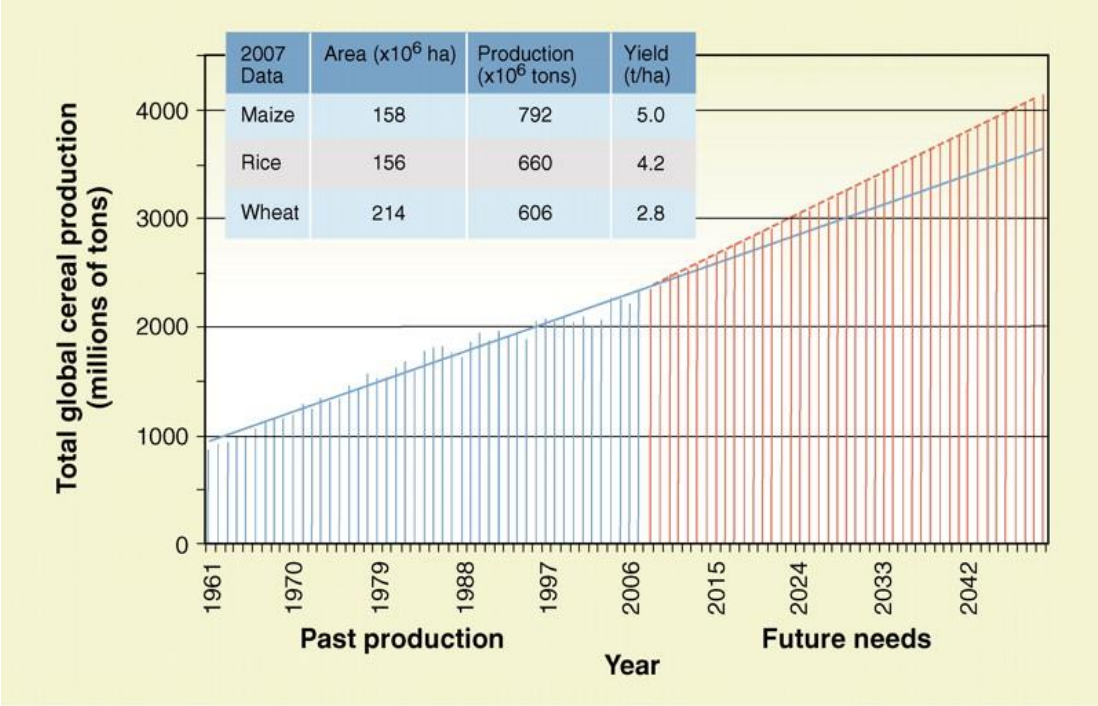


Figure 1.3 Cereal crop production targets (Tester and Langridge, 2010)

Due to breeding and agronomic improvements, the global cereal production has risen from 877 million metric tons in 1961 to 2351 million metric tons in 2007 (blue). To meet the future demand of over 4000 metric tons by the year 2050, production needs to be increased by 38% (red) (Tester and Langridge, 2010). The inset table shows the data for three major cereal productions in 2007 (data from FAO: http//faostat.fao.org/).

In the current century, achieving this target of increased food production in a stable environment will be quite challenging. Besides the limited availability of arable land

and water, environmental threats posed by biotic (e.g. pest and diseases) and abiotic (e.g. drought, salinity, extreme light, nutrient deficiency, cold) stresses are major challenges, and it is obvious that global climate changes will put more pressure on the situation. To be successful in this unprecedented target of food production, substantial changes in methods for agronomic practices and crop improvement are required (Tester and Langridge, 2010). Thus, it is worthwhile to study harmful effects of these environmental stresses on plants, the tolerance mechanisms plants use to withstand them, and the strategies that can be applied to overcome the situation, ensuring normal growth and development of plants. Salinity is one of the serious constraints responsible for the major loss of global cereal production including rice. The current study focuses specifically on salinity stress and tolerance mechanisms cereals use to overcome the situation, using the model cereal rice.

1.4 Salinity as a limiting factor in crop production

Among the various abiotic stress factors, soil salinization has been identified as one of the biggest threats to inland agriculture (Tester and Davenport, 2003). It has been estimated that 20% of total cultivated and 33% of irrigated agricultural lands worldwide are afflicted by high salinity and these salinized areas are increasing at a rate of 10% annually due to low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices. It has also been estimated that more than half of the arable land would be salinized by the year 2050 (Jacoby et al., 2011; Jamil et al., 2011).

When crop plants are under salt stress, yield reduction mainly results from alteration of various metabolic processes in plants. Crop yields are significantly reduced in salt affected areas with an estimation of 30-50% of yield loss for rice, 10-90% yield loss for wheat and 50-70% for cotton and 30-90% for sugarcane (Eynard et al., 2005). This occurs due to the increased osmotic pressure limiting water uptake (physiological drought), extreme pH, and ionic competition limiting nutrient uptake created by excess

of salts in the soil solution (Eynard et al., 2005). The effect of salinity stress on rice depends on the plant's developmental stage (Rad et al., 2012; Balkan et al., 2015). Primary growth stages such as tillering and panicle initiation are more sensitive to salinity, but panicle emergence and ripening are much more tolerant to salinity (Rad et al., 2012).

Australian agriculture is also greatly affected by salt. Naturally occurring large salt deposits stored deep in soils or as surface salt deposits are common features in the Australian landscape. Altered land use and management practices such as widespread vegetation clearance, poor land use, irrigation and industrial practices have facilitated the transport of salts to the soil surface or to waterways resulting in excessive amounts of dissolved salt in water, which affects agriculture and the quality of drinking water. It has been estimated that a further 5.7 million hectares of Australia have a high potential to develop salinity. Unless effective solutions are implemented, it is predicted that the salinity affected area could increase to 17 million hectares by 2050, most of which is agricultural land (NLWRA, 2001).

Soil salinity not only stresses plants, it also challenges human life and the world economy through severely damaging agriculture. As predicted by The Food and Agricultural Organization of the United Nations (FAO), global population will reach to 9.1 billion by 2050 and world food production will need to be raised by 70% to meet the increasing demand. To achieve the projected increase in food production is quite challenging as it will have to overcome number of hurdles blocking its way like rising energy prices, growing depletion of underground aquifers, the continuing loss of farmland to urbanization, and increased drought and flooding resulting from climate change (Steduto et al., 2012). Therefore, it is important to understand the tolerance mechanisms of plants that allow them to grow in saline soil (Ismail et al., 2014) and to develop crop varieties which can withstand such hazardous environmental conditions while maintaining their normal growth and yield.

1.5 Mechanisms of salinity tolerance

Salinity tolerance in plants depends on a range of anatomical, physiological, biochemical and molecular adaptations, which help them to survive under the stress. Salinity has different effects on plants. It affects plant growth, as well as developmental processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set (Sairam and Tyagi, 2004). In salt-affected plants, the result is primarily the result of ionic imbalance and hyperosmotic stress. The effect of this imbalance or disruption in homeostasis occurs at the cell level as well as at the whole-plant level (Zhu et al., 1997; Xiong and Zhu, 2002). The most common effect of soil salinity is growth inhibition by Na^+ and Cl^-, which interferes with the normal physiological processes, such as enzyme activities and protein synthesis, as well as causing osmotic imbalances (Munns and Tester, 2008). For many plants, such as cereal crops, Na^+ is the primary cause of ion-specific damage (Tester and Davenport, 2003). Normal growth and development of plants is dependent on the capacity of plants to overcome salinity stress and this capacity has enormous significance for crop yield.

An obvious response of plants to salinity is reduction of shoot growth, which occurs in two phases, an osmotic stress phase and an ion specific effect phase (Figures 1.4 and 1.5). The osmotic phase is independent of Na^+ accumulation while the ion specific effect phase is associated with Na^+ accumulation in plant tissues (Munns, 1993). As a rapid response to the increase in external osmotic pressure, the plant inhibits the growth of young leaves, which can be seen as a reduction of leaf area, number of leaves or number of tillers produced in case of cereal crops. This effect occurs in all plant varieties equally. The second response is a slower reaction, which is visible in mature leaves. Due to accumulation of Na^+ in leaves, plants accelerate the senescence of mature leaves (Tester and Davenport, 2003). This has an additional impact on their growth, and differences in salt tolerance capacity between plant varieties as salt-tolerant or salt-sensitive, emerge (Figure 1.4). Tolerant plants are

those that can maintain higher growth rates and higher yield performance when grown in saline conditions compared to susceptible plants experiencing the same conditions (Tester and Davenport, 2003). However, the final consequence of stress-induced responses is reduction of plant growth and productivity. The growth and yield reduction occur as a result of the shortening of the life time of individual leaves which leads to the reduction of net productivity and crop yield (Munns, 1993).

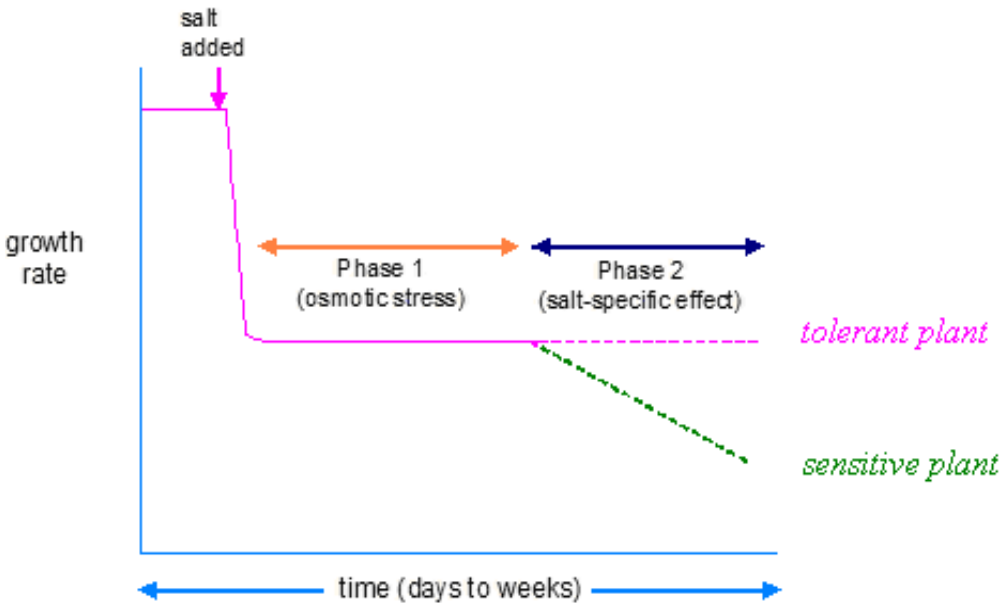


Figure 1.4 Illustration of the two phase growth response of plant under salinity stress (Munns, 1993; Carillo et al., 2011)

There are several predominant salt tolerance mechanisms operating in plants: restriction of the entry of toxic ions at root level (exclusion), transporting the toxic ions to stem, leaf sheath or older leaves (plant level compartmentation), sequestration of the toxic ions to vacuole or cell wall (cell level compartmetation), scavenging ROS using antixidative enzyme, and excretion of salt through glands, salt hairs or bladders by halophytes (Rajendran et al., 2009; Roy and Chakraborty, 2014). There may be a significant contribution of all these mechanisms to overall salt tolerance of a plant and the contribution of an individual mechanism varies between different plant species and even within genotypes (Tester and Davenport, 2003). However, plants which use more than one mechanism are more salinity tolelant than plants which rely on only one mechnism (Figure 1.5) (Rajendran et al., 2009; Horie et al., 2012).

Genes control all salt tolerance mechanisms and a clear understanding of how different genes contribute to stress tolerance opens the opportunity to engineer them to develop crop varieties with better performance. Human interference is considered as one of the major cause of salinity through the use of poor-quality irrigation systems around the world. Therefore, apart from the need for proper irrigation practices, a concerted effort to understand the effects of salinity on plants, development of genetically engineered crop varieties and superior salt-tolerant cultivars are essential to combat the world's salinization problems (Rengasamy, 2006).

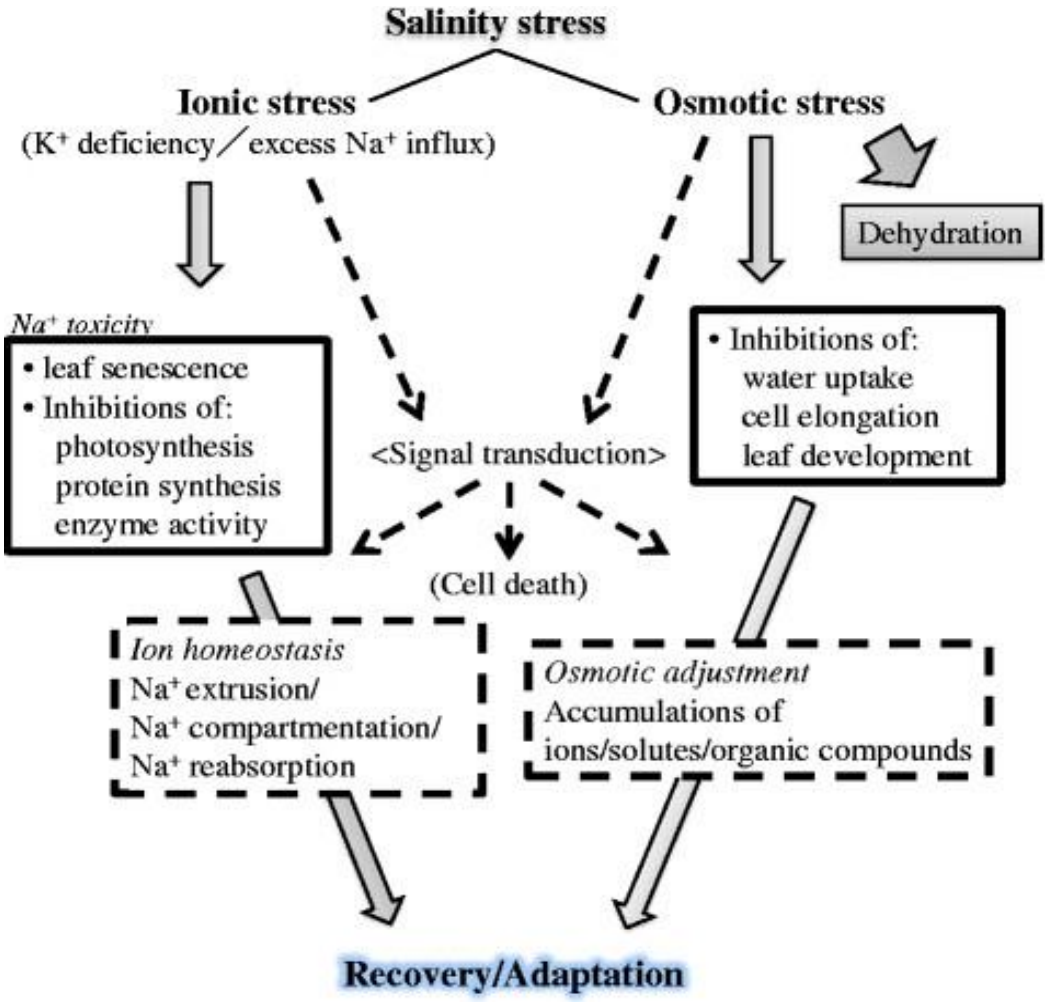


Figure 1.5 A schematic diagram showing major stresses that plants suffer under high salinity growth condition and the corresponding responses plants use in order to survive these detrimental effects (Horie et al., 2012)

Bold arrows and boxes with bold line indicate the harmful effects of salt stress; dash arrows and boxes with dash line indicate the physiological approaches adapted by plants to overcome the harmful effect of stress; Adaptation: Phenotypic changes that occur within individual plants to overcome salinity stress.

Considerable understanding has been gained on the behaviour of plants at the molecular level in response to environmental stresses. It is obvious that plant cells increase the expression of some genes while at the same time they decrease expression of others. New genes that are otherwise not expressed in the absence of osmotic stress may also begin to be expressed (Zhu et al., 1997; Xiong and Zhu, 2002). Different genes coordinate different functions to acclimate to the adverse effect of stress condition and they are localized to various cell organelles/subunits or

compartments. Salt tolerance is a complex trait involving the coordinated action of many gene families that perform a variety of functions such as control of water loss through stomata, ion sequestration, metabolic adjustment, osmotic adjustment and antioxidative defense (Abogadallah, 2010).

There is evidence that plant cellular respiratory proteins, especially proteins associated with the alternative pathway of respiration, such as alternative oxidase and alternative dehydrogenases, play a regulatory role in plant abiotic stress tolerance, not only by maintaining reactive oxygen species at non-deleterious levels within the cell, but also by giving metabolic flexibility that may be required for biosynthesis (Vanlerberghe, 2013). The present study is focused on identifying the possible role of rice alternative respiratory proteins when plants are exposed to saline conditions.

1.6 Oxygen and nitrogen reactive species and oxidative stress in plants

Reactive oxygen species (ROS) is a term used to describe a number of reactive molecules and free radicals derived from molecular oxygen. Non-radical reactive molecules include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3) etc., and superoxide (O_2•−), hydroxyl (OH•) and peroxyl (ROO·) radicals are commonly found free radicals (Halliwell, 2006; Considine et al., 2015; Del Rio, 2015). Chloroplast (photosystem I and II), mitochondria (complex I, ubiquinone and complex III of electron transport chain (ETC)) and peroxisomes are the main sites of ROS production in plants (Figure 1.6), while endoplasmic reticulum, cell membrane, cell wall and the apoplast are considered as secondary sites (Gill and Tuteja, 2010; Das and Roychoudhury, 2014). The term reactive nitrogen species (RNS) includes radicals like nitric oxide (NO•) and nitric dioxide (NO_2•), as well as non-radicals such as nitrous acid (HNO_2) and dinitrogen tetroxide (N_2O_4), among others. RNS are also produced in plants although the generating systems have still not been fully characterized (Del Rio, 2015).

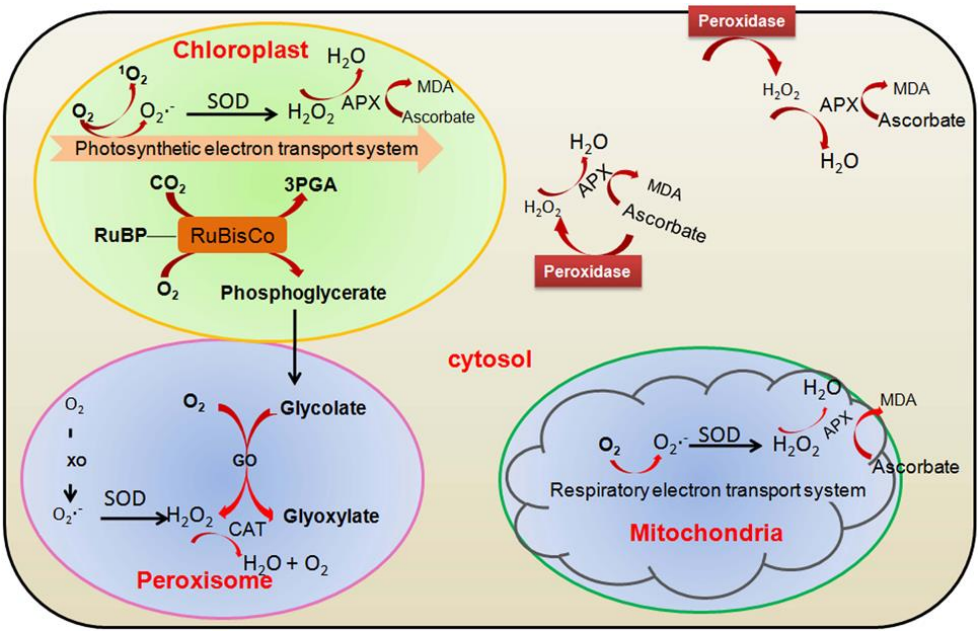


Figure 1.6 Main sites of reactive oxygen species (ROS) production in plant cells and involvement of major antioxidative enzymes (Das et al., 2015)

GO, glycolate oxidase; 3PGA, 3-phosphoglycerate; RuBisCo, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; SOD, superoxide dismutase; XO, xanthine oxidase; CAT, catalase; APX, ascorbate peroxidase (Das et al., 2015).

Production of oxygen-based radicals is common to all aerobic species and these molecules have the potential to cause a number of deleterious events in cells, including peroxidation of lipids, denaturation of proteins, mutation of DNA and various types of cellular oxidative damage. During biotic and abiotic stresses ROS are significantly accumulated in plant cells resulting an oxidative damage even ending up with cell death (Krishnamurthy and Rathinasabapathi, 2013b; Das and Roychoudhury, 2014). On the other hand, the increased production of ROS during stresses is also thought to act as a signal for the activation of stress-response pathways (Krishnamurthy and Rathinasabapathi, 2013b). Plants possess efficient enzymatic and non-enzymatic antioxidative systems to protect themselves against oxidative damage and also to control ROS at low levels for signal transduction. Apart from being potentially harmful molecules, a number of studies showed that ROS plays a key role in the signalling network supporting and regulating numerous biological

processes such as growth, development, and physiological responses to biotic and environmental stresses, and programmed cell death (Figure 1.7) (Considine et al., 2015; Del Rio, 2015). It is not yet understood how ROS plays these two contrasting functional roles of causing oxidative damage to the cell and signalling for stress protection, but it obviously depends on the concentration of ROS.

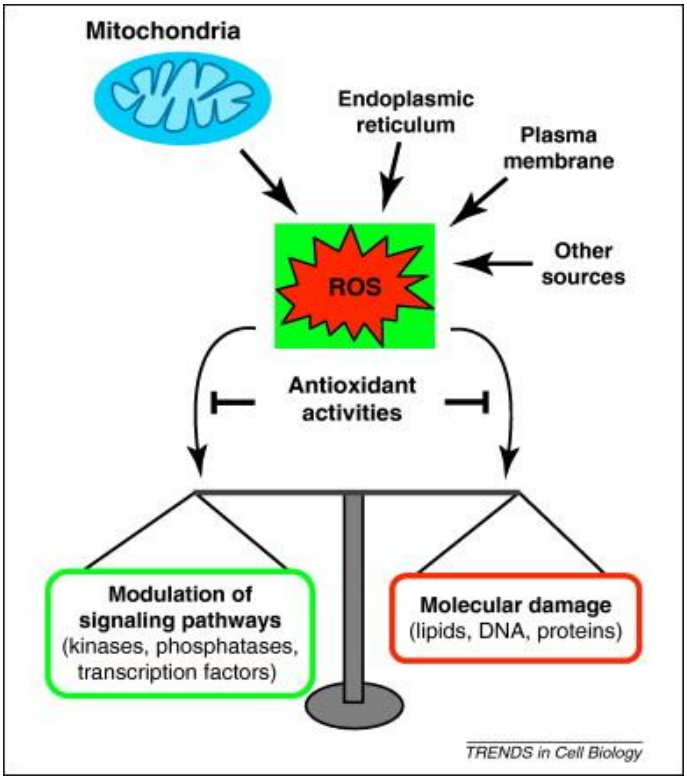


Figure 1.7 Sources and targets of reactive oxygen species (ROS) (Hekimi et al., 2011)

A number of exogenous and endogenous factors can stimulate ROS production from the mitochondria and other compartments. Cellular ROS levels are controlled by a complex network of antioxidant activities which help to maintain the ROS levels either to act as modulators in signalling pathways involved in stress-responses and other functions or causing damage to lipids, nucleic acids, and proteins (Hekimi et al., 2011).

Accumulating evidence from different plant species suggest that in stressed plants, the growth regulator auxin may be the mediator in regulating the level of ROS and directing its role in oxidative damage or signalling (Figure 1.8) (Zhang et al., 2009; Krishnamurthy and Rathinasabapathi, 2013a; Krishnamurthy and Rathinasabapathi, 2013b).

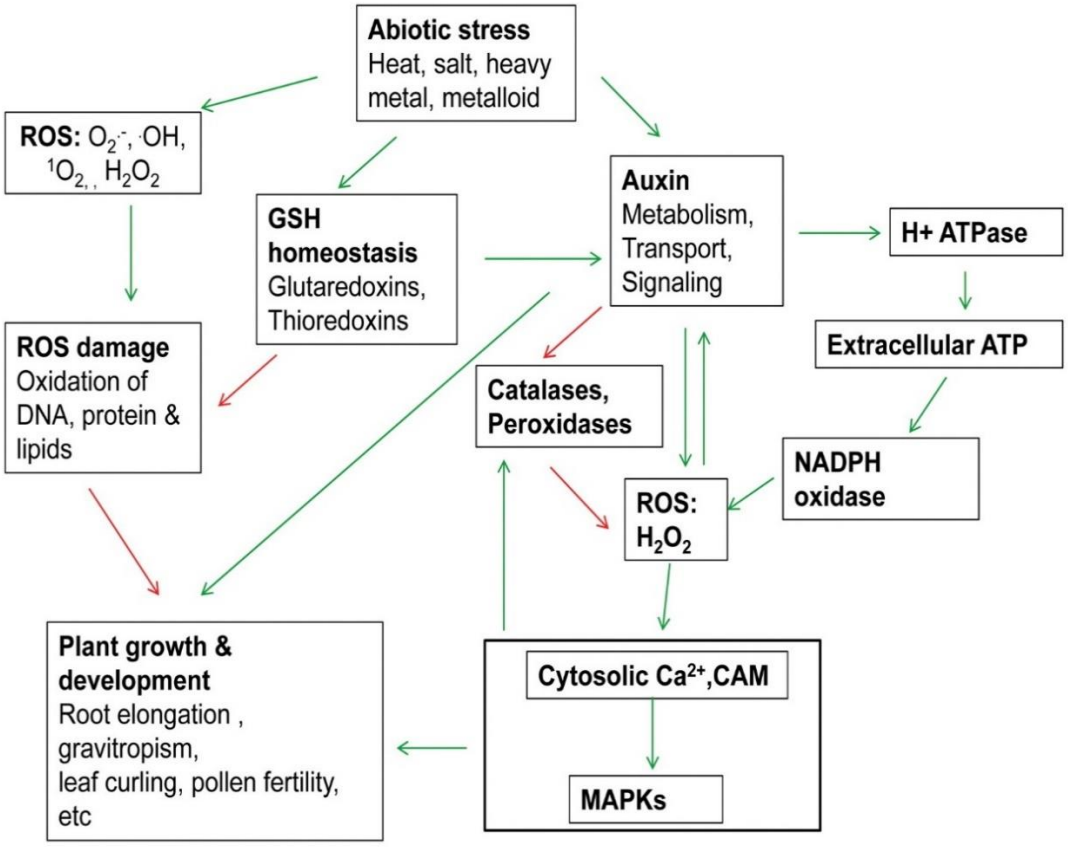


Figure 1.8 The metabolic interplay between auxin and hydrogen peroxide to control plant growth, development and stress tolerance (Krishnamurthy and Rathinasabapathi, 2013b)

Green arrows indicate positive effects and red negative effects (Krishnamurthy and Rathinasabapathi, 2013b). (MAPKs - mitogen-activated protein kinases).

Plants use different strategies for defense against oxidative stress, such as avoidance of ROS production, detoxification of ROS and repairing ROS-mediated damage. Plants use several mechanisms to avoid ROS, including maintaining a balance between substrate availability and ATP production, activation of AOX, uncoupler proteins and alternative dehydrogenase to keep ETC adequately oxidized (Moller, 2001). Detoxification of ROS is an important process for the survival of living cells. There are a number of enzymatic (eg. superoxide dismutase) and non-enzymatic (eg. glutathione) defense mechanisms in cells to detoxify the harmful effect of ROS. These mechanisms help to maintain the balance between production and removal of ROS in the cell. The imbalance of ROS production and removal creates an oxidative stress within the cell.

When plants are subjected to environmental, biotic and chemical stresses, oxidative stress is induced in plant cells. These stresses alter plant metabolism, growth and development and, at their extremes, can lead to death (Jacoby et al., 2012). Recently, a number of studies have examined the changes that occur within plant mitochondria following the induction of oxidative stress. The main sites of ROS production in the ETC in both animal and plant mitochondria appear to be complexes I and III. ROS production is higher when the ETC is most reduced (Moller and Kristensen, 2004). It is a widely accepted fact that the stress responsive alternative respiratory enzymes such as AOX regulate the level of reducing power in the mitochondrial electron transport chain when ROS accumulation is accelerated by environmental stresses (Vanlerberghe and McIntosh, 1997), as well as during biotic stresses such as pathogen attack (Gilliland, 2003). AOX plays a role in modulating ROS production in cells. Similarly, there is evidence showing that AOX is playing a similar role in modulating reactive nitrogen species (RNS) in cells (Cvetkovska and Vanlerberghe, 2012).

High levels of salt stress can induce the cell death of tissues and this process is mostly regulated by the functions associated with mitochondria and seems to be linked to the production of ROS (Rhoads et al., 2006). Many cell death inducing stress factors, such as pathogen infection, H_2O_2 and O_3, cause an increase in the activity of cyanide resistant respiration, suggesting that there is a role of AOX in regulating cell death in plants (Feng et al., 2013). Van Aken et al. (2009) proposed that AOX acts as a buffer that determines the threshold for the induction of programmed cell death in plants. This programming role of AOX can be acquired directly by its ability to suppress the induction of ROS and indirectly by causing changes in the energy status of cells due to the non-phosphorylating nature of the alternative respiratory pathway. Changes to the energy state of the cell are likely achieved in combination with alternative NAD(P)H dehydrogenases, which are co-regulated with AOX (Van Aken et al., 2009).

1.7 Plant cellular respiration

Cellular respiration, also known as aerobic respiration, is a very important process common to almost all eukaryotic organisms, which involves the controlled oxidation of reduced organic substrates to CO_2 and H_2O. This is a sequential set of processes that transport reduced glycolytic products from the cytosol into the mitochondrion followed by a series of reactions leading to the production of CO_2 and reduction of oxygen to water (Siedow and Day, 2000). Three major steps are involved in this process of cellular respiration viz. glycolysis, tricarboxylic acid cycle (TCA) (Citric Acid Cycle/Krebs cycle) and Electron Transport Chain (ETC) (Figure 1.9). Glycolysis takes place in the cytoplasm and TCA and the ETC is localized in the mitochondrion. During glycolysis, sucrose is converted to pyruvate as a main product, but also malate, as well as reduced cofactor NADH and ATP. A set of carriers and channels allow substrates and cofactors from the cytosol to enter to the mitochondrion. In the TCA, the associated enzymes undertake the oxidative decarboxylation of organic acids producing CO_2, NAD(P)H and $FADH_2$, as well as ATP. Finally, in the inner mitochondrial membrane, the reduced co-enzymes generated through glycolysis and the TCA are oxidized by a set of electron transfer proteins that ultimately donate electrons to O_2 producing H_2O. This process releases a large amount of free energy, which is conserved in the acid anhydride linkages of ATP molecules (Siedow and Day, 2000; Jacoby et al., 2012), which is synthesized during this process. Both electron transport and ATP synthesis are collectively known as oxidative phosphorylation. The energy produced during oxidative phosphorylation drives metabolic reactions involved in plant growth, development and other maintenance activities. Changes in this process will affect the growth and productivity of plants. Generally, the process of respiration in plants is the same as other eukaryotic organisms, but there are some special characteristics that can be found in the plants' respiratory pathway to cope with the unique environmental and metabolic conditions faced by plants (Siedow and Day, 2000).

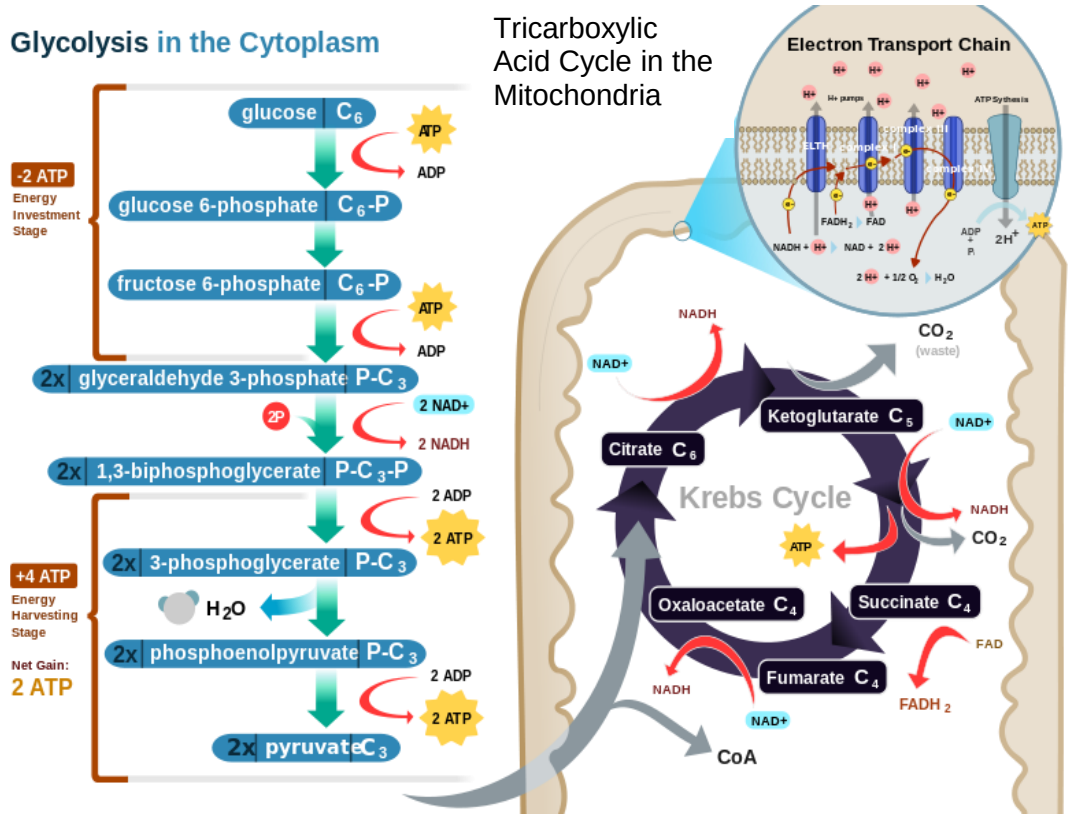


Figure 1.9 Diagram of cellular respiration

Three major steps; glycolysis, Krebs cycle and electron transport chain, are involved in the cellular respiration (https://en.wikipedia.org/wiki/Cellular_respiration)

1.8 Mitochondrial electron transport chain (mETC) in Plants

The key role of mitochondria in living cells is to produce energy through oxidative phosphorylation. This is done through a serious of events catalyzed by several enzyme complexes in the inner mitochondrial membrane forming the ETC.

The ETC of plant mitochondria contains a series of membrane bound redox centers that maintain electron flow from NADH and $FADH_2$ to oxygen, ultimately forming water (Figure 1.10). The classical mitochondrial ETC conserved among eukaryotes consists of four multiple subunits protein complexes commonly known as Complexes I through IV. Complex I (NADH dehydrogenase) oxidizes matrix NADH and donates electron to

Ubiquinone producing NAD^+. The electron flow through complex I is accompanied by H^+ translocation across the membrane which finally contributes to ATP synthesis. Rotenone, a flavonoid, inhibits the activity of Complex I. Complex II which represents succinate dehydrogenase oxidizes the Krebs cycle intermediate succinate to fumarate and donates electrons to Ubiquinone. As Complex II does not translocate H^+ across the membrane it contributes less to ATP production than Complex I. Malonate is a strong competitive inhibitor of succinate dehydrogenase activity (Siedow and Day, 2000). Ubiquinone reduced by these dehydrogenases is oxidized by complex III (UQ-cytochrome *c* oxidoreductase) and transfers electrons to cytochrome c, a peripheral membrane protein located on the outer surface of the inner membrane. Cytochrome c carries electrons from Complex III to complex IV (cytochrome c oxidase), the terminal electron carrier in the classical ETC. These two complexes pump protons across the mitochondrial membrane and thus contribute to the production of ATP. Antimycin A and myxothiazol are complex III activity inhibitors while chemicals like cyanide, azide and CO inhibit the activity of Complex IV. Finally, oxygen accepts electrons from Complex IV and is reduced to H_2O. Electron flow through proton pumping complexes I, III and IV creates an electrochemical gradient across the mitochondrial inner membrane that finally ends up with production of ATP.

1.9 Alternative pathway for respiration in pants

In addition to classical respiratory complexes, plant mETC contains five additional enzymes not present in mammalian mitochondria: four NAD(P)H dehydrogenases (NDHs) and an alternative oxidase (AOX), forming an alternative respiratory pathway (Moller, 2001; Finnegan et al., 2004; Rasmusson et al., 2004) (Figure 1.10). The NDHs oxidize NAD(P)H and donate electrons to the common pool of Ubiquinone, by-passing respiratory Complex I while AOX transfers electrons from Ubiquinol to oxygen, bypassing the cytochrome pathway. As alternative respiratory proteins do not contribute to the proton pumping system, these processes are not coupled to ATP

production. Therefore, the alternative pathway of respiration is a non-energy conserving process (Siedow and Day, 2000; Finnegan et al., 2004; Millar et al., 2011).

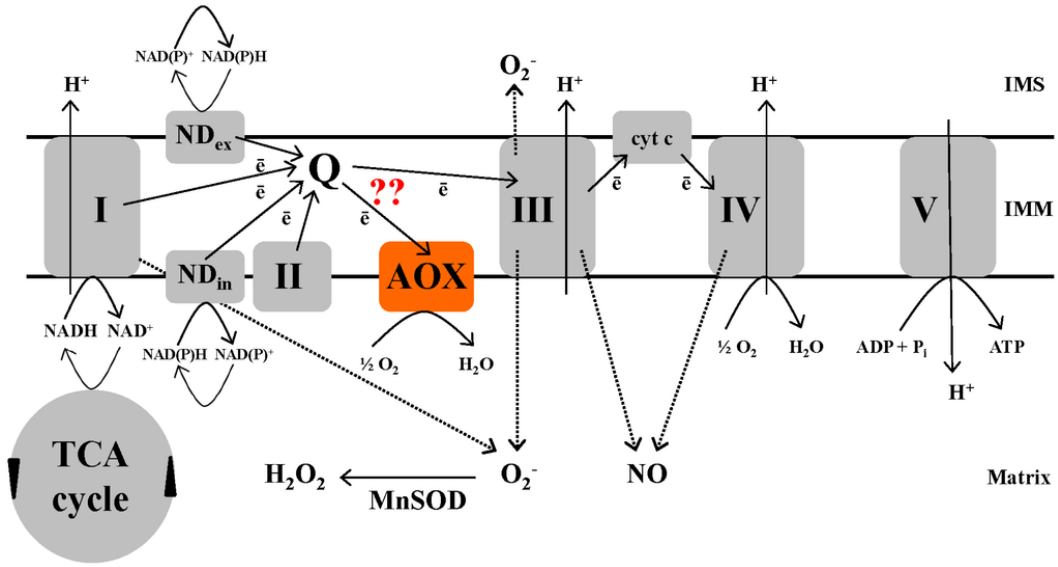


Figure 1.10 The alternative respiratory pathway in the plant mitochondrial electron transport chain (Vanlerberghe, 2013)

Abbreviations: I, II, III, IV: respiratory complexes I to IV; IMM, inner mitochondrial membrane; IMS, inner membrane space; AOX, alternative oxidase; NDex, external NAD(P)H dehydrogenases; NDin, internal NAD(P)H dehydrogenases; MnSOD, manganese superoxide dismutase; Q, ubiquinone pool (Vanlerberghe, 2013).

1.9.1 Alternative oxidase (AOX)

The alternative oxidase pathway, also known as cyanide-resistant respiration, was discovered at the beginning of 20th century as a curiosity in thermogenesis in voodoo lily (*Sauromatum guttatum*) during anthesis (Juszczuk & Rychter, 2003) and later was found to be a typical feature of plant respiration. From the start of the purification of AOX protein from *S. guttatum* (Elthon and McIntosh, 1987), considerable progress has been made in AOX research in the directions of identification of *AOX* gene families and the regulation of its expression during the past two decades. The most extensively studied component of the plant mitochondrial electron transport chain is AOX (Kuhn et al., 2015). The current knowledge on AOX includes a detailed knowledge of its structure including the detailed understanding of the crystal structure

(Shiba et al., 2013), biochemical characteristics (Siedow and Umbach, 2000; Millar et al., 2011), evolution and regulation of AOXs (McDonald, 2008; Pu et al., 2015), and transcriptional and post-transcriptional regulation (Vanlerberghe and McIntosh, 1997; Millar et al., 2011).

The structure of AOX is very similar in all organisms so far investigated (Wagner and Moore, 1997). It is an interfacial protein peripherally associated with the matrix side of the inner mitochondrial membrane (Siedow and Umbach, 2000). Shiba et al. (2013) published the crystal structure of trypanosomal AOX and revealed that it is a homodimer with the non-haem di-iron carboxylate active site buried within a four-helix bundle. It contains four monomers per asymmetric unit that associate to form homodimers. Each monomer consists of a long N-terminal arm, six long α-helices and four short α-helices (Figure 1.11). The four long α-helices (2, 3, 5 and 6) are arranged in an antiparallel fashion and form a four-helix bundle which acts as a scaffold to bind the two iron atoms (Shiba et al., 2013).

AOX is encoded by a small nuclear gene family (Considine et al., 2002). Two separate gene subfamilies have been identified in higher plants, *AOX1*-type and *AOX2*-type. In dicots, both subfamilies are present while in monocots only *AOX1*-type is present (Considine et al., 2002). The expression of gene family members depends on the plant, type of tissue, growth and development stage, and environment. Induction of some isoforms of *AOX1* is linked to stress while the expression of *AOX2* is related to some specific tissues and development (Considine et al., 2002; Arnholdt-Schmitt et al., 2006). *AOX* is present in all higher plants and also in some algae, fungi, eubacteria and protists. The presence of *AOX* was also found in animal phyla, including Mollusca, Nematoda and Chordata (McDonald and Vanlerberghe, 2004).

The existence of three *AOX* genes in gymnosperms has also been reported (Frederico et al., 2009). Two of them belong to the subfamily AOX1 while the other one belong to the subfamily AOX2. The presence of all subfamilies of AOX was

reported in soybean (Whelan et al., 1996; McCabe et al., 1998; Tanudji et al., 1998). Four isoforms of *AOX1* (*a*, *b*, *c* and *d*) are present in rice (*O. sativa*) (Considine et al., 2002; Costa et al., 2014) whereas *Arabidopsis thaliana* contains four *AOX1* isoforms (*a*, *b*, *c* and *d*) and one *AOX2* gene.

A number of studies have been done to examine changes in AOX capacity and activity in response to different abiotic stresses such as cold (Lei et al., 2010; Wang et al., 2011), light (Feng et al., 2007; Florenz-Sarasa et al., 2011), high temperature (Armstrong et al., 2008; Murakami and Toriyama, 2008), high salt concentration (Ferreira et al., 2008; Smith et al., 2009) and heavy metal ions, Cd^{2+} (Wang et al., 2013) as well as biotic stresses like bacterial infections (Vanlerberghe, 2013). The current study focuses on the expression of rice AOXs, *OsAOX1a*, *OsAOX1b* and *OsAOX1c*, under salinity stress in controlled environmental conditions and their potential role in abiotic stress tolerance.

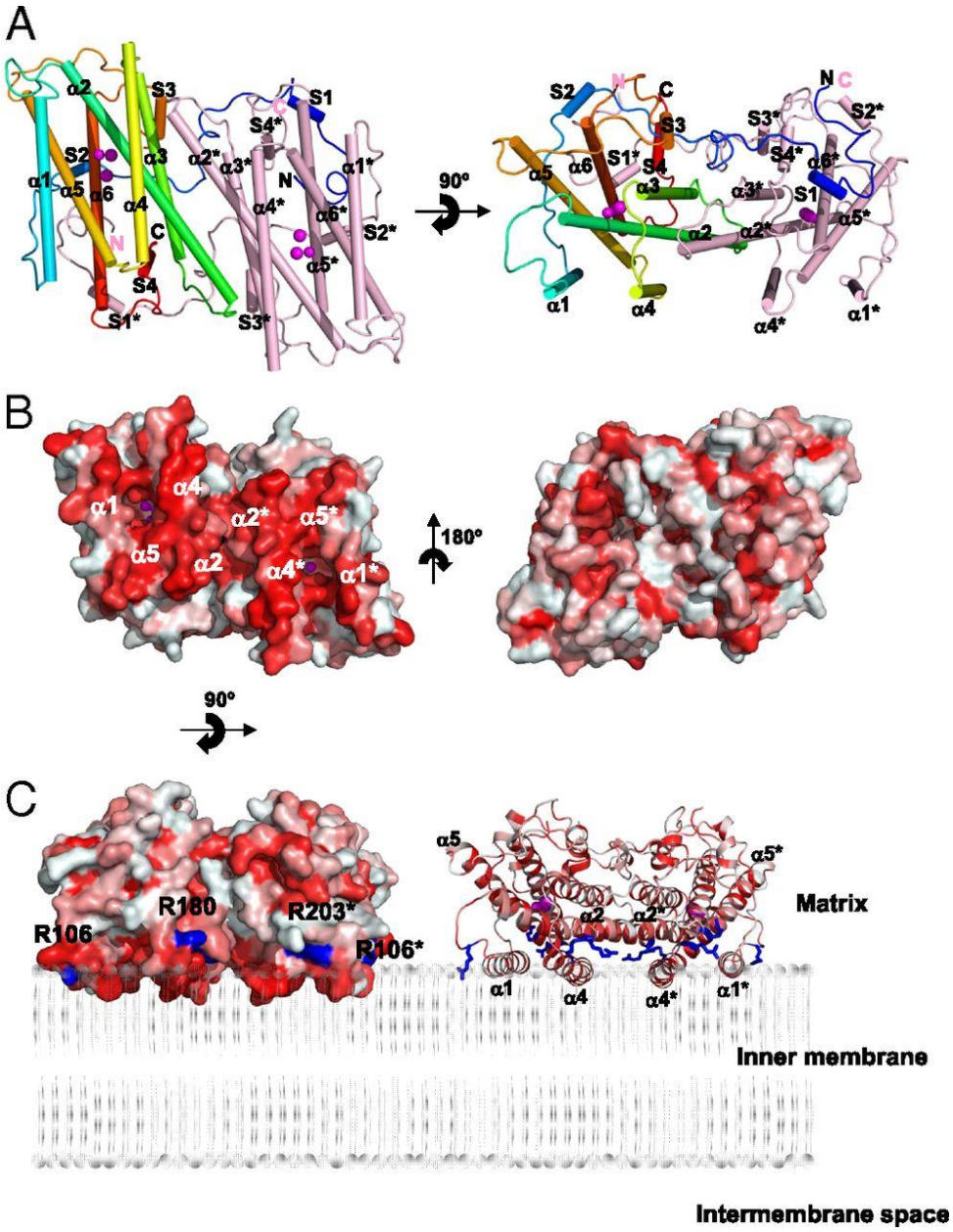


Figure 1.11 Crystal structure of trypansomal alternative oxidase (TAO) (Shiba et al., 2013)

A: Dimeric structure of TAO viewed roughly perpendicular (Left) and parallel (Right) to the helix axes; **B**: Surface representation of dimers showing the hydrophobic (Left) and hydrophilic (Right) surfaces; **C**: Proposed binding model of the TAO dimer to membranes shown by surface (Left) and cartoon (Right) representations

1.9.2 Alternative NAD(P)H dehydrogenases (NDHs)

Apart from the proton-pumping respiratory complex I of the classical ETC, plant mitochondria contain an additional type of proteins which facilitate the electron transfer from cytoplasmic and matrix NAD(P)H to ubiquinone in a non-energy conserving manner (Michalecka et al., 2003). These extra enzymes are known as type II NAD(P)H dehydrogenases or alternative NAD(P)H dehydrogenases (NDHs). Biochemical characterization of isolated plant mitochondria has revealed the presence of internal as well as external types of NDHs, operating in parallel with complex I (Rasmusson and Moller, 1991; Roberts et al., 1995). The distinguishing feature of NDHs is that they are insensitive to rotenone and thus referred as rotenone-insensitive dehydrogenases. The rotenone-insensitive NAD(P)H dehydrogenase has a lower affinity for NAD(P)H compared to complex I and operates only when the matrix NAD(P)H concentration is high (Siedow and Day, 2000). The activity of external NDHs is strongly depended on the presence of calcium (Ca^{2+}) (Moller, 2001; Moller and Kristensen, 2004; Millar et al., 2011).

The NDHs also occur as gene families. Arabidopsis contains seven nuclear encoded NDHs which have been clustered into three gene families NDA (*AtNDA1* and *AtNDA2*), NDB (*AtNDB1*, *AtNDB2*, *AtNDB3* and *AtNDB4*) and NDC (*AtNDC1*) (Michalecka et al., 2003). In potato, two genes, *StNDA1* and *StNDB1*, are shown to encode proteins directed to the internal and external sides of the inner mitochondrial membrane, respectively (Rasmusson and Agius, 2001). Phylogenetic analyses and subcellular targeting prediction studies carried out by Xu et al. (2013) revealed the presence of five NDH proteins in rice which are also clustered into three families as in Arabidopsis, NDA (*OsNDA1* and *OsNDA2*), NDB (*OsNDB1* and *OsNDB2*) and NDC (*OsNDC1*). They further described that in addition to their mitochondrial location, some of them, such as *OsNDA2*, *OsNDB1* and *OsNDB2*, can be found in peroxisomes as well as in chloroplasts (*OsNDC1*).

As was suggested for the AOXs, NDHs are also considered to be present in cells to allow flexible tuning of the redox balance in the cytosol and matrix, and to adjust to the changing need for ATP synthesis (Svensson et al., 2002). The NDHs may also help to avoid ROS formation from complex I. It was shown that the additional NAD(P)H dehydrogenases may be up-regulated under stress conditions and a number of studies have been done to understand their role in abiotic stress tolerance, such as low temperature (Svensson et al., 2002) and light (Michalecka et al., 2003), and also to study the growth and stress tolerance changes when these genes are altered (Smith et al., 2011).

1.10 Mitochondrial retrograde regulation

The nucleus, the chloroplast and the mitochondrion are major storage sites of genetic materials in plant cells. The nucleus possesses the majority of genetic information, including genes that encode organelle proteins, and therefore plays a key role in controlling most aspects of organelles' gene expression, growth, and development. Generally, the signal flow is from nucleus to organelle and the process is defined as anterograde signalling or anterograde regulation (Yang et al., 2008; Giraud et al., 2009; Ng et al., 2014). Environmental stresses, biotic stresses or mutations can alter the functions of cellular organelles like mitochondria and chloroplast and in response these organelles direct changes in nuclear gene expression. Nuclear gene expression alterations directed by affected organelles through organelle-to-nucleus signalling is known as retrograde signalling or retrograde regulation (Butow and Avadhani, 2004; Rhoads and Vanlerberghe, 2004; Rhoads and Subbaiah, 2007; Yang et al., 2008). Transduction of signals from affected plant mitochondria to nucleus in order to activate nuclear gene expression is referred to as mitochondrial retrograde regulation (MRR) (Rhoads and Subbaiah, 2007; Li et al., 2013a). Mitochondrial retrograde signalling can be triggered by dysfunction of mitochondria and could link to metabolic signalling pathways or normal ROS signalling pathways to induce nuclear gene expression

(Rhoads and Subbaiah, 2007). Retrograde signalling is believed to be an important regulatory mechanism involved in plant development as well as in abiotic/biotic stress responses (Rhoads and Subbaiah, 2007; Li et al., 2013a). Although the underlying mechanism is not yet fully understood, there is evidence that the responses of mitochondria to stresses, together with the specific target genes of MRR in individual stress responses, may determine the fate of plant cells, resulting in either recovery or cell death (Figure 1.12).

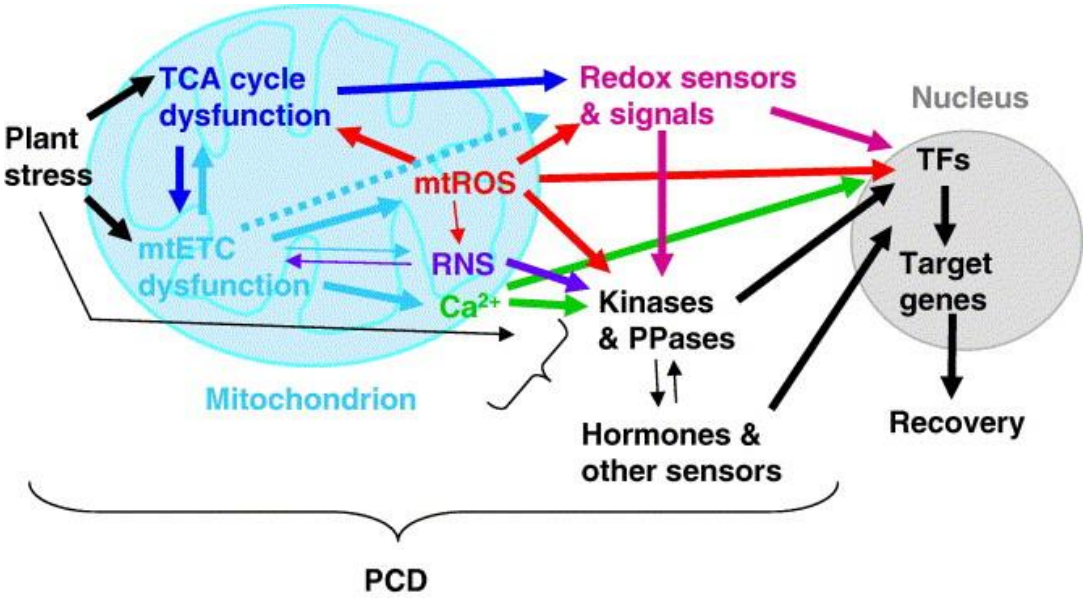


Figure 1.12 Potential signalling pathways for plant MRR (Rhoads and Subbaiah, 2007)

These include signals such as lipid peroxidation products, calmodulin, cyclic nucleotides, and phospholipases. mtETC, mitochondrial electron transport chain; mtROS, mitochondrial reactive oxygen species; PPases, protein phosphatases; RNS, reactive nitrogen species; TFs, transcription factors (Rhoads and Subbaiah, 2007).

The general process of MRR is conserved among yeast, mammals, and plants, but, the molecular mechanisms of signalling and signal transduction are not necessarily conserved across species (Liu and Butow, 2006). Currently, chloroplast retrograde regulation is the best studied retrograde signalling pathway in plants. MRR is poorly understood and centres on dysfunction of mitochondria (Rhoads and Subbaiah, 2007). Dysfunction of mitochondria resulting from ROS damage and changes in the

redox state of the cell, ultimately activates MRR to induce nuclear genes to produce proteins involved in the recovery of mitochondrial functions (Rhoads and Subbaiah, 2007; Yang et al., 2008). The induction of a large number of abiotic stress responsive genes has been reported in a variety of plants including rice. These stress-induced genes not only function to protect cells from stress by production of important metabolic proteins, but also in the regulation of genes for signal transduction in the stress response (Todaka et al., 2012).

Increasing evidence shows that expression of AOXs and NDHs, as well as genes encoding enzymes aimed at regaining ROS level/redox homeostasis, such as glutathione transferase, catalase, ascorbate peroxidase and superoxide dismutase, are increased by MRR during mETC inhibition (Amirsadeghi et al., 2007; Feng et al., 2010a; Keunen et al., 2011; Li et al., 2013a).

Li et al (2013a) demonstrated that abiotic stresses such as drought, salinity, cold etc., initiate MRR to induce rice alternative respiratory pathway genes *AOX1a* and *AOX1b* via mitochondrial produced O_2^-. The absence of *AOX1a* in Arabidopsis results in acute sensitivity to combined light and drought stress, which triggers altered expression of antioxidant defence components and stress responsive genes (Giraud et al., 2008). The investigation done by Giraud et al. (2009) revealed that ABI4 (ABSCISIC ACID INSENSITIVE4), an ABA-responsive transcription factor, plays a central role in mediating mitochondrial retrograde signals to induce the expression of *AOX1a* in Arabidopsis.

Much information is currently available on plant MRR as a response to dysfunctional mETC and the AOX pathway can be an important model to study MRR (Dojcinovic et al., 2005; Rhoads and Subbaiah, 2007). The current study investigates whether there is a link between tissue salt accumulation, ROS production and expression of rice alternative respiratory genes in salt-stressed seedlings and whether there is varietal dependence of these factors.

1.11 Role of alternative respiratory proteins in stress tolerance

A number of studies have attempted to identify the physiological role of alternative respiratory enzymes and their relationship to abiotic stress tolerance in plants. These studies mainly focus on the role of AOX rather than the role of type II NAD(P)H dehydrogenases.

The role of AOX in modulating abiotic stresses, particularly those expected to generate ROS, has been widely studied. At the whole plant level, it is not yet clearly understood how the alternative pathway of respiration alone contributes to the survival of plant under stress condition, but at the cellular level, it is clear that AOX can limit the production of ROS from the ETC when ROS formation is accelerated by environmental stress (Maxwell et al., 1999). Under normal conditions, Complex I of the ETC is the main enzyme responsible for oxidizing NADH, and is also a major site of ROS production, together with complex III. The AOX and possibly type II NAD(P)H dehydrogenases, located inside of the inner mitochondrial membrane, function to limit mitochondrial ROS production by keeping the ETC relatively oxidized (Moller, 2001).

A number of studies have come up, over the last decade, providing evidence that there is a specific role for the alternative respiratory enzymes in environmental stress tolerance. AOX controls the mitochondrial generation of ROS by stabilizing the reduction state of electron transport, which allows plants to cope with environmental stresses (Mhadhbi et al., 2013). Apart from the role played in the reduction of ROS generation in mitochondria, AOX also acts to reduce the formation of RNS by disturbing the transfer of single electrons from the ETC to nitrite (Cvetkovska and Vanlerberghe, 2012). There are a large number of studies that have been done using different plant species to demonstrate that changes in the abundance of the AOX transcripts lead to different physiological responses to biotic and abiotic stresses.

These studies are predominantly related to the ROS status (Vanlerberghe et al., 2009; Wang et al., 2011). It is also suggested that the activity of AOX controls the level of potential mitochondrial signaling molecules such as superoxide, nitric oxide and important redox couples, providing a degree of *signaling homeostasis* to the organelle. Evidence suggests that AOX function in metabolic signaling homeostasis is particularly important during abiotic stresses such as low temperature, drought, and nutrient deficiency, as well as biotic stresses such as bacterial infection (Vanlerberghe, 2013).

In Arabidopsis, salt stress resulted in an increase of transcriptional levels of *AOX1a*, along with the alternative dehydrogenases, *AtNDB2* and *AtNDB4* genes (Smith et al., 2009) showing that there is a role to play in salinity stress. This study also shows that there may be a certain functional link between AOX and NDH. Clifton et al. (2005) also observed the co-expression of *AOX1a* and *NDB2* under a number of treatments in Arabidopsis. Tolerance to salt stress may be due to the combined result of a number of systems. The salt tolerance showed by *Medicago truncatula* may be in part due to an efficient control of oxidative balance by involvement of both antioxidant systems and alternative respiratory pathway (Mhadhbi et al., 2011; Mhadhbi et al., 2013). Complete light dependence and diurnal changes in expression of AP genes also reported. Svensson and Rasmusson (2001) observed that *NDA1* transcript abundance of potato leaves grown in dark was 500 times lower compared to those grown in light, suggesting that the *NDA1* gene is involved in photosynthetically associated processes, most likely photorespiration (Svensson and Rasmusson, 2001).

The responses of alternative respiratory enzymes seem to be species dependent. In potato leaves, internal NADH dehydrogenase capacity was down regulated during cold treatment (Svensson et al., 2002) and AOX was not induced at either transcript or at protein level. However, the exposure of *Nicotiana tabacum* cells to low temperature resulted in an increase of AOX at both transcript and protein levels

(Wang et al., 2011). The expression of AOX was relatively high in soybean hypocotyls throughout development (McCabe et al., 1998), but, in mung beans, the highest capacity and activity of alternative respiration was observed in the seedling 'true' leaves when compared to other seedling parts tested, such as hypocotyls and cotyledon. Mung bean hypocotyls recorded the lowest capacity and activity of alternative pathway (Li et al., 2000). These studies show that the activity of the alternative respiratory pathway differs even in different parts of the same plant.

Some studies suggest that the expression of alternative respiratory genes is dependent on the intensity of the stress. In rice suspension cultures, expression of AOX gene family members showed different expression patterns in response to varying levels of hydrogen sulfide, an inhibitor of cytochrome pathway (Xiao et al., 2010). This study revealed that with the increase of hydrogen sulfide concentration, transcript levels of *AOX1a* and *AOX1c* increased gradually and remained at high levels. But, although the transcript level of *AOX1b* increased at low concentrations of hydrogen sulfide, its expression was inhibited at high concentrations.

1.12 Alternative pathway of respiration in rice

To date, although plenty of work has been done attempting to understand the role of alternative respiratory pathway in stress tolerance in dicots, less attention has been given to monocots, including rice. Among the few reports available, stress responses of only rice AOXs have been revealed (Abe et al., 2002; Saika et al., 2002a; Xiao et al., 2010). Apart from the phylogenetic analyses and subcellular targeting predictions by Xu et al. (2013), there is no published work on the characterization of rice NDHs or their responses during abiotic/biotic stress conditions so far.

However, there are reports on the expression of *AOX* in rice during abiotic stresses such as low temperature and salinity. A group of researchers (Ito et al., 1997) has demonstrated that there is an increase of the transcription levels of *AOX* genes, both *AOX1a* and *AOX1b*, in etiolated rice seedlings when they were exposed to low temperature. This suggests that AOX was playing a certain role in adjusting the plant to the stress situation. The expression of *AOX1a* and *AOX1b* was also induced significantly in a time-dependent manner when rice seedlings were exposed to salinity, drought and low temperature, whereas *AOX1c* transcript level did not significantly change (Li et al., 2013a). A contrasting expression pattern of AOX family members was observed when rice seedlings were grown under total darkness, dark/light cycles or continuous light (Feng et al., 2007). They observed the highest transcript level of *AOX1c* in seedlings grown under continuous light whereas there was no detectable expression of *AOX1a* and *AOX1b* in the treatment with light.

Studies have attempted to show that AOX could be involved in the prevention of ROS formation in salt-stressed rice seedlings and the expression of *AOX1a* under higher salt could be mediated through H_2O_2 (Feng et al., 2010b). Cell death of rice roots under high salt stress is linked to the accumulation of H_2O_2 *in vivo.* Salt stress increased the activity of cyanide-resistant respiration and enhanced transcript levels of *AOX1a* and *AOX1b,* but not *AOX1c,* in rice roots (Feng et al., 2013).

Some studies demonstrated that the expression of AOX depends on the developmental stage. Abe et al. (1997) reported differential accumulation of AOX protein at three different developmental stages of rice anthers, uninucleate microspore stage, bicellular pollen stage and tricellular pollen stage. The study revealed a higher abundance of active form of AOX at uninucleate microspore stage compared to tricellular pollen stage. Ranawake et al. (2012) observed that levels of expression of the same genes differed under different stress conditions during their study on expression profiles of 12 known stress responsive genes in rice (*O. sativa* cv Nipponbare). They noticed that some genes were positively regulated under some

stresses but negatively regulated under other stresses. For example, *OsAOX1a* did not respond to any of the three stresses (drought, low temperature and NaCl) tested and ABA treatment while *OsAOX1b* was induced by all three stresses but not ABA (Ranawake et al., 2012).

Wang et al. (2013) demonstrated that AOX played a role in acquiring tolerance to heavy metal ions in some rice cultivars. These researchers have investigated the primary mechanisms employed by four rice (*O. sativa* L. japonica group) cultivars in attaining Cd tolerance and revealed that different rice cultivars employed different mechanisms to survive under Cd stress and AOX was among them.

The present study aims to identify all components of the rice alternative respiratory pathway using sequence homology to known Arabidopsis genes and to characterize them under oxidative stress created by exposure of seedling tissues to chemical treatments, which inhibit respiratory complexes of mitochondrial electron transport chain, and by exposure of seedlings to environmental stresses like high salt.

1.13 Genetic engineering and value added plants

The use of modern biotechnology techniques to change the genes of an organism is referred to as genetic engineering, genetic manipulation or genetic modification. The organism that has been modified using these techniques is known as a genetically modified organism (GMO) (Key et al., 2008). Production of plants with favourable characteristics using conventional breeding techniques is a long process and there are barriers due to sexual incompatibility and species differences. Genetic engineering not only allows this process to dramatically speed up but also to overcome barriers of sexual incompatibilities between plant species and enormously increase the size of the available gene pool (Key et al., 2008). This technology has been utilized in a variety of fields to produce plants with added qualities such as resistance to biotic and abiotic stresses, enhanced nutritional quality of the product,

herbicide, pesticides resistance and increased productivity. In addition, there is growing interest in producing drugs and industrial proteins in transgenic plants. There are a number of biotechnology techniques which are being used to develop GMOs in agriculture. The most commonly used methods are biolistic methods (particle gun) or *Agrobacterium tumefaciens* mediated transformation. Gene overexpression and silencing have become common tools for functional characterization of genes in crops. These tools facilitate phenotypic change associated with changing the expression of a gene of interest.

Investigations of the role of the alternative pathway of respiration also have been facilitated by transgenic techniques. Once the transgenic line of a certain crop variety is produced, it can be used to study the functional role of alternative respiratory genes under various stress conditions. Alteration of the *AOX1a* gene in rice with sense cDNA constructs resulted in increased expression of *AOX1a* in leaves while antisense cDNA resulted in decreased expression of *AOX1a* (Abe and Toriyama, 2003). Rice lines overexpressing *AOX1a* proteins exhibited enhanced high temperature tolerance compared with wildtype and antisense transgenic lines (Murakami and Toriyama, 2008). A similar study has been done with transgenic tobacco suspensions cells and showed that the capacity of the alternative pathway can be down-regulated or up-regulated by silencing or overexpressing responsible genes (Vanlerberghe et al., 1994). Li et al., (2013b) also showed that overexpression of *OsAOX1a* in rice strongly enhanced the ability of rice seedlings to grow under cold stress, especially in respect to root extension.

Many studies have demonstrated that increasing the antioxidant capacity of a plant improves abiotic stress tolerance (McKersie et al., 1996; Badawi et al., 2004), and the expression of a combination of antioxidant enzymes was shown to be a promising strategy to enhance abiotic stress tolerance in plants (Reguera et al., 2012). Transgenic rice plants constitutively co-expressing *GST* (glutathione S-transferase) and *CAT* (catalase) genes showed enhanced tolerance to salinity and oxidative

stresses in their vegetative stage (Zhao and Zhang, 2006). Co-expression of three antioxidant enzymes, copper zinc superoxide dismutase (*CuZnSOD*), ascorbate peroxidase (*APX*) and dehydroascorbate reductase (*DHAR*) in tobacco, resulted in a higher tolerance to salt stress (Lee et al., 2007).

Zhang et al. (2012) demonstrated that transient expression systems are effective in functional characterization of genes. They have developed a system for overexpression and silencing genes for *Betula platyphylla* Suk (Chinese birch) by isolating the cinnamoyl-CoA reductase (CCR) gene and its promoter. Overexpression and silencing of the CCR gene were performed on birch seedlings using an *Agrobacterium*-mediated transient expression system resulting in different expression levels of CCR.

Panda et al. (2013) observed enhanced performance of tobacco cells under aluminium stress when cells were transformed by overexpressing the *Ntaox1* gene under the control of the cauliflower mosaic virus (CaMV) 35 S promoter. Normally, Al stress suppresses mitochondrial respiration of tobacco cells (*Nicotiana tabacum* L.) and produces ROS within the cell, disturbing their growth. Transformed cells showed a better growth performance with a decreased inhibition of respiration and reduced ROS production, demonstrating that AOX is playing a critical role in stress tolerance in plant cells. Smith et al (2011) observed that suppression of alternative dehydrogenase, *NDB4*, in Arabidopsis leads to the increased expression of *NDB2* and *AOX1a* at both transcript and protein level. These altered Arabidopsis lines showed altered phenotypic characteristics and lower ROS production in cells. In the current study, selected salt-responsive alternative pathway genes will be overexpressed in rice and transgenic rice will be characterized under stress conditions.

1.14 Gene expression diversity in plants

Gene expression can be modulated at several steps, including transcription, RNA splicing, translation, and post-translational modification. The level of gene expression may vary among individuals as well as populations, leading to a vast diversity in their phenotypic characteristics. Gene expression variations exist widely within and between populations in prokaryotic as well as eukaryotic organisms as a result of either genetic or non-genetic factors (Li et al., 2010a). Genetic factors which cause changes in DNA sequence resulting in expression differences include single nucleotide polymorphisms (SNPs) (Chagné et al., 2007) and copy number variations (CNVs) (Orozco et al., 2009). Non-genetic factors include epigenetic modifications such as DNA methylation, histone modifications and small RNA based mechanisms, which can contribute to create phenotypic variations (Grativol et al., 2012).

Quantitative trait loci (QTL) are genetic regions associated with variation in gene expression among individuals. A single gene can have one or multiple QTLs (Druka et al., 2010). These variations can be due to sequence polymorphisms in target genes either at *cis*-regulatory (proximal) or *trans*-regulatory (distal) regions, leading to phenotypic differences. Identifying variation in gene expression within a segregating mapping population can have several uses (Druka et al., 2010), such as to map the chromosomal positions of genes based on constitutive differences in expression between parents, categorizing QTL into *cis*- or *trans*-QTL effects relative to the physical location of a gene etc. (Hammond et al., 2011).

Plants have developed various anatomical, morphological, cellular, biochemical and molecular adjustments to stresses to guarantee their survival under adverse conditions. Many studies have showed that genotype as well as epigenotype may contribute to phenotypic variation often responding differently to environmental variation (Grativol et al., 2012). Transcript profiling techniques allow the simultaneous

examination of gene expression at different time periods in different tissues.

There is significant phenotypic variation in oxidative stress response and leaf senescence between japonica rice and indica rice. Liu et al. (2010) explored the possible molecular mechanism underlying this phenotypic variation during oxidative stress using methyl viologen as a ROS agent to induce oxidative stress in rice seedlings. They observed that cultivar 93-11 (indica) seedlings exhibited severe leaf senescence under methyl viologen treatment compared to Nipponbare (japonica), which may be caused by genomic and transcriptional variation between the two cultivars.

Quinoa (*Chenopodium* q*uinoa* Willd.) is an Andean plant showing a remarkable variably in salinity tolerance. Ruiz-Carrasco et al. (2011) investigated whether the genetic variability in quinoa is associated with the different degree of salinity tolerance and confirmed the existence of genotype specific responses to high salinity at physiological and molecular levels. In foxtail millet, several key genes have been identified that were differentially expressed in response to salinity stress using salt-sensitive and salt-tolerant cultivars (Jayaraman et al., 2008). In this study, cDNA-AFLP (cDNA Amplified Fragment Length Polymorphism) was employed to identify genes that exhibited modulated expression following salt treatments and their expression patterns were validated through quantitative reverse transcription PCR (qRT-PCR) analysis in the salt-tolerant cultivar compared with a salt-susceptible cultivar.

Many studies have revealed expression variation of genes under different abiotic stresses such as salinity (Jayaraman et al., 2008), drought (Machado and Cruzan, 2010), ion toxicity (Bogacki et al., 2013) etc., in plants. During the current study, physiological and molecular responses of different rice varieties towards the oxidative stress imposed via increased salinity will be studied, with emphasis on expression of alternative pathway genes.

1.15 Aims of the study

Beside their primary role in carbon metabolism, plant mitochondria act as signaling organelles which can influence processes such as gene expression (Vanlerberghe, 2013). Alternative respiratory pathway genes, especially AOX, can control the level of ROS and RNS, potential mitochondrial signaling molecules, in the cell, thereby protecting the cell from oxidative stress (Vanlerberghe et al., 2009; Cvetkovska and Vanlerberghe, 2012). This function of alternative pathway (AP) genes is particularly important during abiotic and biotic stress tolerance in plants (Vanlerberghe, 2013). There have been significant achievements in the understanding of the response mechanisms of AP genes under abiotic stress in various crop varieties. Most studies of AP gene regulation have focused on dicot plants while less work has been reported on alternative respiratory genes in monocots, including rice. In rice, work so far, has predominantly been limited to the studies on the expression of AOX in plants exposed to number of environmental stress conditions and no published work is available on studying the expression of rice NDHs under stress conditions. Therefore, it is important to extend the study towards the expression of NDHs in rice under stress conditions, as well as to test the validity of this approach in commercially important rice cultivars.

The overall aim of the current project was to explore whether modifications in the alternative pathway of respiration can improve tolerance to abiotic stresses in cereals using rice as a model cereal. This overall aim will be achieved through several sub-aims. These sub-aims will be discussed in details in individual chapters of the thesis.

Chapter 3 of the thesis will deal with the identification and characterization of alternative pathway gene homologs present in rice using bioinformatics tools. Protein/genomic sequences of Arabidopsis AP gene homologs will be BLAST searched against protein/genomic sequences in the MSU RGAP Release 7 of the Rice Genome Annotation Project and alternative pathway members in rice will be

identified. Further, the diversity of intron-exon structure of all resulting genes and genetic level variation will be studied compared to Arabidopsis alternative pathway genes. Stress responsive rice AP genes will be identified by exposing rice seedling to oxidative stress created through chemical inhibition of cytochrome respiration.

Chapter 4 of the thesis will be on identification of rice AP genes responsive to salt stress and their possible response mechanisms. This aim will be mainly addressed by identifying the salt-responsive rice AP genes in two rice cultivars *O. sativa* ssp *japonica* cv. Nipponbare and *O. sativa* ssp *indica* cv Langi. Seedlings from two cultivars will be exposed to moderate salt stress under controlled environment and physiological responses of two rice cultivars to applied salt stress and expression of AP genes will be studied using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Chapter 4 will further discuss the detection of alternative pathway enzyme activity and proteins using isolated mitochondria.

Chapter 5 of the thesis will discuss the diversity of AP gene expression, growth responses and tissue ion accumulation in different rice cultivars grown under salinity stress. Initially, 16 rice cultivars, which include both japonica and indica rice, will be screened for growth performance and tissue ion accumulation under moderate salt stress. AP gene expression of cultivars, which exhibited extreme growth performance, will also be discussed.

Chapter 6 of the thesis will be on development of transgenic rice lines carrying salt-responsive Arabidopsis alternative respiratory genes, *AtAOX1a* and *AtNDB2* and evaluation of their growth performance under salt stress. Smith et al. (2009) reported that overexpression of *AtAOX1a* and *AtNDB2* genes in Arabidopsis resulted in increased biomass production and reduced ROS levels in cells under salt stress. This chapter will discuss an attempt of applying the same system into rice, by overexpressing *AtAOX1a* and *AtNDB2* in rice cultivar Nipponbare via *Agrobacterium*-mediated transformation. It will also include the details on the preliminary studies on

evaluation of transgenes' performance under environmentally realistic salt stress conditions.

Finally, I expect that findings of the current study will contribute to the understanding of the response mechanisms of alternative respiratory genes to abiotic stresses in other economically important cereals like wheat and barley. The knowledge of potential salt-responsive alternative pathway genes will be beneficial for future studies and the production of improved rice varieties to abiotic stresses including salt stress.

2 General materials and methods

2.1 Plant growth and salinity analysis

2.1.1 Growth facilities and conditions

Experiments described in Chapter 4 were conducted in a greenhouse (No.06) of the Plant Accelerator, Waite Campus, University of Adelaide, South Australia. The growth conditions at the Plant Accelerator for rice were a 12 hour (hr) photoperiod, temperatures of 28^0C/26^oC (day/night) and 75% relative humidity. Supplementary lighting (~400-500 µE m^{-2} s^{-1}) was used during winter season to get a minimum of a 12 hr photoperiod. Experiments described in Chapter 5 and 6 were conducted at the PC2 greenhouse, School of Biological Sciences, Flinders University of South Australia. Growth conditions at the PC2 greenhouse, Flinders University, were 28^oC/22^0C (day/night) temperature and the photoperiod was the day length during the summer season of the year. Supplementary lighting (~400-500 µE m^{-2} s^{-1}) was used during winter season to get a minimum of a 12 hr photoperiod.

2.1.2 Seed treatment and germination

Rice seeds were sorted for uniform size and sterilized with 70% (v/v) ethanol for 1 min followed by thorough rinsing (x 5) with sterile Milli-Q (ultra-pure) water. Then, seeds were further sterilized with 2.5% sodium hypochlorite (50% v/v commercial bleach with 1 drop of Tween 20 per 50 ml) for 15 min and further washed in sterile Milli-Q water to remove all traces of sodium hypochlorite. Surface sterilized seeds were germinated on wet Whatman (Grade 1) filter paper in Petri plates (150 mm x 15 mm). Plates were sealed with parafilm, to prevent evaporation and contamination, and incubated in a growth chamber (Sanyo Versatile Environmental Test Chamber, Model no MLR 351H) for germination with a 12 hr photoperiod at 28^oC for 7-10 days. Seedlings with good root and shoot systems were transplanted to hydroponic tanks.

2.1.3 Hydroponic systems

Twelve litre blue coloured plastic boxes were used to establish hydroponic systems for growing rice seedlings for salinity tolerance assays. The white lids of these boxes contained 96 small holes at equal distances to hold 1.5 ml microtubes. Rice seedlings were transplanted into 1.5 ml microtubes which had their lids and 6 mm of the bottom removed to allow for the seedling roots to grow out of the tube into the nutrient solution in the box. The tubes were placed into the holes in the lids suspended above the hydroponic solution (Figure 2.1). Continuous aeration of the hydroponic solution was achieved by using a commercially available aquarium pump, plastic tubing and aeration stones (Shavrukov et al., 2012).

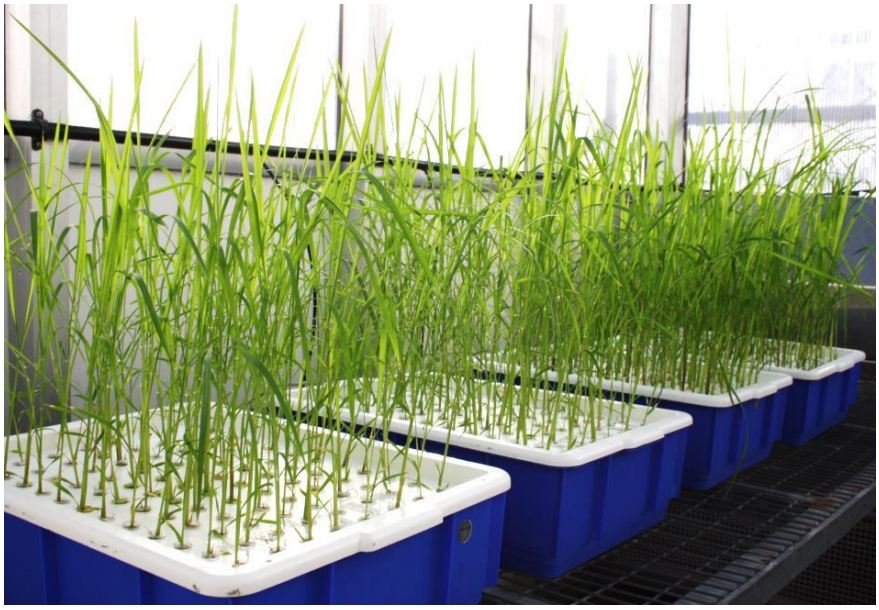


Figure 2.1 Rice seedlings growing on mini hydroponic systems

The hydroponic tanks were filled with nutrient solution prepared using RO water (water filtered through Reverse Osmosis system) and containing 5 mM NH_4NO_3; 5 mM KNO_3; 2 mM $Ca(NO_3)_2.4H_2O$; 2 mM $MgSO_4.7H_2O$; 0.1 mM KH_2PO_4; 0.05 mM NaFe(III)EDTA; 5 µM $MnCl_2.4H_2O$; 5 µM $ZnSO_4.7H_2O$; 50 µM H_3BO_3; 0.5 µM $CuSO_4.5H_2O$; and 0.1 µM Na_2MoO_3; pH 6-7 (Cotsaftis et al., 2011). The H_3BO_3 was

removed from the medium when hydroponics were established at Plant Accelerator, Waite campus as the RO water already rich with boron (Dr. Yuri Shavrukov, University of Adelaide, personnel communication). The nutrient medium was replaced weekly until the experiment was completed and the nutrient solution was topped up regularly with RO water to maintain the total volume.

2.1.4 Measurement of plant biomass and tissue ion concentration

Destructive sampling (complete destruction of whole tissue sample) was carried out to determine the root and shoot biomass from seedlings treated with 120 mM NaCl for 10 days (salt-treated) and from non-treated seedlings grown under the same experimental conditions as salt-treated seedlings. Root samples (cut from ~1 cm below the shoot-root junction) taken from the hydroponic solution were thoroughly washed with water to remove residual NaCl and other nutrients still present on root surface before being blotted dry with paper towels. Root and shoot samples were weighed immediately to determine fresh weight. Dry weights of tissues were measured after samples were dried at 80^{o}C in an oven for two days.

Tissue Na^+ and K^+ concentrations were measured in total root and shoot tissues collected from each replicate. Tissues placed in 50 ml Falcon tubes were digested in 20-40 ml of 1% (v/v) nitric acid (HNO_3) for 5 hours minimum at 70^{o}C in a Hot Block (Model SC 154, Environmental Express, Mt Pleasant, SC, USA) or in an oven. Samples were shaken several times to ensure complete digestion. The concentrations of Na^+ and K^+ were determined on the digest (taken without disturbing plant materials) using a Flame photometer (Model 420; Sherwood Scientific Ltd, Cambridge, UK). The tissue ion concentrations were calculated as sap based (based on the tissue water content) and dry weight based methods.

2.2 Molecular analysis

2.2.1 Extraction of nucleic acid from plant tissues

2.2.1.1 Total RNA

Total RNA from rice leaf and root tissues was extracted using the TRIZOL based extraction method (Chomczynski and Sacchi, 2006). In this study, TRIZOL reagent was replaced with TRIZOL-like reagent, which consisted of 38% (v/v) phenol pH 4.3 (Cat. No. P4682, Sigma Aldrich, St. Louis, MO, USA), 12% (w/v) guanidine thiocyanate (Cat. No. G9277, Sigma Aldrich, St. Louis, MO, USA), 7% (w/v) ammonium thiocyanate (Cat. No. 221988, Sigma Aldrich, St. Louis, MO, USA), 3% (w/v) 3 M sodium acetate, pH 5.0, and 5% (v/v) glycerol. The whole RNA extraction process was carried out under the fume hood.

Fresh rice leaves and roots were sampled into 10 ml yellow cap tubes and snap frozen in liquid nitrogen. Samples were stored at -80^{0}C until use. Liquid nitrogen cooled tissues in yellow cap tubes (root or shoot) were ground to a fine powder by vortexing (Vortex Mixer, Ratex Instruments, Australia) at maximum speed with several (2-3) 9 mm stainless steel balls to maximize grinding. The ball bearings were removed and 50-100 mg of fine powder was transferred to 2 ml microtubes. The plant material was lysed by adding 1 ml of pre-cooled TRIZOL-like reagent. The samples were thoroughly mixed by quick vortexing (for 10 sec) and then incubated at room temperature for 5 min. The mixtures were centrifuged at 11,000 x g for 10 min in a cold room (4^{o}C) and the supernatants were transferred to fresh 1.5 ml microtubes. To remove proteins and other organic contaminants, 200 μl of chloroform was added into each sample and mixed vigorously by quick vortexing (for 10 sec) and incubated at room temperature for 10 min. Samples were again centrifuged at 11,000 x g for 20 min in the cold room. After centrifugation, the colourless upper aqueous layer containing the RNA was transferred into fresh 1.5 ml microtubes without disturbing

the bottom debris. Five hundred ml of iso-propanol was added into each tube and the tubes were mixed well by quick vortexing and then incubated at room temperature for 10 min to precipitate RNA. The tubes were centrifuged at 11,000 x g for 10 min in a cold room to pellet RNA. The supernatants were removed and the RNA pellets were washed by adding 1 ml of 75% (v/v) ethanol. Samples were mixed well and centrifuged at 7,500 x g for 5 min in the cold room. Ethanol was removed without disturbing the RNA pellet. The RNA pellets were air dried in the fume hood for 10-15 min then were redissolved in 25 µl of sterile Milli-Q water at 60^{o}C for 10 min to assist the resuspension of RNA. The quality and quantity of RNA were estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific Pty Ltd, USA). Absorbance was measured at wavelengths of 230, 260 and 280 nm. The purity of the total RNA was assessed using the ratio of absorbance at 260 and 280 and 260 and 230 nm. RNA was stored at -80^{o}C until further use.

2.2.1.2 Genomic DNA

DNA extraction was done according to the method described by Pallotta et al. (2003) from freeze dried leaf tissues with several modifications. Two pieces of 2-3 cm long leaf segments were collected into 2 ml microtubes (Eppendorf) from 3-4 weeks old rice seedlings and stored at -80^{o}C. Tissues were then transferred to a freeze dryer (Christ Beta 2-8 freeze dryer, John Morris Scientific Pty Ltd) to dry the samples overnight. Two 4 mm ball bearings were added into each tube and the tissues ground to a fine powder using a Retsch Mill, MM 400 at a frequency of 30/second for 1-2 min. Ball bearings were then removed from the tubes.

DNA extraction buffer (0.1 M Tris-HCl at pH 7.5, 0.05 M EDTA and 1.25% (w/v) SDS) was preheated to 65^{0}C and 600 µl was added to each tube. The tubes were shaken well and incubated at 65^{0}C for 30 min. Then the tubes were mixed thoroughly and cooled to room temperature before adding 300 µl of 6 M ammonium acetate. The

microtubes were mixed vigorously again to mix plant materials in ammonium acetate and left to stand for 15 min in a fridge at 4ºC, centrifuged at 1,300 x g (4000 rpm) at 4ºC for 15 min to precipitate proteins and plant residues. Six hundred microliters of supernatants were recovered into fresh 1.5 ml tubes and 360 µl of iso-propanol was added into each. The microtubes were mixed vigorously and the DNA was allowed to precipitate for 20-30 min at room temperature. DNA was pelleted by centrifugation at 1,300 x g and the supernatants tipped off. The tubes were inverted on paper towels for less than 1 min to drain off remaining fluid with due care not to lose the DNA pellet. Then the pellets were washed with 70% ethanol by short vortexing and centrifuged at 1,300 x g at 4ºC for 20 min, and the supernatants discarded. The DNA pellets were resuspended in 200 µl of sterile Milli-Q water and left overnight at 4ºC in a fridge to ensure dissolving. The DNA solutions were centrifuged again at 1,300 x g for 20 min to remove undissolved cellular components and 150 µl of each supernatant was transferred into a fresh tube. DNA quantification and quality assessment were carried out using the NanoDrop 1000 spectrophotometer (Thermo Scientific Pty Ltd) and stored at -20ºC until use.

2.2.2 Polymerase chain reaction (PCR)

Go Taq Flexi DNA polymerase and the corresponding $MgCl_2$, 5 x (green or colourless) reaction buffer and dNTP mix from Promega (Madison, WI, USA; www.promega.com) were used in general PCR. PCR reactions consisted of 5 µl of 5 x reaction buffer, 3 µl of 25 mM $MgCl_2$, 0.4 µl of 10 mM dNTP mix, 0.4 µl of 20 µM forward primer, 0.4 µl of 20 µM reverse primer, 0.1µl of Go Taq DNA polymerase, 1-2 µl of template DNA and sterile Milli-Q water to final volume of 25 µl. PCR reactions were carried out in a thermal cycler (MyCyclerTM, Bio-Rad). The PCR cycles were started with an initial denaturation at 94ºC for 2 min, followed by 35-39 cycles of denaturation (94ºC for10 sec), annealing (55-62ºC, depending on the primer pair, for 10 sec) and extension (72ºC for 20 sec). Reactions were completed with a final extension at 72ºC

for 2-5 min depending on the size of the final product. PCR products were separated by gel electrophoresis in 1.5% agarose (Promega, Madison, MI, USA or Bioline, Alexandria, NSW, Australia) gels.

2.2.3 Agarose gel electrophoresis

The DNA products from PCR, RT-PCR, and restriction digestions were separated by agarose gel electrophoresis. The gels were prepared using 1 x TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) and 1-2% (w/v) analytical grade agarose (Promega, Madison, MI, USA or Bioline, Alexandria, NSW, Australia). GelRed (Biotium) or SYBR Safe Gel Stain (Bio-Rad, Hercules, CA, USA) was used to stain DNA. Each gel was cast and run in an EasyCast Mini Gel System (Bio-Rad). Gels were submerged in 1 x TAE buffer. Each PCR product was mixed with loading dye (Promega, www.promega.com) before loading on to the gel (if not PCR buffer with loading dye was used in PCR reaction). DNA marker ladders, 1 kb or 100 bp DNA ladders from Promega, were used to determine the approximate size of the DNA product. Electrophoresis was run at 90 volts for about 45 min to separate DNA. All gel imaging was performed on the GelDoc™ EZ imager (Bio-Rad) or ChemiDoc™ MP Imaging System (Bio-Rad).

2.2.4 Complementary DNA (cDNA) synthesis

The iScript™ cDNA Synthesis Kit (Bio-Rad) was used to reverse transcribe mRNA into cDNA. The reaction was carried our as per the manufacture's guidelines. A 20 µl reaction was made up including 4 µl of 5 x iScript reaction mix, 1 µl iScript reverse transcriptase, 1 µg of DNase-treated RNA and nuclease-free water to a final volume of 20 µl. The reaction mix was incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min in a thermal cycler (MyCyclerTM, Bio-Rad). The synthesised cDNA was diluted 1:9 in sterile Milli-Q water before being used in qPCR/RT-PCR reactions.

2.2.5 Reverse transcription polymerase chain reaction (RT- PCR)

RT-PCR was performed to check the expression of transgenes. The RT-PCR reaction was set up in the same manner as the general polymerase chain reaction described in Section 2.2.2 using cDNA (Section 2.2.4) as template.

2.2.6 Colony PCR

Glycerol stock cultures (-80^{o}C) of bacteria carrying entry clones of genes of interest, and the empty binary vector pIPKb003 were used to initiate plate cultures. Colony PCR was performed to select bacterial colonies carrying *AtAOX1a* and *AtNDB2* entry clones and expression clones using gene-specific primers (Table 2.1). A single colony grown on Luria Betani (LB) plates (tryptone (Sigma-Aldrich, Australia) 10 g/l, yeast extract 5 g/l and NaCl 5 g/l, pH 7.5, supplemented with the relevant antibiotic) was picked with the help of a pipette tip and swirled in sterile TE buffer. This cell suspension was used as DNA template in a colony PCR. The reaction set up and thermocycler conditions were as in Section 2.2.2. The PCR products were visualized on a 1.5% agarose gel (Section 2.2.3) in order to check the presence of gene of interest. 5-8 PCR positive colonies were selected for diagnostic restriction digestions.

Table 2.1 Primers used in molecular analysis of *AtAOX1a* and *AtNDB2* genes

Gene name	Forward primer sequence	Reverse primer sequence	Product size (bp)
AtAOX1a (At3g22370)	CTGGACCACGTTTGTTC	ACACCCCAATAGCTCG	277
AtNDB2 (At4g05020)	CCGAAACTGATGATGTATCTAAG	CCGTATGGAAGAGTGTGAGAA	172

2.2.7 Isolation and purification of plasmid DNA

Plasmid DNA was isolated from various bacteria carrying the pIPKb003 empty vector, entry vectors, and expression vectors for diagnostic restriction digestion, transformation reactions as well as for DNA sequencing. The Wizard® Plus SV Minipreps DNA Purification System (Promega) was used as per manufacture's guidelines.

Plasmid DNA was isolated from 10 ml of overnight grown bacterial cultures in LB liquid medium which were initiated from single colonies raised on selection medium. The bacterial cells were harvested by centrifugation at 4000 x g for 2 min in a benchtop centrifuge and used to produce a clear cell lysate. The supernatant was poured off and the pellet was completely resuspended in 250 µl cell resuspension solution with vigorous vortexing and transferred into 1.5 ml Eppendorf tubes. Then, 250 µl cell lysis solution was added and mixed well by inverting tubes 5-6 times. The cell lysate was incubated at room temperature (RT) for about 5 min until the solution cleared. Ten microliters of alkaline protease solution was added and mixed well by inverting tubes 5-6 times and incubated at RT for 5 min. The alkaline protease inactivates endonucleases and other proteins released during the lysis of the bacterial cells that can adversely affect the quality of the isolated DNA. Then, 350 µl of neutralization solution was added and mixed well. The lysate was centrifuged at 14,000 x g in a microcentrifuge for 10 min at RT. The cleared lysate was applied directly to a spin column without disturbing the white precipitate and centrifuged for 1 min. The filtrate in the collection tube was discarded and 750 µl of column wash solution was added and centrifuged at maximum speed for 1 min. This step was repeated with 250 µl of column wash solution. The columns were centrifuged for a further 2 min to remove residual ethanol. The spin columns were transferred into new 1.5 ml Eppendorf tubes and 50 µl nuclease free water added then incubated for 5 min at RT. Plasmid DNA was eluted by centrifugation at 14,000 x g for 1 min and stored at -20^{o}C until use.

2.2.8 Diagnostic restriction digestion

Restriction digests were conducted as per the guidelines recommended by the manufacturer; New England BioLabs. *BamHI*, *PstI* and *EcoRV* restriction enzymes were used in the study. Custom digestions of plasmid DNA were performed with the selected restriction enzymes and cut sites and expected lengths of DNA fragments were determined using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/ index.php) computer program (Vincze et al., 2003). The expected DNA fragment patterns were compared with the DNA band patterns resulting from the actual restriction digests by visualizing on a 0.8% (w/v) agarose gel.

2.2.9 Preparation of electro-competent cells

Electro-competent cells of both *E. coli* strain, DB10B *and Agrobacterium tumefaciens* strain, AGL1 were prepared following a standard protocol:

A loop full of bacterial cells from a -80ºC glycerol stock was used to establish a 10 ml LB culture containing the appropriate antibiotic and incubated overnight at 37ºC (or 30ºC if *A. tumefaciens* was used) with shaking. On the following day, 1 ml of the overnight culture was used to inoculate 250 ml of LB and incubated at 37ºC (or 30ºC if *A. tumefaciens* was used) with shaking for 4-5 hours until the optical density reached 0.6 at 600 nm (measured in a Beckman DU 640 spectrophotometer, USA). The culture was then chilled on ice for 10 min and then transferred aseptically into 50 ml ice-cold Falcon tubes prior to centrifugation at 3000 x g for 20 min at 4ºC. The pellet was resuspended in 50 ml of sterile Milli-Q water and centrifuged for 15 min at 4ºC. The pellet was then resuspended in 5 ml of 10% (v/v) autoclaved glycerol and centrifuged at 3000 x g for 30 min. Finally, the pellet was resuspended in 200 µl of ice-cold sterile 10% (v/v) glycerol. The cells were divided into aliquots (20 µl), snap frozen in liquid nitrogen and stored at -80ºC.

2.2.10 Gateway cloning system

The Gateway® cloning system (Invitrogen) was used to transfer genes of interest from Gateway entry clones into pIPKb003 destination vector (Himmelbach et al., 2007), using the LR Clonase® II. Reactions were performed according to the manufacturer's guidelines. Two microliter of LR Clonase II enzyme mix (Cat. No. 11791-100) was added to the reaction mix containing 1-7 µl of plasmid DNA from entry clone (50-150 ng/µl) and TE buffer at pH 8.0 up to a final reaction volume of 10 µl. The mixtures were incubated at 25^{o}C overnight (~16 hrs). Then, 1 µl proteinase K was added to the mixtures and they were incubated at 37^{o}C for 10 min to inactivate LR Clonase II enzyme and to terminate the reaction. The recombinant plasmid DNA mixtures were used to transform *E.coli* strains DH10B through electroporation.

2.2.11 Transformation of plasmid DNA into *E.coli* strain DH10B

The plasmid DNA carrying genes of interest were introduced into *E.coli* strain DB10B cells by electroporation. Plasmid DNA (1-100 ng) was mixed with 20 µl of electro-competent *E. coli* strain DH10B cells and the electroporation was performed with a Gene Pulser II Porator Electroporation System (Bio-Rad) according to the manufacturer's instructions. Cells were incubated with 250 µl of liquid LB medium (without antibiotics) at 37^{o}C for 45 min with shaking. A 50 µl volume of bacterial culture was then spread on LB agar plates supplemented with the appropriate antibiotic (usually 100 µg/ml spectinomycin) and the plates were incubated at 37^{o}C overnight. Several relatively large single colonies were selected and colony PCR (Section 2.2.6) was carried out with gene-specific primers (Table 2.1) to confirm the presence of the gene of interest. The PCR-positive colonies were selected to establish liquid LB cultures containing the appropriate antibiotic. The overnight grown cultures were used to prepare glycerol stocks or to isolate plasmid DNA for diagnostic restriction digestion, and for DNA sequencing.

2.2.12 Transformation of plasmid DNA into *A. tumefaciens* strain AGL1

Genetic constructs were introduced into *Agrobacterium* strain AGL1 by electroporation as above (Section 2.2.11) except using 20 µg/ml rifampicin and 100 µg/ml spectinomycin and plates were incubated at 30^{o}C overnight. Several relatively larger colonies were selected and colony PCR (Section 2.2.6) was performed with gene-specific primers (Table 2.1) to confirm the presence of the gene of interest. The PCR-positive colonies were selected to establish LB liquid cultures. The overnight grown cultures were used to prepare glycerol stock cultures which were then stored at -80^{o}C for future use in callus transformation.

2.2.13 DNA sequencing

Sequence analysis of the IPKb003 destination vectors, carrying coding DNA sequences (CDS) of *AtAOX1a* and *AtNDB2* genes, were done by using vector specific primers. (Forward primer sequence: TGCGGAGCTTTTTTGTAGGCGCG; Reverse primer sequence: CCGGCAACAGGATTCAATCTTAAG). The primers were designed about 40-50 bases away from the ATG start codon and stop codon of the gene of interest. Plasmid DNA was isolated and purified as described in Section 2.2.7. The purified DNA samples were prepared as per the guidelines provided by Australian Genome Research Facility (AGRF), Waite campus, Urrbrae, South Australia and submitted for sequencing. DNA sequence data (chromatograms) were checked for accuracy using the Chromas Lite 2.0 (chromas-lite.software.informer.com/2.0/) computer program and sequence alignments were done using the Clustal Omega multiple sequence alignment program (www.ebi.ac.uk/Tools/msa/clustalo).

2.2.14 Preparation of glycerol stock cultures

Overnight-grown bacterial cultures carrying the genetic constructs were used to establish glycerol stock cultures. Five hundred microliter of bacterial culture was mixed well with 500 µl of sterile glycerol (50% (v/v)) in 1.5 ml screw capped tubes.

The tubes were snap frozen in liquid nitrogen and stored in -80ºC for future use.

2.3 Gene expression analysis

2.3.1 Gene nomenclature

The genomic sequences and other relevant details of all the alternative oxidases (AOX) and alternative dehydrogenases (NDH) identified in rice were obtained from the Rice Genome Annotation Project (rice.plantbiology.msu.edu) and Gramene (www.gramene.org) databases. The gene nomenclature used in the present work followed the guidelines described by McCouch (2008). Details of the alternative respiratory genes identified in rice can be found in Table 3.2 and will be discussed in Sections 3.3.1 and 3.3.2.

2.3.2 Primer designing

Primers were designed for qRT-PCR analysis, general PCR and for sequencing of plasmid clones. The qRT-PCR primers were manually designed to span a 100-200 bp product. The melting temperatures of primers were calculated by using OligoCalc: Oligonucleotide Properties Calculator computer software (www.basic.northWestern.edu/biotools/OligoCalc.html). The self-complementarity (possibility of forming primer dimers, potential hairpin formation and 3' complementarity) of primers were checked with the same computer software as above. Primers were designed to span an intron-exon boundary and close to the 3'-end of the gene where possible. The genomic sequences of AOXs and NDHs were BLAST against corresponding coding DNA sequences (CDS) to locate introns and exons using the SDSC (San Diego Supercomputer Centre) Biology Workbench (workbench.sdsc.edu/.). All primer pairs were tested by standard RT-PCR using Go Taq Flexi DNA polymerase and the presence of a single amplification product of the expected size for each gene was verified by electrophoresis.

For confirmation of the sequence of expression clones, both forward and reverse primers were designed from the vector backbone 40-50 bases away from the point where gene of interest was inserted. All primers were synthesized either by GeneWorks Pty Ltd (Australia) or Sigma-Aldrich Australia. The details of the primers used in this study including sequences for the reference genes can be found in Table 2.2 and 2.3, respectively.

2.3.3 Preparation of qRT-PCR standards

qRT-PCR standards were prepared for three rice AOXs, (*OsAOX1a*, *OsAOX1b* and *OsAOX1c*), six rice NDHs (*OsNDA1-2*, *OSNDB1-3* and *OsNDC1*), six selected reference genes (*OsActin*, *OsElf1*, *OsPpIase*, *OsGAPDH*, *Ostubulin*, *Os18S*) and for two antioxidant defence genes, *OsMnSOD* and *OsCAT-A*. PCR was performed with the respective primer pairs using cDNA as templates, the presence of a single product at expected size was verified for each gene by visualizing on 1.5 % agarose gel. The PCR products were purified using the Wizard SV Gel and PCR Clean-UP system (Promega, USA) and quantified using a Nanodrop spectrophotometer (ThermoScientific, Waltham, USA). Standards for qRT-PCR were prepared with purified products at concentrations of 10^{-2}, 10^{-4} and 10^{-6} fmol/µl for subsequent qRT-PCR analysis.

Table 2.2 Details of primers used for qRT-PCR analysis

Gene name	Gene description	Forward primer sequence	Tm (°C)	Reverse primer sequence	Tm (°C)	Expected product size (bp)
OsAOX1a	Alternative oxidase 1a	CGTCAATCACTTCGCATCGG	53.8	GCAAATCCTCGGCAGTAGAC	53.8	105
OsAOX1b	Alternative oxidase 1b	AGGGGATGAAGCTGAAGGAT	51.8	TACGCATGTAGTAGCAGTGAT	50.5	109
OsAOX1c	Alternative oxidase 1c	AAGATCGAGAACGTCCCCGCG	58.3	TCCATCCCCTGAAAATGAACAT	51.1	158
OsNDA1	Alternative dehydrogenase A1	GGTGGACCTGAGGGAGAACAAG	58.6	CAGTTGACCGCCACGTAGAACC	58.6	132
OsNDA2	Alternative dehydrogenase A2	GATGTTAAACAGCGTTATTCACATGT	53.2	ACCTCTTCTCCATTGTCCAGGAT	55.3	197
OsNDB1	Alternative dehydrogenase B1	TGTGGATTCCCAAGTTAAAAGT	49.2	AATTGTGTCGTCCTGATCCTGT	53.0	163
OsNDB2	Alternative dehydrogenase B2	AGGATCAGAGGCACAGGGCGTC	60.4	TGGCATAGACAGAATACCATAG	51.1	165
OsNDB3	Alternative dehydrogenase B3	ATTTGCTGAGGATAAGTTTGGA	49.2	CAATTTGTTTCATGAACTCTGA	47.4	191
OsNDC1	Alternative dehydrogenase C1	CTACTATTGGAACTTCAACCTGCT	54.0	CTACCTGGCCCCGACCATTTAA	56.7	157
OsMnSOD	Manganese-superoxide dismutase	AACTTGGTTCCTTTGTTGGGAATT	52.3	AACATCAAGCAGTCGCATTTTCGTA	54.4	151
OsCAT-A	Catalase isozyme A	ATAGACAGGAGAGGTTCATCCG	54.8	TCACATGCTTGGCTTCACGTTGA	55.3	158

Table 2.3 Details of reference gene primers used in the study

Gene name	Gene description	Forward primer sequence	Tm (°C)	Reverse primer sequence	Tm (°C)	Product size (bp)	Reference
OsActin	Actin	GAAGATCACTGCCTTGCTCC	53.8	CGATAACAGCTCCTCTTGGC	53.8	249	(Kim et al., 2003)
OsElf1	Elongation factor 1 alpha	ATCTGGGAAATCATCGGTTCTG	53.0	AGATCGTCCACAATGGTCATCA	53.0	189	(Cotsaftis et al., 2011)
OsGAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GGGCTGCTAGCTTCAACATC	53.8	TTGATTGCAGCCTTGATCTG	49.7	190	(Kim et al., 2003)
OsTubulin	Alpha tubulin	TACCGTGCCCTTACTGTTCC	53.8	CGGTGGAATGTCACAGACAC	53.8	234	(Kim et al., 2003)
OsPplase	Peptidylprolyl isomerase	CTGGCAAAGAACCTAAACCTCATC	55.7	TGCTCCTGTGGTGTAACTGCTTT	55.3	194	(Cotsaftis et al., 2011)
Os18S	18S ribosomal RNA	ATGATAACTCGACGGATCGC	51.8	CTTGGATGTGGTAGCCGTTT	51.8	169	(Kim et al., 2003)

2.3.4 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Gene expression analysis was carried out with three AOXs (*AOX1a*, *AOX1b* and *AOX1c*) and six NDHs (*NDA1-2*, *NDB1-3*, and *NDC1*) and antioxidant defence genes. The qRT-PCR analysis was performed with a CFX96 Real Time PCR Detection System (Bio-Rad, Hercules, California, USA). Reactions in final volume of 10 µl were set up with the 5 µl SsoFast EvaGreen Supermix Master Mix (Bio-Rad, Hercules, California, USA), 2 µl cDNA sample, 0.3 µl of 20 µM gene-specific forward and reverse primers, and sterile Milli-Q water to a total volume 10 µl.

All the PCR reactions were performed using the following conditions: 3 min at 95°C and 39 cycles of 10 s at 95°C, 30s at 55-60°C and 10 s at 95°C, and then a melt gradient from 65ºC to 95ºC for 5s, in 96-well white colour reaction plates (Bio-Rad, California, USA). The specificity of the amplification was tested by dissociation curve analysis and agarose gel electrophoresis.

2.3.5 Reference gene selection for normalization of qRT-PCR data

Six rice reference genes, *OsActin*, *OsTubulin*, *OsGAPDH*, *OsPpIase*, *OsEIF1* and *Os18S*, which have already been widely used in qRT-PCR studies in rice (Table 2.3), were tested with selected cDNA samples from cultivar Nipponbare and Langi. Samples were selected representing different time points, different tissue types (root and shoot) as well as treated and untreated samples. Final absolute quantification cycle (Cq) values from qRT-PCR (Section 2.3.4) representing the mean of three biological replicates with three technical replicates (Fan et al., 2013) were used to compare expression stability of reference genes across samples tested. Three reference genes with least variation across tested samples (based on coefficient of variance) were selected as reference genes for subsequent gene expression investigations.

2.4 Protein analysis and enzyme activity assays in isolated mitochondria

2.4.1 Isolation and purification of mitochondria

For enzyme activity assays and mitochondrial protein analysis it was necessary to isolate mitochondria from salt-treated and untreated root and shoot tissues from rice. Seedlings of cv. Nipponbare growing at their early vegetative stage (4-week-old) were used to isolate mitochondria. Nipponbare is a model japonica rice cultivar which has been used as a standard rice cultivar in a variety of abiotic and biotic stress studies and was used in this study.

Mitochondrial isolation was carried out following the method described by Kristensen et al. (2004) with slight modifications. Shoots and roots (30-40 g fresh weight) were harvested separately from salt-treated and control hydroponically-grown rice seedlings nine days after salt application. The roots taken out from the hydroponic solution were thoroughly washed with water to remove residual NaCl and other nutrients and blotted dry with paper towels. Mitochondrial isolation was carried out separately for shoot and root tissues and all the steps were done at 4^{o}C in the cold room. Shoots/roots were cut into 2-3 cm pieces and homogenized with a Polytron PT10-35 GT dispersing unit (Kinematica, Australia) by 5 x 10 s bursts at around 5000 rpm in the isolation medium (30 g fresh weight/300 ml of medium; 0.3 M mannitol, 5 mM EDTA, 30 mM MOPS, 1% (w/v) BSA, 5 mM DTT, 1% PVP, pH 7.3). The homogenate was strained through two layers of Mira-cloth (Cat No. 475855-IR, Calbiochem, USA) and centrifuged (Avanti J-26 XPI centrifuge, Beckman Coulter, USA using JA 25.50 rotor) at 1,935 x g (4000 rpm) for 5 min. The supernatant was transferred into fresh tubes and centrifuged at 17,418 x g (12,000 rpm) for 20 min. Then the supernatant was discarded and the crude mitochondrial pellet was resuspended in wash medium (0.3M mannitol, 1mM EDTA, 10mM MOPS, pH 7.2) and centrifuged at 17,418 x g for 20 min. The crude mitochondrial pellet was again

resuspended in 1-2 ml of wash medium and layered on the top of discontinuous Percoll gradient which consisted of four layers: 6 ml of 60% (v/v) Percoll, 8 ml of 45% (v/v) Percoll, 10 ml of 28% (v/v) Percoll and 10 ml of 5% (v/v) Percoll. The gradient was centrifuged at 30,000 x g (13,200 rpm) for 45 min (Optima L-100 XP centrifuge, Beckman Coulter, USA using SW32T1 rotor) so that the cellular organelles and debris were separated according to their density. In this gradient, mitochondria were located in between the 28% Percoll layer and the 45% Percoll layer (Figure 2.2). The layers above the mitochondrial band were removed carefully by aspiration without disturbing the mitochondrial band. The mitochondrial band was taken out with a pasture pipette and Percoll contamination of pellet was completely removed by repeated dilution (with wash medium) and pelletation (Avanti J-26 XPI centrifuge, Beckman Coulter, USA using JA 25.50 rotor, 39,191 x g (18,000 rpm) for 20 min).

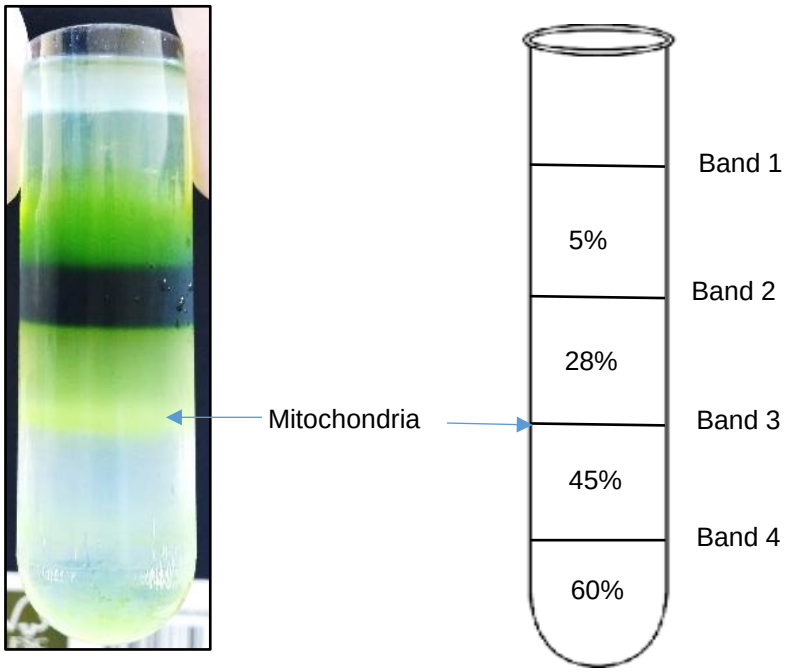


Figure 2.2 Separation of mitochondria by Percoll discontinuous density gradient centrifugation

The Percoll gradient centrifugation technique separates suspensions of organelles and cell debris from tissue homogenate according to their density.

The final pellet was resuspended in a small volume (~0.8 ml) of wash medium. Isolated mitochondria were immediately used for biochemical assays or snap frozen in liquid nitrogen and stored at -80^{o}C for protein quantification and Western blot analysis.

2.4.2 Respiratory Assays

Mitochondrial respiratory assays were carried out with a Clark-type Oxygraph Plus oxygen electrode system (Hansatech Instruments Ltd). Calibration of the instrument was carried out according to the manufacturer's guidelines and oxygen consumption rates of mitochondria isolated from control and salt-treated shoot and root tissues were investigated at 25^{o}C.

2.4.2.1 Alternative oxidase activity assay

The oxygen uptake rate of mitochondria isolated from shoot or root was measured in 1 ml total volume of a standard reaction medium (0.3 M mannitol, 10 mM TES, 10 mM KH_2PO_4, 2 mM $MgCl_2$, pH 7.2) containing 200 µl mitochondria (equivalent to 0.4-0.8 mg mitochondrial protein). Total oxygen consumption rate was measured in the presence of following components in final concentration; 10 mM succinate, 5 mM pyruvate, 1 mM NADH, 1mM ATP, 1 mM ADP, 5 mM DTT. The cyanide resistant and the residual oxygen consumption rates were determined in the presence of 1 mM KCN and 250 nM OG (Octyl Gallate) in final concentrations, respectively.

2.4.2.2 Alternative dehydrogenase activity assay

The oxygen uptake rate of mitochondria isolated from root or shoot was measured in 1 ml total volume of standard reaction medium (as above) containing 200 µl mitochondria (equivalent to 0.4-0.8 mg mitochondrial protein). to determine activities of total external NADH and NADPH dehydrogenases, Ca^{2+} dependent and Ca^{2+} independent external NADH and NADPH dehydrogenases.

Initially, EGTA was added into the reaction medium at 3 mM final concentration and allowed 2 min to chelate Ca^{2+}. Then, oxygen consumption rate by Ca^{2+} independent NADPH dehydrogenase was measured in the presence of NADPH and ADP at 2 mM final concentration. The maximal rate of NADPH oxidation (total oxygen consumption by Ca^{2+} independent and Ca^{2+} dependent NADPH dehydrogenases) was measured by addition of $CaCl_2$ at 2 mM final concentration. The oxygen consumption rate by Ca^{2+} dependent NADPH dehydrogenase was calculated as total oxygen consumption – oxygen consumption by Ca^{2+} independent dehydrogenase. Similarly, total NADH oxidation, Ca^{2+} independent and Ca^{2+} dependent NADH oxidation were determined using NADH and ADP as substrates.

2.4.3 Determination of protein concentration

The concentration of isolated mitochondrial protein was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) as per manufacturer's guidelines using a microplate method. Absorbance was measured at 572 nm using a POLARstar Omega microplate reader (BMG LABTEC, The Microplate Reader Company).

2.4.4 Antibody designing and testing

Polyclonal antibodies were derived from peptides to identify rice NDB2 (OsNDB2) protein. The specificity of the peptide sequences were tested by Clustal analysis (http://www.ebi.ac.uk/Tools/msa/clustalo/) of protein sequences with other rice alternative dehydrogenases. Antibody production was done by BioMatiks (www.biomatik.com).

2.4.5 Antibody dot blot technique

The effectiveness of antibodies was evaluated by dot blot technique. A range of primary antibody dilutions was tested at a range of antigen (peptide) dilutions. Using a narrow-mouth pipette, 2 µl from undiluted, 1/2, 1/4, 1/8 and 1/16 -fold diluted peptide solutions were applied to nitrocellulose membrane strips. The area of solution binding

to the membrane was minimized by applying the solution slowly. Then, the membrane strips were allowed to air dry for 10 min. Strips were placed in individual plastic containers and non-specific sites were blocked by soaking in blocking buffer (20 mM Tris base, 150 mM NaCl, 5% skim milk, 0.1% Tween 20 pH 7.4) at RT for 1 hr with gentle agitation on a rotary platform. Membrane strips were then incubated with range of primary antibody dilutions (1:2000 to 1:12,000 dilutions) in blocking buffer for 1 hr with gentle agitation. The membrane strips were washed three times (5 min each) in TBST buffer (20 mM Tris base, 150 mM NaCl, and 0.1% (v/v) Tween 20 pH 7.4) while agitating, to remove excess primary antibody. Then membrane strips were incubated with secondary antibody (HRP conjugated anti rabbit) in blocking buffer at RT for 1 hr with gentle agitation. The secondary antibody dilution was done following the manufacturer's instructions. The membrane strips were washed three times with TBST buffer (20 mM Tris base, 150 mM NaCl, and 0.1% (v/v) Tween 20, pH 7.4) to remove excess secondary antibody while agitating as described above. Once drained, membrane strips were developed in Immun-Star™ Western™ Chemiluminescent substrate (Bio-Rad) at RT and visualized using a ChemiDoc™ MP Western blot imaging system.

2.4.6 Western blot analysis

Comparison of the alternative respiratory pathway protein levels in salt-treated and untreated tissues was done by using 10% SDS-PAGE (Sodium Dodecyl Sulphate–Polyacrylamides Gel Electrophoresis) followed by Western blotting. Selected proteins, having been separated according to their molecular weight by SDS-PAGE, were identified using specific antibodies.

2.4.6.1 Preparation of 10% SDS-PAGE gel

The glass cassette sandwich for SDS-PAGE gel casting was prepared by placing two cleaned glass plates, spacer plate and short plate, in the casting frame according to

manufacturer's instructions. The 10% (v/v) resolving gel monomer solution (40% acrylamide, 10% SDS, 1.5 M Tris pH 8.8, 10% APS, TEMED) was poured into the gel cassette up to a pre-marked level, about 1 cm away from the comb teeth. The monomer solution was immediately overlayed with water to prevent oxidation and allowed to polymerize for about 30-45 min. The top of the resolving gel was dried with filter papers and a 4% (v/v) stacking gel monomer solution (40% acrylamide, 10% SDS, 1.0 M Tris pH 6.8, 10% APS, TEMED) was poured on to the polymerized resolving gel. The desired comb was inserted and aligned. The stacking gel was left for about 30 min to polymerize. The gels were wrapped with wet filter papers and stored at 4^{o}C until required.

2.4.6.2 Sample preparation and separation

Samples (80 µg) of purified mitochondrial protein were used in the Western blot analysis. Equal volumes of 2 x loading buffer (100 mM Tris HCl pH 6.8, 200 mM DTT, 4% SDS, 0.5% bromophenol blue, 20% glycerol) was added to each sample, mixed well and the mixtures heated at 95^{o}C for 5 min in a Single Dry Block Heater (Ratex Instruments, Australia).

Precast 10% SDS-PAGE gels were assembled in electrode assembly module and placed in a Mini-Protean Tetra cell tank (Bio-Rad) following the manufacturer's instructions. Gels were submerged in SDS-PAGE running buffer (25 mM Tris base, 192 mM Glycine, 0.1% SDS pH 8.3) and equal volumes of protein samples were loaded, electrophoresed at 170 volts for 60 min to separate proteins. Molecular weight marker (PrecisionPlus Protein^{TM} Dual colour Protein Marker, Bio-Rad) or NEB unstained protein marker was also run with each gel to assess the size of the target protein.

2.4.6.3 Transferring the protein from the gel to the membrane

A wet transfer method was used to transfer separated proteins from the gel to the nitrocellulose membrane. After electrophoresis, the stacking gel was removed from the resolving gel. The resolving gel and the nitrocellulose membrane were sandwiched between wet filter papers and wet sponges (Figure 2.3). After ensuring no air bubbles were formed between the gel and the membrane, all parts of the gel sandwich were clamped tightly together using a gel holder cassette (Bio-Rad). Then the cassette was placed in the electrode module and the Mini Trans-Blot® Cell (Bio-Rad) was assembled following the manufacturer's instructions. The gel sandwich was submerged in transfer buffer (25 mM Tris base, 152 mM Glycine pH 8.3) and transfer was conducted at 60 volts for 90 min.

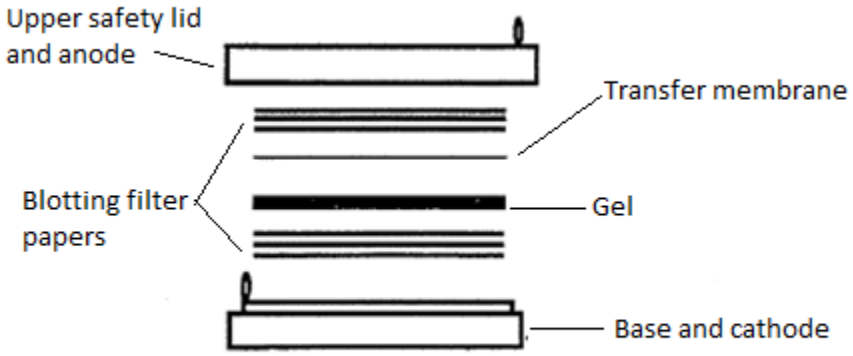


Figure 2.3 Set up for a gel "sandwich" to transfer protein from the SDS-PAGE gel to the nitrocellulose membrane

(http://www.ou.edu/OpenEducation/ou-resources/biochemical-methods/index.html)

2.4.6.4 Visualization of proteins in membranes

Locating the protein bands on the membrane as well as to check the success of protein transfer from the gel to the membrane were done by staining with Ponceau S (1% Ponceau stain, 2% acetic acid). The membrane was unloaded from the transfer chamber and briefly washed with TBST (20 mM Tris base, 150 mM NaCl, 0.1% Tween

20 pH 7.4), then the membrane was immersed in the Ponceau stain for about 15-30 min with gentle shaking and subsequently was washed in water to remove excess stain until the background of the membrane was clear and the protein bands were well defined. Then the membrane was destained completely by repeated washings in TBST before staining with antibody.

2.4.6.5 Antibody staining

The membrane was blocked for one hour at RT using blocking buffer (20 mM Tris base, 150 mM NaCl, 5% skim milk, 0.1% Tween 20 pH 7.4), which prevents non-specific background binding of primary and/or secondary antibodies to the membrane. Then the membrane was incubated overnight at 4^{o}C with an appropriate dilution of primary antibody in blocking buffer with gentle agitation. On the following day, the membrane was washed three times, with a 15 min wash followed by two 5 min washes with TBST buffer. The washing was accelerated by gentle agitation of the membrane in the buffer. The membrane was then incubated with a pre-determined dilution of secondary antibody in blocking buffer at RT for one hour. Excess secondary antibody was removed from the membrane by washing three times in TBST buffer, as described above. The membrane was drained and developed in Immun-Star^{TM} Western^{TM} Chemiluminescent substrate (Bio-Rad) at RT and visualized using a ChemiDoc^{TM} MP Western blot imaging system.

2.4.7 Stripping membrane for re-probing

The membrane was stripped using stripping buffer (50 mM Tris base, 100 µM β-mercaptoethanol, 2% SDS pH 6.8) for 30 min and washed thoroughly to remove stripping buffer with TBST three times (5 min each) with gentle agitation. The membrane was blocked with blocking buffer for 1 hr. Re-probing and visualization of the membrane was carried out following the same procedure described in Section 2.4.6.5.

2.4.8 Coomassie blue staining

Coomassie blue staining of gels were done to confirm the transfer of proteins to membrane or to locate protein bands. Polyacrylamide gels were fixed in destain solution (10% (v/v) glacial acetic acid and 10% (v/v) methanol) for 10-30 min, and then incubated with Coomassie blue stain (0.1% (w/v) Coomassie blue, 40% (v/v) methanol, 10% (v/v) glacial acetic acid) for 60 min. Gels were immersed in destain solution until the background of the gel was fully destained with the solution being replaced several times.

2.5 Data analysis

Graphs were plotted using either GraphPad prism version 7 or Microsoft Excel 2010 programs. Data are generally expressed as mean ± SEM (Standard Error of the Mean). Analysis of variance or *t*-test was performed to determine whether there were significant differences between means of control and treated samples at 95% confidence level using GraphPad prism (http://www.graphpad.com/scientific-software/prism/). Further details of statistical analytical methods used for each data set can be found with relevant figures and tables in results sections.

3 Alternative pathway of respiration in rice: Identification and characterization

3.1 Introduction

Two distinct pathways exist in the plant mitochondrial electron transport chain (ETC) to oxidise NAD(P)H produced through the central respiratory pathways which include the pentose phosphate and tricarboxylic acid pathways. Initially, the NAD(P)H produced from both pathways feed into either Complex I or the alternative dehydrogenases (NDH), to reduce the common pool of ubiquinone, which is then oxidized by either the traditional cytochrome oxidase or alternative oxidase (AOX) (Rasmusson et al., 2004). Alternative respiratory enzymes allow plant mitochondria to respire in the presence of inhibitors of traditional respiratory complexes I, III, and IV; rotenone, antimycin A (AA), and cyanide (CN), respectively (Finnegan et al., 2004). It is proposed that these enzymes provide metabolic homeostasis of carbon and energy metabolism, and signalling homeostasis in the organelle (Vanlerberghe, 2013). Evidence suggests that this is particularly important during abiotic and biotic stress tolerance (Vanlerberghe, 2013). Consequently, the alternative pathway has become an area of research interest due to its potential role during stress tolerance.

Initially, our limited knowledge of genomic data hindered exploring the distribution of alternative respiratory pathway components in different organisms. In earlier studies, enzyme activity assays and inhibitor studies were used to show the presence of the alternative respiratory enzyme activities. Yeast (*Saccharomyces cerevisiae*), which lacks Complex I, has at least three non-phosphorylating NAD(P)H dehydrogenases in its respiratory chain; two of these are external facing (*ScNDE1*, *ScNDE2*) and the other is internal facing *ScNDI1* (Luttik et al., 1998). These findings paved the way to explore the distribution of NDHs in both animal and plant kingdoms. Enzyme activity assays have shown that the alternative pathway enzyme activities to be present in mitochondria from fungi such as *Neurospora crassa* (Weiss et al., 1970) as well as in

plants like potato (Rasmusson et al., 1999). The availability of sequence data and bioinformatics tools accelerated the process of identification and characterization of alternative pathway genes in many organisms from the plant and animal kingdoms, making it an area of research interest.

Initially, AOX was thought to be limited to plants and some fungi and protists. But bioinformatics studies have found AOX sequences in organisms from all kingdoms (except Archaebacteria) including animals (McDonald et al., 2003; McDonald and Vanlerberghe, 2006). Taking advantage of publicly available genomic sequence data and bioinformatics tools, it has been shown that alternative pathway genes, both AOXs and NDHs, are present in most animal taxa except Arthropoda and Vertebrata (McDonald and Vanlerberghe, 2006).

The alternative respiratory pathway in Arabidopsis has been well studied. It contains five AOX genes, *AOX1a-d* and *AOX2* (Thirkettle-Watts et al., 2003) and seven NDH genes which are belong to three subfamilies, NDA (*AtNDA1-2*), NDB (*AtNDB1-4*) and NDC (*AtNDC1*) (Michalecka et al., 2003). For all these genes, encoded proteins have been localized to mitochondria and within the mitochondria, all four members of NDB group located on the outside of the inner mitochondrial membrane while NDA family and *NDC1* located on the inside of the inner mitochondrial membrane (Figure 3.1). However, some of these NDHs have been identified to be localized into more than one organelle. Some are localized to peroxisomes (*AtNDA1*, *AtNDA2* and *AtNDB1*) while *AtNDC1* is localized to chloroplast other than mitochondria (Michalecka et al., 2003; Xu et al., 2013).

There is a wide distribution of alternative respiratory pathway genes in plant as well as animal kingdoms. It has also been suggested, in a number of plant species, that alternative pathway proteins play a crucial role in abiotic stress tolerance by regulating the production of reactive oxygen species (ROS) in cells during environmental stress. Therefore, it is worthwhile to investigate whether different plant species, especially

crop species, share common AOX and NDH structure and expression profiles, which will be important in improved varietal development programs.

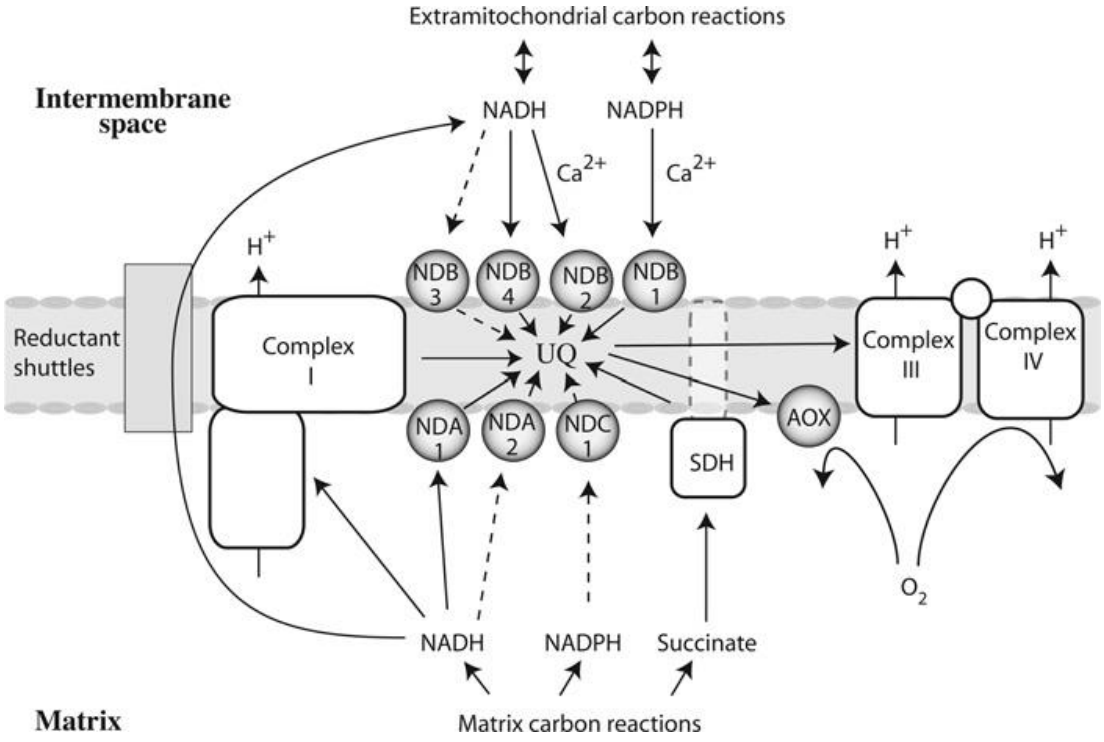


Figure 3.1 The traditional and alternative respiratory enzymes in plant mitochondrial electron transport chain

The traditional respiratory complexes are depicted in white, including the proton-pumping complexes I, III and IV. Type II NAD(P)H dehydrogenases and AOX are depicted in grey shading. Broken lines denote presently unclear enzymatic properties. SDH, succinate dehydrogenase (Rasmusson and Wallstrom, 2010).

The overall aim of the current project was to explore whether modifications in the alternative pathway of respiration can improve tolerance to abiotic stresses in cereals using rice as a model cereal. There are several approches to achieve this target and this chapter describes the identification of the complete rice alternative pathway genes using publicly available databases and bioinformatics tools and assessing which of these are the stress-responsive genes. Sequences of Arabidopsis alternative pathway genes were used to mine databases to identify alternative pathway gene sequence homologs in japonica rice and indica rice. There were no published works on the study of rice alternative dehydrogenases (NDH) until the start of the current work. However,

later Xu et al. (2013) published on the dual targeting ability of rice NDHs. The present study was carried out extending the work towards the comparative analysis of gene sequence and gene structural diversity of identified rice AOXs and NDHs in two rice sub-species and Arabidopsis using publicly available databases and bioinformatics programs. Further, the stress responsive genes were identified by generating gene expression profiles in root and shoot tissues exposed to oxidative stress induced by chemical inhibition of traditional respiratory pathway.

3.2 Materials and methods

3.2.1 Identification of alternative pathway (AP) genes in rice

Protein/genomic sequences for all Arabidopsis AOXs (*AtAOX1a*, At3g22370; *AtAOX1b*, At3g22360; *AtAOX1c,* At3g27620; *AtAOX1d*, At1g32350; *AtAOX2*, At5g64210) and NDHs (*AtNDA1,* At1g07180; *AtNDA2*, At2g29990; *AtNDB1*, At4g28220; *AtNDB2*, At4g05020; *AtNDB3*, At4g21490; *AtNDB3*, At2g20800; *AtNDC1*, At5g08740) were downloaded from the TAIR (The Arabidopsis Information Resource) web site (https://www.arabidopsis.org) and used for BLASTp/BLASTn searches against protein/genomic reference sequences of genes in the MSU RGAP Release 7 on Rice Genome Annotation Project database (rice.plantbiology.msu.edu) to identify alternative pathway homologs in japonica rice. Protein/genomic sequences of identified japonica rice AP genes were used in BLASTp/BLASTn searches against *O. sativa* ssp *indica* group protein/genomic sequences on the Gramene database (www.gramene.org) to identify AOX and NDH homologs in indica rice.

3.2.2 Identification of conserved regions/domains in rice AP genes

Conserved domain search of identified proteins were carried out to find the protein families that they belong to and to check whether the proteins have been grouped correctly. A conserved regions/domains search of identified AP genes from both rice sub-species was carried out using the “InterPro” protein sequence analysis and classification computer program (http://www.ebi.ac.uk/interpro/) and results were confirmed with the NCBI (National Centre for Biotechnology Information) conserved domain database search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple alignments and pairwise alignments of protein/genomic sequences were carried out with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and resultant aligned sequences were visualized using GenDoc computer software (http://genedoc.software.informer.com/) to identify variations/similarities in sequences of both rice sub-species and compared with Arabidopsis.

3.2.3 Determination of subcellular localizations of AP genes

Computational determination of the subcellular localization of identified AP genes from both rice groups was carried out using publicly available subcellular localization prediction programs; TargetP (http://www.cbs.dtu.dk/services/TargetP/), Predotar (http://urgi.versailles.inra.fr/Tools/), ChloroP1, (http://www.cbs.dtu.dk/Servies), and Plant-mPLOC (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). Proteins targeting to the peroxisomes were predicted using PredPlantPTS1 (http://ppp.gobics.de/).

3.2.4 Phylogenetic analysis

AOX and NDH protein phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987) in the MEGA7 (Molecular Evolutionary Genetic Analysis version 7) computer program (Tamura et al., 2013). The alignments of protein sequences were performed using the Clustal Omega multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/) and data were imported to the MEGA 7 computer program. Phylogenetic tree construction was carried out using the

Bootstrap method with 1000 replications, “number of differences” as the substitution model and “complete deletion” for gaps/missing data treatment. AOX and NDH protein sequences from 10 different monocot species were used for the phylogenetic tree construction. Protein sequences were downloaded from publicly available databases such as NCBI (http://www.ncbi.nlm.nih.gov), Gramene (www.gramene.org), Plaza 3 (http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_monocots/), Phytozome (http://www.phytozome.net), as well as from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/).

3.2.5 Exon-intron structural polymorphism in rice AP genes

A comparative study of the exon-intron structure of AP genes from two rice sub-species and Arabidopsis was carried out to explore the diversity of exon-intron structures in the rice sub-species and to study how they have deviated from dicot species using Arabidopsis as an example. Exon-intron gene structures of AOXs and NDHs were generated using the Gene Structure Display Server 2.0 (GSDS 2.0) computer program (http://gsds.cbi.pku.edu.cn/) and compared with each other. Genomic and coding DNA sequences of japonica rice and indica rice were obtained from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and Gramene databases (www.gramene.org), respectively, while genomic and coding DNA sequences for Arabidopsis were downloaded from the TAIR website (https://www.arabidopsis.org). The results were further confirmed by working out exon and intron regions for all AOX and NDH genes using the BL2SEQ option on the SDSC biology workbench (San-Diego Supercomputer Centre; http://workbench.sdsc.edu/) computer program and by the exon-intron data retrieved from the Gramene database before generating gene structures using the GSDS 2.0 program.

3.2.6 Exon-intron gene structural polymorphism among different rice species

An extended study was carried out to explore the exon-intron structural diversity of *AOX1a* and *NDB2* genes in different rice species (including japonica and indica rice). Identified japonica rice *AOX1a* and *NDB2* sequences were used as queries to perform BLAST searches against the rice database on the Gramene website. The “UniProt” (www.uniprot.org), “InterPro” (www.ebi.ac.uk/interpro/protein/) or NCBI databases were used to confirm the presence of respective conserved domains and molecular functions of putative *AOX1a* and *NDB2* genes. Genomic and CDS sequences of *AOX1a* and *NDB2* genes from 10 rice species (Table 3.1) were obtained from the Gramene database and exon-intron structures were generated using the Gene Structure Display Server 2.0 program (http://gsds.cbi.pku.edu.cn/).

3.2.7 Screening for stress responsive AP genes in rice

Three-week-old rice seedlings (*O. sativa* ssp *japonica* cv Nipponbare), grown on Petri dishes, were exposed to respiratory inhibitors, 20 µM antimycin A or 5 mM potassium cyanide to create oxidative stress in seedling tissues. Roots and shoots were collected before treatment, 6 h and 24 h after treatments, to extract RNA for gene expression analysis. RNA extraction, cDNA synthesis and gene expression analysis using qRT-PCR technique were carried out as described in sections 2.2.1.1, 2.2.4 and 2.3.4, respectively.

3.2.8 Search for conserved *cis*-acting regulatory motifs in rice AP genes

Cis-acting regulatory elements (CAREs) located on promoter regions are responsible for the signalling pathways that regulate stress-induced gene expression in plants (Ho et al., 2008). A number of conserved CAREs have been identified in Arabidopsis *AOX1a* and *NDB2*, which are responsive to oxidative stress. A computational approach was used for identifying similar motifs in rice promoter regions *AOX1a* and *NDB2* genes. The “CentriMo”, Local Motif Enrichment Analysis tool on the motif

prediction algorithm “MEME” (meme-suite.org) was used to detect the presence of Arabidopsis regulatory *cis*-sequence elements in promoter regions of *AOX1a* and *NDB2* from japonica and indica rice. Both directions of the DNA strand were analysed for the presence of motifs. Promoter region sequences of japonica *AOX1a* (1.5 kb) and *NDB2* (1.3 kb) genes were retrieved from the relevant BAC clones while promoter sequences for *AOX1a* (1.5 kb) and *NDB2* (1.5 kb) from indica rice was obtained from the Gramene database (www.gramene.org). For all other AP genes, promoter region sequences (1.5 kb) were downloaded from the Gramene database and presence of CAREs were analysed as described above.

Table 3.1 Details of rice species used in the study of exon-intron structural polymorphism in *AOX1a* and *NDB2* genes. Extracted from Gramene database (www.gramene.org)

	Rice species	Species description
1	*O. barthii*	The progenitor of the West African cultivated rice, *O. glaberrima*. It belongs to the AA genome group and has 12 chromosomes and a genome size of 411Mb.
2	*O. brachyantha*	A wild rice which is a distant relative of cultivated rice (*O. sativa* ssp*. japonica* and *O. sativa* ssp *indica*). Only member in the F genome type. This diploid species is distributed in Western and Central Africa with a genome size of ~362 Mb
3	*O. glaberrima*	African cultivated rice, which was domesticated from extant progenitor, *O. barthii*. Similar to Asian and African rice. Represents diploid AA genome group, having 12 chromosomes and an estimated size of ~358 Mb.
4	*O. glumaepatula*	A wild rice from South America and belongs to the AA genome group and has 12 chromosomes with a genome size of 464 Mb.
5	*O. longistaminata*	A wild rice, perennial, tall (2 m or more), erect, and rhizomatous grass which is belong to AA genome type.
6	*O. meridionalis*	Australian wild rice that belongs to AA genome group. It has 12 chromosomes with a genome size of 435 Mb.
7	*O. nivara*	A wild rice from India that belongs to AA genome group. It has 12 chromosomes and a genome size of 448 Mb.
8	*O. punctata*	A wild native rice species from Africa. A diploid species belongs to BB genome type. It has 12 chromosomes and a genome size of 423 Mb.
9	*O. rufipogon*	A tetraploid wild rice with BBCC genome type. A perennial, tufted, and scrambling grass with nodal tillering; plant height variable (1-5 m) depending on the depth of water.
10	*O. sativa* ssp*. indica*	One of the two most commonly cultivated sub-species of rice (along with *O. sativa* ssp *japonica*) and is the most widely grown in hot climates of Southern Asia. The genome is very similar to that of japonica.
11	*O. sativa* ssp*. japonica*	Important model species for monocot plants and cereals and has a compact diploid genome of ~ 500 Mb (n=12). Fully sequenced.

3.3 Results

3.3.1 Both AOXs and NDHs were identified in japonica rice

Both type 1 AOXs and NDHs were identified in japonica rice. Sequence search (BLAST) against the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/) resulted in four gene sequences homologous to Arabidopsis AOXs: *AOX1a* (LOC_Os04g51150), *AOX1b* (LOC_Os04g51160), *AOX1c* (LOC_Os02g47200), and *AOX1d* (LOC_Os02g21300), and six sequence homologous to NDHs: *NDA1* (LOC_Os07g37730), *NDA2* (LOC_Os01g61410), *NDB1 (*LOC_Os06g47000), *NDB2 (*LOC_Os05g26660), *NDB3* (LOC_Os08g04630) and *NDC1* (LOC_Os06g11140) in rice sub-species japonica (Table 3.2). No hits were found for sequences corresponding to *AtAOX2*, confirming the fact that the AOX2 type is absent in monocots (Considine et al., 2002).

3.3.2 Both AOXs and NDHs were identified in indica rice

Homologous sequences for all AOXs and NDHs identified in japonica rice were also present in indica rice. A BLAST search against protein/genomic sequence database of the *O. sativa indica* group on the Gramene website identified four AOX isoforms: *AOX1a* (BGIOSGA014422), *AOX1b* (BGIOSGA014421), *AOX1c* (BGIOSGA005788) and *AOX1d* (BGIOSGA008063), and also six NDHs: *NDA1* (BGISOGA025955), *NDA2* (BGIOSGA004734), *NDB1* (BGIOSGA020655), *NDB2* (BGIOSGA018290), *NDB3* (BGIOSGA027677) and *NDC1* (BGIOSGA021679) in indica rice (Table 3.2). Pairwise alignment of CDS sequences revealed that all AOX and NDH isoforms from japonica rice were very similar to those of indica rice except *NDB3*. *NDB3* from japonica rice consisted of a shorter sequence compared to indica rice (Table 3.2).

Table 3.2 Alternative respiratory pathway genes identified in japonica and indica rice

Four isoforms of type 1 AOXs and six NDHs were identified in japonica rice as well as in indica rice. Sequence alignment studies showed that AOXs and NDHs from japonica rice were very similar to those from indica rice except *NBD3*. Sequences for japonica rice were downloaded from the Rice Genome Annotation Project website (rice.plantbiology.msu.edu) while sequences for indica were downloaded from the Gramene website (www.gramene.org). aa-amino acids.

Alternative oxidases					Alternative dehydrogenases				
Gene name	Japonica rice		Indica rice		Gene name	Japonica rice		Indica rice	
	Gene Locus/ID	Protein length (aa)	Gene Locus/ID	Protein length (aa)		Gene Locus/ID	Protein length (aa)	Gene Locus/ID	Protein length (aa)
AOx1a	LOC_Os04g51150	332	BGIOSGA014422	332	*NDA1*	LOC_Os07g37730	562	BGISOGA025955	561
AOX1b	LOC_Os04g51160	335	BGIOSGA014421	335	*NDA2*	LOC_Os01g61410	499	BGIOSGA004734	497
AOX1c	LOC_Os02g47200	345	BGIOSGA005788	345	*NDB1*	LOC_Os06g47000	588	BGIOSGA020655	588
AOX1d	LOC_Os02g21300	339	BGIOSGA008063	339	*NDB2*	LOC_Os05g26660	575	BGIOSGA018290	575
					NDB3	LOC_Os08g04630	357	BGIOSGA027677	580
					NDC1	LOC_Os06g11140	548	BGIOSGA021679	548

3.3.3 AOX proteins were identified as members of the Ferritin-like superfamily

Conserved domain searches confirmed that all japonica and indica putative AOX proteins belong to the Ferritin-like superfamily of di-iron containing four-helix-bundle proteins (http://www.ncbi.nlm.nih.gov/Structure/cdd/) as of Arabidopsis AOXs (Figure 3.2). This confirms the presence of iron-binding sites in all identified AOX proteins in both rice groups similar to Arabidopsis AOXs, which is considered as a positive identification characteristic of AOXs (McDonald et al., 2003). Pairwise alignment of amino acid sequences showed that AOXs from both rice sub-species share 100% amino acid (aa) residue identity (Appendix A) while alignment of rice and Arabidopsis AOX sequences showed that rice AOX1a, AOX1b, AOX1c and AOX1d share 85%, 73%, 77% and 70% of amino acid residue identity with corresponding Arabidopsis AOXs.

There are two highly conserved cysteine (Cys) residues, CysI and CysII, in most plant AOXs, which are important in the regulation of AOX activity (Umbach et al., 2006; McDonald, 2008). Although rice AOX1a, AOX1c and AOX1d proteins shared these conserved Cys residues similar to Arabidopsis AOXs, they were replaced by serine (Ser) in AOX1b in both japonica and indica rice (Umbach et al., 2006; McDonald, 2009) (Figure 3.2).

3.3.4 NDH proteins were identified as members of the Pyr-redox superfamily

All three groups of NDH proteins; NDA, NDB and NDC from both groups of rice were identified as members of pyridine nucleotide disulphide oxidoreductase (Pyr-redox) superfamily as of Arabidopsis NDHs, in which two characteristic GXGXXG motifs are present. The first motif facilitates NAD(P)H binding (NADB domain) while the second motif is responsible for specifically binding a substrate and catalyzing a particular enzymatic reaction (http://www.ncbi.nlm.nih.gov/Structure/cdd/). Both of these domains were present in NDA (Figures 3.3), NDB (Figure 3.4) and NDC (Figure 3.5) family members from both rice groups as well as in Arabidopsis. In addition to the

NADB domain, NDB proteins from Arabidopsis, indica and japonica contained EF-hand domains, except japonica NDB3, that facilitate binding of Ca^{2+} (Figure 3.4). Ca^{2+} binding EF-hand motifs were also absent in both NDA and NDC group members. Pairwise alignment of protein sequences of NDHs from japonica and indica rice showed that they share very similar sequences except that NDB3 having shorter sequence (Appendix B). NDB3 protein sequence of japonica rice is shorter (357 aa) compared to indica NDB3 (580 aa) and that of Arabidopsis (580 aa) (Figure 3.4). Protein sequence alignment analysis of rice and Arabidopsis revealed that rice NDA1 and NDA2 share 71% and 77% of amino acid residue identity with AtNDA1 and AtNDA2 respectively while NDB1, NDB2 and NDB3 share 65%, 64% and 63% amino acid residue identity with corresponding Arabidopsis NDBs. Rice NDC1 also shares 65% of amino acid sequence identity with AtNDC1.

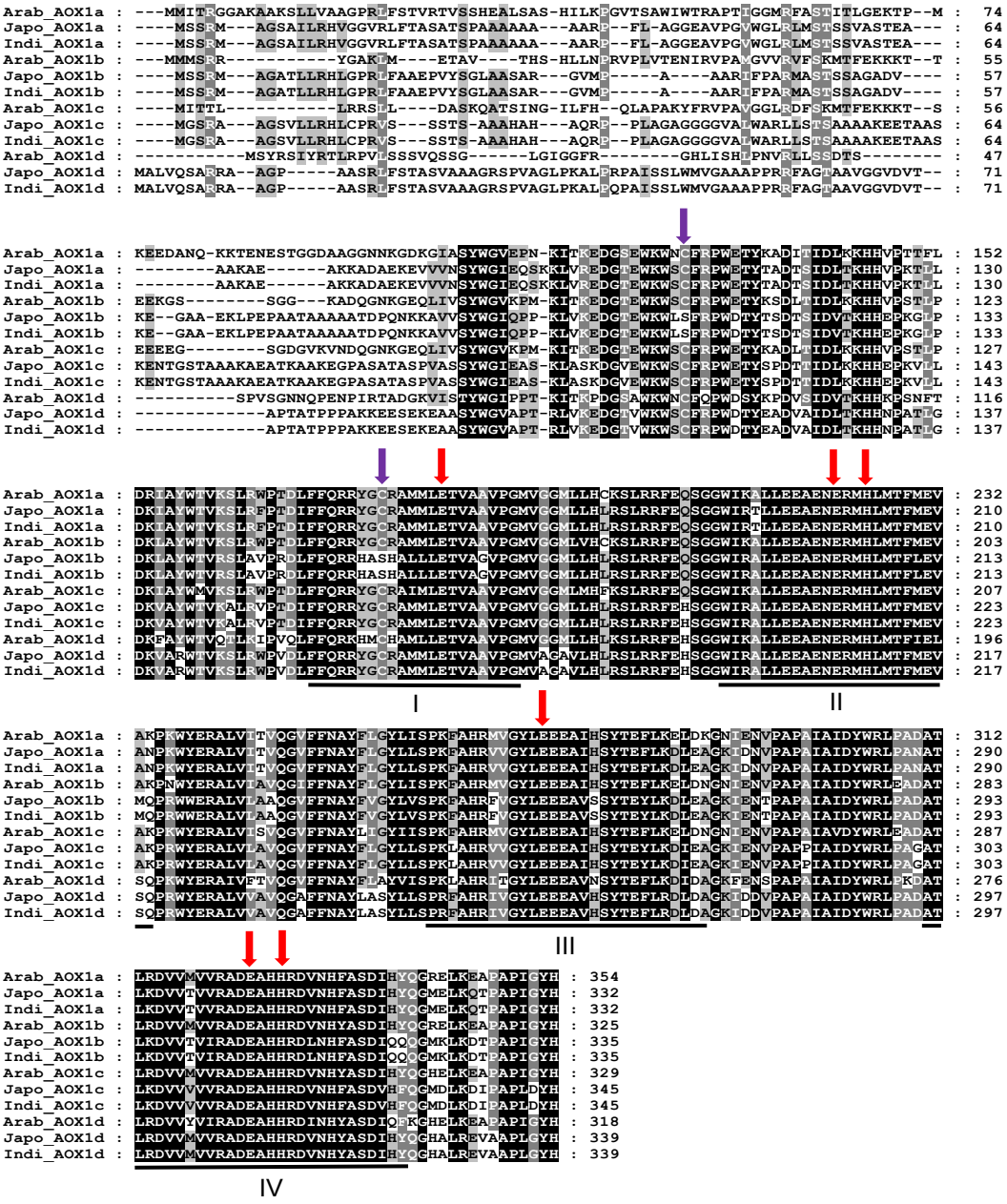


Figure 3.2 A multiple sequence alignment of full length AOX proteins from Arabidopsis, japonica and indica rice

Purple arrows point to conserved Cysteine (C) or substitute Serine (S) residues (in rice AOX1b), which are responsible for the regulation of AOX activity. Underlines indicate four alpha-helix bundles found in the core of AOX and numbered as I–IV. The red arrows point to conserved duplicate metal ligands, glutamate and histidine (EXXH) and two additional glutamate (E) residues within four-helix bundle. Three consecutive glutamate (E) residues are located on the third ion-binding site in all AOXs from Arabidopsis as well as two rice groups, which are conserved in most AOX proteins (McDonald, 2009). Rice AOX1a, AOX1b, AOX1c and AOX1d share 85%, 73%, 77% and 70% of amino acid residue identity with corresponding Arabidopsis AOXs.

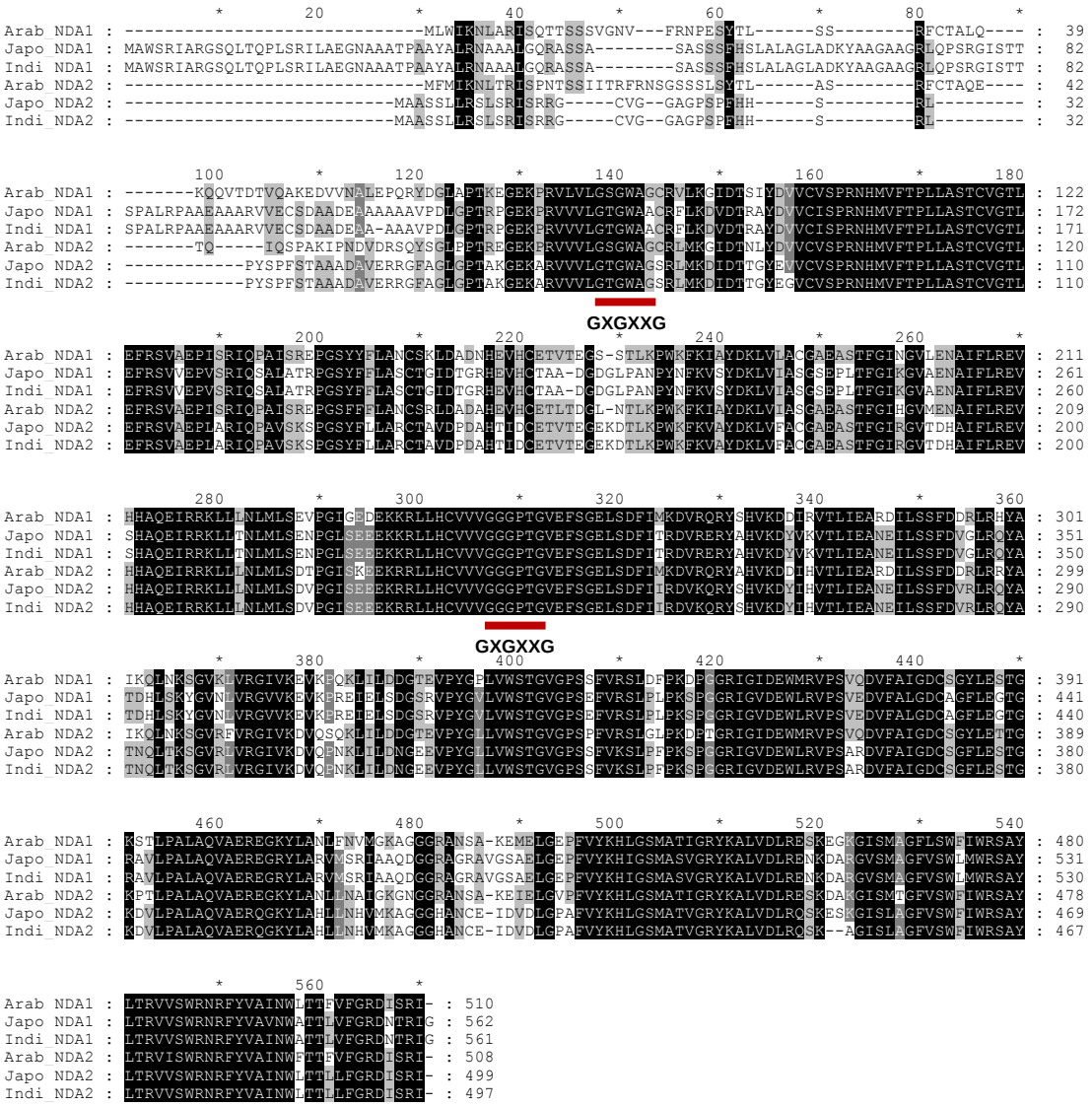


Figure 3.3 A multiple sequence alignment of full length NDA proteins from Arabidopsis, japonica and indica rice

The positions of the GXGXXG conserved domains are indicated by the red bars below the alignment. The first GXGXXG motif facilitates NAD(P)H binding (NADB domain) and the second is responsible for specifically binding a substrate and catalyzing a particular enzymatic reaction. EF-hand motifs are absent form NDA proteins. Rice NDA1 and NDA2 share 71%, and 77% of amino acid residue identity with Arabidopsis NDA1 and NDA2 respectively.

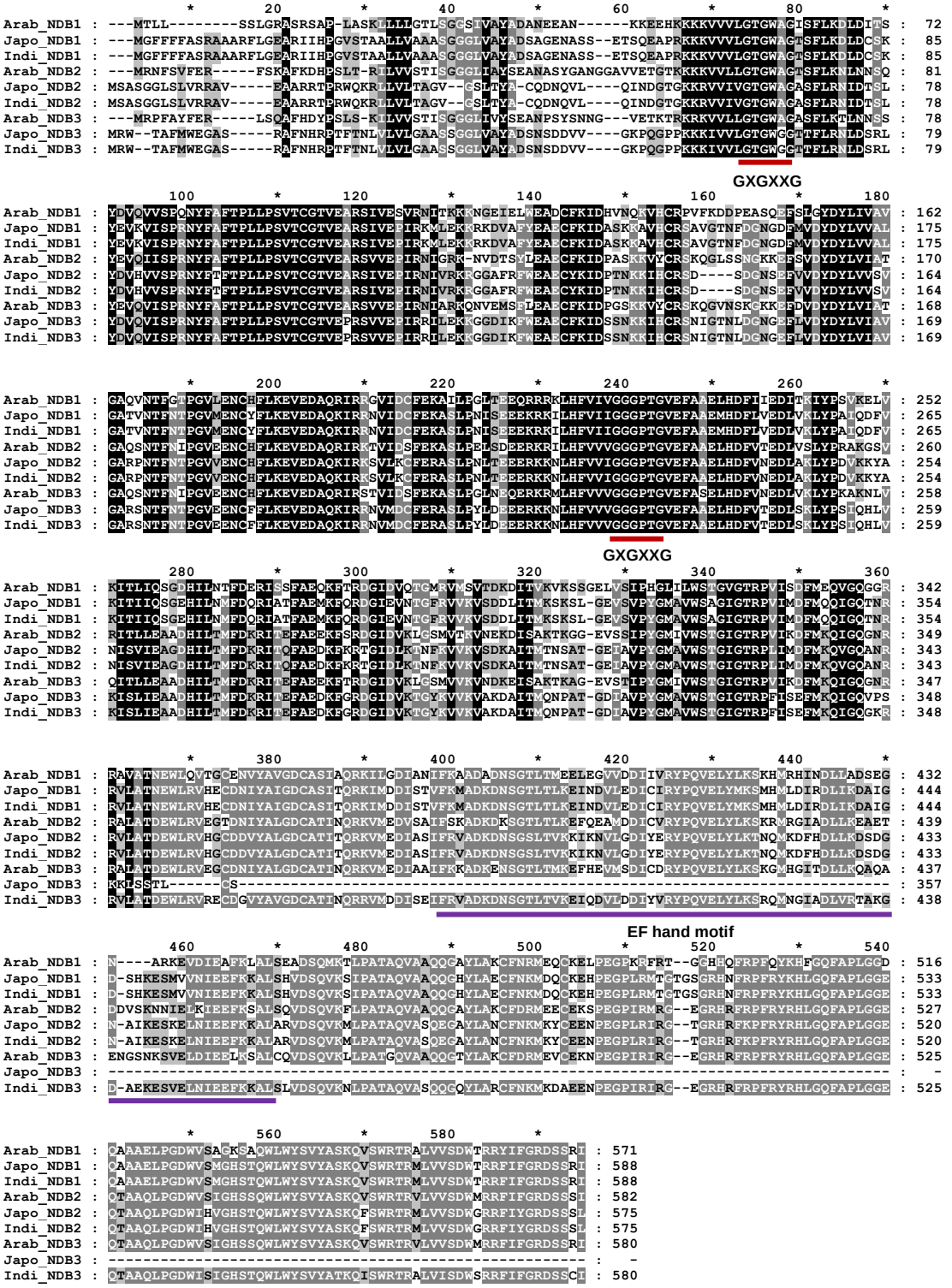


Figure 3.4 A multiple sequence alignment of full length NDB proteins from Arabidopsis, japonica and indica rice

The position of the GXGXXG conserved domains; characteristic of the family “Pyr-redox”, are indicated by the red bars below the alignment. The purple bar below the alignment represents the EF-hand motif which is responsible for Ca^{2+} binding. EF-hand sequence is absent from japonica rice NDB3 and it has relatively shorter sequence compared to indica rice and Arabidopsis. Rice NDB1, NDB2 and NDB3 share 66%, 64% and 63% amino acid residue identity with Arabidopsis NDB1, NDB2 and NDB3 respectively.

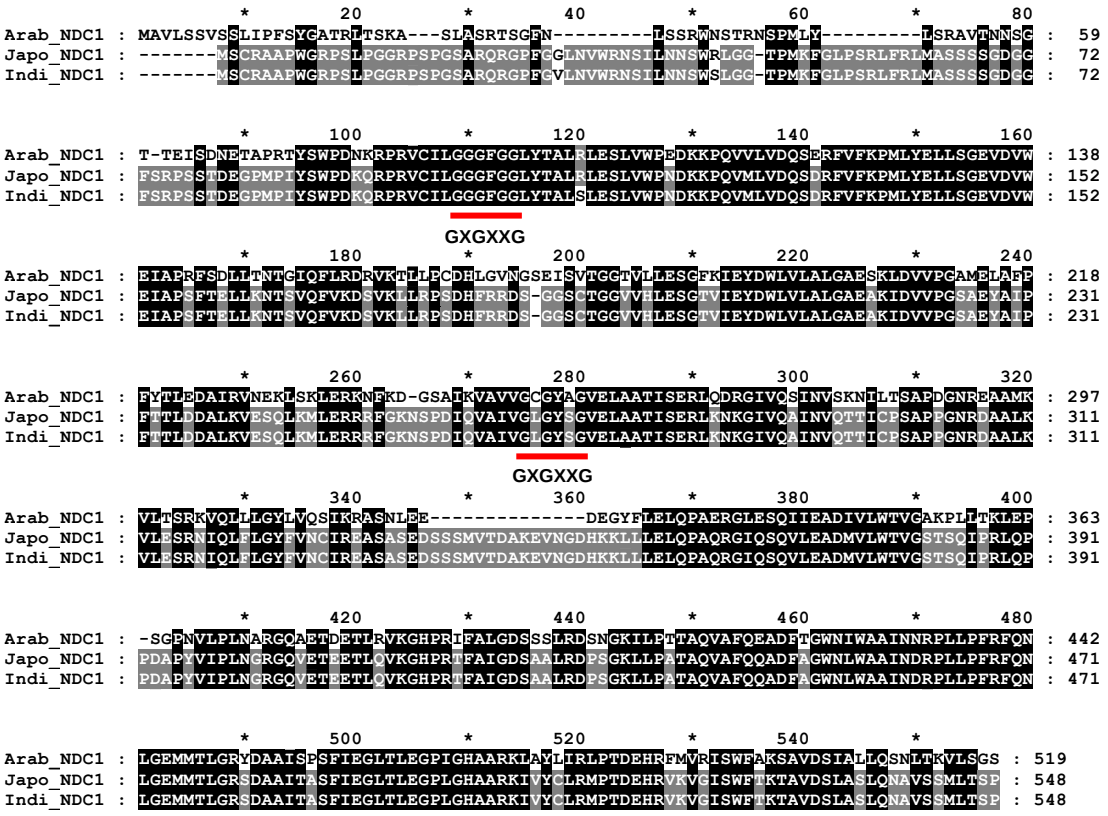


Figure 3.5 A multiple sequence alignment of full length NDC proteins from Arabidopsis, japonica and indica rice

The position of the GXGXXG conserved domains are denoted by red bars bellow the alignment. The first GXGXXG motif facilitates NAD(P)H binding (NADB domain) and the second is responsible for specifically binding a substrate and catalyzing a particular enzymatic reaction. EF-hand motifs are absent from NDC proteins in Arabidopsis as well as in rice. NDC1 sequence from Arabidopsis is shorter than rice NDC1 sequences**.** Rice NDC1 sequences share 65% of amino acid residue identity with Arabidopsis NDC1.

3.3.5 All rice AOX proteins are predicted be targeted only to mitochondria while some NDH proteins are predicted to be dual targeted

Some nuclear encoded proteins appear to be targeted to more than one organelle and these are designated as dual targeted proteins (Elhafez et al., 2006; Carrie et al., 2008). The subcellular locations of rice AOX and NDH proteins were assessed using a number of subcellular prediction programs. From these programs, AOXs from both japonica and indica rice were predicted to be targeted to mitochondria, having cleavable N-terminal targeting signals (Table 3.3). No peroxisomal type 1 targeting sequences (PTS1) at the C-terminus were identified according to the prediction program “PredplantPTS1” confirming that AOXs do not target to peroxisomes. Whilst NDA1 protein from both rice sub-species was predicted to be targeted to mitochondria, NDA2 was predicted to be dual targeted to both mitochondria and peroxisomes based on predicted PTS1 signals. It is interesting to note that NDB1 and NDB2 from both rice sub-species were predicted to be dual targeted to mitochondria and peroxisomes but NDB3 was predicted to be localized only to mitochondria. NDC1 from both rice groups was predicted to be dual targeted to both mitochondria and chloroplasts but not targeted to peroxisomes (Table 3.3).

Table 3.3 Subcellular localization of identified alternative respiratory proteins from japonica and indica rice

Prediction of subcellular targeting of rice alternative respiratory proteins using publicly available subcellular localization prediction programs; TargetP (http://www.cbs.dtu.dk/services/TargetP/), Predotar (http://urgi.versailles.inra.fr/Tools/), Plant-mPLOC (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) and ChloroP1, (http://www.cbs.dtu.dk/Servies). Proteins targeting to the peroxisomes were predicted using PredPlantPTS1 (http://ppp.gobics.de/).

Protein	Targeting organelle predicted by different programs					
	Plant- mPLOC	**TargetP**	**MitoProtII**	**ChloroP1**	**Predotar**	**PredPlantPTS1**
AOX1a	Mitochondria	Mitochondria	Mitochondria	Chloroplast	Mitochondria	Not targeted to peroxisomes
AOX1b	Mitochondria	Mitochondria	Mitochondria	Chloroplast	Mitochondria	Not targeted to peroxisomes
AOX1c	Mitochondria	Mitochondria	Mitochondria	Chloroplast	Mitochondria	Not targeted to peroxisomes
AOX1d	Mitochondria	Mitochondria/ Chloroplast	Mitochondria	Chloroplast	Mitochondria	Not targeted to peroxisomes
NDA1	Mitochondria	Mitochondria	Mitochondria	Chloroplast	Mitochondria	Not targeted to peroxisomes
NDA2	Mitochondria	Mitochondria	Mitochondria	Chloroplast	Mitochondria	Peroxisomes
NDB1	Mitochondria	Mitochondria	Mitochondria	Not targeted to chloroplast	Probably mitochondria	Peroxisomes
NDB2	Mitochondria	Mitochondria	Mitochondria	Not targeted to chloroplast	Possibly mitochondria	Peroxisomes
NDB3	Mitochondria	Mitochondria	Mitochondria	Not targeted to chloroplast	Possibly ER	Not targeted to peroxisomes
NDC1	Mitochondria	Chloroplast	Mitochondria	Chloroplast	Possibly plastid	Not targeted to peroxisomes

3.3.6 Protein phylogeny reveals that rice AOX proteins can be grouped into four subfamilies along with other monocot species

Protein phylogeny was used in order to identify different AOX subfamilies in selected monocot species and to see whether the identified rice AOX sequences were grouped accordingly. Phylogenetic analysis showed that all AOX orthologs from selected monocot species were clustered into four distinct clades (Figure 3.6); type a, b, c and d. Rice AOXs from both sub-species were also grouped into four clades accordingly (indicated by red arrows) and showed very close similarity. The phylogenetic tree also revealed that there was a close similarity between type “a” and type “c” group members compared to type “b” or type “d”. There was obvious further clustering of members within the main clades. For example, the type “b” clade was further divided into four sub-groups and AOX1b from the two rice sub-species were grouped within the type “b” clade as a separate group. Similarly, type “a” members were grouped into two major sub-groups (Figure 3.6).

3.3.7 Protein phylogeny reveals that rice NDH proteins can be grouped into three families along with other monocot species

Phylogenetic analysis of NDHs showed clustering of monocot alternative dehydrogenases into three discrete clades; NDA, NDB and NDC (Figure 3.7). There was very clear further division of the NDB clade into three sub-groups as NDB1, NDB2 and NDB3. The NDA clade was further divided into two groups as NDA1 and NDA2. The rice NDH proteins also clustered into these NDA, NDB and NDC families (indicated by red arrows). It was very clear that NDA and NDB families had a higher degree of homology with each other than with the NDC family, as reported for Arabidopsis (Michalecka et al., 2003).

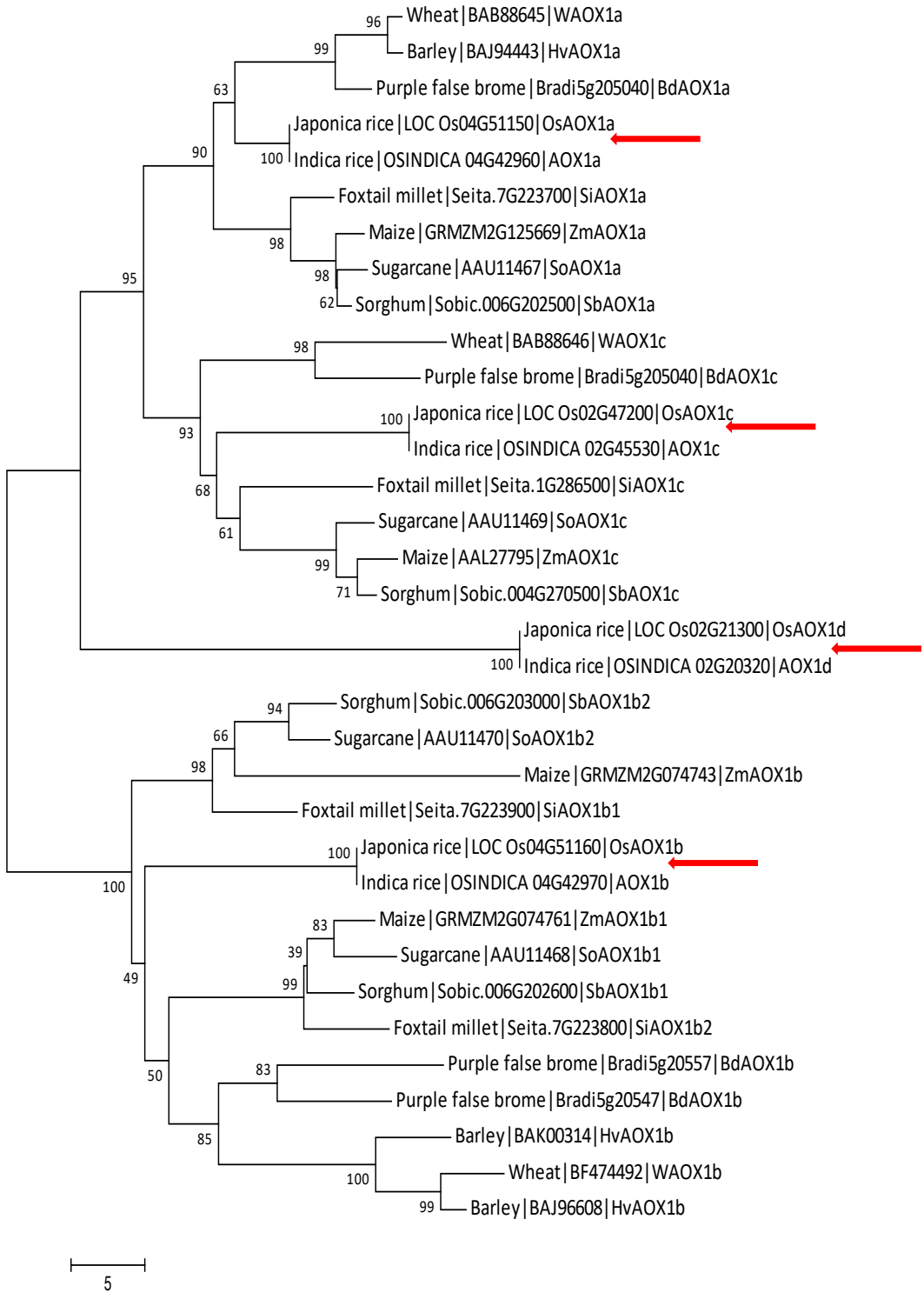


Figure 3.6 Representative protein phylogenetic tree of monocot species with AOX protein sequences showing four major clades, AOX1a, AOX1b, AOX1c and AOX1d

The phylogenetic tree was constructed using the Neighbor-Joining method in MEGA 7 (www.megasoftware.net/). Numbers at each node are the percentage bootstrap values of 1000 replicates. The scale bar indicates the number of amino acid substitutions at each site.

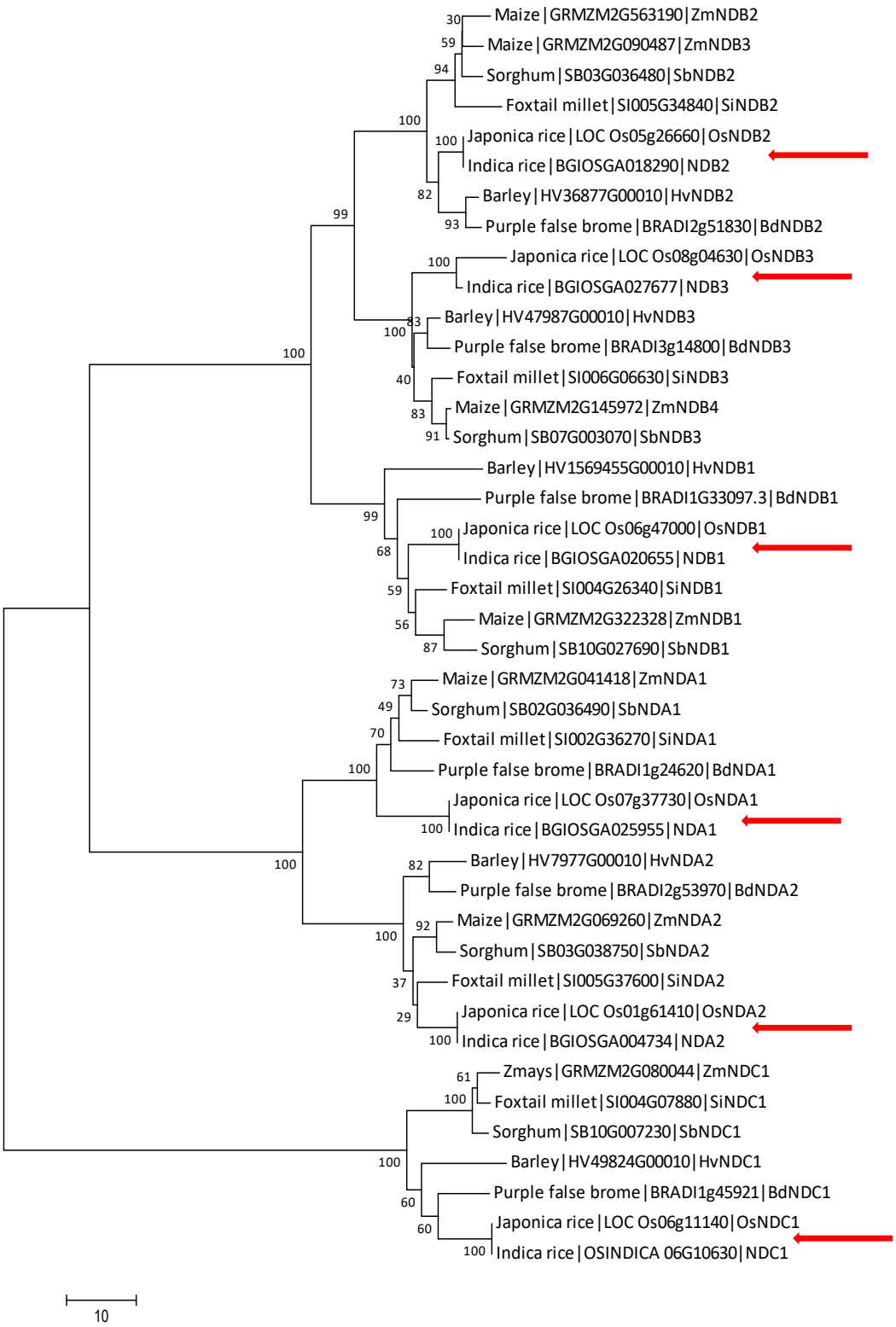


Figure 3.7 Representative protein phylogenetic tree of monocot species with NDH protein sequences showing three major clades, NDA, NDB and NDC

The phylogenetic tree was constructed using the Neighbor-Joining method in MEGA 7 (www.megasoftware.net/). Numbers at each node are the percentage bootstrap values of 1000 replicates. The scale bar indicates the number of amino acid substitutions at each site.

3.3.8 Exon-intron structural polymorphism in AP genes is present within and between species

A comparative study of the exon-intron structures of alternative respiratory pathway genes in japonica and indica rice was conducted to explore gene structure polymorphism in two rice sub-species. Information on Arabidopsis exon-intron structures were also included in the study to explore how monocot gene structures have deviated from those in the dicots. The study found that there are variations in exon-intron gene structure between dicots and monocots as well as within the two rice sub-species.

AOX1 genes from Arabidopsis, japonica and indica rice groups have a conserved structure consisting of four exons interrupted by three introns, with the exception of rice *AOX1b* (Figures 3.8, 3.9, 3.10 and 3.11). The *AOX1b* genes from both rice sub-species have three exons separated by two introns (Figure 3.9). It is suggested that this could be due to the loss of the second intron in the other *AOX* genes (Considine et al., 2002).

NDA gene structures from Arabidopsis and the two rice groups also show variation. *AtNDA1* has 8 coding sequences separated by 7 non-coding regions, while *NDA1* from both japonica and indica rice have three coding regions separated by two non-coding regions (Figure 3.12). The exon-intron structure of *NDA1* from both rice groups is similar to that of rice *AOX1b* (Figure 3.9). All three plant species, Arabidopsis, japonica and indica rice, share a similar *NDA2* gene structure: 8 exons interrupted by 7 introns (Figure 3.13). A similar number of exons and introns were present in NDA gene family members in Arabidopsis (Figures 3.12 and 3.13), but in the two rice groups, there is a reduction of number of exons and introns in *NDA1* compared to *NDA2* (Figures 3.12 and 3.13).

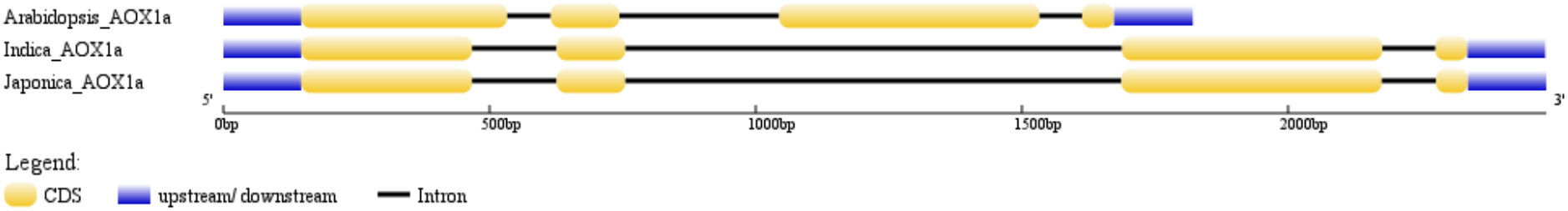


Figure 3.8 Comparison of exon-intron structure of *AOX1a* gene from Arabidopsis, japonica and indica rice

AOX1a from all three plant groups are consisted of conserved structure of plant AOXs; four exons interrupted by three introns.

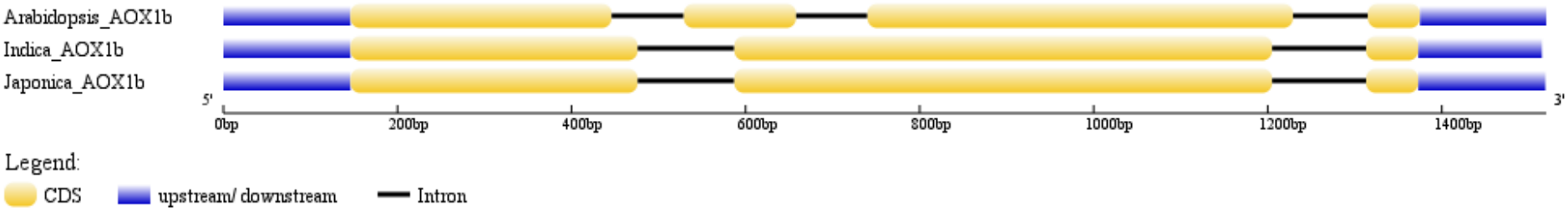


Figure 3.9 Comparison of exon-intron structure of *AOX1b* gene from Arabidopsis, japonica and indica rice

AOX1b from Arabidopsis is consisted of conserved structure of plant AOXs; four exons interrupted by three introns. But, gene structure from both rice groups are consisted of three exons separated by two introns.

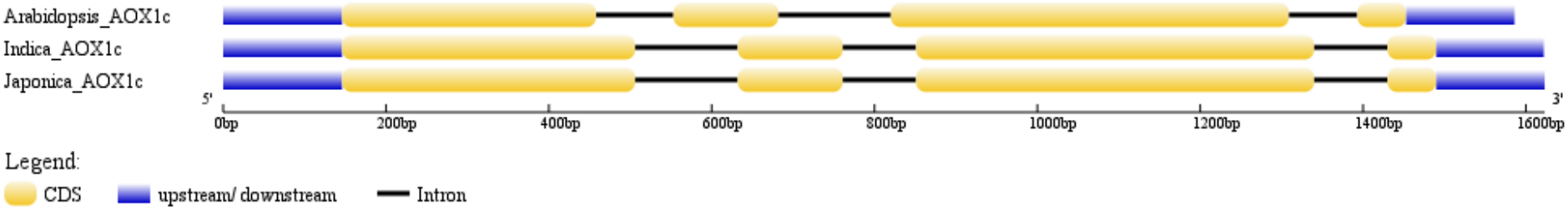


Figure 3.10 Comparison of exon-intron structure of *AOX1c* gene from Arabidopsis, japonica and indica rice

AOX1c from all three plant groups are consisted of conserved structure of plant AOXs; four exons interrupted by three introns.

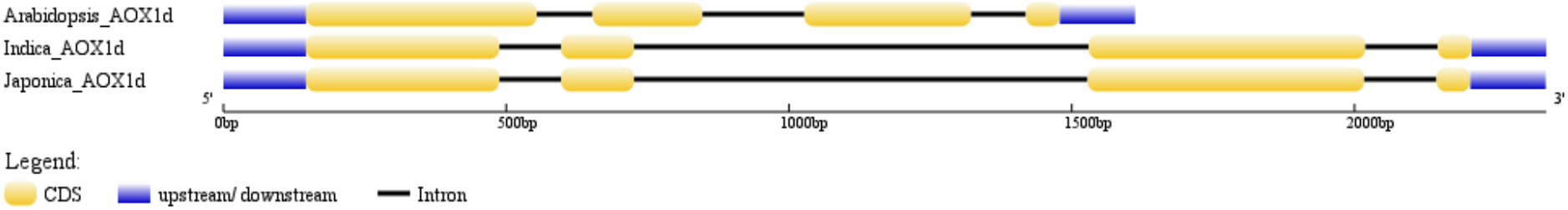


Figure 3.11 Comparison of exon-intron structure of *AOX1d* gene from Arabidopsis, japonica and indica rice

AOX1d from all three plant groups are consisted of conserved structure of plant AOXs; four exons interrupted by three introns.

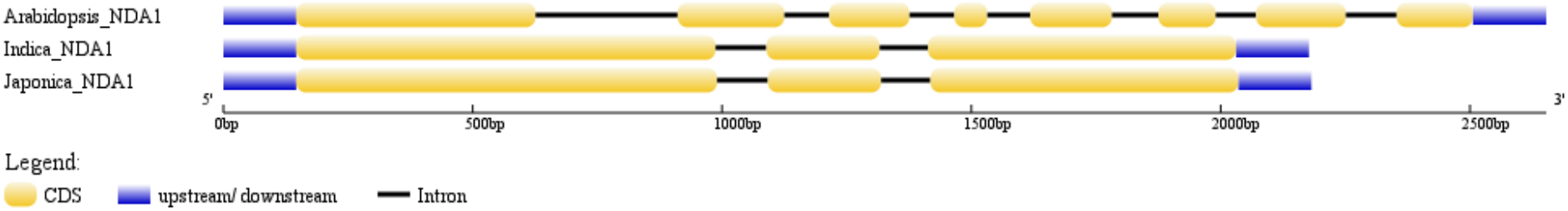


Figure 3.12 Comparison of exon-intron structure of *NDA1* gene from Arabidopsis, japonica and indica rice

Exon-intron structure of *NDA1* from Arabidopsis is consisted of 8 exons separated by 7 introns. However, exon-intron structure of *NDA1* from both rice groups contains 3 exons separated by two exons showing the similar structure as seen in rice *AOX1b* in Figure 3.9.

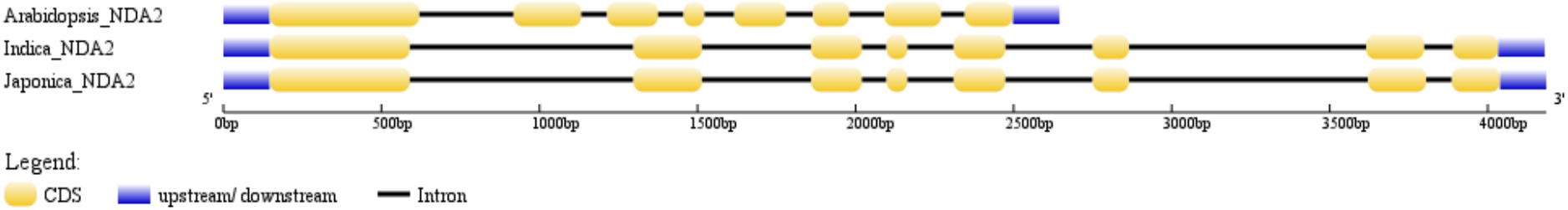


Figure 3.13 Comparison of exon-intron structure of *NDA2* gene from Arabidopsis, japonica and indica rice

Exon-intron structure of *NDA2* from all three plant groups, Arabidopsis, japonica and indica rice are similar with 8 exons separated by 7 introns. Exon-intron structure of *NDA1* and *NDA2* from Arabidopsis shares similar structure (Figure 3.12).

The comparison of exon-intron structures of the NDB gene family also revealed variations in the number of exons and introns between Arabidopsis and the two rice groups (Figures 3.14, 3.15, 3.16 and 3.17). Japonica and indica rice share similar exon-intron gene structure for *NDB1* and *NDB2*, 10 exons separated by 9 introns (Figures 3.15 and 3.16). *NDB3* from indica rice is also has a similar exon-intron structure, but that from japonica rice has a different structure. Although it has 9 exons, only 5 of them are coding exons (www.gramene.org, http://rice.plantbiology.msu.edu/) and the rest are situated in the 3' UTR as non-coding exons. As a result, the japonica NDB3 protein sequence is shorter (357 aa) than the indica NDB3 protein (580 aa), which has 10 coding exons (Figures 3.14 and 3.17). However, analysis of organization of *NDB3* from 10 other rice species revealed that they all share the exon-intron structure similar to indica rice with 10 exons divided by 9 introns mostly producing translation length of 580 aa. This confirms that this is the predominant structure amongst rice species. Therefore, it is suspicious that only japonica rice deviates from this structure and appears truncated and may reflect a sequencing error for the sequence uploaded into the database.

NDB gene family members from Arabidopsis show varying numbers of exons and introns in their gene structure. *NDB1* has 9 exons interrupted by 8 introns while *NDB2* and *NDB3* have 11 and 10 exons, respectively. It is reported that *AtNDB2* has two splice variants (www.gramene.org), one with 10 coding exons producing a translation length of 582 aa, while the second splice variant has 11 exons resulting in a translation length of 619 aa. NDC family members from both rice groups showed similar exon-intron structures with 11 exons separated by 10 introns, while *NDC1* from Arabidopsis has 10 exons (Figure 3.18).

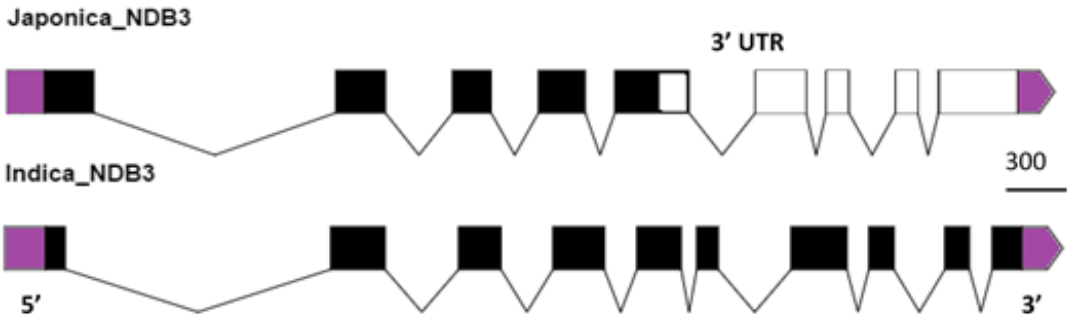


Figure 3.14 Exon-intron structure of *NDB3* in japonica and indica rice

Exon-intron structure of *NDB3* from indica rice is consisted of 10 coding exons while that from japonica rice contains only 5 coding exons (www.gramene.org). Rest of them are located in the UTR as non-coding exons. Coding exons are indicated by black boxes and non-coding exons are indicated by empty boxes. Introns are indicated by black lines. Purple colour indicates untranslated regions.

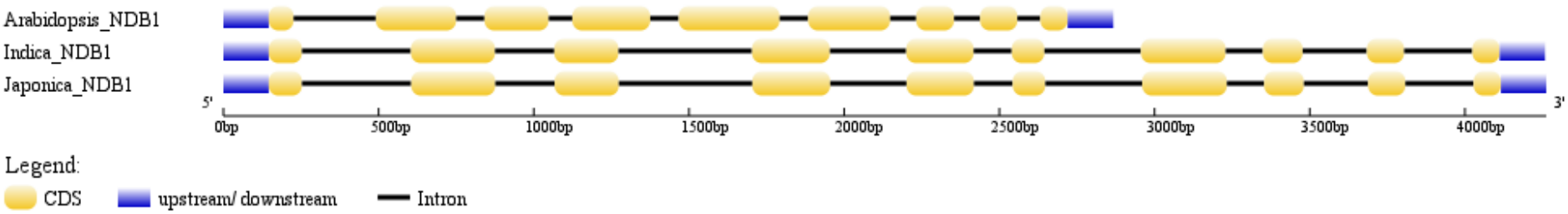


Figure 3.15 Comparison of exon-intron structure of *NDB1* gene from Arabidopsis, japonica and indica rice

Exon-intron structure of *NDB1* from Arabidopsis is consisted of 9 exons separated by 8 introns. Two rice groups share a similar exon-intron structure for *NDB1* with 10 exons separated by 9 introns. Arabidopsis *NDB1* is lack of one exon compared to two rice groups.

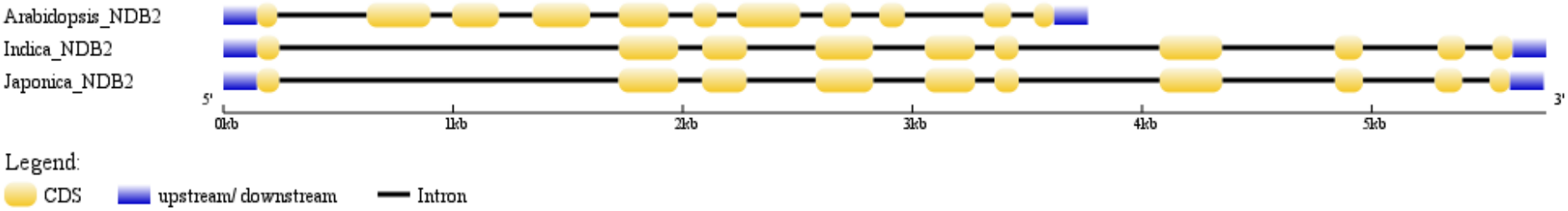


Figure 3.16 Comparison of exon-intron structure of *NDB2* gene from Arabidopsis, japonica and indica rice

Arabidopsis is consisted of an exon-intron structure of *NDB2* with 11 exons interrupted by 10 introns while two rice groups share similar structure with 10 exons separated by 9 introns similar to their NDB1 exon-intron structure (Figure 3.15).

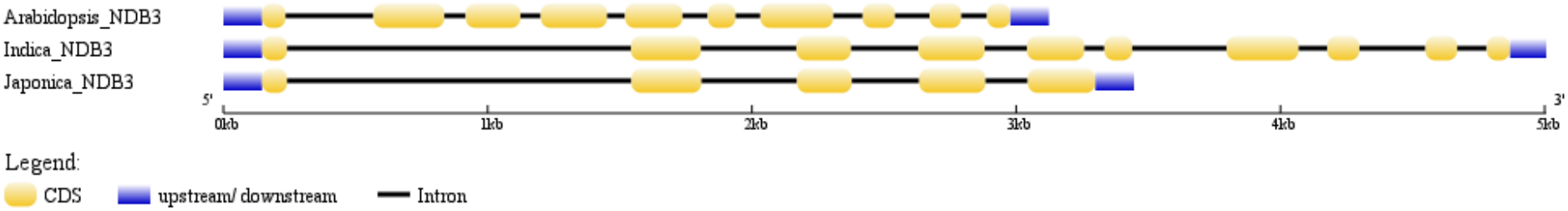


Figure 3.17 Comparison of exon-intron structure of *NDB3* gene from Arabidopsis, japonica and indica rice

Arabidopsis and indica rice is consisted of an exon-intron structure of *NDB3* with 10 exons interrupted by 9 introns. *NDB3* from japonica rice group is consisted of 9 exons, only 5 of them are coding exons (www.gramene.org).

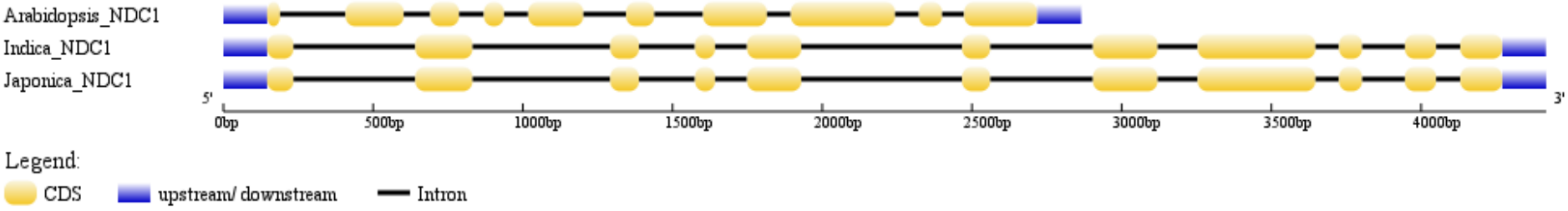


Figure 3.18 Comparison of exon-intron structure of *NDC1* gene from Arabidopsis, japonica and indica rice

Two rice groups share similar exon-intron structure of *NDC1* with 11 exons separated by 10 introns while Arabidopsis has gene structure with 10 exons separated by 9 introns.

3.3.9 Exon-intron structural polymorphism in *AOX1a* is higher than that of *NDB2* among different rice species

The loss or gain of introns creates complexity of gene structure, which is a key evolutionary mechanism of most gene families (Cao, 2016). To understand the evolution and diversity of rice AP genes, exon-intron structures of *AOX1a* and *NDB2* from 11 rice species were investigated. This analysis revealed that cultivated rice, *O. glaberrima* (African rice) and two Asian rice sub-species of *O.sativa* ssp *indica* and *O. sativa* ssp*. japonica*, share a common exon-intron gene structure for *AOX1a* with four coding exons separated by three introns (Figure 3.19). *O. brachyantha*, a wild rice species, also shared this structure, but it has three more non-coding exons in the 5' UTR (www.gramene.org). Multiple alignment of protein sequences from these four rice species showed a very similar sequence pattern with only a few amino acid residue mismatches. All other wild rice species have 5-8 exons, except *O. punctata* (BB genome type), which has only two coding exons (Figure 3.19).

NDB2 structures showed less diversity compared to those of *AOX1a*. Apart from *O. longistaminata* and *O. punctata*, all other rice species shared a similar exon-intron organization in their *NDB2* gene, with 10 exons separated by 9 introns (Figure 3.20). *O. longistaminata NDB2* has 9 exons interrupted by 8 introns, while that of *O. punctata* has 13 exons separated by 12 introns, the highest number of exons and intron found in rice NDB2 genes. Multiple alignment of amino acid sequences showed all rice species have very similar protein sequences with 575 amino acid residues, except *O. brachyantha* (579 aa), *O. longistaminata* (547 aa) and *O. punctata* (913 aa).

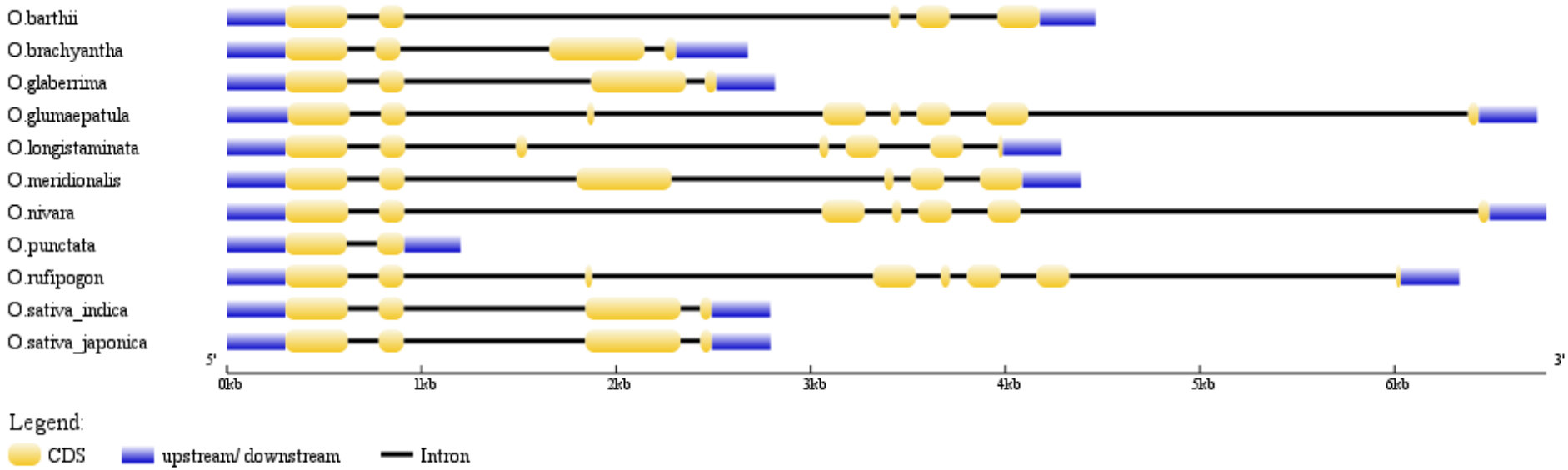


Figure 3.19 Exon-intron structural organization of *AOX1a* gene in different rice species

Exon-intron structural organization of *AOX1a* gene showed high polymorphism among different rice species. Cultivated rice, *O. glaberrima* (African rice), two Asian rice sub-species of *O.sativa* ssp *indica* and *O. sativa* ssp. *japonica* and *O. brachyantha* (a wild rice) shared exon-intron gene structure with four coding exons separated by three introns, which is the conserved AOX structure in plants (Considine et al., 2002). *O. punctata* has the exon-intron structure with the lowest number of exons (2) while all other wild rice species were consisted of 5-8 number of exons.

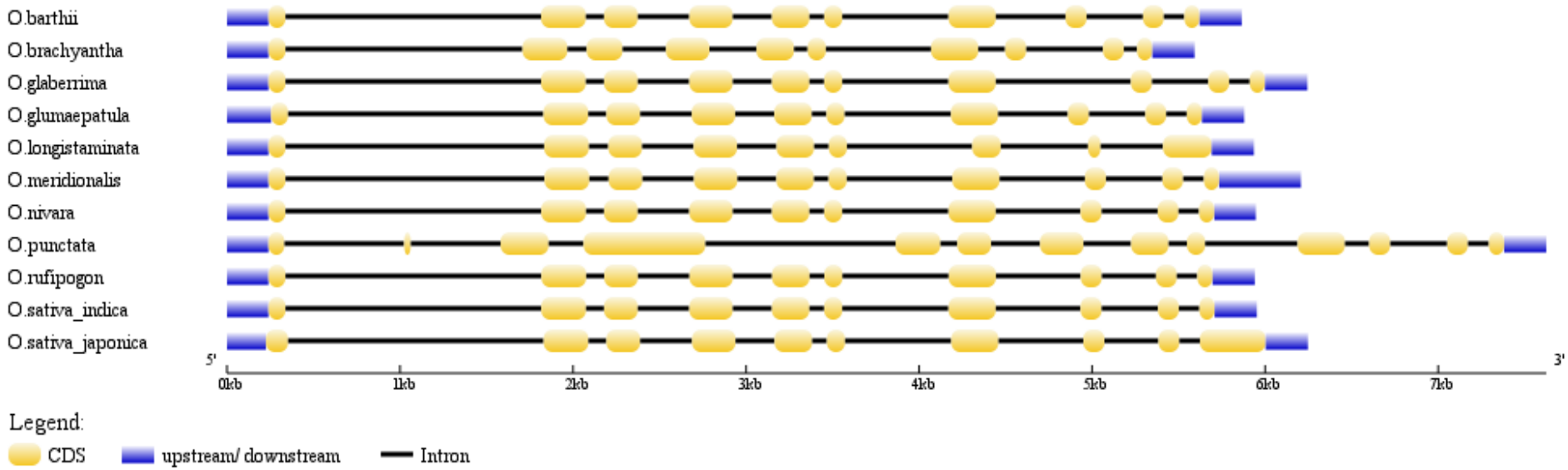


Figure 3.20 Exon-intron structural organization of *NDB2* gene in different rice species

All rice species except *O. longistaminata* and *O. punctata* share a similar exon-intron structure with 10 exons separated by 9 introns. The exon-intron structures of *O. longistaminata* and *O. punctata* are consisted of 9 exons separated by 8 introns and 13 exons separated by 12 introns respectively.

3.3.10 Three reference genes were selected for normalization of qRT-PCR data

The accuracy of results obtained by qRT-PCR depends on the accurate transcript normalization using stably expressed genes, reference genes (Guenin et al., 2009), across tested samples. Therefore, systematic validation of reference is important for generating reliable, accurate data in qRT-PCR analyses. qRT-PCR assay was carried out to examine the expression stability of 6 candidate reference genes (Table 2.3) across a range of cDNA samples by comparing quantification cycle (Cq) values (Sections 2.3.4 and 2.3.5). The coefficient of variance (CV) of Cq values was used as the indicator of the expression stability of a particular gene (Fan et al., 2013) and three genes with lowest CV values were selected as reference genes for normalization of qRT-PCR data in subsequent gene expression analyses.

Table 3.4 Coefficient of variation (CV) of Cq values from different reference genes

Reference gene	Cq range	Mean Cq	CV
Os18S	14.6-22.7	19.1	0.16
OsGAPDH	22.5-34.1	26.4	0.18
OsPpIase	27.6-31.2	29.9	0.05
OsTubulin	21.8-31.6	26.2	0.12
OsEIF1	24.8-27.2	26.3	0.03
OsActin	25.5-33.4	28.4	0.10

The expression levels of 6 reference genes varied widely, with Cq values ranging from 14 to 34 (Table 3.4 and Figure 3.21). Although *Os18S* was the most highly expressed candidate reference gene, with a mean Cq value of 19.1, its expression variability in different tissue samples was also higher with Cq values ranging from 14.6 to 22.7. Three genes, *OsEIF1*, *OsPpIase* and *OsActin* maintained a relatively constant expression levels in different tissue samples tested (Figure 3.21) and therefore they were selected as reference genes to normalize gene expression data.

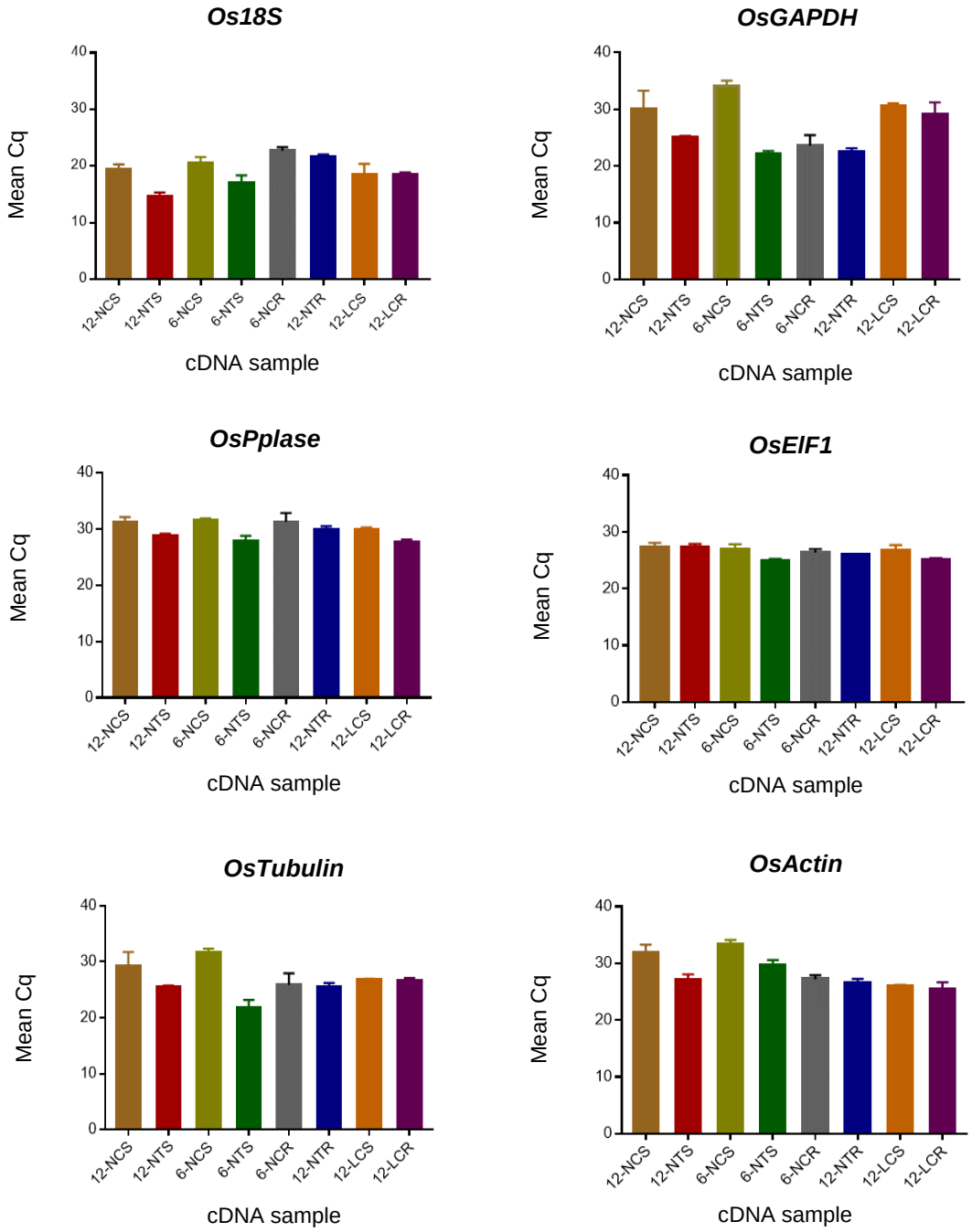


Figure 3.21 Mean Cq values in different cDNA samples from different candidate reference genes

qRT-PCR assay was carried out to examine the expression stability of 6 candidate reference genes, *OsActin*, *OsTubulin*, *OsGAPDH*, *OsPplase*, *OsElF1* and *Os18S*, across a range of cDNA samples, representing salt-treated and control shoots and roots from cultivar Nipponbare and Langi. Samples were collected 6 and 12 days after the salt treatment.

NCS, Nipponbare control shoots; NTS, Nipponbare treated shoots; NCR, Nipponbare control root; Nipponbare treated roots; LCS, Langi control shoots; LCR, Langi control roots.

3.3.11 Transcript abundance of rice AP genes change in response to chemical disruption of the mETC

All rice AOX and NDH sequences were found in expression sequence tag (EST) databases (http://www.ncbi.nlm.nih.gov) confirming that all sequences have been detected at the transcript level in certain tissue type under stressed or non-stressed conditions. Expression patterns of alternative pathway genes under oxidative stress were studied to identify the stress-responsive genes in rice. Antimycin A (AA) and KCN, inhibitors of traditional electron transport pathway components, Complex III and IV respectively, were used to create oxidative stress in plants. Rice seedlings were exposed to 20 µM AA or 5 mM KCN and changes in transcription levels in roots and shoots were determined using qRT-PCR technique at predetermined time-points as described in Section 3.2.7. Transcript data were normalized against geometric average (Vandesompele et al., 2002) of three selected reference genes, *OsActin*, *OsEIF1* and *OsPlase* (Sections 2.3.5 and 3.3.10), and used to calculate transcript level of each gene relative to the control at each time point, set at 1.00 (Table 3.5 and 3.6). The preliminary transcript level data normalized against geometric mean of three reference genes is also shown as relative transcript abundance for each gene in control and treated shoots and roots in Supplementary Figures C1-C6 (Appendix C).

Transcript abundance of *AOX1a* was significantly enhanced in shoots 6 h after exposing to 5 mM KCN as well as 20 µM AA. There was a 58-fold increase of transcript abundance in KCN-treated shoots compared to control while the increase was 2.3-fold in AA-treated shoots (Table 3.5). Transcript levels of *AOX1b* were also significantly higher in shoots exposed to KCN but not AA. Transcript levels of *AOX1b* increased 248- and 348-fold, 6 h and 24 h after treatment compared to their corresponding controls (Table 3.5). No noticeable changes of *AOX1c* transcript levels were observed (Table 3.5). Of the NDH genes, *NDB2* transcript levels were significantly higher in shoots 6 h after seedlings were exposed to KCN as well as AA. There was a significant increase also in *NDA1* and *NDB3* transcript levels in KCN-treated shoots after 6 h of treatment (Table 3.5). Although there were higher transcript levels of *NDA1*, *NDA2*,

NDB1, *NDB3* and *NDC1* in AA-treated shoots 6 h or/and 24 h after treatment, the increases were not significant compared to their controls (Table 3.5).

Transcript abundance of all AOX isoforms (*AOX1a*, *AOX1b* and *AOX1c*) and NDH isoforms, except *NDB3* and *NDC1*, were significantly increased in roots either 6 h or 24 h after AA treatment (Table 3.6). Of the NDH genes, only *NDB2* maintained the higher level of transcript abundance up to 24 h after AA treatment, while transcript levels of all other NDH genes decreased over the longer time. Although transcript abundance of *NDB3* and *NDC1* in roots increased during AA treatment, but not significantly compared to their controls. In KCN-treated roots, the transcript abundance of two AOX isoforms (*AOX1a* and *AOX1b*), two NDA isoforms (*NDA1* and *NDA2*) and two NDB isoforms (*NDB2* and *NDB3*) were significantly increased either 6 h or 24 h after the treatment (Table 3.6). The transcript abundance for treatment with KCN was not significantly increased for *NDA2* and *NDB2* in roots at 6 h, but significantly increased 24 h after treatment. Transcript abundance of *AOX1c*, *NDA2*, and *NDB1* were decreased in KCN treated shoots while transcript levels of *NDC1* were decreased both in KCN treated roots and shoots (Table 3.5. and 3.6).

Interestingly, transcript abundance of *AOX1a* and *NDB2* increased significantly in both roots and shoots when seedlings were exposed either KCN or AA treatments and showed a similar pattern. In shoots, significantly higher transcript abundances of both genes were observed 6 h after KCN or AA treatments. In roots also, significantly high transcript abundance was detected in both *AOX1a* and *NDB2* 6 h after AA treatment and maintained until 24 h. Only in KCN-treated roots, *AOX1a* showed an early increase of transcript levels (at 6 h) while higher transcript abundance of *NDB2* was observed 24 h after treatment (Table 3.6).

Table 3.5 Transcript level changes of alternative respiratory genes in shoots when traditional ETC was chemically inhibited

Exposure to potassium cyanide (5 mM) and antimycin A (20 µM) treatments up-regulates AOX and NDH gene expression in rice (*O. sativa* ssp *japonica* cv Nipponbare). Gene expression analyses were carried out using qRT-PCR and transcript levels were determined in both shoot and root tissues exposed to treatments for 6 h and 24 h or grown under control conditions. Data are shown as mean ± SEM of three biological replicates relative to the control at each point set at 1.00. Significant transcript level changes relative to the control according to two way ANOVA are indicated by green boxes ($p \leq 0.05$). Blue boxes indicate increased transcript levels relative to control but not significant.

	Potassium cyanide (KCN) treatment (5 mM)				Antimycin A (AA) treatment (20 µM)			
Shoots								
Gene	KCN (mM)	0 h	6 h	24 h	AA (µM)	0 h	6 h	24 h
AOX1a	0	1.0 ± 0.4	1.0 ± 0.37	1.0 ± 0.50	0	1.0 ± 0.18	1.0 ± 0.23	1.0 ± 0.07
	5		58.2 ± 15.51	1.0 ± 0.29	20		2.3 ± 0.73	1.2 ± 0.13
AOX1b	0	1.0 ± 0.4	1.0 ± 0.33	1.0 ± 0.88	0	1.0 ± 0.12	1.0 ± 0.29	1.0 ± 0.15
	5		248.6 ± 63.97	348.6 ± 78.37	20		1.2 ± 0.62	0.9 ± 0.19
AOX1c	0	1.0 ± 0.4	1.0 ± 0.32	1.0 ± 0.53	0	1.0 ± 0.22	1.0 ± 0.46	1.0 ± 0.20
	5		0.3 ± 0.08	0.1 ± 0.02	20		1.0 ± 0.59	0.7 ± 0.14
NDA1	0	1.0 ± 0.7	1.0 ± 0.53	1.0 ± 0.57	0	1.0 ± 0.28	1.0 ± 0.83	1.0 ± 0.44
	5		17.4 ± 3.13	0.7 ± 0.05	20		1.3 ± 0.77	2.2 ± 0.18
NDA2	0	1.0 ± 0.2	1.0 ± 0.70	1.0 ± 0.36	0	1.0 ± 0.13	1.0 ± 0.40	1.0 ± 0.18
	5		0.2 ± 0.04	1.1 ± 0.20	20		1.4 ± 0.52	1.5 ± 0.49
NDB1	0	1.0 ± 0.35	1.0 ± 0.40	1.0 ± 0.26	0	1.0 ± 0.09	1.0 ± 0.22	1.0 ± 0.04
	5		0.2 ± 0.11	0.9 ± 0.66	20		1.3 ± 0.45	2.1 ± 0.50
NDB2	0	1.0 ± 0.12	1.0 ± 0.41	1.0 ± 0.27	0	1.0 ± 0.11	1.0 ± 0.62	1.0 ± 0.12
	5		5.2 ± 0.91	1.9 ± 0.42	20		2.8 ± 0.79	1.4 ± 0.06
NDB3	0	1.0 ± 0.36	1.0 ± 0.37	1.0 ± 0.07	0	1.0 ± 0.21	1.0 ± 0.85	1.0 ± 0.43
	5		18.3 ± 0.7.5	0.7 ± 0.05	20		1.3 ± 0.82	1.6 ± 0.41
NDC1	0	1.0 ± 0.33	1.0 ± 0.39	1.0 ± 0.02	0	1.0 ± 0.02	1.0 ± 0.31	1.0 ± 0.21
	5		0.6 ± 0.07	0.2 ± 0.11	20		1.0 ± 0.47	1.7 ± 0.38

Table 3.6 Transcript level changes of alternative respiratory genes in roots when traditional ETC was chemically inhibited

Exposure to potassium cyanide (5 mM) and antimycin A (20 µM) treatments up-regulates AOX and NDH gene expression in rice (*O. sativa* ssp *japonica* cv Nipponbare). Gene expression analyses were carried out using qRT-PCR and transcript levels were determined in both shoot and root tissues exposed to treatments for 6 h and 24 h or grown under control conditions. Data are shown as mean ± SEM of three biological replicates relative to the control at each point set at 1.00. Significant transcript level changes relative to the control according to two way ANOVA are indicated by green boxes (p≤0.05). Blue boxes indicate increased transcript levels relative to control but not significant.

	Potassium cyanide treatment (5 mM)				Antimycin A treatment (20 µM)			
Roots								
Gene	KCN (mM)	0 h	6 h	24 h	AA (µM)	0 h	6 h	24 h
AOX1a	0	1.0 ± 0.12	1.0 ± 0.22	1.0 ± 0.20	0	1.0 ± 0.31	1.0 ± 0.27	1.0 ± 0.29
	5		44.0 ± 4.52	5.1 ± 1.01	20		11.53 ± 1.60	6.5 ± 0.76
AOX1b	0	1.0 ± 0.51	1.0 ± 0.06	1.0 ± 0.34	0	1.0 ± 0.21	1.0 ± 0.26	1.0 ± 0.11
	5		355.4 ± 46.55	11.3 ± 1.36	20		5.9 ± 1.88	6.5 ± 2.16
AOX1c	0	1.0 ± 0.42	1.0 ± 0.14	1.0 ± 0.25	0	1.0 ± 0.50	1.0 ± 0.39	1.0 ± 0.23
	5		1.7 ± 0.25	0.1 ± 0.02	20		3.0 ± 0.65	1.0 ± 0.30
NDA1	0	1.0 ± 0.19	1.0 ± 0.20	1.0 ± 0.25	0	1.0 ± 0.73	1.0 ± 0.28	1.0 ± 0.42
	5		56.9 ± 13.04	2.0 ± 0.15	20		6.5 ± 0.46	0.5 ± 0.02
NDA2	0	1.0 ± 0.66	1.0 ± 0.19	1.0 ± 0.28	0	1.0 ± 0.26	1.0 ± 0.10	1.0 ± 0.07
	5		3.0 ± 0.81	6.0 ± 1.33	20		3.1± 0.39	0.7 ± 0.17
NDB1	0	1.0 ± 0.68	1.0 ± 0.35	1.0 ± 0.63	0	1.0 ± 0.02	1.0 ± 0.30	1.0 ± 0.17
	5		1.6 ± 1.13	0.9 ± 0.23	20		2.4 ± 0.71	0.7± 0.12
NDB2	0	1.0 ± 0.48	1.0 ± 0.27	1.0 ± 0.10	0	1.0 ± 0.31	1.0 ± 0.09	1.0 ± 0.32
	5		2.0 ± 0.37	7.1 ± 1.63	20		2.8 ± 0.18	3.8 ± 0.42
NDB3	0	1.0 ± 0.46	1.0 ± 0.54	1.0 ± 0.14	0	1.0 ± 0.64	1.0 ± 0.06	1.0 ± 0.12
	5		29.7 ± 5.64	1.0 ± 0.38	20		2.4 ± 0.85	1.2 ± 0..06
NDC1	0	1.0 ± 0.47	1.0 ± 0.21	1.0 ± 0.10	0	1.0 ± 0.09	1.0 ± 0.17	1.0 ± 0.22
	5		1.0 ± 0.19	0.4 ± 0.14	20		1.8 ± 0.85	0.8 ± 0.26

3.3.12 Functional elements of Arabidopsis *AOX1a* and *NDB2* promoters were also found to be present in rice *AOX1a* and *NDB2*

The search within the promoter region of *AOX1a* from japonica and indica rice for the presence of the *cis*-acting regulatory elements (CAREs) that were functional in Arabidopsis *AOX1a* revealed that the most of them were also present in rice *AOX1a* (Table 3.7). Seven out of the ten functional elements in Arabidopsis *AOX1a* were also found be present in both japonica and indica rice suggesting that these regulatory element sequences were conserved among plant species. All functional CAREs found in promoter region of Arabidopsis *NDB2* were also found to be present in the promoter region of *NDB2* from both rice groups. As in Arabidopsis, it seems that rice *AOX1a* and *NDB2* promoter regions were also sharing number of regulatory elements which are responsible for controlling stress tolerance responses (Table 3.7). However, it is yet to be investigated whether these regulatory elements are functional in rice.

The promoter region search for the presence of CAREs were extended to other rice AP genes as it was found that some other AP genes were also co-induced with *AOX1a* and *NDB2*. Similar to rice *AOX1a* and *NDB2*, functional elements were present in the 1.5 kb upstream regions of other AP genes from japonica and indica (Table 3.7). It is interesting to note that all 6 functional elements found in Arabidopsis *NDB2* were predicted to be present in rice *NDA2*, *NDB1*, *NDB2*, *NDB3* while only five of them were predicted to be present in *NDA1* and *NDC1* (Table 3.7).

Table 3.7 Summary of the conserved *cis*–acting regulatory motifs predicted to be present in rice AOX and NDH promoter regions, which were found to be functional in Arabidopsis *AOX1a* and *NDB2*

Putative CAREs in rice AP genes were identified using the computer program “CentriMo”, Local Motif Enrichment Analysis tool on the motif prediction algorithm “MEME” (meme-suite.org) with default settings.

Motif Sequence	Arabidopsis		Rice								
	AOX1a	*NDB2*	*AOX1a*	*AOX1b*	*AOX1c*	*NDA1*	*NDA2*	*NDB1*	*NDB2*	*NDB3*	*NDC1*
TGAAGC	✓		✓	✓	✓						
CGTGAT	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
ATCCG	✓	✓			✓		✓	✓	✓	✓	
CACACA	✓		✓	✓	✓						
CGGCTTT	✓		✓	✓							
TCGTAAA	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
TCTCT	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓
GTCATC	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
ACGTG	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
TTCGATCA	✓			✓	✓						

‘✓’ motif signal detected in promoter region

3.4 Discussion

The objectives of this Chapter were to identify alternative pathway homologs in two rice sub-species using sequence similarity to Arabidopsis alternative pathway genes, to characterize them, and to identify genes that respond to oxidative stress. The bioinformatics survey revealed that both AOXs and NDHs are present in both japonica and indica rice. Only *AOX1*-type genes were present, while *AOX2*-types were absent in rice, as reported from other monocot species (Considine et al., 2002). Four isoforms of *AOX1* (*AOX1a*, *AOX1b*, *AOX1c* and *AOX1d*) and 6 members of NDHs (*NDA1*, *NDA2*, *NDB1*, *NDB2*, *NDB3* and *NDC1*) homologous to Arabidopsis genes were identified. All NDH gene families found in dicot plants so far were present in both japonica and indica rice. Genes from all AOX and NDH families encode proteins predicted be targeted to mitochondria, while some of the NDH proteins are predicted to also localise to other organelles, as suggested previously by (Xu et al., 2013). Further analysis of rice AP gene sequences confirmed that all AOX proteins belong to the Ferritin-like protein superfamily with characteristic iron binding sites, while NDH proteins were found to belong to the pyr-redox superfamily with NADP(H) binding domains. Phylogenetic analysis revealed the separation of AOXs into four major groups (type a, b, c and d) and NDHs into three major families, NDA, NDB and NDC. Japonica and indica rice share very high similarity between AP protein sequences.

Dual targeting of NDH proteins has been reported in many plant species including Arabidopsis, rice, grapes and potato (Carrie et al., 2008; Xu et al., 2013). It has been experimentally shown that *AtNDA1*, *AtNDA2* and *AtNDB1* are dual targeted to mitochondria and peroxisomes while *AtNDC1* is dual targeted to mitochondria and chloroplasts (Michalecka et al., 2003; Xu et al., 2013). In the current study, the prediction of subcellular locations of identified NDH proteins from japonica and indica rice revealed that some might be targeted to more than one organelle. The current study demonstrated that *NDA2*, *NDB1* and *NDB2* from both rice sub-species were

predicted to be dual targeted to mitochondria and peroxisomes, while *NDC1* from both rice sub-species was predicted to be dual targeted to mitochondria and chloroplasts. Similar results were reported for dual targeting of NDH proteins from rice by Xu et al. (2013) using GFP (Green Florescence Protein) fusion analysis. In Arabidopsis, both NDA family members were targeted to peroxisomes in addition to mitochondria while only one member from the NDB family (NDB1) was dual targeted to mitochondria and peroxisomes (Xu et al., 2013). In contrast, in rice two NDB family members (NDB1 and NDB2) were dual targeted to mitochondria and peroxisomes while only one member from the NDA family (NDA2) dual targeted to mitochondria and peroxisomes. These results are summarised in Figure 3.22.

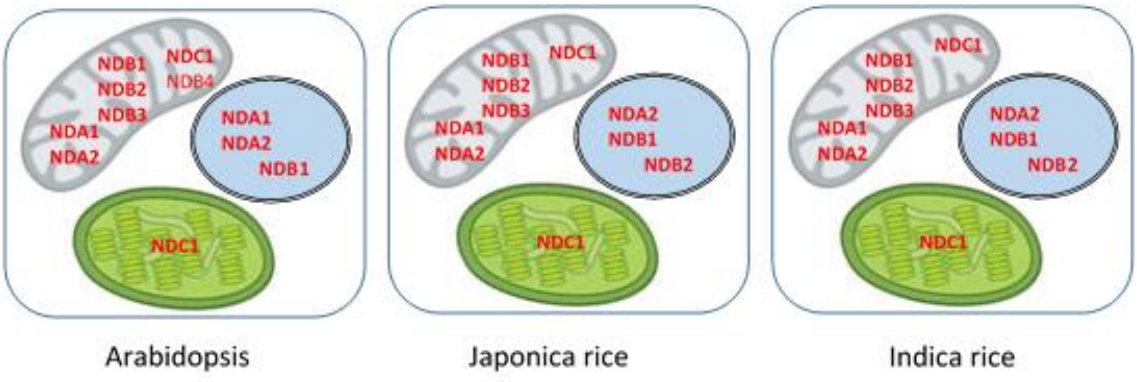


Figure 3.22 Summary of the subcellular localization of NDH proteins in Arabidopsis, japonica and indica rice

In both rice groups, NDA2, NDB1 and NDB2 were dual targeted to mitochondria and peroxisomes while NDC1 was dual targeted to mitochondria and chloroplast. In Arabidopsis, NDA1, NDA2 and NDB1 were dual targeted to mitochondria and peroxisomes while NDC1 was dual targeted to mitochondria and chloroplast (Xu et al., 2013). Chloroplasts-green, Mitochondria-grey and Peroxisomes-blue.

The organization of genomic sequences and expression of AP genes has also been found to be highly variable among plant species. In the present study, the exon-intron organization of AOX and NDH genes from japonica and indica rice was studied and compared with Arabidopsis AP genes to explore the polymorphisms between two rice sub-species as well as between monocots and dicots. Plant AOXs have been

extensively studied in terms of biochemistry as well as gene expression and regulation under stress conditions. However, little information about gene structure especially for NDHs is available. A structure of four exons interrupted by three introns has been recognized as the conserved AOX gene structure in plants (Considine et al., 2002; Polidoros et al., 2009). Although all four isoforms of Arabidopsis *AOX1* shared a conserved gene structure, polymorphisms were apparent in rice. The exon-intron organization of *AOX1a*, *AOX1c* and *AOX1d* from japonica and indica rice have the predominant exon-intron structure of four exons interrupted by three introns, but that of *AOX1b* from both rice groups consisted of 3 exons separated by 2 introns. The occurrence of an exon-intron gene structural polymorphism was reported in other plant species also. For example, the exon-intron structure of *AOX1a* from potato (*Solanum tuberosum*) consists of 3 exons interrupted by 2 introns (Polidoros et al., 2009) while other isoforms shared the conserved gene structure for AOX. It is suggested that variations in exon-intron organization have evolved through loss or gain of introns, exon recombination, exon gain or deletion of fragments (Polidoros et al., 2009; Pu et al., 2015). The possible responsible mechanism for the variation in rice *AOX1b* has been suggested as loss of the second intron (Figure 3.23) (Considine et al., 2002). Similarly, the possible mechanism for the variation in potato *AOX1a* gene structure has been suggested as loss of the third intron (Figure 3.24) (Polidoros et al., 2009).

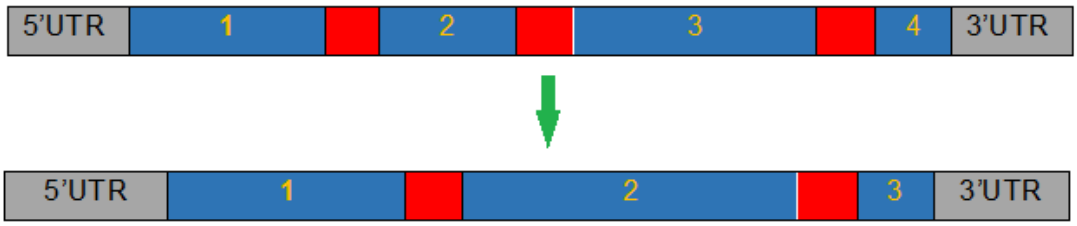


Figure 3.23 Loss of intron 2 from the conserved structure and evolution of exon-intron structure with 3 exons interrupted by 2 introns eg: rice *AOX1b*

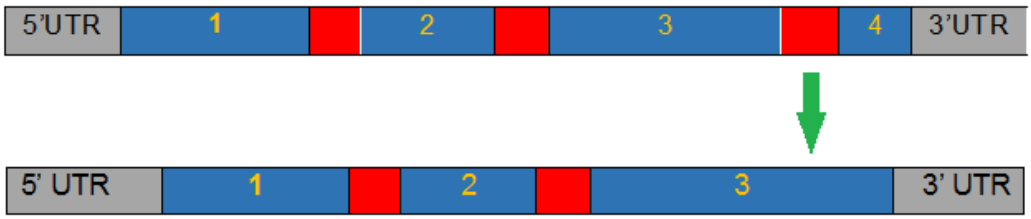


Figure 3.24 Loss of intron 3 from the conserved structure and evolution of exon-intron structure with 3 exons interrupted by 2 introns eg: potato *AOX1a*

As it was found that the exon-intron organization of rice *AOX1b* is different from Arabidopsis, to check whether this is common in rice species, the gene structure of *AOX1b* from 8 rice species (Table 3.1), including both cultivated and wild types, was studied using the genomic data available on the Gramene database (www.gramene.org). The results revealed that 3 exons separated by 2 introns was the predominant form for *AOX1b* (Figure 3.25). In gene evolution studies, it would be very useful to explore whether the 3 exons and 2 introns structure of *AOX1b* gene is limited to rice species or a common feature in monocots. However, formation of the gene structure of 3 exons divided by 2 introns through the loss of second intron has been observed only in Poaceae members (*O. sativa*, *Sorghum bicolor*, *Zea mays* and *Panicum virgatum)* (Pu et al., 2015). In this study, further investigation revealed that there is a very high polymorphism in *AOX1a* gene structure among different rice species. Although cultivated species shared the conserved structure for *AOX1a*, the structure of *AOX1a* from all wild types consisted of 5-8 exons except *O. punctata,* which had only two exons (Figure 3.19).

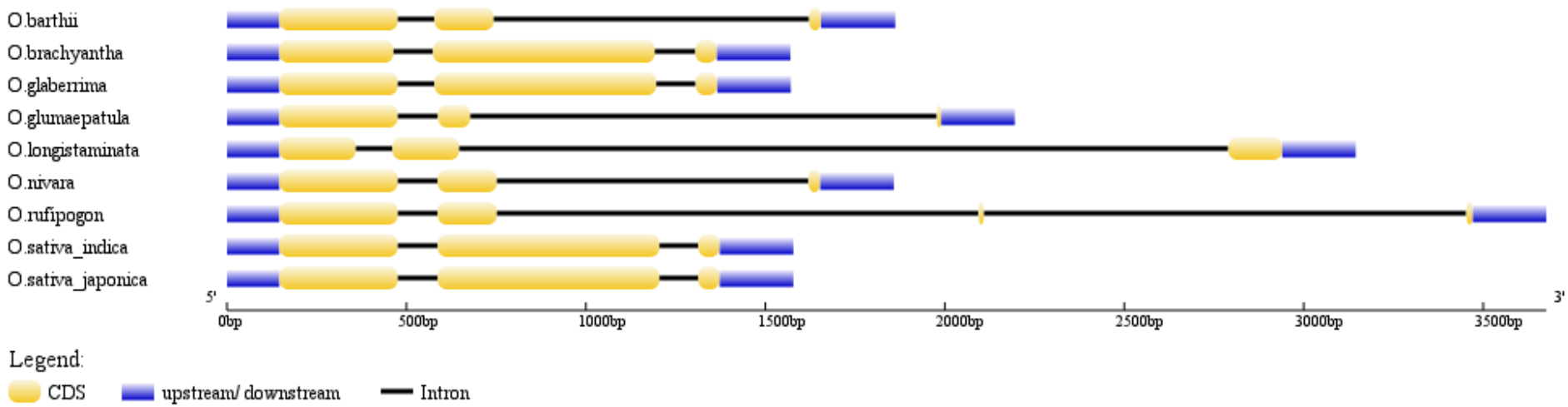


Figure 3.25 Exon-intron structural organization of *AOX1b* gene in different rice species

Three exons separated by 2 introns found to be the predominant gene structure for *AOX1b* in rice. Out of 8 rice species investigated, 7 species share this structure except *O. rufipogen,* which has 4 exons. Both japonica and indica rice groups share the predominant gene structure with 3 exons separated by 2 introns.

Less is known about the biochemistry, and gene expression and regulation of plant NDHs under stress conditions, especially in monocots. A study of exon-intron organization of Arabidopsis NDHs has been reported (Michalecka et al., 2003) but no work has been reported so far on rice NDHs except their dual targeting ability (Xu et al., 2013). The current study focused on the structural polymorphism of rice NDHs as well as their role under abiotic stress tolerance. The current bioinformatics analysis revealed that exon-intron organization of *NDA1* in japonica and indica rice is completely different from that of rice *NDA2* as well as from Arabidopsis NDA family members. The rice *NDA1* consists of 3 exons separated by 2 introns in contrast to *NDA2,* which has 8 exons. A comparative study of exon-intron structure of *NDA1* from 10 rice species, including japonica and indica rice (Table 3.1), using the data available on Gramene database, confirmed that it was the predominant structure of *NDA1* among rice species including wild rice. The majority of rice species (8 out of 10) shared common exon-intron structure of 3 exons divided by 2 introns. Two species, *O. barthii* and *O. longistaminata* consist of 4 exons divided by 3 introns (Figure 3.26). Although it is hard to predict the exact evolutionary mechanism, there appears to have been a reduction of the number of exons as well as introns. The presence of fewer exons and introns in *NDA1* appears to be specific to rice amongst the species studied to date and it is worthwhile to explore whether this is a feature of all monocot species. A study of *NDA2* from 10 rice species demonstrated that 7 species carry 8 exons separated by 7 introns while the other 3 species have 9 or 10 exons. This suggests that 8 exons separated by 7 introns is predominant in rice *NDA2.* All NDB family members from japonica and indica rice share similar gene structure, which consisted of 10 exons divided by 9 introns. A study of the *NDB2* from 10 rice species showed that all except two species, *O. longistaminata* and *O*. *punctata* share the common structure of 10 exons separated by 9 introns, suggesting this is the predominant in rice NDB family members (Figure 3.20).

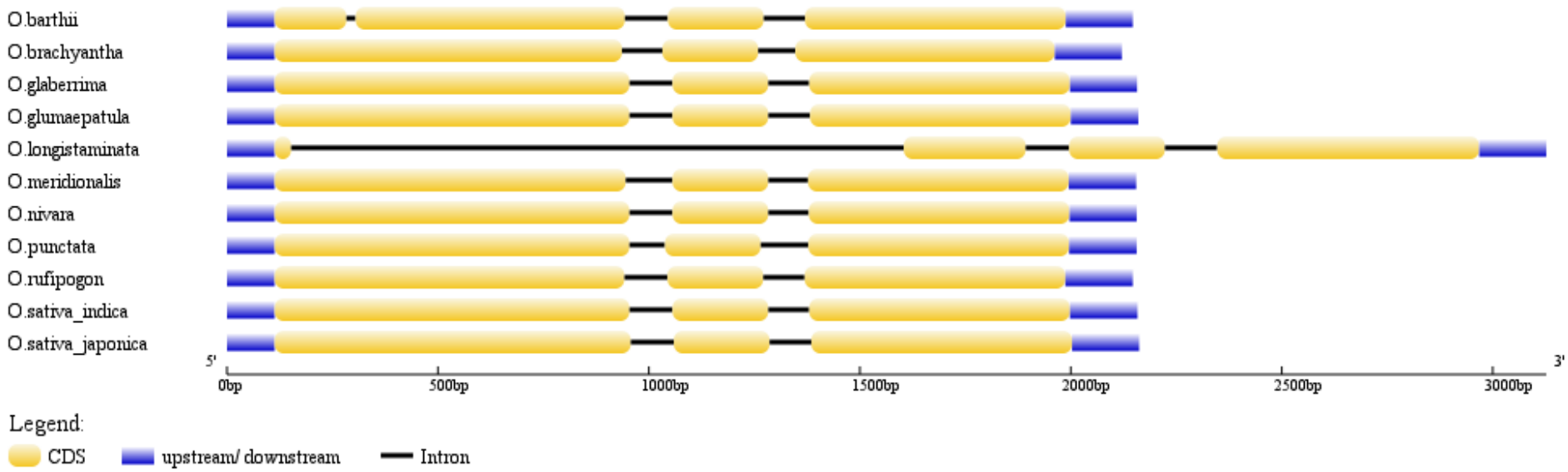


Figure 3.26 Exon-intron structural organization of *NDA1* gene in different rice species

Three exons separated by 2 introns found to be the predominant gene structure for *NDA1* in rice. Out of 10 rice species investigated, 8 species share this structure except *O. barthii and O. longistaminata* which share 4 exons separated by 3 introns. Both japonica and indica rice groups share the predominant gene structure with 3 exons separated by 2 introns**.**

Potassium cyanide (KCN) and antimycin A (AA) are well-known inhibitors of the traditional mitochondrial electron transport chain (mETC) (Moller, 2001). KCN inhibits respiration through the cytochrome pathway by blocking a proton translocation site in complex IV while AA inhibits complex III (Moller, 2001; Takumi et al., 2002; Schwarzlander et al., 2009; Ma et al., 2011). Disruption of mETC activity by AA or KCN can increase ROS production in plant cells leading to oxidative stress (Moller, 2001; Umbach et al., 2012). Changes in transcript levels of AP genes have been reported from a variety of plant species treated with AA or KCN suggesting that AP genes are responsive to oxidative stress created though inhibition of mETC activity and play a role in controlling ROS in plant cells (Vanlerberghe and McIntosh, 1996; Karpova, 2002; Geisler et al., 2004; Umbach et al., 2012). In Arabidopsis cells, *NDB2* and *AOX1a* are consistently up-regulated co-ordinately during oxidative stress (Clifton et al., 2005). During the current study, rice seedlings were exposed to AA or KCN, and changes in transcript levels in roots and shoots were studied using qRT-PCR. Results showed that a number of rice AP genes were responsive to oxidative stress. Although *AOX1a* and *NDB2* appeared to be the most commonly stress-responsive, *AOX1b*, *NDA1*, *NDA2* as well as *NDB3* also responded in some treatments. In Arabidopsis, leaves sprayed with 10 µM AA specifically up-regulated the transcript level of *AOX1a* but other AOX genes were unaffected (Saisho et al., 1997). Similarly, in the current study, 20 µM AA treatment up-regulated the transcript abundance of rice *AOX1a* in shoots but *AOX1b* or *AOX1c* were not affected. In contrast, there was a significant increase of transcript level of all AOX isoforms tested in AA-treated roots. This could be due to the fact that though the AA concentration tested was strong enough to induce all three AOX isoforms in roots, it might not be strong enough to induce *AOX1b* or *AOX1c* in shoots other than *AOX1a* reflecting the difference in sensitivity to stresses between shoots and roots, and between the AOX isoforms (Ohtsu et al., 2002). Alternatively, regulation of AOXs might be different in roots and

shoots as it is suggested that multiple signalling pathways might be involved in the induction of AOXs in plants (Thirkettle-Watts et al., 2003). In the present study, when rice seedlings were treated with 5 mM KCN, the transcript abundance of *AOX1a* and *AOX1b* increased dramatically in both shoots and roots while a slight reduction and negligible changes were observed in *AOX1c* transcript levels in shoots and roots respectively. Similarly, a rapid increase of *AOX1a* transcript level was also observed when 5-day old rice seedlings were exposed to 100 µM sodium azide, an inhibitor of cytochrome oxidase (Saika et al., 2002b). When wheat seedlings were exposed 3 mM KCN, an increase of *WAOX1a* transcript abundance was observed but not *WAOX1c* suggesting that two *WAOX1* genes were transcriptionally controlled by two different regulatory pathways (Takumi et al., 2002). It is likely that different regulatory pathways to control gene expression at transcriptional level exist also in rice.

Compared to AOX, less work has been done on NDHs. No study has been reported so far on the expression pattern of rice NDH genes under abiotic stress conditions. The current chapter focused on identifying stress-responsive rice NDH genes by exposing seedlings to AA or KCN treatments. A change of transcript abundance was observed in number of rice NDH genes in both shoots and roots suggesting that NDHs are also responsive to oxidative stress along with AOXs. It was clear that tissue type as well as type of treatment affected gene induction. The induction magnitude of some NDH genes (*NDA1* and *NDB3*) were very high in KCN treated shoots as well as in roots compared to AA treated tissues. *AOX1a* and *NDB2* appeared to be co-expressed in AA treated shoots while transcript abundance of all five genes; *AOX1a*, *AOX1b*, *NDA1*, *NDB2* and *NDB3* were observed in KCN-treated shoots. Although the expression magnitude was lower, the number of co-expressed NDH genes and AOX was higher in AA treated roots compared to KCN treated roots.

Overall, the transcriptional analysis of rice alternative respiratory pathway genes under oxidative stress revealed that a number of genes are stress-responsive. However, *AOX1a* and *NDB2* appeared to be most consistently responsive. Transcript level responses are important in identifying the molecular mechanisms underlying signalling and for understanding the role and regulation of the gene in cellular responses (Van Aken et al., 2009). The regulatory regions of genes, generally known as promoters, are the key players of any signalling pathway for induction of gene(s) at the transcriptional level (Ho et al., 2008). *Cis*-acting regulatory elements (CAREs), which are located on the promoter region, play a role in regulating the expression of mitochondrial-encoded genes under stress conditions (Ho et al., 2008). To gain a better understanding of induction of rice AP genes under oxidative stress, promoter regions of AP genes were searched for the presence of functional elements. *AOX1a* and *NDB2* genes have been initially selected to identify sequence elements responsible for the regulating stress response as they appeared to be highly responsive when mETC was chemically inhibited. Also, it is reported that *AOX1a* and *NDB2* in Arabidopsis are likely co-regulated by sharing common CAREs in the promoter region (Ho et al., 2008). Functional analysis of the *AtAOX1a* promoter region revealed the presence of 10 CAREs and six of them were also found to be functional in *AtNDB2* (Ho et al., 2008). In rice, although there was a difference in magnitude, *AOX1a* and *NDB2* showed similar patterns of transcript abundance under tested conditions in both roots and shoots. Subsequent analyses of promoters of other co-induced rice AP genes also revealed the sharing of similar CAREs as *AOX1a* and *NDB2*.

The regulation of *AOX* gene expression generally appears to involve the same signal pathways in different plant species (Thirkettle-Watts et al., 2003) and it was found that *AOX* gene paralogs which showed similar expression pattern in soybean and Arabidopsis shared common regulatory motifs (Thirkettle-Watts et al., 2003). Therefore, it is worthwhile to investigate whether the promoter regions of rice *AOX* also share the same regulatory motifs that are responsible for signalling during stress conditions as in other plant species and to see whether there is a co-regulation of rice *AOX1a* and *NDB2* by sharing common CAREs. As a start, promoter regions of *AOX1a* and *NDB2* genes from japonica and indica rice were analysed and the results revealed that most of the functional CAREs present in the promoter of Arabidopsis *AOX1a* were also present in rice. As in Arabidopsis, six of the functional elements were also found to be present in the upstream region of rice *NDB2* suggesting that these co-induced genes share common regulatory motifs. Not only *NDB2*, but there were some other rice AP genes co-induced with *AOX1a* or other AOX isoforms under oxidative stress created via disrupting the function of mETC. Therefore, the presence of functional elements in other co-induced genes were also investigated and results revealed the presence of functional elements similar to Arabidopsis, further confirming the sharing of common regulatory elements in co-induced genes under stress conditions. However, it is yet to be confirmed whether these CAREs are functional in rice. The use of information on promoter activity together with transcript analysis and mutants is important in understanding the underlying regulatory mechanisms in gene expression under stress conditions (Ho et al., 2008). Therefore, functional analysis of *cis*-regulatory elements present in upstream regions of rice AP genes would be a vital achievement for understanding of regulatory mechanisms of rice AP gene expression under environmental stresses.

4 Expression of rice alternative respiratory pathway genes under salt stress

4.1 Introduction

High salt concentrations in plant cells result in an excessive production of ROS, such as $O_2^{\cdot-}$, ·OH and H_2O_2 etc., by impairment of electron transport within different subcellular organelles such as chloroplasts and mitochondria (Sharma et al., 2012). It is a well-established fact that plants use several enzymatic and non-enzymatic antioxidants to scavenge ROS induced during environmental stress conditions. Evidence suggests that alternative respiratory pathway genes, AOXs along with NDHs, play a regulatory role in plant abiotic stress tolerance through maintaining ROS at non deleterious level within the mitochondrion (Vanlerberghe, 2013).

Most stressful conditions, including drought (Bartoli et al., 2005; Feng et al., 2009), low temperature (Ito et al., 1997; Mizuno et al., 2008), high salt (Ferreira et al., 2008; Smith et al., 2009; Feng et al., 2010b; Feng et al., 2013), and heavy metal toxicity (Panda et al., 2013; Wang et al., 2013), increase AOX transcript abundance, AOX protein and the capacity of alternative respiratory pathway. Increases in AOX (*AOX1a* and *AOX1b*) transcript abundance as well capacity of AOX respiration were observed in rice leaves when rice seedlings were exposed to 300 mM NaCl (Feng et al., 2010b). This study further revealed that the induction of AOXs in tissues treated with high salt (300 mM) was more intense compared to tissues treated with low salt concentrations (150 mM), suggesting that AOX response is dependent on the intensity of the stress. Similarly, enhancement of cyanide-resistant respiration and an increase in AOX transcript levels were observed in rice roots treated with 200 mM or 300 mM NaCl for 12 h (Feng et al., 2013). Similarly, induction of rice AOX isoforms, especially *AOX1a* and *AOX1b*, were reported when seedlings were treated with 300 mM NaCl (Li et al., 2013a) and 250 mM NaCl (Ohtsu et al., 2002).

Although the AOX has received considerable attention from researchers, less attention has been given to the alternative dehydrogenases. There is no published work so far on responses of rice non-energy conserving alternative dehydrogenases under environmental stress conditions. The previous chapter on bioinformatics revealed that there are 6 NDHs in rice along with four isoforms of AOXs. Further characterization of these demonstrated that transcript levels of rice NDHs as well as AOXs were up-regulated when the cytochrome pathway was chemically inhibited. This suggests that a number of rice alternative dehydrogenases are also stress-responsive and co-expressed with AOXs when plants are under stress. The current chapter describes the characterization of rice alternative pathway components, AOXs and NDHs, under salinity stress. A comparative study was carried out to explore the diversity in physiological and gene expression responses of rice seedlings from two different rice cultivars; Nipponbare (japonica rice) and Langi (indica rice) grown under high salinity. Rice seedlings grown on hydroponic cultures were exposed to salt stress in their early vegetative stage and cultivar differences in physiological responses and in expression of alternative pathway genes in shoots and roots were investigated. The study was extended to investigate the correlation of changes in transcript abundance with the changes in corresponding protein level and enzyme activity in shoot and root tissues in response to salinity stress using cultivar Nipponbare.

4.2 Materials and methods

4.2.1 Plant material and growth conditions

Experiments were conducted at the Plant Accelerator, Waite Campus, University of Adelaide, Australia from March to April 2013 under the growth conditions described in Section 2.1.1. Cultivar Nipponbare (japonica rice) was selected for the study and cultivar Langi (indica rice) was used for a comparison in salt screening and gene expression analysis.

4.2.2 Screening for salt-tolerance

The salt screening experiment was performed using a hydroponic system established as described in Section 2.1.3. Seedlings grown hydroponically for 3 weeks (~4 weeks old seedlings) were exposed to 120 mM NaCl added in two increments (50 mM NaCl + 0.75 mM $CaCl_2$ and 70 mM NaCl + 1.0 mM $CaCl_2$). The second salt addition was made two days after the first. $CaCl_2$ was added to the nutrient medium to maintain a constant Ca^{2+} activity of 1 mM throughout the experiment.

4.2.3 Measurement of biomass and tissue ion concentrations

Shoots and roots were harvested 10 days after the salt application for biomass measurements and for tissue ion analysis. FW, DW and Na^+ and K^+ concentrations of shoots and roots were measured as described in Section 2.1.4. The growth responses to salinity was shown by 'relative growth in dry weight', which is the growth in dry weight of the NaCl-treated seedlings relative to that in the control (%). Five biological replicates were used from each treatment; salt-treated and control.

4.2.4 Analysis of transcript abundance

Fresh shoot and root samples were harvested at predetermined time points (1, 2, 6, 12 days after salt application) for RNA extraction. Samples were snap frozen in liquid N_2 and stored at -80^{o}C until further use. Three biological replicates were collected from salt-treated and from non-treated plants at each time point. RNA extraction and cDNA synthesis were performed as described in Sections 2.2.1.1 and 2.2.4 respectively. Transcript abundance of genes was investigated using qRT-PCR as described in Section 2.3.4. For accurate quantification of gene expression data, it is essential to normalize qRT-PCR data to a fixed reference; one that is not affected by the experimental conditions. Therefore, qRT-PCR data obtained were normalized to the geometric mean expression of three stable reference genes, *OsEIF1*, *OsPpIase* and *OsActin*, selected out of a set of 6 as described in Section 3.3.10 to remove systematic variation in experiments which affects the measured gene expression levels.

4.2.5 Analysis of relative abundance of proteins in isolated mitochondria

The abundance of AOX and NDB2 proteins were estimated in mitochondria purified from salt-treated and non-treated shoot and root tissues of Nipponbare, using the Western blot technique. Separation of proteins using SDS-PAGE, electro-blotting onto a nitrocellulose membrane, probing with primary antibody and visualization of immuno-reactive peptides were carried out as described in Sections 2.4.6.

The abundance of AOX protein in salt-treated and non-treated tissues was estimated by immunoblotting purified mitochondrial proteins with monoclonal AOA antibody, with the peptide recognition sequence of RADEAHHRDVNH (Finnegan et al., 1999), against AOX. Electro-blotted membranes were incubated with the AOA primary antibody (1:10000) as described in Section 2.4.6.5 followed by rabbit anti-mouse IgG horseradish peroxidase conjugated secondary antibody (Rockland Immunochemicals Inc.) (1:10000) and visualized by chemiluminescence. The abundance of NDB2 protein was estimated by using the polyclonal antibody, developed during the current study, with the peptide recognition sequence of CQDNQVLQINDGTGKKR. Separated mitochondrial proteins were electro-botted on to nitrocellulose membrane and probed with OsNDB2 primary antibody (1:8000) as described in Section 2.4.6.5 followed by Goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (1:10000) (Rockland Immunochemicals Inc.), and visualized by chemiluminescence. Protein loading was determined either by re-probing the same membrane or duplicate membrane with the monoclonal antibody porin (1:10000) (loading control).

4.2.6 Enzyme activity assays in isolated mitochondria

Relative changes in AOX and Ca^{2+} dependent and Ca^{2+} independent alternative dehydrogenase enzyme activities were estimated in salt-treated and non-treated shoot and root tissues using purified mitochondria as described in Section 2.4.2.

4.3 Results

4.3.1 Physiological responses of two rice cultivars under salinity stress

Salt affected seedlings showed obvious reduction in plant size compared to their controls and displayed leaf tip burning and some necrosis. There was a significant reduction of dry biomass production in both shoots and roots in response to elevated salt in Langi compared to respective controls. However, roots of Nipponbare showed non-significant reduction of biomass in response to salinity (Figure 4.1A). It is interesting to note that there was a tissue specific nature of Na^+ accumulation in the two cultivars. The Na^+ concentration in shoots was higher compared to roots in both cultivars (Figure 4.2A) and Nipponbare accumulated more Na^+ in shoots than did Langi. In contrast, the ratio of Na^+/K^+ was higher in the roots of both cultivars, indicating that roots accumulated less K^+ compared to shoots (Figure 4.2B). A comparatively higher shoot Na^+/K^+ ratio was recorded in Nipponbare while that in roots was lower, compared to Langi (Figure 4.2B).

Despite higher Na^+ accumulation and higher Na^+/K^+ ratios, Nipponbare had a significantly higher relative dry shoot biomass production compared to that of Langi (75.73% and 47.75% respectively: Figure 4.1B). Nipponbare also had higher relative root growth (56.75% and 44.33% respectively). This indicates that Nipponbare is capable of tolerating more Na^+ compared to Langi.

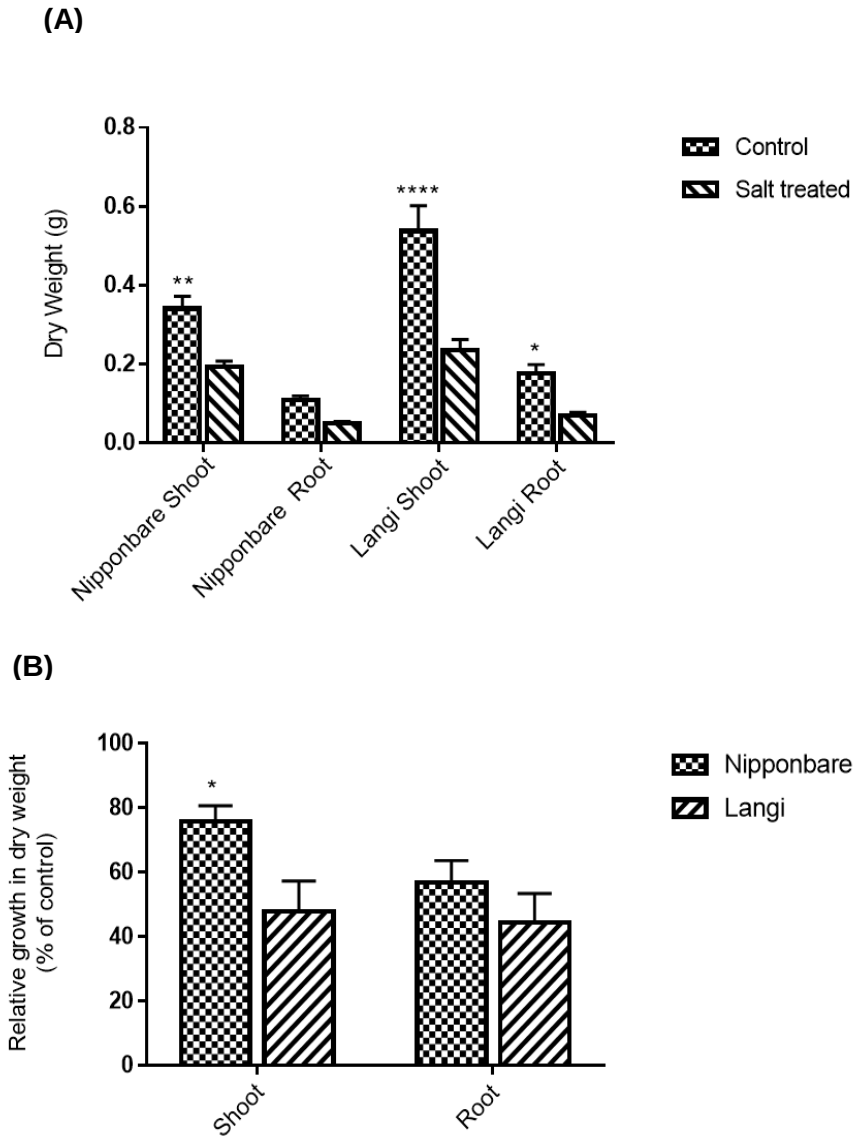


Figure 4.1 Comparative growth analysis for assessing relative salinity tolerance in seedlings of two rice cultivars, Nipponbare and Langi

Four weeks old seedlings grown on hydroponic culture system were exposed to 50 mM NaCl stress for 2 days followed by 120 mM NaCl for further 8 days and growth was assessed in term of fresh and dry biomass production. Data were compared as absolute dry weight (A) and relative dry weight (B) in shoot and root tissues of two cultivars. Values are shown as mean + SEM (n=5). *, ** and **** represent the statistical significant difference at P<0.01, P<0.001 and P<0.00001 according to *t*-test, respectively. Relative growth in dry weight: the growth in dry weight of the NaCl-treated shoots or roots relative to that in the control.

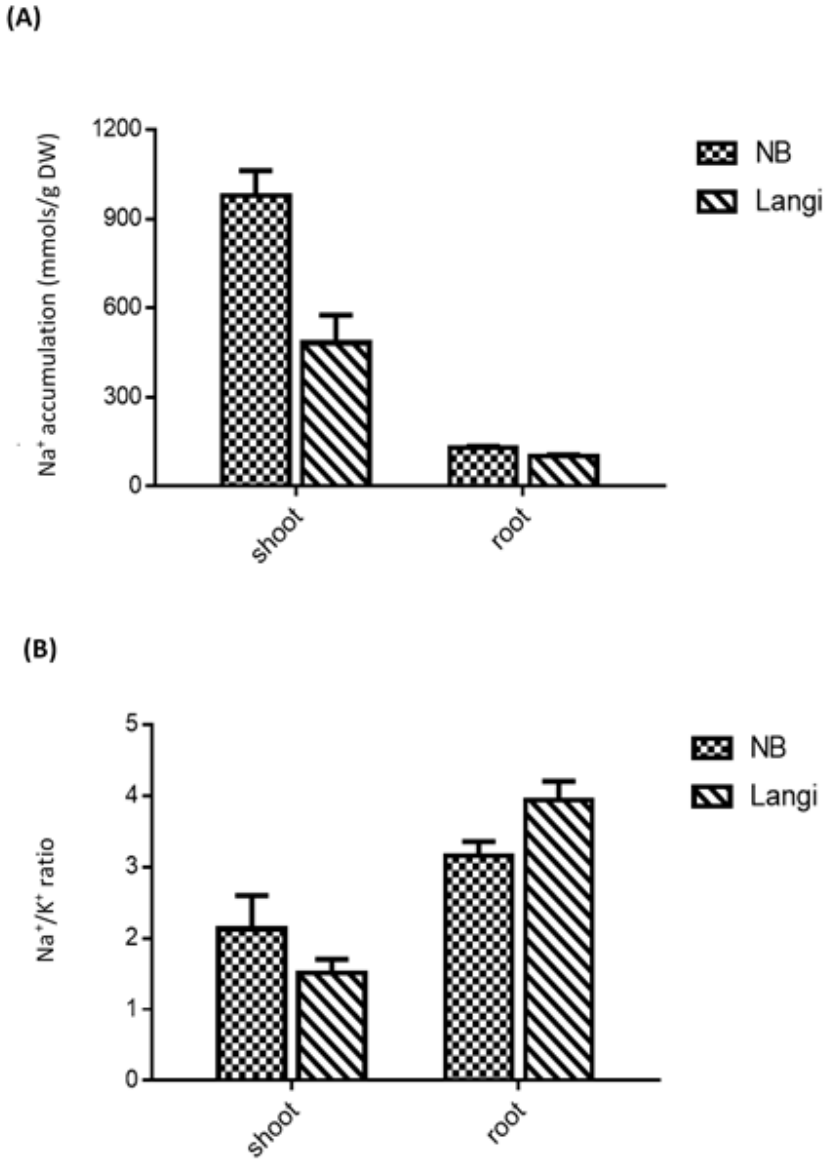


Figure 4.2 Comparative analysis of tissue ion accumulation for assessing relative salinity tolerance in seedlings of two rice cultivars, Nipponbare and Langi

Four weeks old seedlings grown on hydroponic culture system were exposed to 50 mM NaCl stress for 2 days followed by 120 mM NaCl for further 8 days and Na^+ and K^+ accumulation in tissues were measured using Flame photometer. Accumulation of Na^+ (A) and ratio of Na^+/K^+ (B) in tissues were compared. Data are shown as mean + SEM from five biological replicates.

4.3.2 Alteration of transcript abundance of alternative respiratory pathway genes in response to salinity stress

Results presented in Chapter 3 demonstrated that both AOX and NDH genes were stress-responsive with *AOX1a* and *NDB2* the most responsive to the direct inhibition of cytochrome pathway by antimycin A and KCN. Here, I examine the responsiveness of rice AOXs and NDHs in response to the environmental stress of salinity, widening the study to the expression of AP genes in different cultivars. Expression of three AOX isoforms (*AOX1a*, *AOX1b* and *AOX1c*) and six NDHs (*NDA1-2*, *NDB1-3*, and *NDC1*) in root and shoot tissues from cultivars Nipponbare and Langi were measured using qRT-PCR.

Transcript abundance of *AOX1a* and *AOX1b* from both cultivars increased in shoots and decreased in the roots in response to salinity stress (Figures 4.3A and B). However, the magnitude of responses differed between cultivars. There was a significant reduction of transcript levels of all three AOX isoforms in salt-treated Nipponbare roots over 12 day period, but in Langi roots, only *AOX1a* and *AOX1b* decreased. Both cultivars recorded their highest *AOX1c* transcript abundance 12 days after the salt application (Figure 4.3C). In general, in both cultivars, AOX transcripts increased in shoots and decreased in roots in response to salinity.

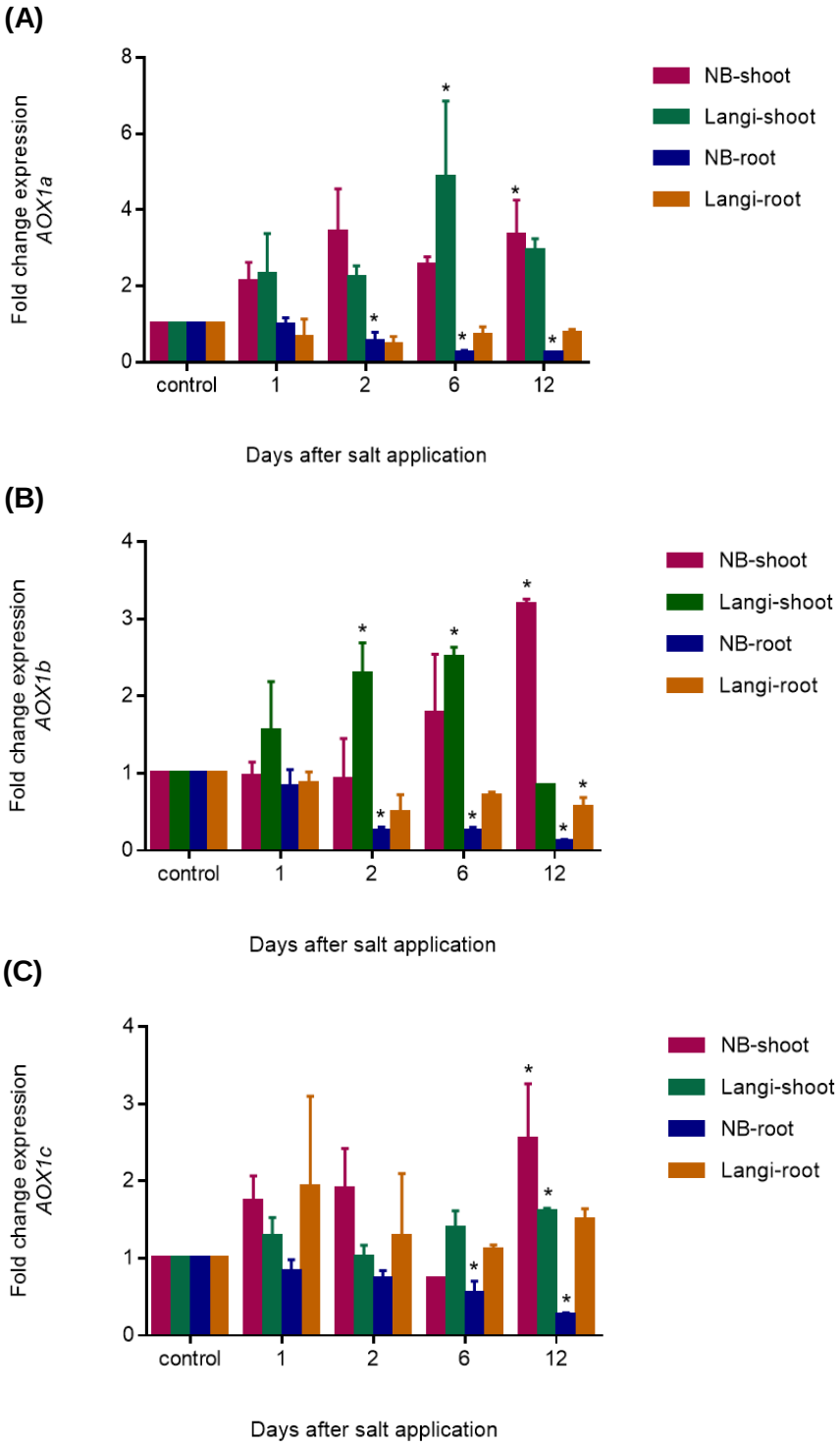


Figure 4.3 Comparative study of expression of AOX isoforms in two rice cultivars, Nipponbare and Langi, under salt-treated and non-treated conditions

Fold change expression of AOX isoforms, *AOX1a* (A), *AOX1b* (B) and *AOX1c* (C), in seedling tissues were analysed in response to 120 mM NaCl using qRT-PCR over a period of 12 days. Transcript levels in salt-treated or in control tissues were determined at 1, 2, 6 and 12 day(s) after the start of salt application and normalized against the geometric mean of three stable reference genes. Data are shown as the mean + SEM of three biological replicates relative to the control at each time point set as 1.00. * indicates a significant difference at $p<0.01$ according to *t*-test.

The most dramatic change in transcript abundance in response to salinity was of *NDB1* in Nipponbare-roots, with the highest abundance of (7.4-fold) at day 2. The response of *NDB3* transcripts in Nipponbare-roots was similar to that of *NDB1* except that *NDB3* responded to salt stress earlier (Figures 4.4A and C). NDB2 transcript abundance increased significantly only in Nipponbare-shoots, 12 days after the salt treatment (Figure 4.4B). *NDB3* transcript abundance increased in Langi-shoots and Nipponbare-shoots, several days after salt treatment. These results clearly indicate the diversity of expression of NDB isoforms in rice in response to salinity stress.

Transcript abundance of *NDA1* increased in shoots and decreased in roots of Nipponbare several days after salt treatment. *NDA1* transcript abundance decreased somewhat in roots of Langi (Figure 4.5A). In Langi, in contrast to *NDA1*, *NDA2* transcript level increased in shoots, peaking at day 6, while that in roots decreased initially and then levelled (Figure 4.5B). A significantly higher abundance of root *NDC1* was recorded in Langi at day 6 while, in shoots, it peaked at day 12. In Nipponbare, the transcript abundance of *NDC1* peaked at 12 days after the salt application in both roots and shoots (Figure 4.5C). Overall, these results show that rice alternative respiratory pathway genes are responsive to salinity stress but responses varied with cultivar as well as with tissue type.

It has been reported that the changes in the alternative pathway gene transcript abundance under environmental stress conditions correlates with the changes in the corresponding protein abundance and enzyme activity in plant tissues (Smith et al., 2009; Wang et al., 2011). With the aim of investigating the potential correlation of transcriptional level changes and corresponding protein levels and enzyme activity in response to salinity stress, the current study was extended with relevant experiments continued with cultivar Nipponbare.

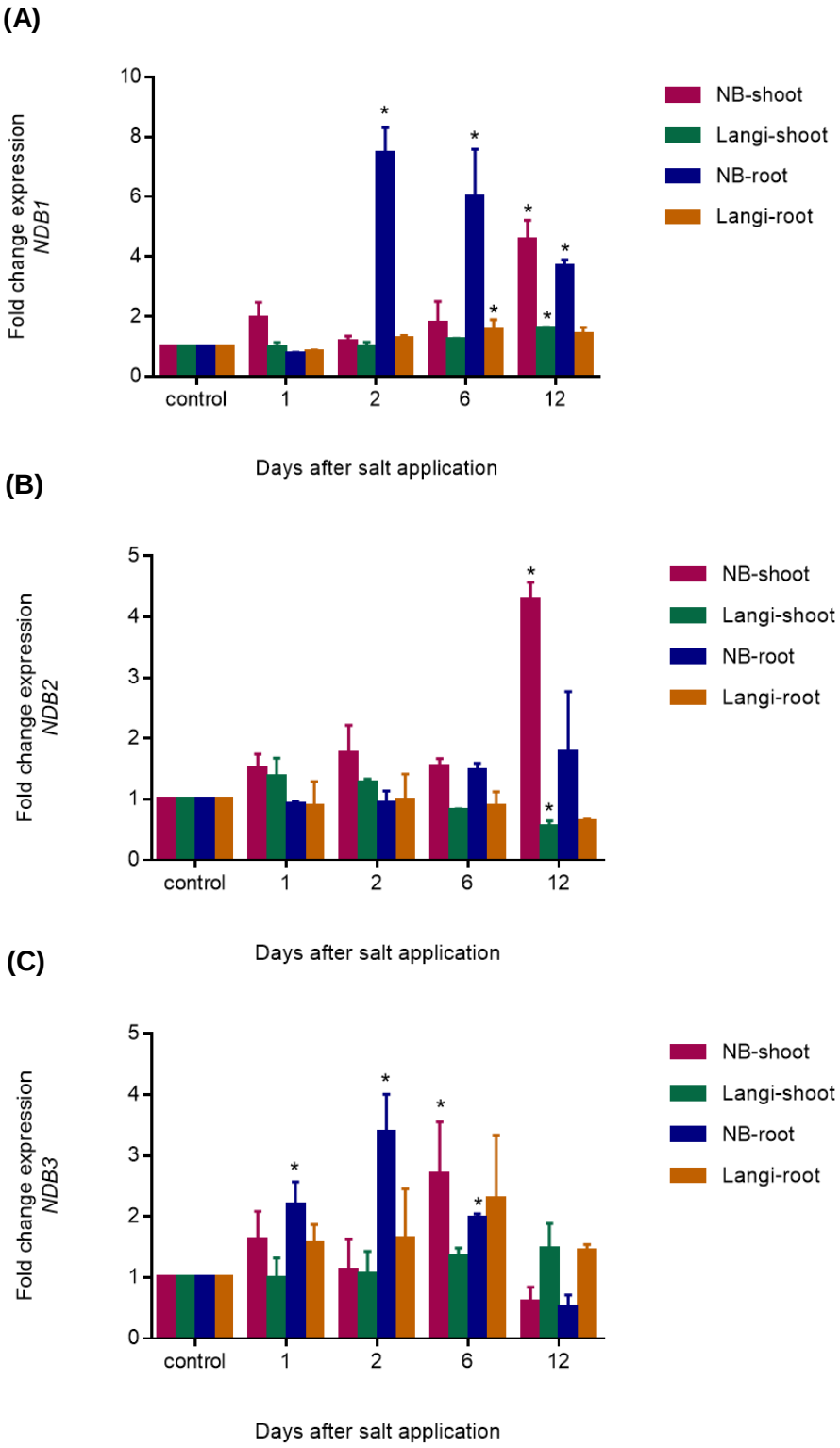


Figure 4.4 Comparative study of expression of NDB isoforms in two rice cultivars, Nipponbare and Langi, under salt-treated and non-treated conditions

Fold change expression of NDB isoforms, *NDB1* (A), *NDB2* (B) and *NDB3* (C), in seedling tissues were analysed in response to 120 mM NaCl using qRT-PCR over a period of 12 days. Transcript levels were determined in salt-treated or in control tissues at 1, 2, 6 and 12 day(s) after the start of salt application and normalized against the geometric mean of three stable reference genes. Data are shown as the mean + SEM of three biological replicates relative to the control at each time point set as 1.00. * indicates a significant difference at $p<0.01$ according to *t*-test.

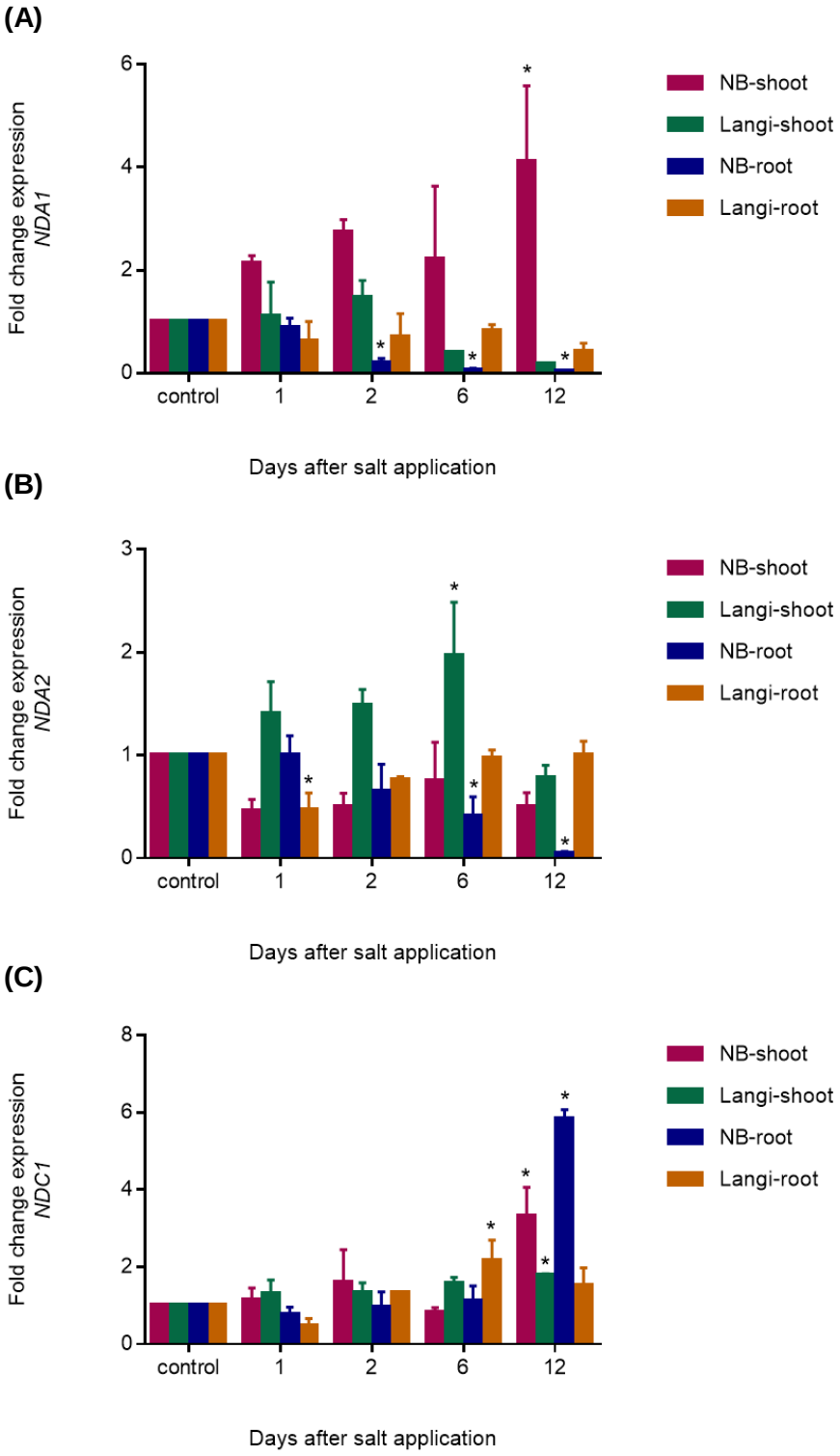


Figure 4.5 Comparative study of expression of NDA and NDC isoforms in two rice cultivars, Nipponbare and Langi, under salt-treated and non-treated conditions

Fold change expression of NDA isoforms, *NDA1* (A), *NDA2* (B), and *NDC1* (C), in seedling tissues were analysed in response to 120 mM NaCl using qRT-PCR over a period of 12 days. Transcript levels were determined in salt-treated or in control tissues at 1, 2, 6 and 12 day(s) after the start of salt application and normalized against the geometric mean of three stable reference genes. Data are shown as the mean + SEM of three biological replicates relative to the control at each time point set as 1.00. * indicates a significant difference at $p<0.01$ according to *t*-test.

4.3.3 Protein levels of alternative respiratory pathway in response to salinity stress

Using an immunoblotting strategy, the relative abundance of AOX proteins as well as NDB2 protein were investigated in mitochondrial preparations isolated from salt-stressed and non-stressed root and shoot tissues of cultivar Nipponbare.

4.3.3.1 Changes in AOX protein abundance

The abundance of AOX protein was estimated by immunoblotting mitochondrial proteins with a monoclonal antibody (AOA) raised against AOX from the aroid lily (*Sauromatum guttatum*), which can recognize AOX subunits in a variety of plant species (Elthon et al., 1989; Finnegan et al., 1999). It has been documented that AOX protein is present as a homodimer and capable of existing in either an oxidized or reduced form. When participating monomeric subunits in the dimer are covalently linked by an intermolecular disulphide bond, the protein is inactive (oxidized form). The reduction of this disulphide bond to its component sulfhydryl converts it to a form that can be activated by keto-acids such as pyruvate (Umbach and Siedow, 1993; Siedow and Umbach, 2000). These forms of AOX can be interconverted by the treatment with reductants such as dithiothreitol (DTT) or diamide and visualized on a membrane where the oxidized form has a molecular mass twice that of the reduced form (Umbach and Siedow, 1993).

Consistent with previous findings, both oxidized and reduced forms of the AOX protein were detected in mitochondrial proteins isolated from rice roots as well as from shoots (Figures 4.6B and 4.7). The oxidized form of the protein was detected close to the 75 KD band of the molecular weight marker while the reduced form was detected close to the 37 KD band, which is the calculated molecular weight of rice AOX proteins based on their peptide sequences. When protein samples from roots were treated with reducing agents (DTT), only the reduced form of the protein was detected on the membrane (Figure 4.6A).

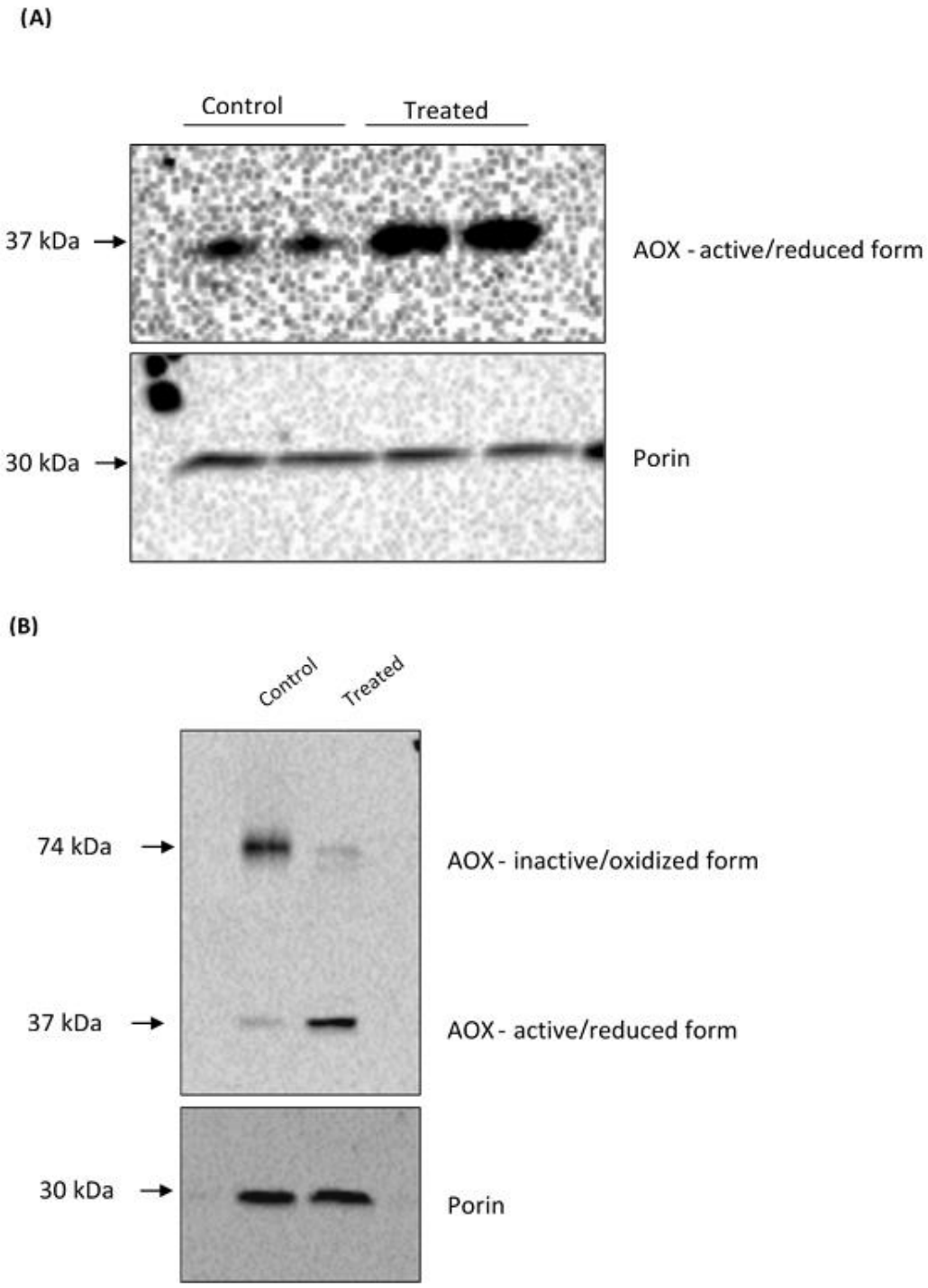


Figure 4.6 Detection of rice AOX protein in mitochondria isolated from salt-treated and control roots by the monoclonal AOA antibody

Immunoblots of the AOX protein present in mitochondria isolated from salt-treated and non-treated (control) roots harvested 9 days after the start of salt application. (A)-samples treated with reductant (DTT) and (B) samples not treated with reductants. Pre-stained markers were imaged with white light and aligned with chemiluminescent images to determine molecular weights of bands. Duplicate blots were prepared, probed with porin antibody and visualized by chemiluminescence to indicate the equal loading of samples. KD-kilodaltons.

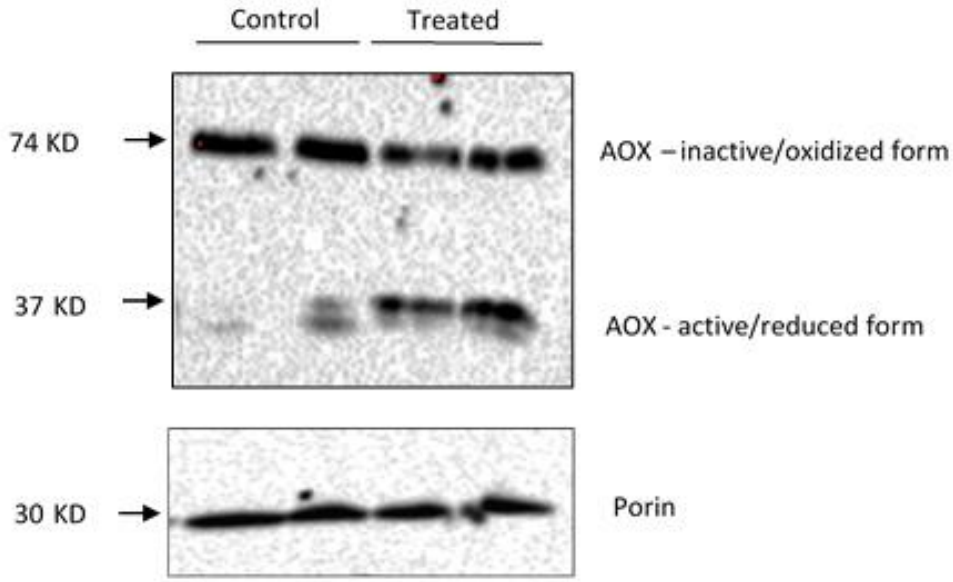


Figure 4.7 Detection of rice AOX protein in mitochondria isolated from salt-treated and control shoots by the monoclonal AOA antibody

An immunoblot of the AOX protein present in mitochondria isolated from salt-treated and non-treated (control) shoots harvested 9 days after the star of salt application Pre-stained markers were imaged with white light and aligned with chemiluminescent images to determine molecular weights of bands. Duplicate blots were prepared, probed with porin antibody and visualized by chemiluminescence to indicate the equal loading of samples. KD-kilodaltons.

While transcript abundance of AOX genes tended to increase in shoots of Nipponbare under salt stress (Figure 4.8), total protein abundance of AOX (using an antibody that does not distinguish between AOX isoforms) was approximately the same in mitochondria isolated from control and salt-treated plants (Figures 4.6B and 4.7). However, in mitochondria from salt-stressed plants, AOX protein was predominately in the reduced (active) form while in mitochondria from control plants it was largely in the oxidised (inactive) form (Figures 4.6B and 4.7). This suggests that post-translational regulation of AOX may be more important for that protein *per se* in rice.

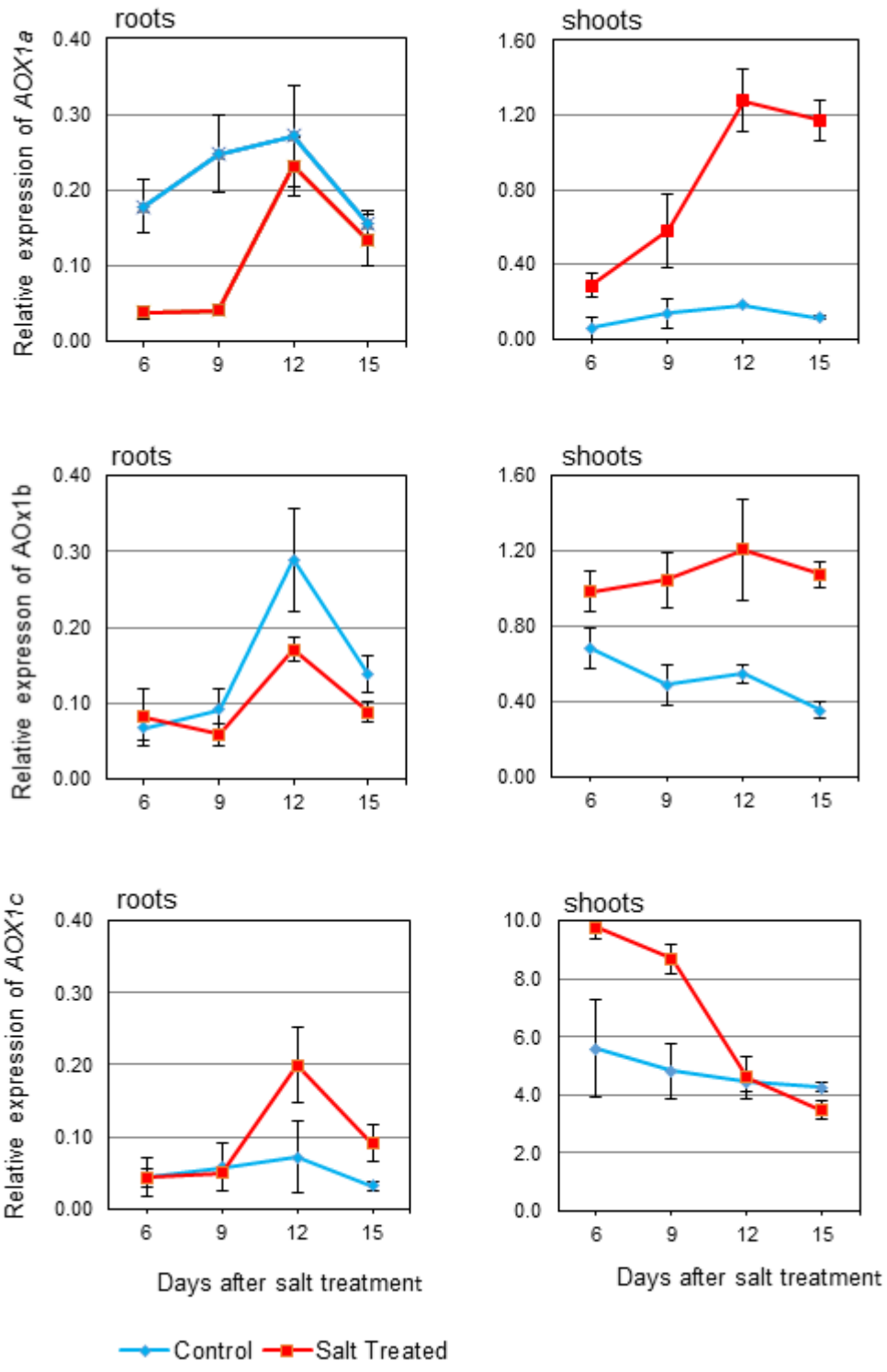


Figure 4.8 Transcript abundance of AOX isoforms in salt-treated and non-treated roots and shoots of cv Nipponbare over a period of 15 days

Seedlings (cv Nipponbare) grown on hydroponic cultures were exposed to 120 mM NaCl and transcript levels were determined in salt-treated or control tissues 6, 9, 12 and 15 days after the start of salt application. Transcript data were normalized against the geometric mean of three reference genes. Data are shown as the mean relative gene expression ± SEM of three biological replicates. Tissues for qRT-PCR and protein assay were sampled from the same experiment.

4.3.3.2 Changes in NDB2 protein abundance

The abundance of NDB2 protein in salt-treated and control tissues was also investigated by immunoblotting with mitochondria isolated from salt-treated and non-treated tissues harvested 9 days after the start of salt application. Affinity purified polyclonal antibodies were synthesized against NDB2 peptide (Biomatik, http://www.biomatik.com) and the concentration of NDB2 specific antibody was titrated against dilutions of unconjugated peptide antigen by dot blot analysis to determine the antibody dilution suitable for use in immunoblotting (not shown). Before being tested with experimental samples, NDB2 specific antibodies were tested with purified mitochondria isolated from etiolated rice seedlings. Immunoblots of mitochondria from etiolated rice seedlings produced a sharp band at around the predicted molecular weight of rice NDB2 (~64 KD). (Image of the pre-stained protein marker run parallel to samples was aligned with the blot to estimate the size of the band) (Figure 4.9).

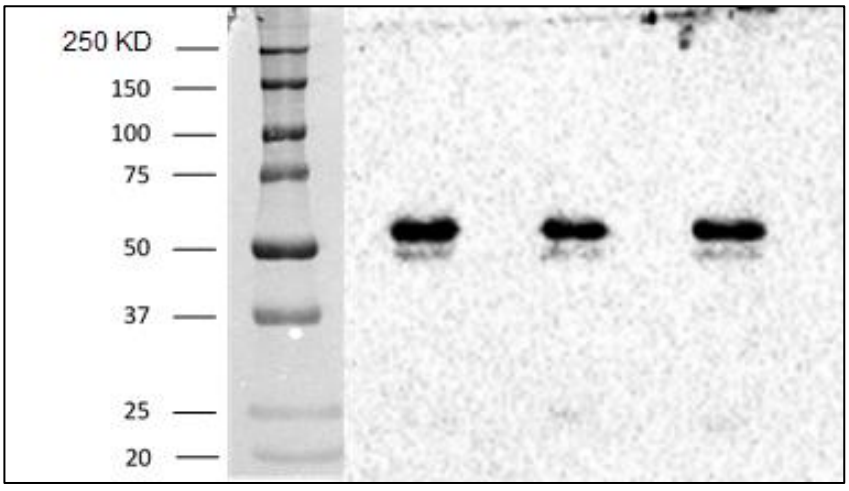


Figure 4.9 Detection of NDB2 protein in seedling mitochondria

Immunoblot image showing the detection of the peptide recognition sequence of NDB2 protein in purified mitochondria isolated form etiolated rice seedlings, by the polyclonal antibody developed during the current study. The blot shows probing of three technical replicates of the sample with 1:10000 dilution of antibody.

Using the antibody tested above, immunoblots showed that NDB2 protein abundance in mitochondria isolated from salt-treated shoots was higher than that in mitochondria from control shoots (Figure 4.10). It is interesting to note that no bands were detected in mitochondria from the roots of either set of plants, even though porin controls showed that the loading of mitochondrial protein was equivalent to that of shoot mitochondria (Figure 4.10) and transcript abundance in both shoots and roots was very similar at 9 days after treatment (Figure 4.11). This was unexpected and a second experiment also showed the same results. However, qRT-PCR analysis showed that transcript abundance in roots did not increase in salt-treated plants until 12 days after treatment, in contrast to the situation in shoots, where NDB2 transcripts were higher in stressed plants from the beginning of the treatment (Figure 4.11). Further investigation is needed to determine why NDB2 protein is so low in root mitochondria, but the results suggest that post-translational events may be different between roots and shoots.

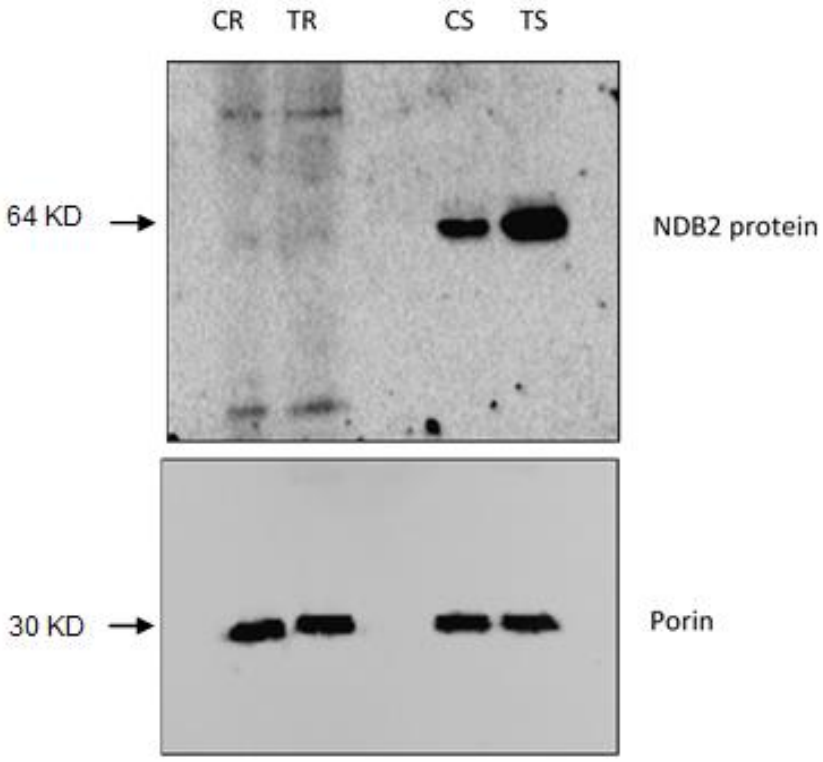


Figure 4.10 Detection of rice NDB2 protein in mitochondria isolated from salt-treated and control shoots by the polyclonal NDB2 antibody developed during the current study

An immunoblot of the NDB2 protein present in mitochondria isolated from salt-treated and control shoots harvested 9 days after the start of salt application. Pre-stained markers were imaged with white light and aligned with chemiluminescent images to determine molecular weights of bands. Duplicate blots were prepared, probed with porin antibody and visualized by chemiluminescence to indicate the equal loading of samples. KD – kilodaltons. CR-control roots; TR-treated roots; CS-control shoots; TS-treated shoots

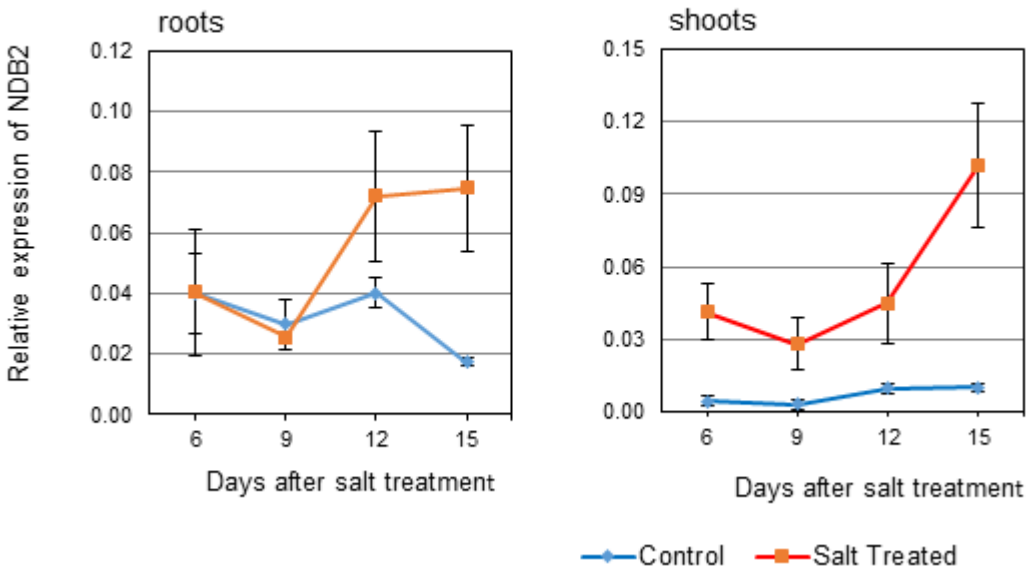


Figure 4.11 Transcript abundance of *NDB2* in salt-treated and non-treated roots and shoots from cv Nipponbare over a period of 15 days

Seedlings (cv Nipponbare) grown on hydroponic cultures were exposed to 120 mM NaCl and transcript levels were determined in salt-treated or control tissues 6, 9, 12 and 15 days after the start of salt application. Transcript data were normalized against the geometric mean of three reference genes. Data are shown as the mean relative gene expression ± SEM of three biological replicates. Tissues for qRT-PCR and protein assay were sampled from the same experiment.

4.3.4 Alternative respiratory pathway enzyme activity in response to salinity stress

The same mitochondria preparations used in the immunoblot analysis were used in enzyme activity assays immediately after the purification of mitochondria. Oxygen uptake rates were used to determine cytochrome pathway, AOX, Ca^{2+} dependent and Ca^{2+} independent external NAD(P)H dehydrogenase activities in a Clark-type oxygen electrode.

4.3.4.1 Changes in oxygen consumption rates across cytochrome and AOX pathway in response to salinity stress

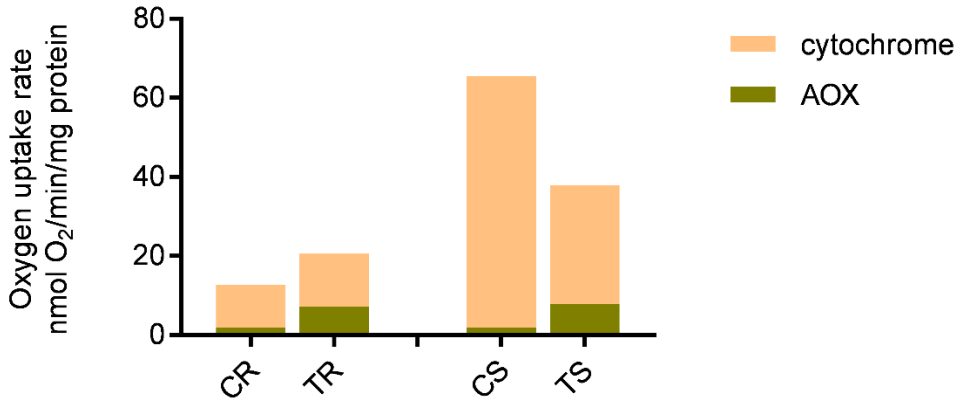


Figure 4.12 Average oxygen consumption rates for mitochondria isolated from salt-treated and non-treated shoot and root tissues

Oxygen consumption rates across cytochrome and AOX pathway were determined for mitochondria isolated from salt-treated and non-treated shoot and root tissues using a Clark-type oxygen electrode. Data are shown as average oxygen uptake rates (nmol O_2/min/mg mitochondrial protein) from two independent experiments. Total oxygen consumption rate was measured in the presence of 10 mM succinate, 5 mM pyruvate, 1 mM NADH, 1mM ATP, 1 mM ADP, 5 mM DTT in final concentrations. The cyanide resistant and the residual oxygen consumption rates were determined in the presence of 1 mM KCN and 250 nM OG (Octyl Gallate) in final concentrations, respectively. CR-control root; TR-treated root; CS-control shoot; TS-treated shoot.

When comparing the inhibitory effect of high salinity on seedling respiration, cytochrome pathway and cyanide resistant (AOX) pathway showed different sensitivities (Figure 4.12). AOX pathway showed an increased activity in both root and shoot tissues compared to their controls. But, though there was a slight increase in activity of cytochrome pathway in roots, it was definitely reduced in shoots

(Figure 4.12). Overall, there was a slight increase of total respiration in roots in response to salinity level tested while it was reduced significantly in shoots ($P<0.03$). It is also worthwhile to note that the increased activity of AOX enzyme in response to salinity stress in shoots corresponded to responses detected at the protein level as well as the transcriptional level. However, in roots, transcript levels did not correlate with protein and activity measurements.

4.3.4.2 Changes in NAD(P)H oxidation in response to salinity stress

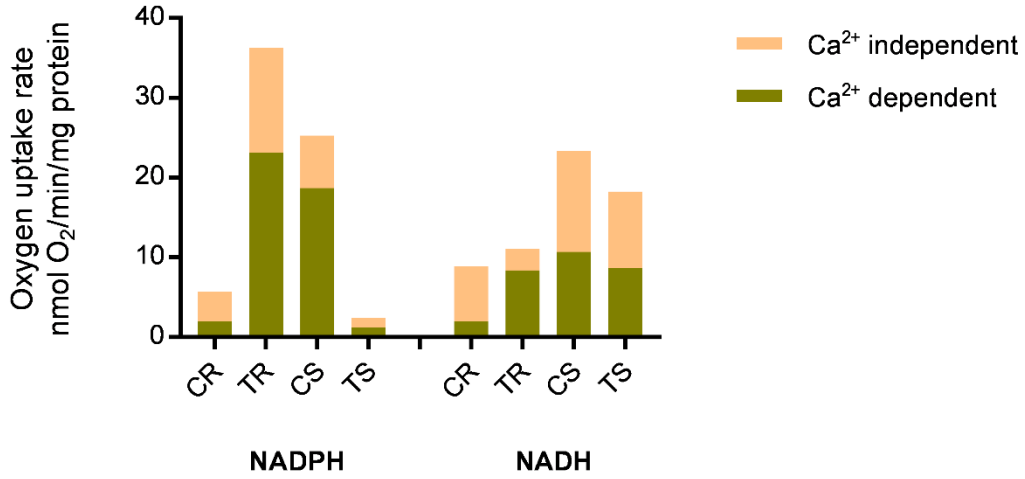


Figure 4.13 NAD(P)H oxidation in mitochondria isolated from salt-treated and non-treated root and shoot tissues

Oxygen consumption rates were determined for mitochondria isolated from salt-treated and non-treated root and shoot tissues using a Clark-type oxygen electrode. NADPH and NADH oxidation were determined in the presence and absence of Ca^{2+}. Data are shown as average oxygen uptake rates (nmol O_2/min/mg mitochondrial protein) from two independent experiments. CR-control root; TR-treated root; CS-control shoot; TS-treated shoot.

Both Ca^{2+} dependent and Ca^{2+} independent NADPH oxidation were increased in salt-treated roots compared to their controls while those in salt-treated shoots were reduced in response to salinity stress (Figure 4.13). For NADH oxidation, the Ca^{2+} dependent NADH oxidation in roots was induced while Ca^{2+} independent NADH oxidation was inhibited by the high salinity compared to their controls. There was only a slight reduction of both Ca^{2+} dependent and independent NADH oxidation in shoots in response to salinity stress (Figure 4.13).

4.4 Discussion

A number of previous studies have documented the induction of AOXs in rice under environmental stresses, such as low temperature (Ito et al., 1997) and high salt (Ohtsu et al., 2002; Feng et al., 2010b; Feng et al., 2013). However, no work has been reported so far on the responses of rice NDHs under environmental stresses. Here, a comparative study of transcriptional level responses of alternative respiratory pathway genes, AOXs and NDHs, has been undertaken in two contrasting rice cultivars under salinity stress and extended investigation of the alteration of AP genes at protein and enzyme activity level in response to salinity stress.

Consistent with previously documented studies, a significant growth reduction in salt-treated seedlings from both cultivars compared to their controls was observed, showing that the seedlings were stressed. Shoots from Nipponbare store more Na^+ than Langi, while roots from both cultivars accumulated less Na^+ compared to shoots. Interestingly, the ratio of Na^+/K^+ in roots was higher than that of shoots in both cultivars suggesting the capability of acquiring K^+ was higher in shoots compared to that of roots. However, Nipponbare showed a higher relative growth in both shoots and roots compared to Langi under the tested salinity stress, suggesting that Nipponbare was more capable of withstanding salt stress. These results demonstrated that there is a cultivar specific variability in salinity tolerance responses in rice. The genetic variation that exists among cultivars can be used to identify the underlying mechanisms and molecular components that create cultivar-specific responses to salinity stress (Kavitha et al., 2012).

The study of expression of AP genes in the two contrasting rice cultivars demonstrated that some genes were up-regulated while others were down-regulated in response to salinity stress. It is also interesting to note that though expression patterns of alternative dehydrogenases varied substantially between the two cultivars, the expression of AOXs, especially *AOX1a* and *AOX1b*, showed a very similar

expression pattern in different tissue types from the two cultivars, with a gradual increase of transcript abundance in shoots and a gradual decrease of transcript abundance in roots (Figure 4.3). The current results with shoots are coincident with those of Ohtsu et al. (2002) and Feng et al. (2010b). With roots, Feng et al. (2013) reported an increase of transcript abundance of *AOX1a* and *AOX1b* when roots were exposed to 200 mM and 300 mM salt stress for 12 h. On the other hand, Ohtsu et al. (2002) reported that there was greater transcript abundance of *AOX1a* and *AOX1b* in roots 6 h after the treatment with 250 mM salt, which then decreased gradually, similar to my results. During the current study, assessment of transcript levels was started a day after the salt treatment and a gradual decrease of transcript abundance in AOX isoforms was observed throughout the testing period showing a similar trend as observed by Ohtsu et al. (2002) in the expression of AOX isoforms in salt-stressed rice roots. An analysis of microarray data on “Genevestigator” database (https://genevestigator.com/) further confirmed the consistency of presented results on transcriptional level expression of AOX isoforms in rice shoots and roots in response to salinity stress (Figure 4.14).

The microarray data from Genevestigator database clearly demonstrate the tissue-specific nature of *AOX1* expression in rice in response to salinity stress and the patterns of *AOX1a* and *AOX1b* genes in different tissue types from different cultivars are consistent with my observations. In particular, there was a reduction of *AOX1a* and *AOX1b* transcriptional abundance in roots from the cultivars Pokkali, IR63731, IR29 and FL478, which were assessed 8 days after the salt application (Figure 4.14). Collectively, all of these findings suggest that there was an immediate increase of *AOX1a* and *AOX1b* at the transcriptional level in roots in response to salinity stress followed by a decrease. Overall, while increases in transcript abundance of the different AOX genes were seen at various times in shoots and roots, there were not significant differences between the salt-sensitive and salt-tolerant rice cultivars.

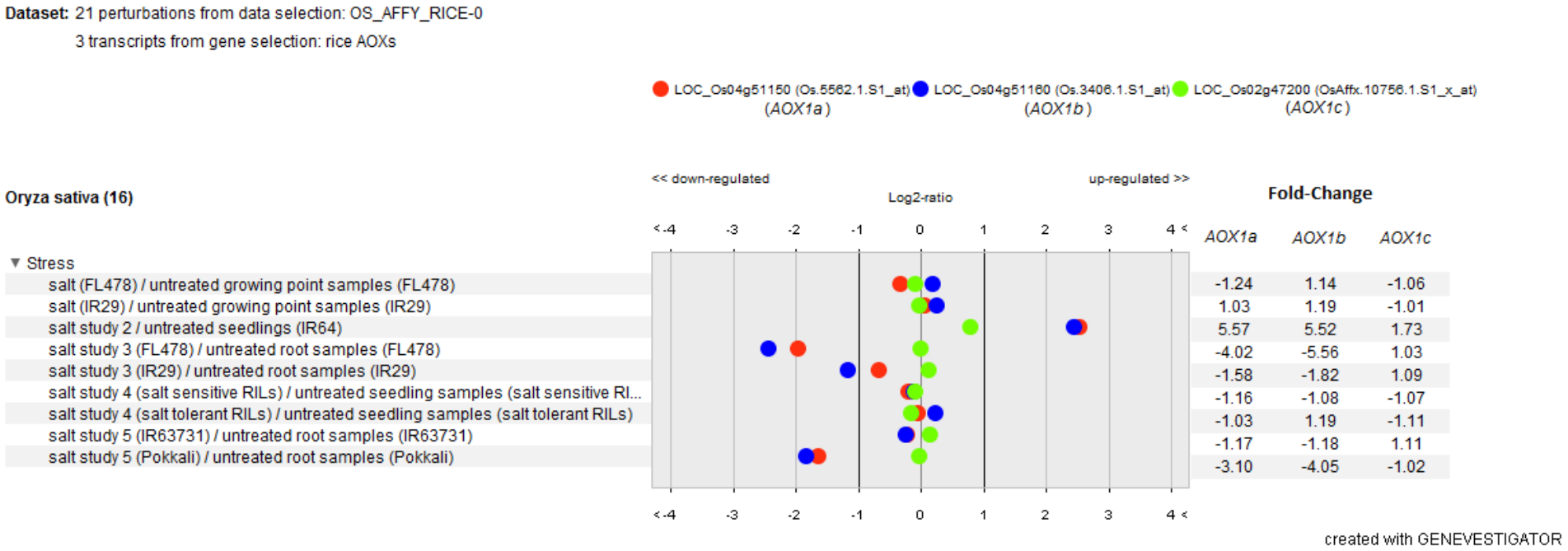


Figure 4.14 Transcriptional level expression of AOX isoforms in different rice cultivars in response to salinity stress (extracted from Genevestigator database)

Data illustrate the fold-change expression of *AOX1a*, *AOX1b* and *AOX1c* in salt-treated tissues compared to their controls in different rice cultivars. Data clearly demonstrate the tissue-specific nature of gene expression and the similar expression pattern of *AOX1a* and *AOX1b* in different tissue types (whole seedlings, roots and growing point/crown tissue) from different cultivars. Comparatively *AOX1c* is less responsive to the salt stress. Different experimental conditions were applied to different rice cultivars. The cultivars Pokkali, IR63731, IR29 and FL478 were subjected to ~60 mM salt for 8 days and roots and crown tissues were used for the transcriptional analysis. The cultivar IR64 and RIL lines were subjected to 200 mM salt for 3 h and 150 mM salt for 24 h respectively and whole seedling was used for the transcriptional analysis (https://genevestigator.com/).

AOX was also followed at the protein level in Nipponbare. Measurements of AOX protein levels showed that while they were similar in total between control and stressed plants, the latter had much more of the protein in its reduced (monomer) active form. Activity assays were consistent with this. It therefore seems that AOX is regulated at both the transcriptional and post-translational levels in rice.

There are a number of documented studies on changes in rice AOXs at enzyme activity level measured using slices of leaf or root tissues. These studies reported that the capacity of AOX respiration was significantly increased in leaves when seedlings were treated with 300 mM salt for 24 h without significant changes to the total respiration compared to their controls (Feng et al., 2010b). Feng et al. (2013) also observed a significant increase of CN-resistant/AOX respiration and significant decrease in the total respiration in response to 200 mM salt. They further observed that increase of salt concentration up to 300 mM further decrease the level of total respiration but CN-resistant respiration did not show any significant changes compared to the control. Although the observations made during the present study on CN-resistant respiration is consistent with the documented results, contrasting observations were made on the changes of total respiration in response to salinity stress. The present data demonstrated that there was a slight increase of total respiration rate in mitochondria isolated from salt-treated roots while the total respiration rate in salt-treated shoots was reduced dramatically due to the reduction of cytochrome respiration rate (Figure 4.12). Similar observations on the responses of total respiration and cytochrome respiration were made by Jolivet et al. (1990) in mitochondria isolated from salt-treated (400 mM for 72 h) barley leaves compared to their controls, but AOX respiratory rates remained unchanged. The same authors further reported that leaf slices also showed the same responses in total respiration and cytochrome respiration but there was an increase of AOX respiration. Collectively, it can be concluded that there is an induction of AOXs in rice at transcriptional and enzyme activity levels in both shoot and root tissues in response

to salinity stress, but the latter seems to be due to post-translational regulation via protein oxidation.

Co-expression of AOXs and NDHs has been reported in a number of plant species under abiotic stresses (Clifton et al., 2005; Smith et al., 2009), which could be a result of co-regulation of these genes by common sequence elements present in the up-stream promoter regions (Clifton et al., 2005; Ho et al., 2008). Co-expression of number of rice NDHs have been observed during the current study when the cytochrome pathway was inhibited either by AA or KCN (Chapter 3). Data from Chapter 3 further revealed that up-stream promoter regions of these co-expressed genes share common sequence elements that have been identified as regulatory elements responsible for the stress tolerance in Arabidopsis *AtAOX1a* and *AtNDB2,* suggesting that these co-expressed genes could be co-regulated under chemical stress. Verification of this is required through functional analysis of the promoter region of the respective genes. To confirm whether NDHs are co-expressed under the environmental stress conditions and to explore the expression diversity of NDHs in different rice cultivars, a comparative study of transcriptional alteration of NDHs were carried out.

The results demonstrated that a number of rice NDHs were also responsive to salt stress, confirming that in rice NDHs and AOXs co-expressed under environmental stresses like salinity. Due to its response to ETC inhibition (Chapter 3), NDB2 was further investigated at the protein level and enzyme activity level. There is no documented work so far on expression patterns of rice NDHs at protein or enzyme activity levels or even at transcriptional levels except the microarray data available on the Genevestigator database. The expression of NDHs exhibited much more variation between the two cultivars. Some NDHs were up-regulated while others were down-regulated in response to salinity. The microarray data available on Genevestigator database demonstrated a similar pattern of expression of rice NDHs, with up- and

down-regulation in response to salinity, as also seen for AOXs (Figure 4.14, 4.15 and 4.16), which is consistent with current observations. My results demonstrated a cultivar- and tissue-specific variation in NDHs expression between japonica and indica rice. This diversity was not so evident in microarray data, which could be due to all investigated cultivars being from the same rice group of indica. However, cultivars IR64 and Pokkali showed contrasting expression of NDHs in root and seedling tissues. The expression of *NDA1*, *NDA2*, *NDB2*, and *NDB3* was up-regulated in IR64 while *NDB1* and *NDC1* were down-regulated in seedling tissues. In contrast, the expression of *NDA1*, *NDA2*, *NDB2*, and *NDB3* were down-regulated in Pokkali while *NDB1* and *NDC1* showed the same pattern of expression as in IR64 (Figure 4.15 and 4.16), suggesting a tissue-type variation even within the same rice group as detected in the current study.

The analysis of transcriptional changes with changes in mitochondrial NAD(P)H oxidation activities and NDB2 protein in roots and shoots, appears to give conflicting evidence. Salt-treatment resulted in an increase in NADPH and calcium-dependent NADH oxidation rates in mitochondria isolated from root tissue and a decline of NADPH oxidation in salt-treated shoot mitochondria (Figure 4.13). In tissue collected at 9 days after salt stress there was no change in *NDB2* transcript in roots and slightly higher in shoots (Figure 4.11). Additionally, no change in the amount of NDB2 protein content in roots. It may be that these activity changes are mediated by products of other genes rather than *NDB2*. For example *NDB1* and *NDB3* transcript levels both increase earlier in salt stress (Figures 4.4A and 4.4C), in potato and Arabidopsis NDB1 protein has been linked to NADPH oxidation in mitochondria (Michalecka et al., 2004; Geisler et al., 2007) and the antibody designed for OsNDB2 protein would not interact with OsNDB1 or OsNDB3.

In conclusion, rice AP genes, including both AOX and NDH, were responsive to salinity stress. There was a cultivar- and tissue-specific variation in expression patterns, which will be further investigated in the next Chapter using a number of salt-tolerant and sensitive cultivars representing both japonica and indica groups. Consistent with previous findings with other plant species, it appears that rice AOX also can exists as a homodimer, which can be reduced or oxidized and an increase of the reduced (active) form of the protein was detected in salt-treated tissues, as was an increase in AOX activity. During the study, a polyclonal antibody was developed to specifically bind to rice NDB2. As the peptide recognition sequence of this particular antibody shares high similarity with other cereals, it could be useful in investigating NDB2 protein from number of other cereals, including wheat and barley.

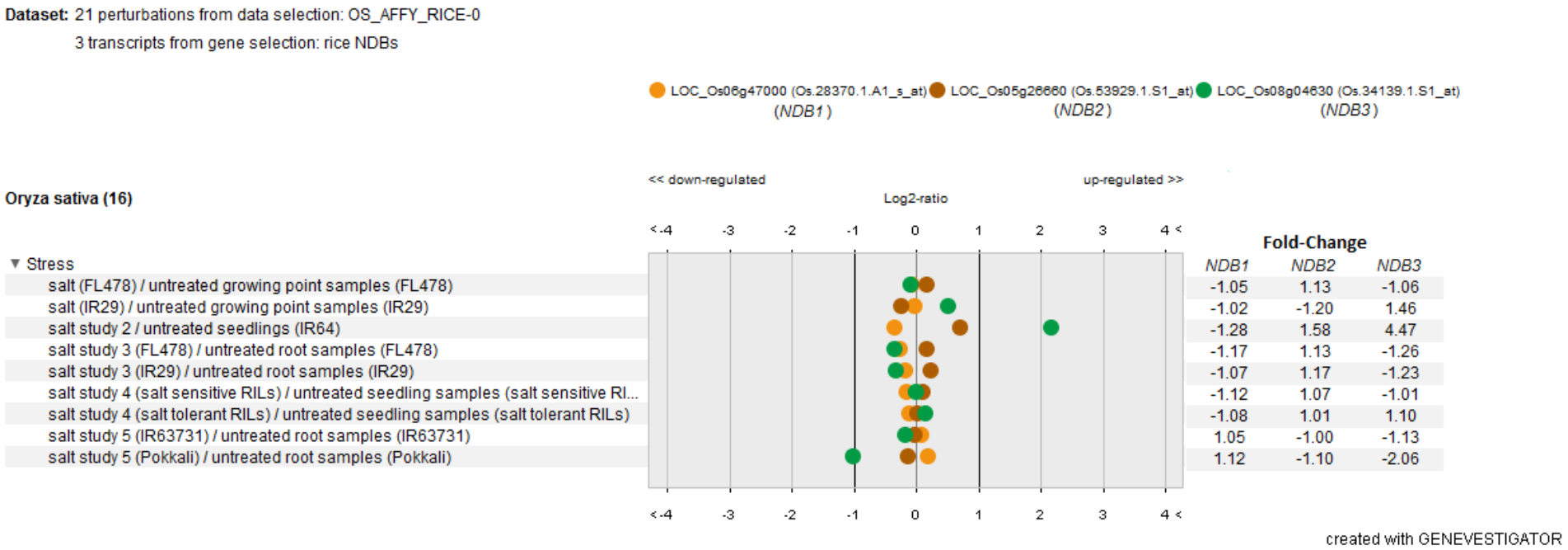


Figure 4.15 Transcriptional level expression of NDB isoforms in different rice cultivars in response to salinity stress (extracted from Genevestigator database)

Data illustrate the fold-change expression of *NDB1*, *NDB2* and *NDB3* in salt-treated tissues compared to their controls in different rice cultivars. Data demonstrate a less cultivar variation in the expression of NDB isoforms among cultivars from indica rice. Different experimental conditions were applied to different rice cultivars. The cultivars Pokkali, IR63731, IR29 and FL478 were subjected to ~60 mM salt for 8 days and roots and crown tissues were used for the transcriptional analysis. The cultivar IR64 and RIL lines were subjected to 200 mM salt for 3 h and 150 mM salt for 24 h respectively and whole seedling was used for the transcriptional analysis (https://genevestigator.com/).

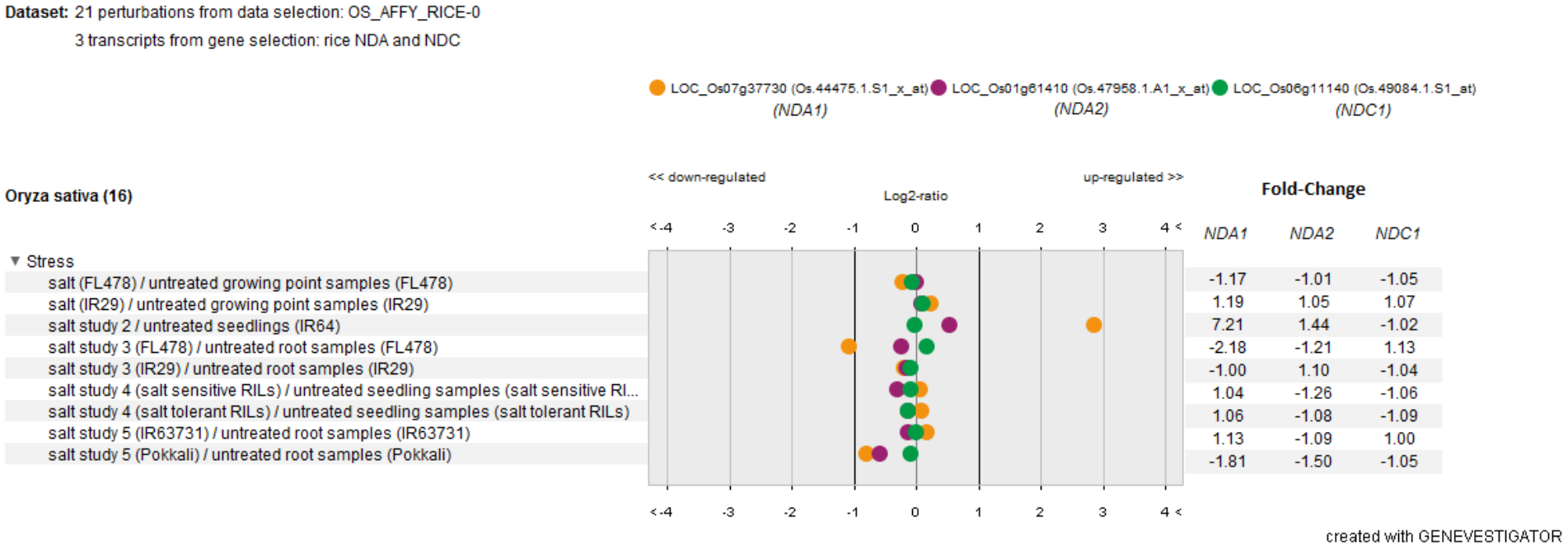


Figure 4.16 Transcriptional level expression of NDA isoforms and *NDC1* in different rice cultivars in response to salinity stress (extracted from Genevestigator database)

Data illustrate the fold-change expression of *NDA1*, *NDA2* and *NDC1* in salt-treated tissues compared to their controls in different rice cultivars. Data demonstrate a less cultivar variation in the expression of *NDA* and *NDC* isoforms among cultivars from indica rice. Comparatively, *NDC1* was less responsive to the salt stress. Different experimental conditions were applied to different rice cultivars. The cultivars Pokkali, IR63731, IR29 and FL478 were subjected to ~60 mM salt for 8 days and roots and crown tissues were used for the transcriptional analysis. The cultivar IR64 and RIL lines were subjected to 200 mM salt for 3 h and 150 mM salt for 24 h respectively and whole seedling was used for the transcriptional analysis (https://genevestigator.com/).

5 Differential sensitivity of rice cultivars to salinity stress and cultivar variation in the expression of alternative respiratory pathway genes

5.1 Introduction

Salinity tolerance in plants greatly varies among species and even cultivars within a species, as reflected in their different growth responses (Munns and Tester, 2008; Rajendran et al., 2009). It ranges from highly salt-sensitive glycophytes to highly salt-tolerant halophytes (Figure 5.1).

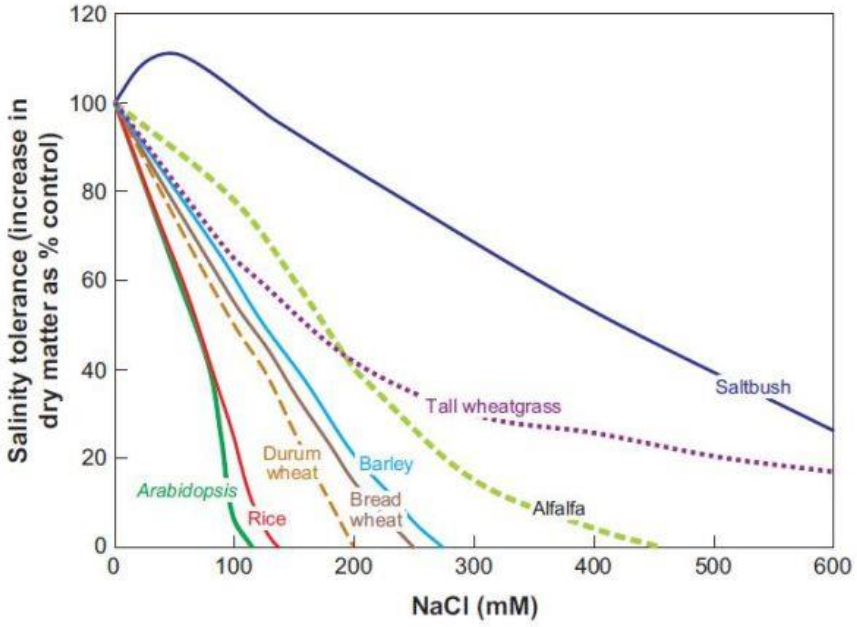


Figure 5.1 The differential sensitivity of plant species to elevated salinity

Salinity tolerance is shown as increases in shoot dry matter after growth in solution or sand culture containing NaCl for at least 3 weeks, relative to plant growth in the absence of NaCl (Munns and Tester, 2008).

Rice (*O. sativa*) is the most salt-sensitive species of the cereals while barley (*Hordeum vulgare*) is the most tolerant. Tall wheatgrass (*Thinopyrum ponticum*) is one of the most salt-tolerant of the monocotyledonous species and is a halophytic relative of wheat (Figure 5.1) (Munns and Tester, 2008). Dicotyledonous species show greater variance in salinity tolerance compared to monocotyledonous species.

Some species, such as Arabidopsis, are more sensitive while legumes like alfalfa (*Medicago sativa*) are very tolerant. Saltbush (*Atriplex* spp.), a halophyte, is able to grow well in salinities greater than that of sea water (Figure 5.1) (Aslam et al., 1986; Munns and Tester, 2008).

Salinity tolerance is a genetically and physiologically complex trait, which exhibits quantitative inheritance. This trait is complex as it is a combined result of number of factors such as plant level and cell level ion compartmentation, minimizing Na^+ uptake by roots, secretion through glands etc. It shows continuous variation and also is highly affected by the environment (Koyama et al., 2001; Kumar et al., 2015). The phenotype of a quantitative trait or characteristic is a collective result of the interaction of many genes (polygenic) and all genes together contribute towards the overall phenotype of the plant despite environmental disturbances (Bartley et al., 2013).

Cultivated rice was domesticated from a common ancestor of wild rice. But, as a result of both natural and human selection, there exists large genetic diversity across thousands of varieties. As described in Section 1.1, rice cultivars are grouped into two subspecies, japonica and indica, with marked differences in plant architecture, agronomic and physiological features (Khush, 1997). As japonica and indica cultivars exhibit clear variation in genome sequences as well as in their morphological and physiological characteristics, it is worthwhile to study the molecular and physiological response variations between japonica and indica rice to environmental stresses, which will be helpful in identifying the mechanism of tolerance.

Rice shows differential sensitivity to salinity stress during its life cycle. Generally, it is considered that rice plants are more sensitive to salt at the young seedling stage and less sensitive at the reproductive stage (Flowers and Yeo, 1981). Nevertheless, there is still a high impact on rice yield when plants are exposed to salinity stress during early reproductive stages, such as panicle initiation or during pollination (Zeng et al., 2001). There is a great variation in salt content and survival rate of individual rice

plants at seedling stage within the same variety as well as between varieties grown under salt stress (Flowers and Yeo, 1981). Differential varietal responses to salinity stress were not limited to seedling stage, but also clearly seen in plants growing at reproductive stage (Mohammadi-Nejad et al., 2010). Ferdose et al. (2015) reported that growth inhibition by salt stress was more prominent at early seedling stage than in the developed seedling stage. They also reported that the growth performances were cultivar dependant and significantly negatively correlated with the Na^+ content and Na^+/K^+ ratio in root and shoot tissues in both developmental stages. Growth reduction under salinity stress was significantly lower in indica rice compared to japonica (Lee et al., 2003). They noticed that indica varieties were good Na^+ excluders and absorb high amount of K^+ to maintain low Na^+/K^+ ratio in shoots. Despite whether they are salt-tolerant or sensitive, cultivars from both *O. sativa* and *O. glaberrima* showed vast diversity in salt tolerance. However, the majority of salt-tolerant cultivars belong to the indica rice group (Platten et al., 2013).

Rice shows considerable genetic variation for salt-tolerance within the cultivated genotypes (Walia et al., 2005; Platten et al., 2013). Comparative transcriptional profiling using shoots of two contrasting indica genotypes showed that a greater number of genes were induced by the salt-sensitive genotype (IR 29) in response to salt stress, compared to a salt-tolerant genotype (FL 478) (Walia et al., 2005). A complementary study on cultivar FL 478 and IR 29 looking at the response in roots revealed that, in contrast to shoots, a greater number of genes were induced by salt-tolerant FL 478 in response to salt stress compared to salt-sensitive cultivar IR 29 (Cotsaftis et al., 2011). Genetic variation in salt-tolerant and susceptible rice genotypes was also observed by Xie et al. (2000) from the study, which investigated the genetic diversity among salt-tolerant and salt-susceptible rice genotypes using random amplified polymorphic DNAs (RAPDs). Their results showed that on the basis of RADP markers, three salt-tolerant rice genotypes, Pokkali, Nona-Bokra and Bicol differed from salt-sensitive IR29.

An understanding of salinity effects on seedling growth and seed production of rice would be beneficial to improve field management practices as well as to increase the understanding of salt-tolerance mechanisms (Mohammadi-Nejad et al., 2010). The current study explores the diversity in growth and molecular responses of different rice cultivars representing both japonica and indica rice grown under salinity stress. Rice seedlings grown as hydroponic cultures were exposed to 120 mM salt and growth performance, Na^+ and K^+ accumulation patterns in root and shoot were studied to explore the salt-tolerance mechanisms in different rice cultivars. The diversity of expression of alternative respiratory pathway (AP) genes in selected rice cultivars representing salt-sensitive and tolerant cultivars from both japonica and indica varieties were studied to investigate whether there is a correlation between alternative pathway gene expression, growth and ion accumulation in tissues, to explore the potential use of AP genes as molecular markers for salt-tolerance in rice.

5.2 Materials and methods

5.2.1 Plant materials

Sixteen rice cultivars (Table 5.1), representing both japonica and indica rice were used in the study. Seeds for all 16 cultivars were kindly provided by the Yanco Agricultural Institute, Department of Primary Industries, New South Wales, Australia.

5.2.2 Salinity tolerance assay

Experiments for investigating salt-tolerance diversity of 16 different rice cultivars were conducted in a PC2 greenhouse, School of Biological Sciences, Flinders University of South Australia during July-August 2015. Growth conditions used were as described in Section 2.1.1. Supplementary light (~400–500 $\mu Em^{-2}s^{-1}$) was supplied to maintain a 12 h photoperiod as it was winter in South Australia.

5.2.2.1 Screening for salt-tolerance

Screening for salt-tolerance was carried out using a hydroponic culture system. Seed sterilization, germination and establishment of hydroponic cultures were carried out as described in Sections 2.1.2 and 2.1.3, respectively. Rice seedlings were exposed to salt stress 3 weeks after transplanting to hydroponic cultures. Salt was applied in two increments (50 mM NaCl + 0.75 mM $CaCl_2$ and 70 mM NaCl + 1.0 mM $CaCl_2$) to achieve a final concentration of 120 mM. The second salt application was added 2 days after the first and salt stress was maintained for 12 days. $CaCl_2$ was added together with NaCl to maintain constant Ca^{2+} activity in the growth solution. Five biological replicates were collected from each cultivar per treatment (salt-treated and control) for the determination of growth and tissue ion accumulation.

5.2.2.2 Measurements of biomass and tissue ion concentration

Destructive sampling was performed to determine root and shoot biomass of seedlings grown under salt-stressed and non-stressed conditions. Data were collected for fresh weight (FW), dry weight (DW) and Na^+ and K^+ accumulation in whole shoot and whole root tissues as described in Section 2.1.4. Biomass data were presented as means of five biological replicates as a percentage relative to their controls and represented as relative dry weight.

5.2.3 Gene expression analysis

Based on the growth performance of 16 different rice cultivars, 7 cultivars representing salt-tolerant and sensitive cultivars from both japonica and indica rice were selected for gene expression analysis. The experiment was conducted under the same growth conditions as for screening for salt-tolerance and hydroponic culture establishment and salt application were carried out as described in Section 5.2.2.1. Two youngest leaves and root samples were collected from three biological replicates for RNA extraction after 9 days of salt application. RNA extraction and cDNA synthesis was carried out as described in Sections 2.2.1.1 and 2.2.4, respectively. The expression levels of AP genes and anti-oxidative enzyme genes in leaf and root tissues were

evaluated by qRT-PCR as explained in Section 2.3.4. The expression data were normalized to the geometric mean expression of three stable reference genes, *OsActin*, *OsEIF1* and *OsPlase*, selected out of a set of 6 (Sections 2.3.5 and 3.3.10).

Table 5.1 Details of rice genotypes/cultivars used in the study

Rice cultivar/genotype	Cultivar/genotype description
Lemont	Tropical japonica variety originated in USA, semi-dwarf, long grain variety.
Doongara	Australian long grain japonica variety, semi-dwarf, very resistant to water lodging, hard cooking variety grown in Australia with low glycaemic index.
Li-Jiang_Xin_Tuan_Hei_Gu (LTH)	Landrace Japonica rice from Yunnan province, China.
Takiminori	Upland japonica variety originated in Japan.
IR 64	Moderate salinity tolerant indica variety developed by IRRI, wide adaptability and high yielding potential, it is also Known as a “Mega variety” because of its huge cultivated area in Asia. Commonly used in salinity testing studies.
Pandang wong-7	Tropical javonica type japonica variety originated in Indonesia.
Pokkali	Traditional indica variety originated from India. Salt-tolerant, commonly grown in coastal areas, poor yielding potential and poor grain quality, susceptible to water lodging. Commonly used in salinity testing studies.
Phka Rumduol	Fragrant rice variety with jasmine type aroma, long slender grain, indica variety originated in Cambodia.
Tequing	Taiwanese tropical japonica.
Kyeema	Australian long grain japonica variety, tall strawed and prone to fall over before harvesting, poor seedling vigour, low yielding fragrant variety.
Milang 23	Korean Tongil type japonica (Tongil is an interesting blend of indica/japonica), high yielding variety.
Reiziq	Australian japonica variety, semi-dwarf, medium grain variety commonly grown in Australia.
Topaz	Australian long grain japonica variety released in 2014, fragrant rice variety commonly grown in Australia.
Nipponbare	Japonica type rice variety originated in Japan. Medium grain, common cultivar used in salinity testing studies.
Koshihikari	Japonica type, tall strawed, and susceptible to water lodging, short grain Japanese quality, low yielding commonly grown in Australia, originated in Japan.
Sherpa	Australian japonica variety, semi-dwarf, medium grain, cold-tolerant variety with good yield potential, released in 2011 and commonly grown in Australia.

5.2.4 Statistical analysis

Where appropriate, results were statistically analysed for differences or correlations using GraphPad statistical software. Data on Na^+ and K^+ concentrations in shoots and roots were subjected to analysis of variance at $p<0.05$ and the differences from the cultivar Nipponbare were statistically analysed using Bonferroni multiple comparison test. A multiple *t*-test was performed to find statistically significant changes in gene expression in treated vs. non-treated samples at 95% confidence level. Pearson's correlation coefficient between the variables was computed to measure the degree of linear relationships between AP gene expression and Na^+ content, K^+ content, ratio of Na^+/K^+ or relative growth (Ferdose et al., 2015).

5.3 Results

5.3.1 Differential responses of rice cultivars to salt stress

Differential sensitivity of 16 different rice cultivars was characterized in terms of their biomass production and tissue ion accumulation under salt stress (120 mM) and control (0 mM NaCl) conditions. Rice seedlings were grown on hydroponic culture system and seedlings were exposed to salinity stress at their early seedling stage (~4 weeks old). The salt-stressed plants showed obvious growth reduction compared to plants grown under control conditions (0 mM NaCl). Stressed plants also initially showed senescence of leaf tips, which is a characteristic symptom of ion toxicity (Figure 5.2). There was a variation in symptom severity across genotypes depending on the individual's vigour to withstand salt stress. Yeo et al (1990) also demonstrated that vigour was the most significantly correlated physiological characteristic of rice genotypes with the survival of seedlings grown under salinity stress using a large scale salt screening study involving 200 rice genotypes. The responses of different rice cultivars/genotypes to salt stress were further evaluated by measuring their biomass production and tissue Na^+ and K^+ accumulation patterns.

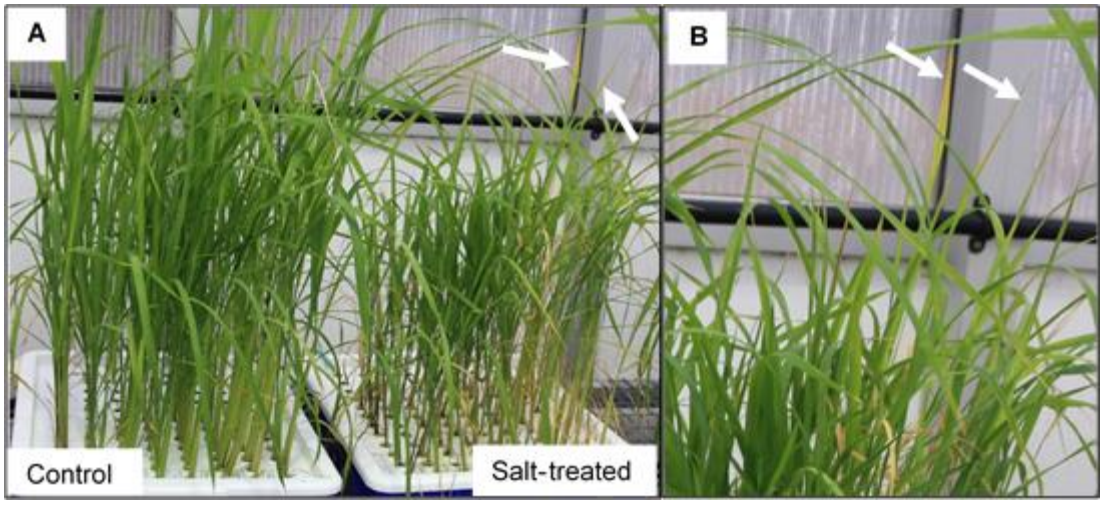


Figure 5.2 Representative image showing growth reduction and leaf tip senescence in salt-treated rice seedlings

Rice seedlings were grown under 50 mM NaCl for 2 days followed by 120 mM NaCl for further 10 days on hydroponic culture system. Photographs were taken 10 days after the start of salt treatment. White arrows indicate leaf tip senescence in salt-treated plants. **A**: Seedlings growing on hydroponic tanks **B**: Enlarged figure showing leaf tip senescence.

5.3.2 Cultivar variation in plant biomass production

Initial data analysis revealed a similar trend in both percentage relative growth in FW and percentage relative growth in DW. Therefore, in the current chapter, the growth responses of seedlings grown under 120 mM NaCl stress are shown as percentage relative growth in DW, which is the growth in DW of the NaCl-treated seedlings relative to that of non-salt-treated seedlings x 100. Initial data on FW and DW of shoot and root tissues are also available in Appendix D. Figure 5.3 shows the variation in the relative shoot and root dry biomass production of 16 cultivars grown for 2 days under 50 mM NaCl stress followed by 10 more days under 120 mM NaCl stress. Rice cultivars showed great variability in shoot dry biomass production as well as in root dry biomass production under stress condition (Figure 5.3). Shoot dry biomass production among cultivars ranged from 54-96% under salt stress compared to controls while that of roots ranged from 40-93%. Seven cultivars (Sherpa, Koshihikari, Kyeema, Nipponbare, Reiziq, Milang 23 and Topaz) grouped together showing the highest shoot as well as root dry biomass producers under the salinity stress investigated (indicated in blue circle) (Figure 5.3). Pokkali showed the second highest

shoot and root dry biomass production (76.68% and 82.03% respectively) while Lemont showed the lowest shoot dry biomass production (54.44%). Doongara recorded the lowest root dry biomass production (40.59%) out of the 16 cultivars tested. It is interesting to note that although the shoot dry biomass production of cultivars Takiminori, Phka Rumduol, Pandang, Tequing, IR 64 and LTH varied in a very narrow range (65-72%), their root dry biomass production showed higher variation ranging from 48-65% (Figure 5.3).

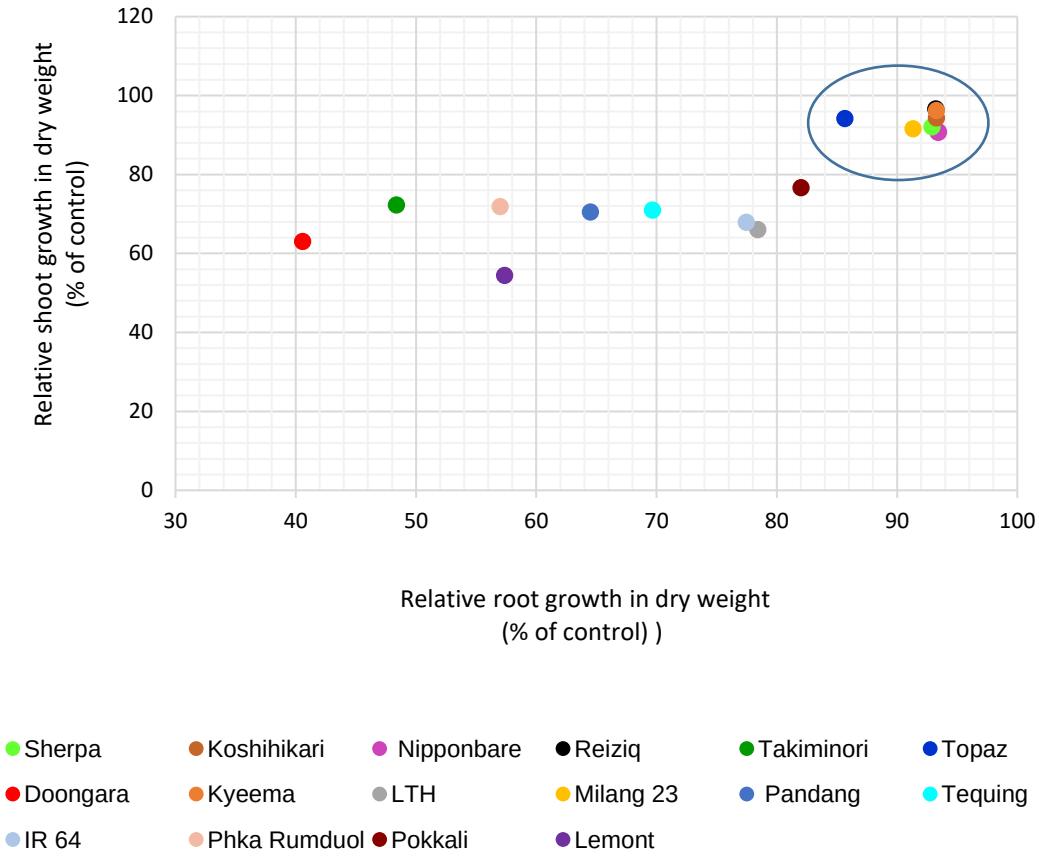


Figure 5.3 Cultivar variation in relative shoot and root dry biomass production of rice seedlings grown under salt stress

Rice seedlings were grown under 50 mM NaCl for 2 days followed by 120 mM NaCl for further 10 days on hydroponic culture system and plants were harvested for measurements. Results are shown as percentage relative dry weight compared to the control.

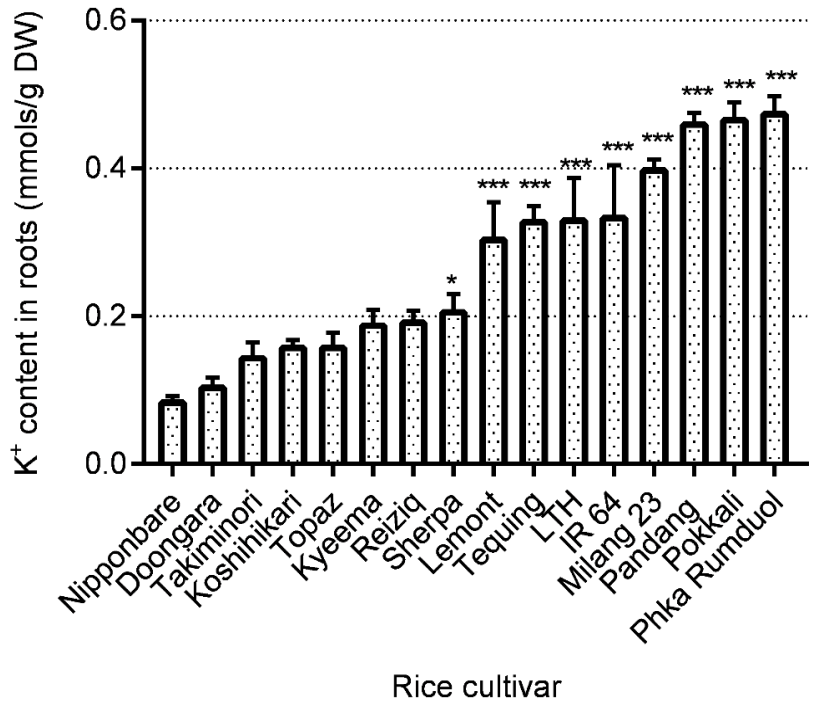
5.3.3 Tissue and cultivar variation in Na^+ and K^+ accumulation

The ion accumulation patterns of root and shoot tissues from 16 rice cultivars were investigated to explore the diversity of K^+ and Na^+ retaining ability of tissues under salt stress and to study the cultivar variation in salt-tolerant mechanisms. The concentrations of Na^+ and K^+ in tissues were calculated based on their FW as well as based on their DW. Evaluation of results from both calculation methods showed positive correlation and therefore data presented in this chapter was based on the DW based calculation method. The japonica rice cultivar Nipponbare was used as the standard cultivar to compare ion accumulation in seedling tissues.

5.3.3.1 Tissue and cultivar variation in K^+ accumulation

The responses of rice cultivars grown on salinized nutrient solution showed extreme variability in terms of K^+ accumulation in roots compared to shoots (Figures 5.4A and B). The accumulation of K^+ in roots ranged from 0.088-0.478 mmols/g DW. The lowest K^+ concentration in roots was recorded in Nipponbare. The root K^+ concentration was significantly higher in the cultivars Sherpa, Lemont, Tequing, LTH, IR 64, Milang 23, Pandang, Pokkali and Phka Rumduol compared to Nipponbare (Figure 5.4A). The accumulation of K^+ in shoots showed less variability compared to roots ranging from 0.704-1.184 mmols/g DW. The cultivar Nipponbare recorded shoot K^+ content of 0.789 mmols/g DW which was significantly lower compared to Pokkali (1.184 mmols/g DW) (Figure 5.4B).

(A)



(B)

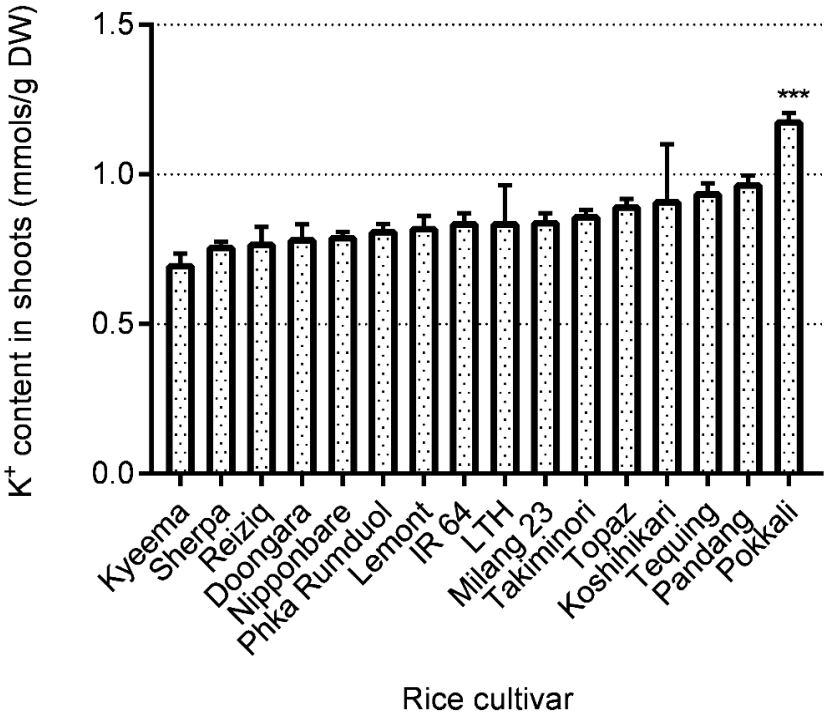


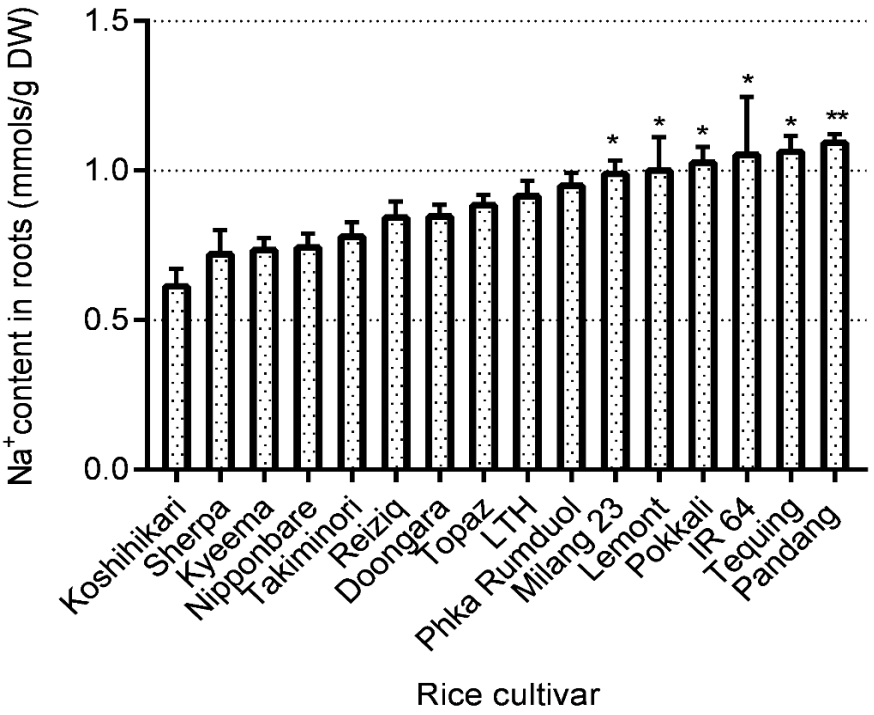
Figure 5.4 K^+ accumulation in roots (A) and shoots (B) from 16 different cultivars grown under 50 mM NaCl for 2 days followed by 120 mM NaCl for further 10 days on hydroponic culture system

Data are shown as mean + SEM from five biological replicates. * and *** represent significant difference from the cultivar Nipponbare at $p<0.01$and $p<0.0001$ respectively by Bonferroni multiple comparisons test.

5.3.3.2 Tissue and cultivar variation in Na^+ accumulation

The responses of rice cultivars grown on salinized nutrient solution showed extreme variability in terms of Na^+ accumulation in shoots compared to roots (Figures 5.5A and B). There was less variation in root Na^+ concentration among cultivars ranging from 0.624-1.104 mmols/g DW compared to the variation observed in shoots. The root Na^+ concentration in cultivars Milang 23, Lemont, Pokkali, IR 64, Tequing, and Pandang was significantly higher compared to Nipponbare (Figure 5.5A). In contrast to roots, seedling shoots showed higher variability in Na^+ accumulation ranging from 0.614-3.276 mmols/g DW (Figure 5.5B). The lowest shoot Na^+ content (0.614 mmols/g DW) was recorded in Pokkali and was significantly lower compared to Nipponbare (2.074 mmols/g DW). The highest shoot Na^+ content (3.276 mmols/g DW) was recorded in Doongara and was significantly higher compared to Nipponbare. In addition to the cultivar Pokkali, significantly lower shoot Na^+ contents were recorded by cultivars Reiziq (0.80 mmols/g DW), Sherpa (0.89 mmols/g DW), Milang 23 (0.99 mmols/g DW) and Koshihikari (1.19 mmols/g DW) compared to Nipponbare.

(A)



(B)

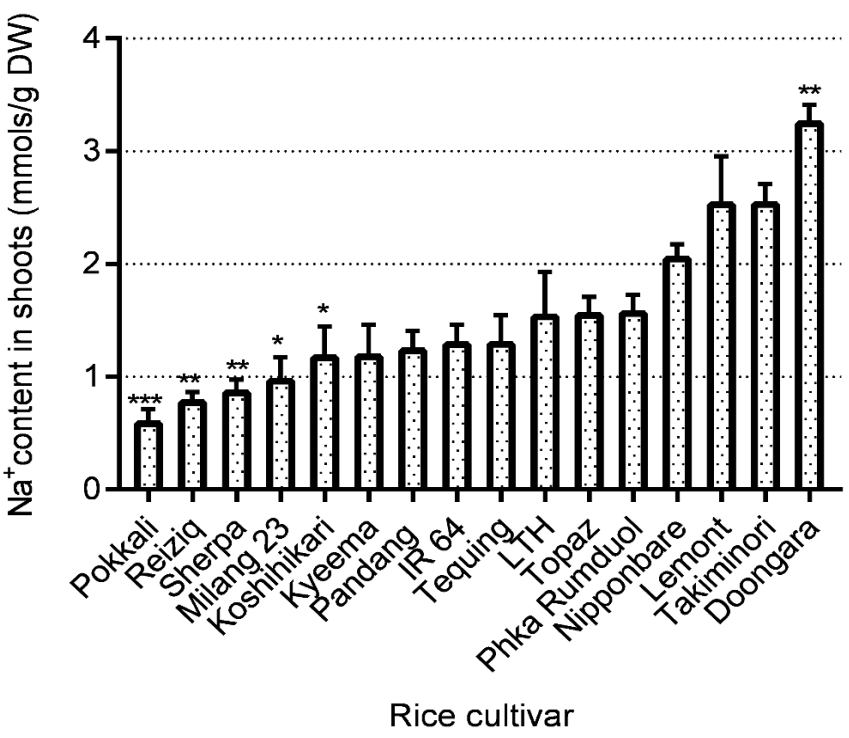


Figure 5.5 Na^+ accumulation in roots (A) and shoots (B) from 16 different cultivars grown under 50 mM NaCl for 2 days followed by 120 mM NaCl for further 10 days on hydroponic culture system

Data are shown as mean + SEM from five biological replicates. *, ** and *** represent significant difference from the cultivar Nipponbare at $p<0.01$, $p<0.001$ and $p<0.0001$ respectively by Bonferroni multiple comparisons test.

5.3.4 Cultivar variation in growth responses to tissue ion accumulation

The study was further extended to explore the correlation between tissue ion accumulation and their biomass production. There was a large cultivar variation in biomass production and tissue ion accumulation in rice seedlings grown under 120 mM salt stress.

5.3.4.1 Cultivar variation in root growth and Na^+ accumulation

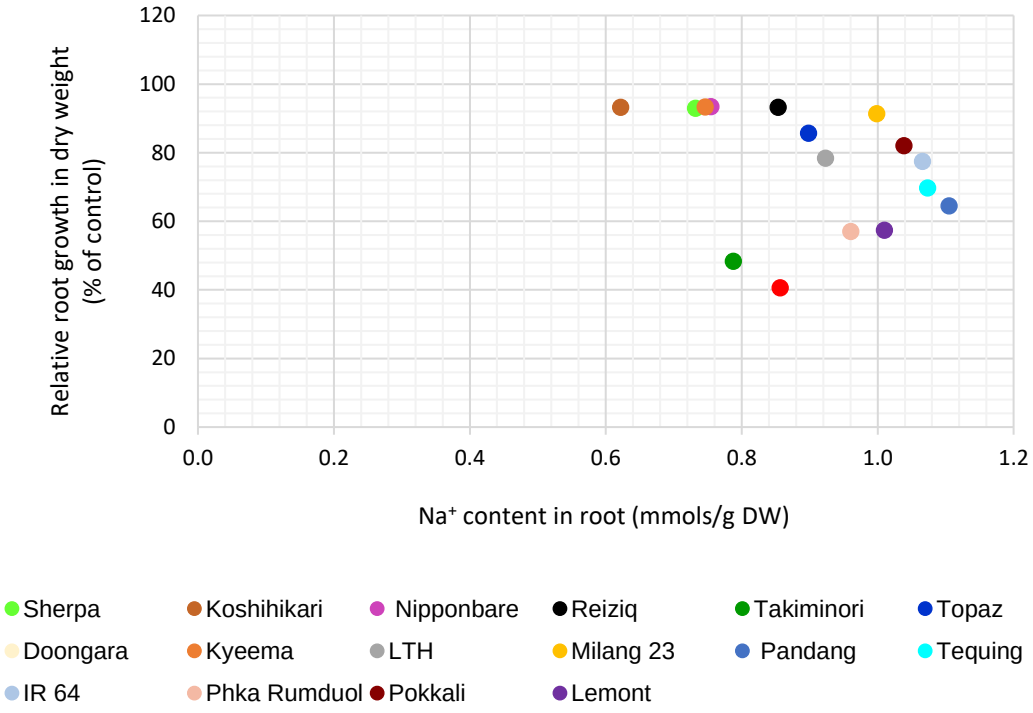


Figure 5.6 Effect of Na^+ accumulation on root growth of seedlings grown under 50 mM NaCl for 2 days followed by 120 mM NaCl for further 10 days on hydroponic culture system

Nipponbare, Milang 23, Topaz, Koshihikari, Reiziq, Kyeema, Sherpa and Pokkali were the cultivars that showed the highest relative root dry biomass production (over 80%) against the Na^+ content out of 16 cultivars examined (Figure 5.6). It is also interesting to note that though all these cultivars were able to maintain the root biomass production in the same range, there was a cultivar variation in root Na^+ accumulation ranging from 0.62-1.04 mmols/g DW. The root Na^+ content in Doongara

was 0.86 mmols/g DW and recorded the lowest root relative growth of 40.59% while Reiziq was one of the highest root dry biomass producers under salt stress tested and retained the same concentration of Na^+ in roots (Figure 5.6). Similarly, cultivars Milang 23 and Lemont had similar root Na^+ accumulation but the relative dry biomass production of Lemont was 57.37% while it was 91.33% for Milang 23. Thus Milang 23 can tolerate higher Na^+ content in the roots, indicating the use of different mechanisms to avoid salt stress.

5.3.4.2 Cultivar variation in shoot growth and Na^+ accumulation

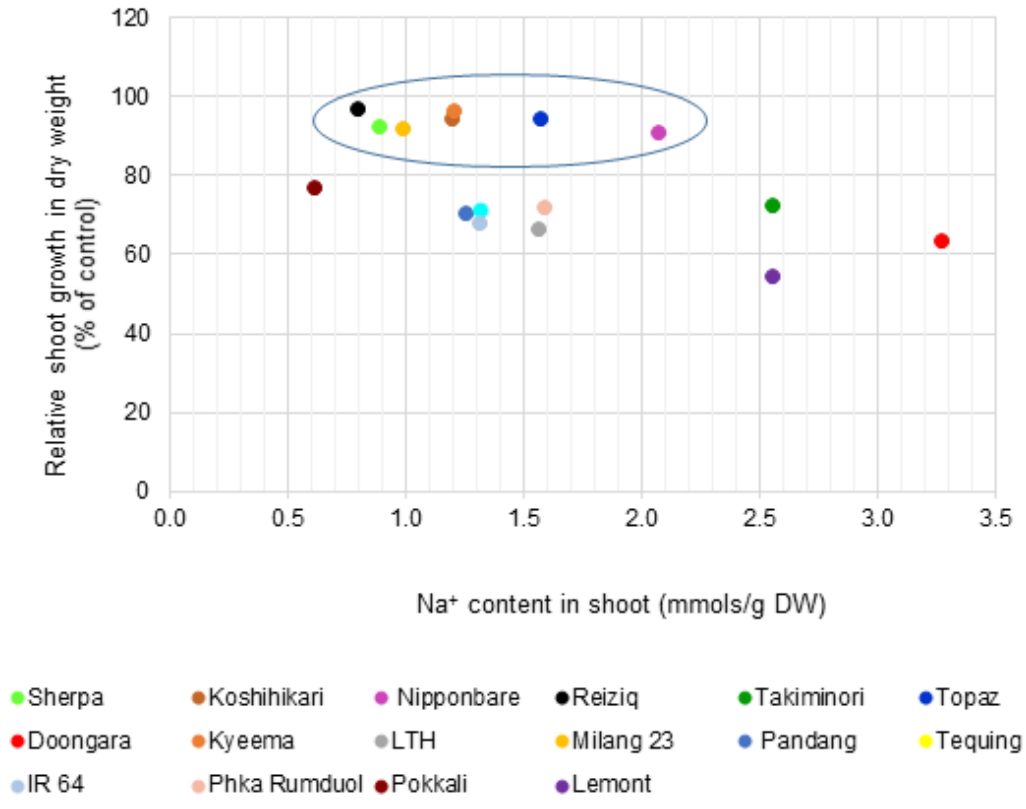


Figure 5.7 Effect of Na^+ accumulation on shoot growth of seedlings grown under 50 mM NaCl for 2 days followed by 120 mM NaCl for further 10 days on hydroponic culture system

Similar to root growth responses, the same group of cultivars (Nipponbare, Milang 23, Topaz, Koshihikari, Reiziq, Kyeema, and Sherpa) showed the highest relative shoot dry biomass production out of 16 cultivars examined (Figure 5.7). All these cultivars (indicated by the circle), were able to maintain over 90% of shoot growth under salt stress. Interestingly, while these cultivars maintained dry biomass production in the same range, there was a great cultivar variation in shoot Na^+ accumulation ranging from 0.8-2.08 mmols/g DW. The cultivars that had the highest shoot Na^+ (Doongara 3.28 mmols/g DW; Lemont 2.56 mmols/g DW) had the poorest shoot growth (Figure 5.7). Interestingly, Pokkali had the lowest shoot Na^+ content but did not show the highest growth (Figure 5.7).

5.3.5 Cultivar variation in plant growth and Na^+/K^+ ratio

Generally, it is considered that cytosolic Na^+/K^+ ratio is a key determinant of plant survival in saline environments (Asch et al., 2000; Pires et al., 2015). The Na^+/K^+ ratio in shoot and root tissues of the 16 cultivars was determined to explore the correlation of Na^+/K^+ ratio and the biomass production of seedlings exposed to 120 mM NaCl stress for 12 days.

5.3.5.1 Cultivar variation in shoot growth and Na^+/K^+ ratio

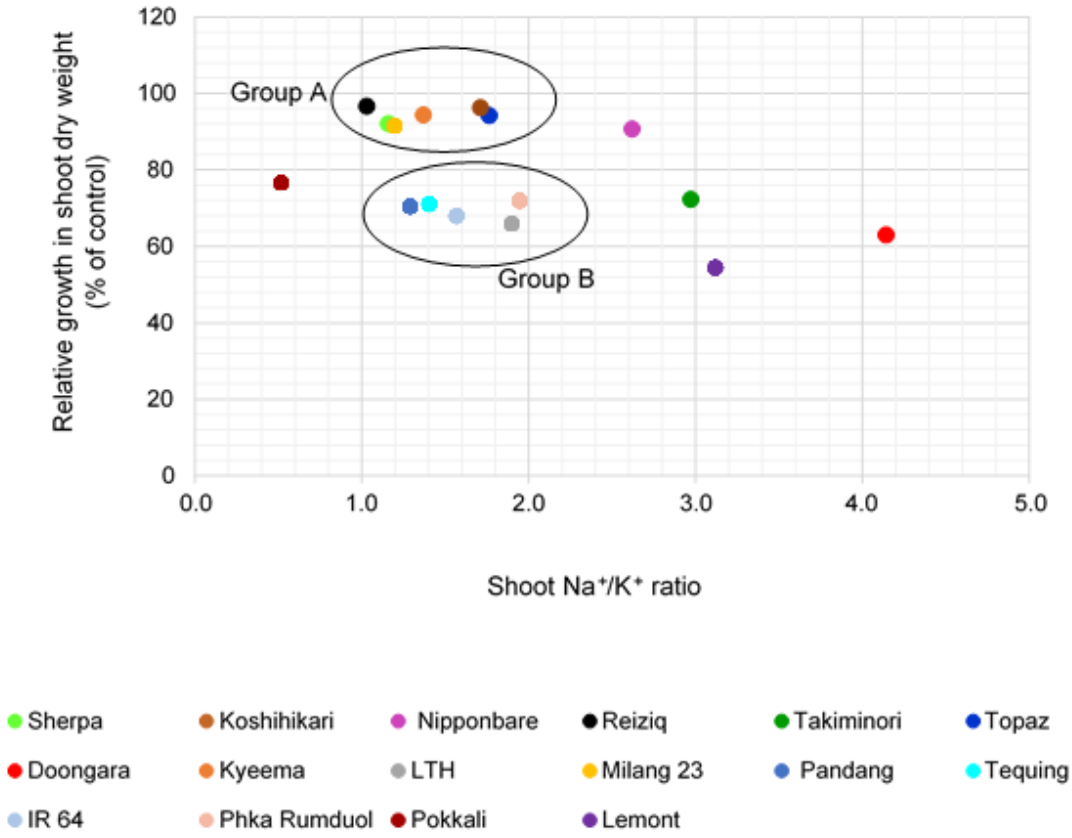


Figure 5.8 Cultivar variation in shoot growth and Na^+/K^+ of 16 cultivars grown under 50 mM NaCl for 2 days followed by 120 mM NaCl for further 10 days on hydroponic culture system

Cultivar Pokkali had the lowest shoot Na^+/K^+ ratio (0.52) but only maintained 76.68% of shoot growth under the salt stress tested. Doongara recorded the highest shoot Na^+/K^+ ratio of 4.14 and was restricted to approximately 63% of control shoot growth (Figure 5.8). It is interesting to note that the shoot Na^+/K^+ ratio of the cultivars Milang 23, Topaz, Koshihikari, Reiziq, Kyeema and Sherpa (indicated as "Group A") and the cultivars Pandang, Phka Rumduol, IR 64, Tequing and LTH (indicated as "Group B") were in the same level ranging from 1-2, but the two groups showed quite distinct differences in shoot biomass, 91-96% and 66-71%, respectively (Figure 5.8). Cultivar Nipponbare recorded over 90% of dry biomass production, which was in the similar range as Group "A" cultivars, but its Na^+/K^+ ratio (2.62) was higher (Figure 5.8). Similarly, Lemont recorded a lower shoot Na^+/K^+ ratio of 3.12 compared to that of

Doongara (4.14), but its shoot dry biomass was lower. These results indicate that the Na^+/K^+ ratio in tissues *per se* is not indicative of the survival of rice seedlings under salt stress.

5.3.5.2 Cultivar variation in root growth and Na^+/K^+ ratio

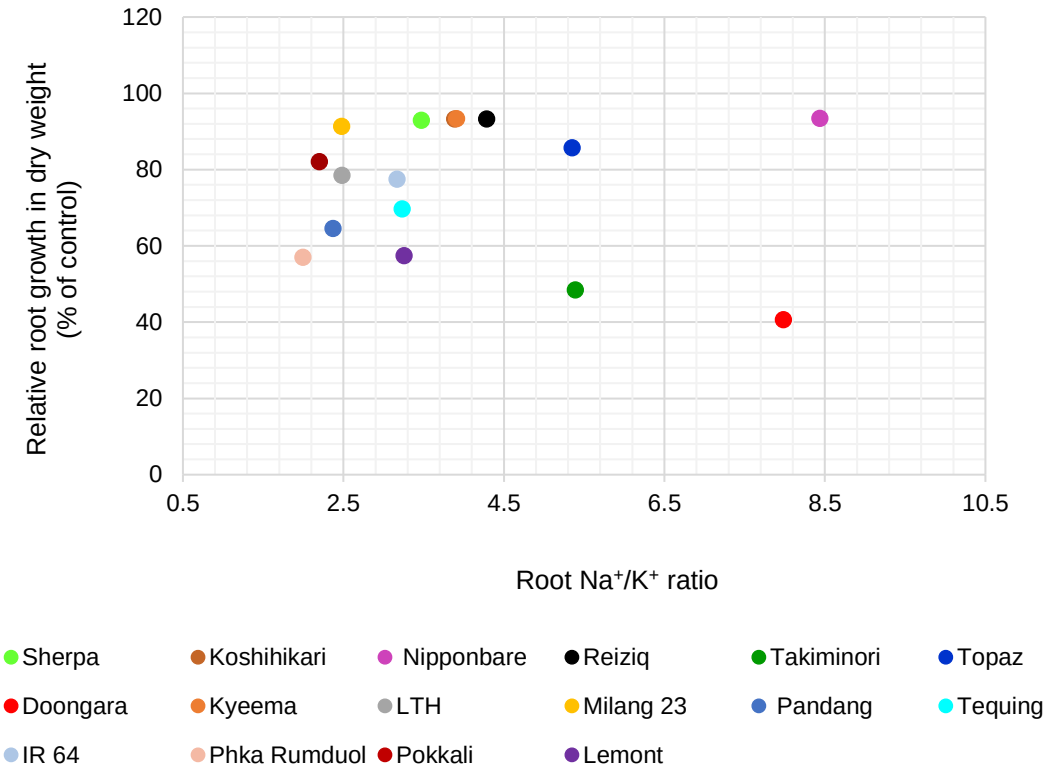


Figure 5.9 Cultivar variation in root growth and Na^+/K^+ of 16 cultivars grown under 50 mM NaCl for 2 days followed by 120 mM NaCl for further 10 days on hydroponic culture system

The importance of tolerance mechanisms in the survival of rice seedlings as opposed to tissue ion accumulation is further supported by the behaviour of the cultivar Nipponbare. Nipponbare recorded the highest Na^+/K^+ ratio (8.44) in roots, but still maintained the highest root biomass production (93.44%) out of the 16 cultivars under NaCl stress (Figure 5.9). The growth of Doongara, on the other hand, with a similar Na^+/K^+ ratio of 7.99, was severely restricted. Although cultivars Nipponbare and Doongara recorded almost identical Na^+/K^+ ratios, Nipponbare maintained twice the root growth than Doongara (Figure 5.9). Likewise, cultivars Takiminori and Topaz

recorded 48.39% and 85.69% of root biomass production, respectively, but the root Na^+/K^+ ratio in both cultivars was the same (~5.4).

5.3.6 Cultivar variation in the expression of alternative pathway genes under salinity stress

Based on the growth performances, seven rice cultivars representing salt-sensitive and salt-tolerant cultivars, were selected for gene expression analysis. The selected group included both indica and japonica rice types. Seedlings were grown hydroponically under the same experimental conditions as the previous growth and ion analysis, as described in Section 5.2.2.1, and were exposed to 50 mM NaCl for 2 days followed by 120 mM NaCl for a further 7 days and the abundance of alternative pathway genes were investigated using qRT-PCR. Two major genes responsible for detoxification of ROS, *MnSOD*, which scavenges superoxide dismutating it into O_2 and H_2O_2, and *CAT-A*, which breaks down H_2O_2 to O_2 and water (Moller, 2001), were also included in the study.

5.3.6.1 Cultivar variation in the expression of AOX1 isoforms

Substantial genotypic variation was observed in the expression of AOX1 isoforms, *AOX1a*, *AOX1b* and *AOX1c*, regardless of the tissue type or the treatment. *AOX1c* transcript abundance was higher in leaves of all rice cultivars investigated under control conditions compared to *AOX1a* and *AOX1b*. No further significant induction of leaf *AOX1c* transcripts was observed upon salt treatment in any of the cultivars, except Phka Rumduol, which significantly ($p<0.04$) increased *AOX1c* transcript abundance in response to salinity stress (Figure 5.10A). Doongara showed a significant ($p<0.001$) increase in transcript levels of both *AOX1a* and *AOX1b* in leaves of plants grown under salt-treated conditions compared to their controls (Figure 5.10A), while cultivars Kyeema ($p<0.04$) and Phka Rumduol ($p<0.02$) recorded significantly higher leaf *AOX1b* transcript levels compared to their controls. Though it was not significant, cultivars Nipponbare, Kyeema and Phka Rumduol also showed an increase in leaf *AOX1a* transcript abundance in response to salt treatment.

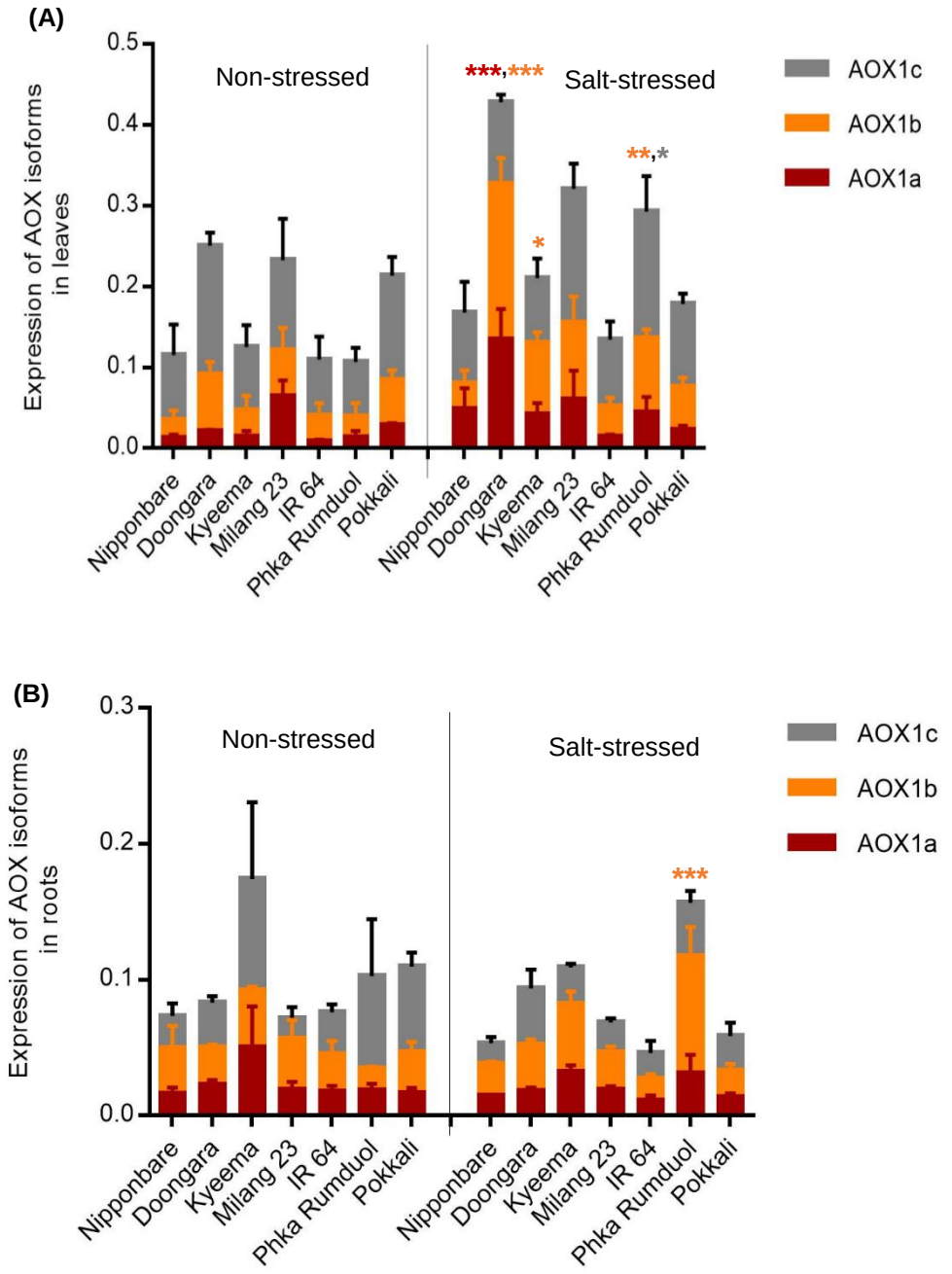


Figure 5.10 Comparative transcriptional study of AOX1 isoforms in non-stressed and salt-stressed tissues from selected rice cultivars grown on hydroponic culture system for nine days under treatment

Relative expression pattern of alternative pathway genes *AOX1a*, *AOX1b* and *AOX1c* was investigated in leaves (A) and roots (B) of seven rice cultivars under control (0 mM NaCl) and salt-stressed (120 mM NaCl) conditions. Transcript levels were determined by qRT-PCR and normalized against the geometric mean of three reference genes. Data are shown as mean + SEM from three biological replicates. Significant differences with control response at $p<0.04$, $p<0.02$ and $p<0.001$ are indicated by *, ** and ***, respectively by multiple *t*-test.

The majority of cultivars showed a slight decrease in root AOX1 isoforms transcript abundance in response to salinity stress except cultivar Phka Rumduol, which showed a significant ($p<0.001$) increase in *AOX1b* transcript abundance compared to control roots (Figure 5.10B). Cultivar Kyeema also recorded a higher *AOX1b* transcript level in salt-stressed roots compared to control roots (Figure 5.10B).

5.3.6.2 Cultivar variation in the expression of NDB isoforms

There was significant cultivar variation in the expression of *NDB* isoforms in leaves compared to roots in the cultivars examined. Some isoforms showed a significant increase of their transcript abundance while others showed reductions in response to salinity stress. Out of the three *NDB* isoforms found in rice, the expression level of *NDB1* was very low in both leaf and root tissues compared to *NDB2* and *NDB3*, regardless of whether the plants were under stress (Figures 5.11 A and B). Statistical analysis showed that leaf *NDB1* transcript abundance in Phka Rumduol significantly ($p<0.02$) increased while in Pokkali it significantly decreased ($p<0.02$) when seedlings were exposed to 120 mM salt stress. None of the other cultivars showed significant induction or reduction of *NDB1* transcript levels in roots in response to salinity stress, except Pokkali, which recorded a significant ($p<0.01$) reduction, as detected in leaves (Figure 5.11A and B).

There was a large cultivar variation in transcript abundance of *NDB2* in both roots and shoots (Figures 5.11A and B). It is interesting to note that there was an up-regulation of leaf *NDB2* transcript levels in some cultivars while others were down-regulated in response to salinity stress (Figure 5.11A).

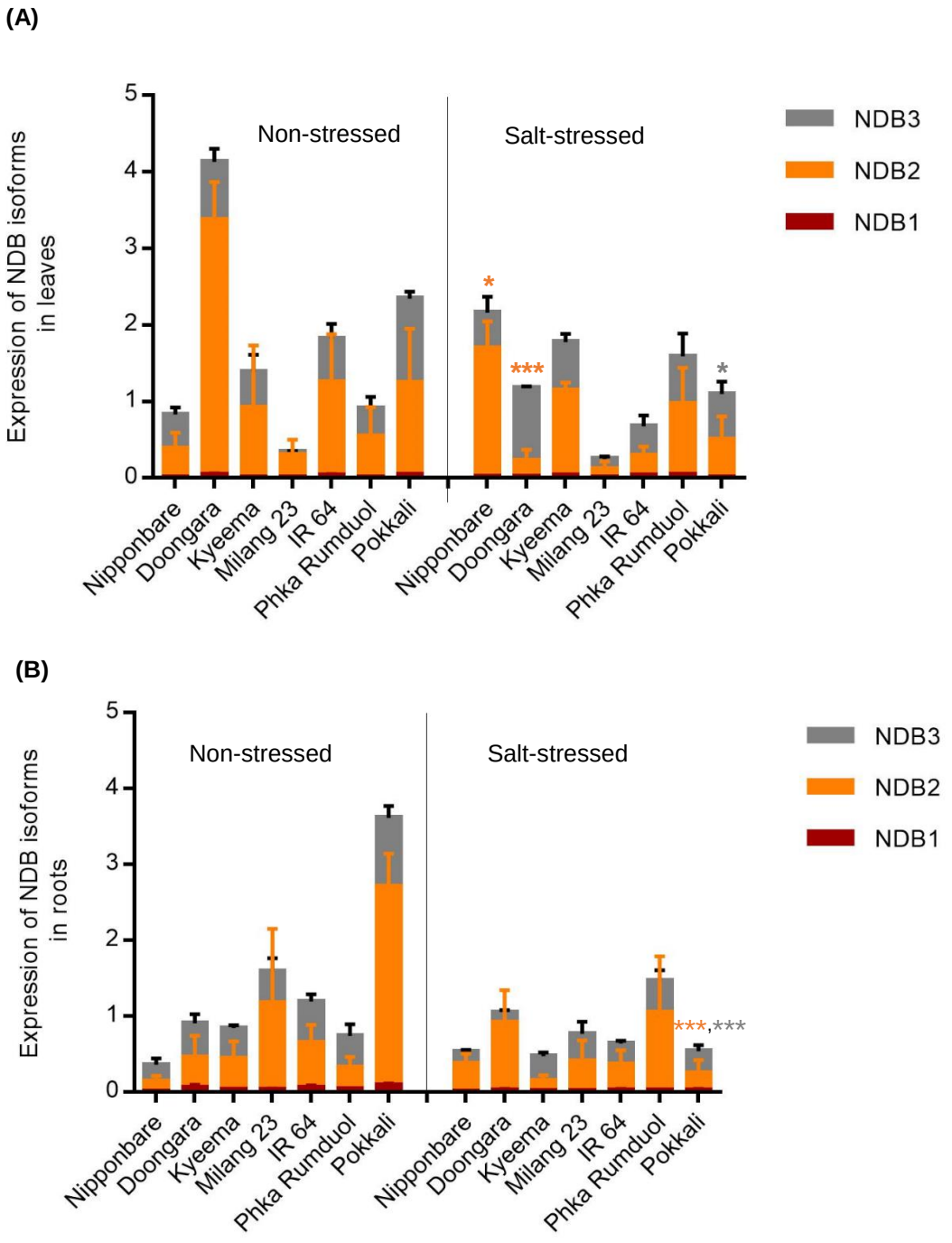


Figure 5.11 Comparative transcriptional study of NDB isoforms in non-stressed and salt-stressed tissues from selected rice cultivars grown on hydroponic culture system for nine days under treatment

Relative expression pattern of alternative pathway genes *NDB1*, *NDB2* and *NDB3* were investigated in leaves (A) and roots (B) of seven rice cultivars under control (0 mM NaCl) and salt-stressed (120 mM NaCl) conditions. Transcript levels were determined by qRT-PCR and normalized against geometric mean of three reference genes. Data are shown as mean + SEM from three biological replicates. Significant differences with control response at $p<0.03$ and $p<0.001$ are indicated by * and ***, respectively by multiple *t*-test.

The abundance of leaf *NDB2* transcript was very high in Doongara grown under non-stressed conditions compared to all other rice cultivars investigated and it significantly decreased ($p<0.001$) in response to salinity stress (Figure 5.11A). In contrast to Doongara, Nipponbare recorded a significant ($p<0.03$) increase of leaf *NDB2* transcripts and cultivars Kyeema and Phka Rumduol showed non-significant increases. Cultivars Milang 23, IR 64 and Pokkali recorded a non-significant decrease of leaf *NDB2* transcript levels in response to salinity stress (Figure 5.11A).

There was a higher abundance of root *NDB2* transcripts in Pokkali under control conditions and with the exposure of seedlings to salt, transcript level was significantly ($p<0.001$) decreased (Figure 5.11B). Cultivars Kyeema, Milang 23 and IR 64 also recorded a slight reduction of root *NDB2* transcript levels in response to salinity stress while cultivars Nipponbare, Doongara and Phka Rumduol grown under salt stress showed slight increase of root *NDB2* transcript levels (Figure 5.11B).

None of the other cultivars, other than Pokkali, recorded significant changes in the *NDB3* transcript abundance in response to salinity stress in both tissue types tested. Pokkali showed a significant decrease in transcript levels in leaves ($p<0.03$) as well as roots ($p<0.001$) when seedlings were exposed to salt stress. It is interesting to note that expression of *NDB* isoforms in leaves as well as in roots in Pokkali was inhibited when seedlings were exposed to salinity stress.

5.3.6.3 Cultivar variation in expression of NDA isoforms and NDC1

Out of two *NDA* isoforms, expression level of *NDA2* was very low compared to the expression level of *NDA1* in both leaves and roots regardless whether plants were under stressed or non-stressed condition, and didn't show any significant changes in response to salinity stress tested.

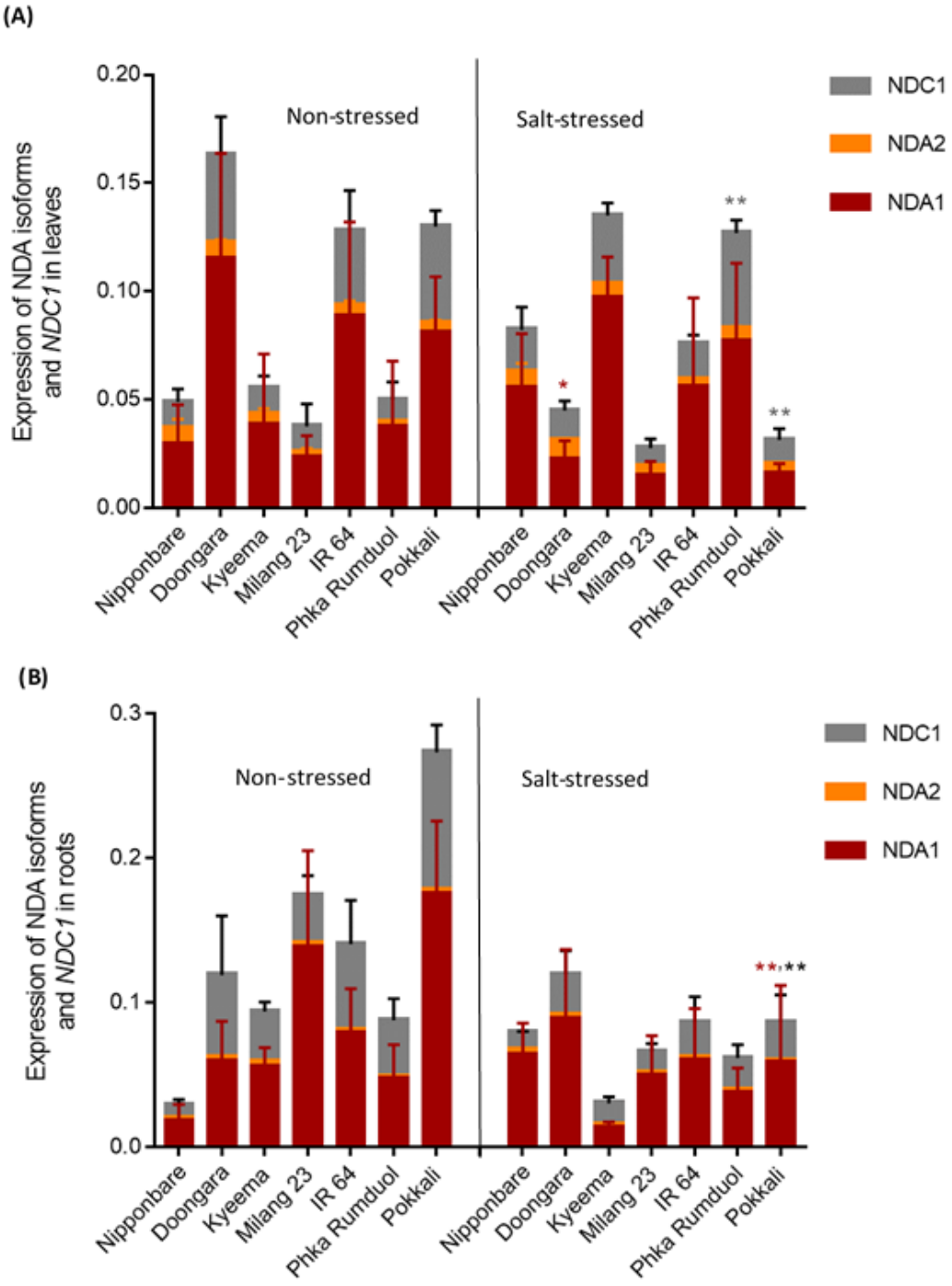


Figure 5.12 Comparative transcriptional study of NDA isoforms and NDC1 in non-stressed and salt-stressed tissues from selected rice cultivars grown on hydroponic culture system for nine days under treatment

Relative expression pattern of alternative pathway genes *NDA1*, *NDA2* and *NDC1* were investigated in leaves (A) and roots (B) of seven rice cultivars under control (0 mM NaCl) and salt-stressed (120 mM NaCl) conditions. Transcript levels were determined by qRT-PCR and normalized against geometric mean of three reference genes. Data are shown as mean + SEM from three biological replicates. Significant differences with control response at $p<0.03$ and $p<0.01$ are indicated by * and **, respectively by multiple *t*-test.

It is interesting that in some cultivars leaf *NDA1* transcript level was higher in control condition while it was low in others. In response to salt stress, the cultivars which had higher transcript abundance showed reduction while those had lower transcript levels showed further induction (Figures 5.12A and B). Cultivars Doongara, IR 64 and Pokkali showed higher leaf *NDA1* transcript abundance under control condition compared to the other cultivars tested (Figure 5.12A), and it was lowered significantly ($p<0.03$) in Doongara and non-significantly in IR 64 and Pokkali. In contrast, leaf *NDA1* transcript levels in cultivars Nipponbare, Kyeema and Phka Rumduol were increased when seedlings were exposed to salt (Figure 5.12A). Milang 23 didn't show any noticeable change when seedlings were treated with salt. Likewise in leaves, the transcript abundance of root *NDA1* was increased in Nipponbare. In contrast to leaves, root *NDA1* transcript level in Doongara was increased when seedlings were exposed salinity stress (Figure 5.12A and B). Higher expression of root *NDA1* was observed in two cultivars, Milang 23 and Pokkali under non-treated conditions and it was down-regulated significantly ($p<0.01$) in Pokkali and non-significantly in Milang 23 when seedling were treated with salt (Figure 5.12B).

There was a significant ($p<0.01$) increase in leaf *NDC1* transcript level in Phka Rumduol when seedlings were treated with 120 mM NaCl. In contrast, there was a significant ($p<0.01$) reduction both leaf and root *NDC1* transcript level in Pokkali.

5.3.7 Cultivar variation in the expression of antioxidant defence genes *MnSOD* and *CAT-A* under salinity stress

Expression of two selected antioxidant defence genes (*MnSOD* and *CAT-A*), which regulate the increased accumulation of ROS during oxidative stress (Moller, 2001), were also investigated in seven rice cultivars under non-stressed and salt-stressed conditions to explore whether there is any correlation between the expression of antioxidant genes and the expression of alternative pathway genes.

Expression level of *MnSOD* was very low compared to that of *CAT-A* in leaves and roots of rice seedlings under stressed as well as non-stressed conditions. Cultivar Milang 23 showed a significant reduction of *MnSOD* transcript level in shoots in response to the NaCl stress applied. Other than that, no noticeable changes were observed in *MnSOD* abundance in leaves or roots (Figures 5.13A and B).

Comparatively higher leaf *CAT-A* transcript levels were observed in cultivars Doongara and Kyeema in non-treated seedling compared to other cultivars investigated and reduced non-significantly in response to salinity stress. In contrast, Phka Rumduol showed a further increase of *CAT-A* transcript abundance in salt-stressed leaves (Figure 5.13A).

In roots, *CAT-A* transcript was significantly increased in cultivars Doongara ($p<0.05$) and decreased in Kyeema ($p<0.005$) while all other cultivars had non-significant increase (Nipponbare, Phka Rumduol, Pokkali) or decrease (Milang 23) in response to salinity stress (Figure 5.13B).

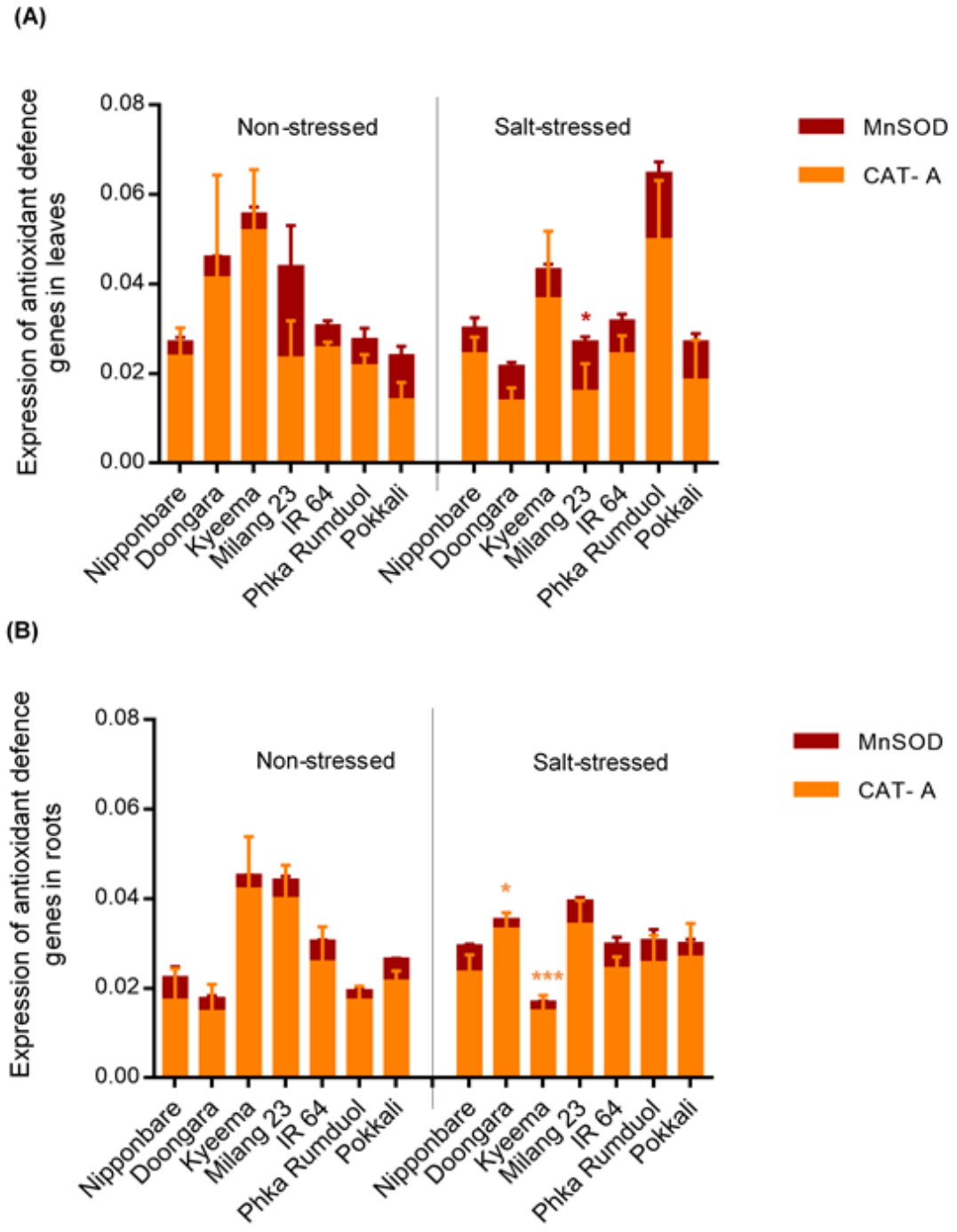


Figure 5.13 Comparative transcriptional study of antioxidant defence genes in non-stressed and salt-stressed tissues from selected rice cultivars grown on hydroponic culture system for nine days under treatment

Relative expression of two antioxidant defence genes encoding mitochondrial manganese superoxide dismutase (MnSOD) and catalase A (CAT-A) enzymes were investigated in leaves (A) and roots (B) of seven rice cultivars under control (0 mM NaCl) and salt-stressed (120 mM NaCl) conditions. Transcript levels were determined by qRT-PCR and normalized against geometric mean of three reference genes. Data are shown as mean + SEM from three biological replicates. Significant differences with control response at $p<0.05$ and $p<0.005$ are indicated by * and ***, respectively by multiple *t*-test.

5.3.8 Correlation between the expression of AP genes, tissue ion accumulation and relative growth

Pearson's correlation coefficient (r) was computed between the expression of AP genes and the tissue Na^+ content, K^+ content and Na^+/K^+ to check whether there was a correlation between AP gene expression and tissue ion accumulation. Strength of the correlation was described following the guidelines suggested by Mukaka (2012).

The expression of three AP genes, *AOX1a*, *NDB3* and *NDA2* showed a strong (positive or negative) correlation with the accumulation of ion in seedling tissues while all others had very weak or no correlation (Table 5.2). The expression of *AOX1a* in leaves showed a significant positive correlation with tissue Na^+ content (r=0.85) and with Na^+/K^+ ratio (r=0.85) while it had weak positive correlation with tissue K^+ content (r=0.46) (Table 5.2). In contrast, expression of *AOX1a* in roots showed moderate negative correlation with tissue Na^+ content (r=-0.58) and very poor positive correlation with tissue K^+ content (r=0.12) and negative correlation with Na^+/K^+ ratio (r=-0.26). The expression of *AOX1b* in leaves showed moderate positive correlation with tissue Na^+ content (r=0.64) and Na^+/K^+ ratio (r=0.67) while it was very weak with K^+ content (r=0.21). Although the expression of *AOX1c* showed a moderate negative correlation with leaf K^+ content, it had very weak correlation with Na^+ content and ratio of Na^+/K^+ (Table 5.2).

Out of NDHs, *NDA2* showed a strong correlation with tissue Na^+ content (r=0.86) and Na^+/K^+ ratio (r=0.83) in leaves. Interestingly, in roots, the expression of *NDA2* had very strong positive correlation (r=0.91) with Na^+/K^+ ratio but the negative association with Na^+ concentration was weak (r=-0.39). There was a moderate positive correlation of leaf *NDA2* and K^+ content but in roots it was strong negative correlation (Table 5.2).

Similar association pattern was observed in leaf *NDB3* expression and tissue ion contents. It showed moderate positive correlation with tissue Na^+ (r=0.66), K^+ (r=0.54) and ratio of Na^+/K^+ (r=0.65), but, in contrast to *NDA2*, in roots, *NDB3* had very strong

negative correlation with the ratio of Na^+/K^+ (r=-0.9) and strong positive relationship with tissue K^+ concentration (r= 0.82).

Expression of some isoforms of AP genes showed little correlation with percentage increase in growth after 12 days exposure to stress under saline conditions (Table 5.2). There were moderate correlations of expression of *NDB2* and *NDB3* with relative shoot growth (r=0.5 and r=-0.51, respectively) and strong negative correlations of *AOX1c* and *NDB2* with relative root growth (r=0.86 and 0.87, respectively).

5.3.9 Correlation between the expression of alternative respiratory pathway genes and the antioxidant defence genes

The association of the expression of the AP genes and antioxidant defence genes was checked by calculating Pearson's correlation coefficient. Results demonstrated that there was a weak negative correlation between the expression of *AOX1a* and the expression of *CAT-A* in both leaf and roots (r=-0.36 and -0.43, respectively). Though there was a moderate negative relationship between expression of *AOX1a* and *MnSOD* in roots (r=-0.35), it was negligible in leaves (r=0.027) (Table 5.3). The association between expression of *AOX1b* and antioxidant genes were negligible in both leaves and roots while *AOX1c* recorded a strong positive correlation with *MnSOD* in leaves (r=0.89) and moderate negative correlation in roots (r=-0.56).

Expression of *NDB1* had very strong positive correlation (r=0.93) with *CAT-A* in leaves while it was moderate in roots (r=0.35). Although, the induction of *NDB2* had moderate positive correlation with *CAT-A* in leaves (r=0.50) and in roots (r=0.46), no association with the expression of *MnSOD*. It is interesting to note that *NDA1* had strong positive correlation with *CAT-A* in both leaves (r=0.84) and roots (r=0.70), but *NDA2* did not show any correlation with antioxidant genes. There was a significant positive correlation between *NDC1* and *CAT-A* in leaves (r=0.98) while it was moderate in roots (r=0.35). The moderate association between *MnSOD* and *NDC1* in leaves was positive while it was negative in roots (Table 5.3).

Table 5.2 Correlation of the expression of alternative respiratory pathway genes and tissue ion accumulation of seven rice cultivars grown under 120 mM salinity stress for 9 days

The values indicate the Pearson's correlation coefficient (r) computed using GraphPad statistical software. * and ** indicate the significant correlation at P<0.05 and P<0.01 respectively.

Leaf									
	AOX1a	***AOX1b***	***AOX1c***	***NDB1***	***NDB2***	***NDB3***	***NDA1***	***NDA2***	***NDC1***
K^+ content	0.46	0.21	-0.52	-0.53	0.28	0.54	-0.34	0.67	-0.39
Na^+ content	0.85 *	0.64	-0.27	-0.15	0.11	0.66	-0.09	0.86 *	-0.07
Na^+/K^+	0.85 *	0.67	-0.19	0.0035	0.085	0.65	0.014	0.83 *	0.06
Relative growth in DW (%)	-0.29	-0.37	0.022	-0.13	0.5	-0.51	0.28	0.022	0.021
Root									
K^+ content	0.12	0.25	0.061	0.21	0.053	0.82*	-0.32	-0.81 *	0.21
Na^+ content	-0.58	-0.4	-0.33	0.16	-0.073	0.24	0.31	-0.39	0.16
Na^+/K^+	-0.26	-0.26	-0.011	-0.15	0.12	-0.9 **	0.55	0.91 **	-0.15
Relative growth in DW (%)	-0.082	-0.38	-0.86 *	-0.74	-0.87 *	0.18	-0.52	-0.0064	-0.74

Table 5.3 Correlation of the expression of alternative pathway genes and the expression of antioxidant defence genes, *MnSOD* and *CAT-A* of seven rice cultivars grown under 120 mM salinity stress for 9 days

The values indicate the Pearson's correlation coefficient (r) computed using GraphPad statistical software. *, ** and *** indicate the significant correlation at P<0.01, P<0.002 and P<0.0002 respectively.

Leaf									
	AOX1a	***AOX1b***	***AOX1c***	***NDB1***	***NDB2***	***NDB3***	***NDA1***	***NDA2***	***NDC1***
CAT-A	-0.36	-0.19	0.18	0.93 **	0.5	0.096	0.84 *	-0.042	0.98 ***
MnSOD	-0.027	0.17	0.89 **	0.36	-0.26	-0.14	-0.028	-0.25	0.44
Root									
CAT-A	-0.43	-0.21	0.25	0.35	0.46	-0.13	0.7	-0.073	0.35
MnSOD	-0.35	-0.11	-0.56	-0.39	0.035	0.16	0.088	0.26	-0.39

5.4 Discussion

Plants exhibit a number of physiological and molecular responses in order to survive under the osmotic and ion toxicity imposed by elevated salinity, and these responses vary greatly within plant species/cultivar (Walia et al., 2005; Munns and Tester, 2008; Platten et al., 2013) and even among individual plants within the same cultivar (Flowers and Yeo, 1981). The current study evaluated the physiological responses of 16 different rice cultivars, representing indica and japonica rice, to salinity stress at early seedling stage using a hydroponic culture system. Based on growth performance under salinity stress, seven cultivars representing salt-tolerant and salt-sensitive cultivars, were selected for further transcriptional analysis. The study demonstrated contrasting cultivar variations in the physiological as well as molecular responses in response to salinity stress. Out of 16 cultivars used in the current study, the cultivars Pokkali, IR 64 and Nipponbare have been commonly used in salinity tolerance studies (Kumari et al., 2009; Cotsaftis et al., 2011; Kavitha et al., 2012; Ferdose et al., 2015). However, comparative growth and molecular responses of other cultivars, especially Australian japonica rice, to salinity stress have not yet been reported.

During the current study, a great variation between cultivars was observed in shoot as well as in root biomass production under the salinity stress imposed. A group of seven japonica rice cultivars (Sherpa, Koshihikari, Kyeema, Nipponbare, Reiziq, Milang 23 and Topaz), which mostly consisted of Australian japonica rice cultivars, showed the highest biomass production in terms of both shoots and roots (Figure 5.3). Doongara, which is also an Australian japonica cultivar, showed the lowest root biomass production while Lemont (USA tropical japonica) had the lowest shoot biomass production (Figure 5.3). While shoot biomass production of some cultivars, Takiminori, Phka Rumduol, IR 64, Pandang and LTH, which represent both japonica

and indica rice cultivars, was found to be in a similar range but these cultivars showed a large variation in root biomass production (Figure 5.3). Generally, rice is considered to be the most salt-sensitive cereal crop especially at the early seedling stage (Munns and Tester, 2008; Ferdose et al., 2015). My results demonstrate that despite this, rice shows contrasting genotypic as well as tissue-wide variation in the sensitivity to salinity stress as reflected by growth performance. Such variation in salinity tolerance among accessions from cultivated *Oryza* species, *O. sativa* and *O. glaberrima*, has been reported earlier (Platten et al., 2013). These authors showed that, despite the diverse origin and relationships of the accessions, salinity tolerance in almost all accessions from both *O. sativa* and *O. glaberrima* correlated with leaf Na^+ accumulation. They suggested that the mechanisms proposed to influence Na^+ concentrations in leaves, such as Na^+ exclusion from roots, sequestration of Na^+ in roots, stems and leaf sheath, partitioning of Na^+ from leaf to leaf and dilution of Na^+ content in a large biomass, can all play a major role in salinity tolerance. Lee et al. (2003) also observed extreme cultivar variability between indica and japonica rice groups in response to salinity stress at the seedling stage. These varietal differences in salinity tolerance were highly significant in the context of plant growth (height, shoot weight, root weight) as well as tissue ion accumulation. Remarkable sensitivity differences to salinity stress were reported even within closely related indica cultivars (Walia et al., 2005; Kumari et al., 2009). It has been observed that rice plants with more biomass had more inherent vigour which allows them to tolerate salinity stress better than smaller plants (Yeo and Flowers, 1986). Therefore, plant size might also be important when comparing salinity tolerance between a number of genotypes with very different plant biomass (Pires et al., 2015). But, the current study did not investigate the effect of plant size on salinity tolerance.

The results presented also demonstrated the existence of extreme cultivar variation in ion accumulation in tissues. Pokkali recorded the lowest concentration of shoot Na^+

out of 16 cultivars tested, which was significantly lower than that of Nipponbare (Figure 5.5B). In contrast, the root Na^+ concentration of Pokkali was significantly higher compared to that of Nipponbare (Figure 5.5A), yet both cultivars were able to maintain over 75% of relative root and shoot biomass production under 120 mM salt over 12 days (Figures 5.6 and 5.7). These results indicate that the two different cultivars use different tolerance mechanisms to survive under saline conditions. Cotsaftis et al. (2011), who obtained similar results in cultivar Pokkali, suggested that the possible mechanisms used by this cultivar could be sequestration of Na^+ in root tissues, preventing transfer to shoot tissues, or reduction of Na^+ transport to shoots. Pokkali is generally a large biomass producer compared to others, which may have increased Na^+ dilution in the shoot tissue (Yeo et al., 1990; Platten et al., 2013). Nipponbare is a poor Na^+ excluder and accumulates substantial Na^+ in its tissues, probably sequestered in vacuoles since its relative biomass production was maintained. Doongara recorded the highest Na^+ accumulation in shoots while Lemont was the second highest, they also had the lowest relative shoot biomass production, suggesting that these two cultivars were not only poor Na^+ excluders but probably accumulated Na^+ in cells, possibly in the cytoplasm, which inhibited normal cell functions, that is, they had poor tolerance mechanisms.

It is a widely known fact that high concentrations of Na^+ in the cytoplasm creates metabolic toxicity in cells, possibly as a result of its ability to compete with K^+ for binding sites essential of cellular functions (Tester and Davenport, 2003). K^+ is required for the activation of some enzymes and Na^+ cannot substitute. Protein synthesis also requires high concentrations of K^+. Therefore, high levels of Na^+ or a high Na^+/K^+ ratio, disrupt cellular enzymatic processes including protein synthesis (Tester and Davenport, 2003), which in turn has a negative effect on plant growth and development. The Na^+/K^+ ratio in a plant is considered to be one of the most important

factors in deciding the tolerance of a plant to salinity stress (Asch et al., 2000; Pires et al., 2015). The data presented here show significant variation in Na^+/K^+ ratios among the 16 rice cultivars. The traditional salt-tolerant indica cultivar, Pokkali, recorded the lowest Na^+/K^+ ratio in shoots (Figure 5.8) and maintained comparatively higher (~80%) relative shoot dry biomass production. Doongara lost ~40% of shoot and ~60% and root biomass production as a consequence of higher Na^+/K^+ ratio in tissues (Figure 5.8 and 5.9), in line with it being a salt-sensitive cultivar. In contrast to these two cultivars, Nipponbare had higher Na^+/K^+ ratios in both shoots and roots but maintained over 90% of relative dry matter production in both tissue types, showing that it can tolerate higher Na^+ levels in its tissues, probably by sequestering Na^+ in shoots and roots away from the cytoplasm.

Comparing physiological and transcriptional parameters in rice cultivars with divergent level of salinity tolerance can improve our knowledge of salt-tolerance mechanisms (Senadheera et al., 2009). There are a number of studies which focused on the genotypic variations in transcriptional and physiological responses of rice cultivars to salinity stress (Walia et al., 2005; Senadheera et al., 2009; Cotsaftis et al., 2011). Transcriptional analysis in most of these studies was based on genome-wide gene expression analysis to explore the genes that are up-regulated or down-regulated in response to salinity stress and mostly focused on indica rice cultivars. In the current study, seven cultivars, representing diverse rice germplasm (both indica and japonica rice were included), were selected for transcriptional analysis based on their initial physiological characterization. Unlike other studies, this study compared the expression of alternative respiratory pathway genes, AOXs and NDHs, using qRT-PCR. To my knowledge, this is the first such study in cultivars with divergent germplasm variation to salinity stress. The data presented here demonstrate that there is a wide variation between cultivars in alternative pathway gene expression

under control as well as salt-treated conditions. Some genes were activated under non-salt-treated condition and inhibited in response to salinity stress while others were induced further. For example, *AOX1a* and *AOX1b* in Doongara were significantly induced in response to salinity stress while *AOX1c* expression was inhibited (Figure 5.10A). Likewise, there was a higher abundance of shoot *NDB2* transcripts in Doongara under control conditions but it was dramatically decreased in response to salinity stress. In contrast, the abundance of *NDB2* transcript in roots was less under control conditions and increased in salt-treated roots (Figure 5.11). Cultivar variation and tissue-specificity of other transcriptional responses of rice in response to salinity stress have been reported previously (Walia et al., 2005; Cotsaftis et al., 2011). Walia et al. (2005) observed that a relatively large number of genes were expressed in the shoots of sensitive indica cultivar, IR 29, compared to tolerant indica type, FL478, in response to salinity stress. In contrast, Cotsaftis et al. (2011) observed the expression of large number of genes in the roots of salt-tolerant cultivar, FL478 compared to the salt-sensitive IR 29 under the same experimental conditions, showing the tissue-specific nature of transcriptional responses.

Other studies have shown that tolerant cultivars maintain higher transcript levels of genes related to salt-tolerance even under non-stressed conditions, to be “well prepared” for the subsequent stress (Kumar et al., 2009). Kumari et al. (2009) reported that the salt-tolerant rice cultivar, Pokkali maintained high level of expression of stress inducible genes in the absence of stress, which in turn resulted in expression of fewer genes in response to salinity stress compared to IR 64. Similarly, current results also revealed high level of transcript abundance of some AP genes (*NDA1*, *NDB2*, and *NDC1*) in Pokkali in the absence of stress, and down-regulated in response to salinity stress while some other cultivars (Nipponbare, Kyeema and Milang 23) had low level of expression of these genes in the absence of stress and

up-regulated in response to high salt (Figure 5.12). But, in contrast, salt-sensitive cultivars like Doongara and even IR 64 also maintained high levels of expression of *NDA1*, *NDB2* and *NDC1* in non-stressed tissues, and down-regulated in response to elevated salinity.

Although expression of *AOX1a* and *NDA2* in leaf tissues showed a strong positive correlation with tissue Na^+ content, there was no correlation between AP gene expression and shoot growth. In fact, the expression of *AOX1c* and *NDB2* in roots revealed strong negative correlation with relative growth of roots. It must be kept in mind that this study has only determine gene expression levels and not protein levels or activity. As AOX can be post-transcriptionally regulated it is still possible that alternative pathway components may play a role in salt-tolerance in some cultivars, the data presented here imply that AP gene expression levels may not be good molecular markers for salt-tolerance in general in rice.

6 Generation and characterization of transgenic rice carrying salt-responsive Arabidopsis alternative respiratory genes, *AtAOX1a* and *AtNDB2*

6.1 Introduction

The application of genetic methods has the potential to upgrade existing crops and to provide incremental improvements. Therefore, plant genetic engineering has become one of the important molecular tools in molecular breeding of crops (Sah et al., 2014). Once the genes responsible for controlling mechanisms of abiotic stresses have been identified, they can be manipulated to develop improved crop varieties using genetic recombination technology. Overexpression of stress responsive alternative respiratory genes has been shown to improve growth and to reduce ROS production under stress conditions compared to their wild types. Li et al (2013) showed that overexpression of *OsAOX1a* gene in rice could strongly increase the growth performance, especially the root growth, of rice seedlings grown under cold stress due to the reduction of ROS generation within cells and thereby minimizing oxidative damage. Smith et al (2009) provided evidence that overexpression of *AtAOX1a* in Arabidopsis seedlings improved the growth of seedlings under salt stress by reducing the production of ROS within tissues. And also they further showed that *AtNDB4* knockdown lines of Arabidopsis showed the increased expression of *AtAOX1a* and *AtNDB2* genes in seedlings grown under salinity stress giving evidences that Arabidopsis *AOX1a* and *NDB2* are playing a role during salinity stress (Smith et al., 2011).

Overexpression of dicot stress responsive genes in cereals provided enhanced tolerance to respective stress conditions. Schilling et al. (2014) showed that transgenic barley expressing Arabidopsis vacuolar H^+ pyrophosphatase (*AVP1*) gene produced a larger shoot biomass when grown in soil with 150 mM NaCl. And also these transgenic barley produced a greater grain yield per plant when grown in a

saline field compared to their wild type. Transgenic rice co-expressing *Suaeda salsa* vacuolar membrane Na^+/H^+ antiporter (*SsNHX1*) and Arabidopsis *AVP1* also showed enhanced salt-tolerance during 3 days of 300 mM NaCl treatment under outdoor growth conditions (Zhao et al., 2006). In contrast, there are some reports which use the model plant Arabidopsis to study the function of monocot genes under abiotic stresses. Sugie *et al* (2006) developed a system to study the functions of wheat *AOX1a* gene under low temperature by cloning wheat *AOX1a* into Arabidopsis. They noticed that there was rapid recovery of total respiration capacity of transgenic plants as well as decreased levels of ROS under low temperature stress compared to their wild type.

The main objective of this chapter is to test the potential of applying the knowledge gained through the model system Arabidopsis, to improve cereal crop productivity under salinity stress using rice as model cereal. The chapter discusses an attempt towards generating transgenic rice with improved salinity tolerance by inserting salt-responsive *AtNDB2* and *AtAOX1a* genes under the control of rice actin-1 promoter. The work here describes the molecular cloning of two plasmid constructs carrying salt-responsive Arabidopsis *AOX1a* or *NDB2* genes into the pIPKb003 overexpression vector and the subsequent process of producing transgenic rice carrying *AtAOX1a* or *AtNDB2* through *Agrobacterium*-mediated transformation. The chapter will further describe the preliminary evaluation and characterization of putative salt-tolerant transgenic rice under greenhouse conditions by exposing to environmentally realistic salt concentrations (eg 30 mM) during panicle initiation stage.

6.2 Materials and methods

The processes of developing genetic constructs, generating transgenic rice plants and their evaluation studies were carried out in the OGTR (Office of the Gene Technology Regulator) licensed PC2 laboratory and plant facility at Flinders university of South Australia.

6.2.1 Plasmid vectors used in the study

Gateway® entry clones carrying the *AtAOX1a* gene and *AtNDB2* gene (Flinders University) and the pIPKb003 overexpression/destination vector (Himmelbach et al., 2007) were used as plasmid vectors in the generation of two genetic constructs, either an *AtAOX1a* or an *AtNDB2* overexpression/destination clone, to transform rice scutellum-derived embryogenic calli. The Gateway® entry clone carrying the *AtAOX1a* gene was generated by insertion of the *AtAOX1a* coding DNA sequence (CDS) (1065 bp) into the pDONR™207 donor vector via the Gateway® BP reaction, which carries a gentamycin resistance gene as a bacterial selection marker (Figure 6.1). The Gateway® entry clone carrying *AtNDB2* gene was generated by insertion of the *AtNDB2* CDS sequence (1749 bp) into the pDONR™221 donor vector via the Gateway® BP reaction. It carries a kanamycin resistance gene as a bacterial selection marker (Figure 6.2). pIPKb003 overexpression vector (Acc. EU161569), which carries a spectinomycin resistance gene for bacterial selection and a hygromycin resistance gene for plant selection, was used as the destination vector (Figure 6.3). This plasmid vector was specifically designed for cereal transformation and was Gateway® compatible.

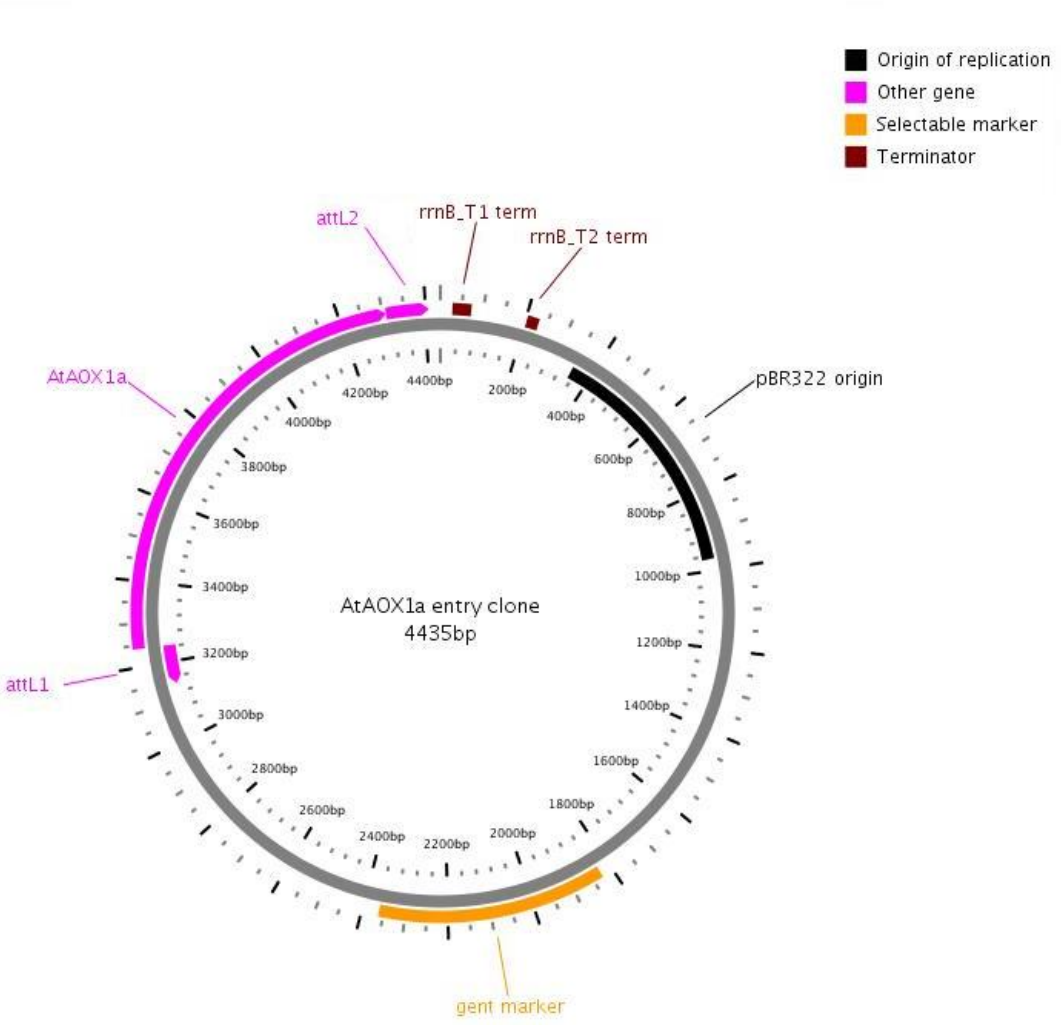


Figure 6.1 Schematic representation of Gateway® entry clone carrying *AtAOX1a* gene (created with PlasMapper - http://wishart.biology.ualberta.ca/PlasMapper/)

A gateway entry clone carrying the *AtAOX1a* gene (At3g22370) was used to generate an *AtAOX1a* overexpression clone in the current study. The entry clone was generated by insertion of the *AtAOX1a* CDS sequence (1065 bp) into the pDONR™207 donor vector via Gateway® BP reaction. The entry clone backbone consisted of gentamycin resistance marker gene (gent marker) for the selection of plasmid in *E.coli*, transcription termination sequences (rrnB_T1term and rrnB_T2 term), CDS sequence of Arabidopsis *AOX1a* gene (*AtAOX1a*), Gateway recombination sites (attL1 and attL2), and pBR322 origin of replication for high plasmid yield.

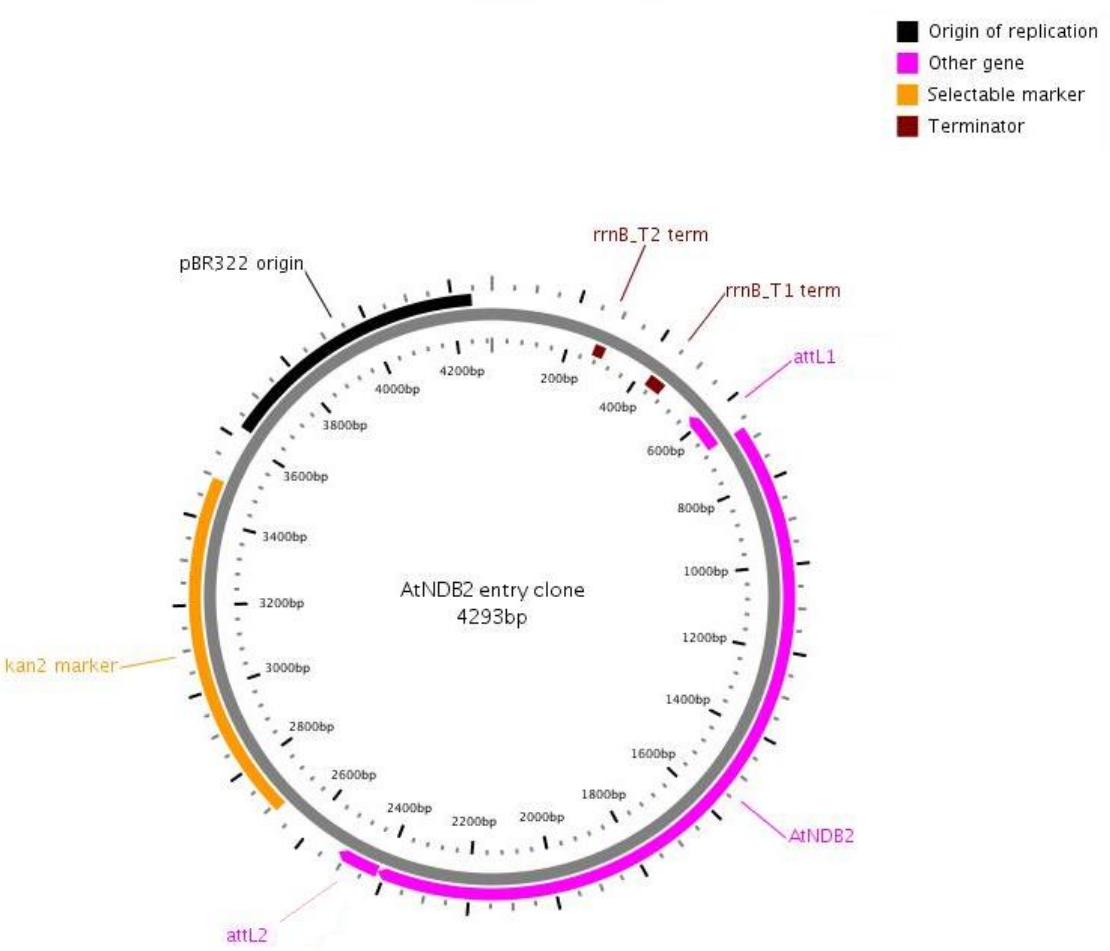


Figure 6.2 Schematic representation of Gateway® entry clone carrying *AtNDB2* gene (created with PlasMapper - http://wishart.biology.ualberta.ca/PlasMapper/)

A gateway® entry clone carrying the *AtNDB2* gene (At4g05020) was used to generate an *AtNDB2* overexpression clone in the study. The entry clone was generated by insertion of the Arabidopsis *NDB2* CDS sequence (1749 bp) into the pDONR™221 entry vector via Gateway® BP reaction. The clone backbone consisted of the kanamycin resistance marker gene (kan2 marker) for the selection of plasmid in *E.coli*, transcription termination sequences (rrnB_T1term and rrnB_T2 term), CDS sequence of Arabidopsis *NDB2* gene (*AtNDB2*), Gateway recombination sites (attL1 and attL2), and pBR322 origin of replication for high plasmid yield.

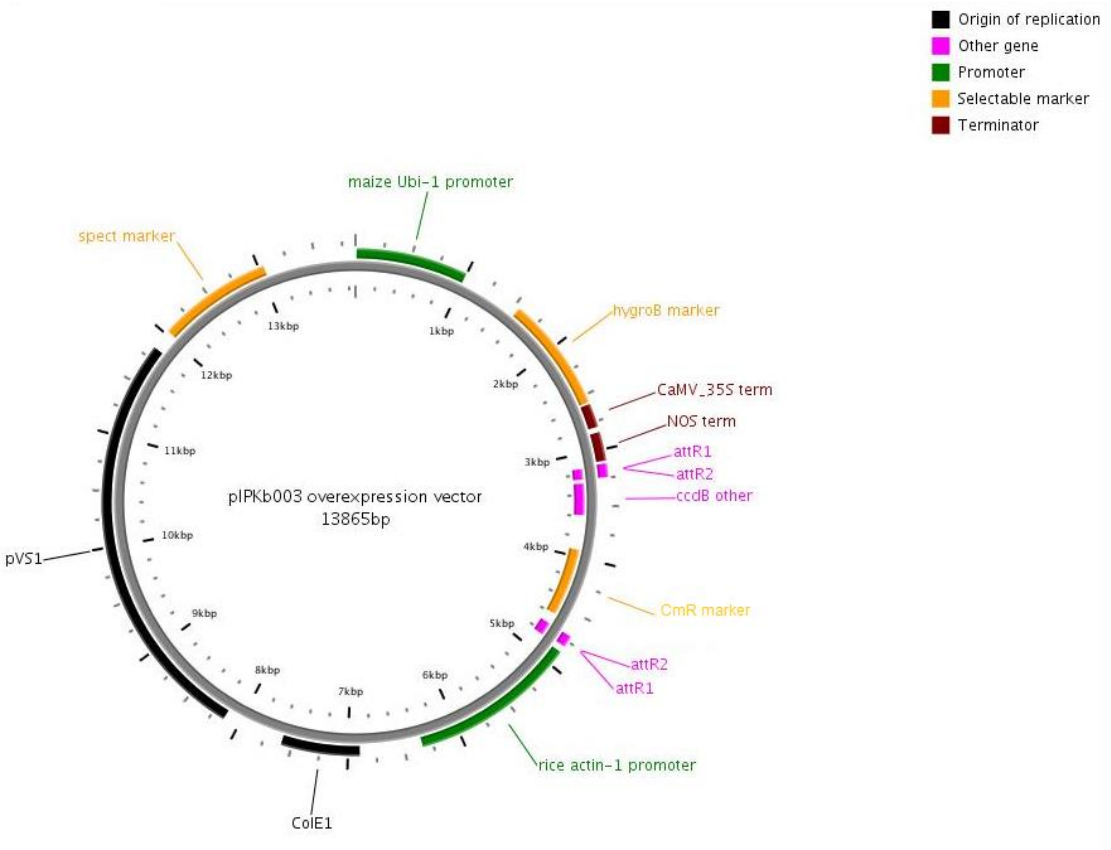


Figure 6.3 Schematic representation of pIPKb003 overexpression/destination vector (created with PlasMapper - http://wishart.biology.ualberta.ca/PlasMapper/)

The pIPKb003 overexpression vector (binary vector), specifically designed for cereal transformation (Himmelbach et al., 2007), was used as the destination vector. It is Gateway compatible and the vector backbone consisted of the following components: Gateway recombination sites (attR1 and attR2), which facilitate the exchange of a gene sequence of interest (GOI) between an appropriate entry vector and an IPKb003 destination vector using the Gateway recombination system, a chloramphenicol resistance gene (CmR marker) for selection of bacteria, ccdB toxin gene for negative selection of bacteria, *E. coli* origin of replication (ColE1), *Agrobacterium* origin of replication (pVS1), a spectinomycin resistance gene for selection of bacteria (spec marker), hygromycin resistance gene (hygroB marker) controlled by maize ubiquitin 1 promoter (maize Ubi-1 promoter) for plant selection, rice actin-1 promoter to control the expression of inserted GOI, CaMV 35S and nopaline synthase (NOS) terminators.

6.2.2 Identity verification of plasmid vectors

Colony PCR and/or analytical/diagnostic restriction digestion were performed to verify the identity of the Gateway® entry vector carrying *AtNDB2* gene, the Gateway® entry vector carrying *AtAOX1a* and the pIPKb003 overexpression vector before being used in Gateway LR cloning reaction.

6.2.2.1 Identity verification of Gateway® entry clone carrying *AtAOX1a*

Gateway® entry clones carrying the *AtAOX1a* gene maintained in *E. coli* strain DH5α cells, were streaked on LB selection plates supplemented with 25 µg/ml of gentamycin and incubated at 37ºC for 16-20 hrs. Several single colonies were selected and colony PCR was performed with gene-specific primers (Section 2.2.6). PCR positive colonies were used to establish 10 ml LB liquid cultures in Falcon tubes and incubated overnight at 37ºC with shaking. Plasmid DNA was isolated and purified as described in Section 2.2.7 and plasmid identity was verified by diagnostic restriction digestion (Section 2.2.8) with *BseR1*. Digests were run on 0.8% agarose gel and compared with expected DNA fragment pattern. Cut sites and length of expected DNA fragments were determined using NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/).

6.2.2.2 Identity verification of Gateway® entry clone carrying *AtNDB2*

Gateway® entry clones carrying the *AtNDB2* gene maintained in *E. coli* strain DH5α cells, were streaked on LB plates supplemented with 25 µg/ml of kanamycin and incubated at 37ºC for 16-20 hrs. Several single colonies were selected and colony PCR was performed with gene-specific primers (Section 2.2.6). PCR positive colonies were used to establish 10 ml LB liquid cultures in Falcon tubes and incubated overnight at 37ºC with shaking. Plasmid DNA was isolated and purified as described in Section 2.2.7 and plasmid identity was verified by diagnostic restriction digestion (Section 2.2.8) with *EcoRV.* Products were run on 0.8% agarose gel and compared with the expected banding pattern determined by NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/).

6.2.2.3 Identity verification of pIPKb003 overexpression vector

The pIPKb003 (Acc. EU161569) plasmid vector maintained in *E.coli* strain in DB3.1 cells was streaked on to LB selection media plates containing 50 µg/ml spectinomycin and incubated at 37ºC for 16-20 hrs. Several single colonies were selected and 10 ml LB liquid cultures in Falcon tubes were inoculated and incubated overnight at 37ºC with shaking. Plasmid DNA was isolated and purified as described in Section 2.2.7 and plasmid was digested (Section 2.2.8) with *Pst1* and products were visualized on 0.8% agarose gel. Identity of the plasmid DNA was verified by comparing the resulted DNA banding pattern with the expected banding pattern determined by NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/).

6.2.3 Generation of genetic constructs via LR cloning

LR cloning (Section 2.2.10) was performed to transfer the Arabidopsis salt-responsive *AtAOX1a* gene from the Gateway® entry clone into the pIPKb003 binary vector and to generate a Gateway® overexpression clone carrying an *AtAOX1a* gene (Figure 6.6). *E.coli* strain DH10B cells were transformed with the plasmid construct through electroporation (Section 2.2.11). Resulting DH10B cells were grown on LB selection media plates containing 100 µg/ml of spectinomycin at 37ºC to select single transformed colonies. Colony PCR was performed with gene-specific primers with selected colonies (Section 2.2.6) and confirmed the presence of the *AtAOX1a* genetic construct. LB liquid cultures in 10 ml Falcon tubes were inoculated with colony PCR positive cells and incubated at 37ºC with shaking. Plasmid DNA was isolated and purified as described in Section 2.2.7. Identity of plasmid DNA was verified by diagnostic restriction digestion with *PstI* run on 0.8% agarose gel and by sequencing with vector-specific forward and reverse primers (Section 2.2.13). The same procedure was carried out to generate and to verify identity of the Gateway® overexpression clone carrying *AtNDB2* gene (Figure 6.7). Identity validated plasmid constructs were maintained in glycerol stocks at -80ºC for future use.

6.2.4 Generation of transgenic rice carrying *AtAOX1a* and *AtNDB2*

6.2.4.1 Callus induction and proliferation

Embryogenic callus (scutellum-derived callus) was generated from mature seeds of *O. sativa* L spp *japonica* cv Nipponbare using N6D medium (Appendix E). Seeds were dehusked and surface sterilized following the steps described in Section 2.1.2. The sterilized seeds were inoculated, facing embryo up, on callus induction (N6D) medium solidified with 0.4% Gelrite and incubated under continuous light at 32^{o}C for 2 weeks. Proliferating calli arising from the scutellum were transferred to fresh N6D medium and allowed to grow for a further week before being used in the transformation process.

6.2.4.2 Production and selection of transgenic rice carrying *AtAOX1a*

Agrobacterium-mediated rice transformation was carried out following the methods described by Hiei et al. (1997) and Toki (1997) with minor modifications.

Agrobacterium strain, AGL1, harbouring the pIPKb003+*AtAOX1a* expression clone (Figure 6.6), was cultured on AB medium (Appendix E) supplemented with 20 µg/mg rifampicin and 100 µg/mg spectinomycin solidified with 1.5% agar and incubated for 3 days at 30^{o}C in the dark. One large loop of *Agrobacterium* culture was scraped from the plates and suspended in AAM medium (Appendix E) to yield an OD_{600} of approximately 0.1. Proliferating calli were immersed in the *Agrobacterium* suspension by gentle shaking for 1.5 min, then calli were blotted dry with a sterilized filter paper to remove excess bacteria.

These calli were transferred on to a sterilized filter paper (9 cm diameter) that had been moistened with 0.5 ml of AAM medium and placed on 2N6-AS medium (Appendix E) solidified with 0.4% Gelrite. After 3 days of co-cultivation at 25^{o}C in the dark, calli were washed five times in sterilized water and then washed once in sterilized water containing 150 µg/ml timentin to suppress the growth of *Agrobacterium*. The calli were rapidly blotted dry on a sterilized filter paper and further

cultured on N6D selection medium containing 50 µg/ml hygromycin B (to select transformed calli) and 150 µg/ml timentin (to suppress the growth of *Agrobacterium*) under continuous light at 32ºC for 2 weeks. Healthy proliferating calli were transferred to regeneration medium (Appendix E) containing 50 µg/ml hygromycin B and 150 µg/ml timentin for plantlet regeneration. Individual plantlets were acclimatized in tap water for a day at room temperature before being transferred to soil. Confirmation of transgene integration into the rice genome was done by performing PCR (Section 2.2.2) using rice genomic DNA isolated from T_0 plants; and the expression of the gene of interest (*AtAOX1a*) in rice plants was confirmed by performing RT-PCR (Section 2.2.5) with cDNA synthesized from RNA isolated from T_0 plants. In both PCR and RT-PCR reactions, gene-specific primers were used (Table 2.1).

6.2.4.3 Production and selection of transgenic rice carrying *AtNDB2*

The same procedure was followed as described in Section 6.2.4.2 to generate *AtNDB2* transgenes starting with *Agrobacterium* strain, AGL1, harbouring the pIPKb003+*AtNDB2* expression clone (Figure 6.7). The integration of the transgene into the rice genome was confirmed by performing PCR (Section 2.2.2) using rice genomic DNA isolated from T_0 plants and the expression of the gene of interest (*AtNDB2*) in rice plants was confirmed by performing RT-PCR (Section 2.2.5) with cDNA synthesized from RNA isolated fromT_0 plants. In both PCR and RT-PCR reactions the same gene-specific primers were used (Table 2.1).

6.2.5 Segregation analysis of putative T_1 transgenic rice

The inheritance of the gene of interest (*AtAOX1a* or *AtNDB2*) into the T_1 generation was tested by performing PCR (Section 2.2.2) with genomic DNA isolated from leaves of T_1 plants with respective gene-specific primers. In order to perform Mendelian segregation analysis, the number of PCR positive plants was counted and Mendelian inheritance pattern was evaluated by the Chi-square test (Goodness of Fit test) at 0.05 probability level.

6.2.6 Evaluation of transgenic rice carrying *AtAOX1a* or *AtNDB2* genes under salt stress

Evaluation of transgenic rice under salt stress was done by applying NaCl (30 mM) to growing seedlings at their panicle initiation stage. Three independent transgenic lines from each transgenic rice group (plants carrying *AtAOX1a* or *AtNDB2*) were selected for the evaluation studies. T_1 transgenic rice seeds were surface sterilized and germinated on wet filter papers in Petri dishes as described in Section 2.1.2. Approximately 10 days after sowing, healthy seedlings were transferred to 180 mm plastic pots containing potting mix supplemented with Osmocote fertilizer which consisted of N, P, K (19.4 : 1.6 : 5) and other trace elements (Scotts Australia Pty Ltd). The pots were placed in containers filled with tap water ($\sim 2-3$ cm below the soil level in pots) and allowed to grow to maturity. Then the pots were transferred to big plastic tanks filled with half strength nutrient/growth medium (Section 2.1.3) and allowed to grow several weeks while maintaining the nutrient medium level ~2 cm above the soil level (Figure 6.4). Water level was topped up with tap water and new growth medium was added weekly. Salt application was started when panicle emergence was noticed in 4-5 plants. Salt was applied into the surrounding nutrient solution in two increments (15 mM each time, the 2nd application was done 2 days after the first) to reach the final concentration of 30 mM and maintained up to one month. During the salt application period, the water level in tanks was maintained by adding tap water. Leaf samples were collected for the measurement of Na^+ and K^+ content after one month of salt application and ion contents were measured as described in Section 2.1.4.

Figure 6.4 Experimental set up for the evaluation of transgenic rice under salinity stress in controlled (greenhouse) conditions

30 mM NaCl was applied to seedlings at their panicle initiation stage and they were allowed to grow under salt-stressed conditions for one month. Then the seedlings were released from the stress and grown under normal conditions until maturity. Data related to yield performances were recorded at harvesting.

After one month of treatment, salt solution was replaced with half strength nutrient solution and seedlings were allowed to grow until harvesting time. In the meantime, the water level in tanks was maintained as explained above and fresh nutrient medium was added weekly. The number of panicles per plant, number of tillers per plant, number of filled and unfilled (empty) seeds per panicle and seed weight per panicle, were recorded at harvesting to evaluate the effect of salt stress on plant growth and seed yield.

6.3 Results

6.3.1 Identities of Gateway® entry clones and pIPKb003 overexpression vector were confirmed by both colony PCR and diagnostic restriction digestion

Before starting molecular cloning of Arabidopsis salt-responsive genes into the pIPKb003 overexpression vector, the identity of all plasmid vectors was verified by colony PCR and/or analytical/diagnostic restriction digestion. Gateway® entry clones carrying *AtAOX1a* were digested with *BseR1* (GAGGAG$(N)_8$▲NN▼) while the *AtNDB2* entry clone was digested with *EcoRV* (GAT▼▲ATC). As determined by NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/), digestion of the *AtAOX1a* entry clone plasmid DNA resulted in three DNA fragments of 2864 bp, 1281 bp and 290 bp, confirming the identity of plasmid DNA (Figure 6.5 A). The *AtNDB2* entry clone contained only one *EcoRV* cut site, which resulted in one DNA fragment when digested products were run on the 0.8% agarose gels (Figure 6.5 B). Digestion of the overexpression vector (pIPKb003) with *PstI* resulted in the expected band pattern, confirming the presence of correct plasmid DNA of pIPKb003 (Figure 6.5 C). The pIPKb003 overexpression vector backbone has six cut sites for *PstI* (C▲TGCA▼G) and resulted in six DNA fragments at lengths of 9027 bp, 1591 bp, 1501 bp, 921 bp, 452 bp and 366 bp, as determined by NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/). These verified plasmid DNAs were used to generate overexpression/destination clones carrying salt-responsive Arabidopsis *AtAOX1a* or *AtNDB2* overexpression clones (Figure 6.6 or 6.7) via Gateway LR reaction.

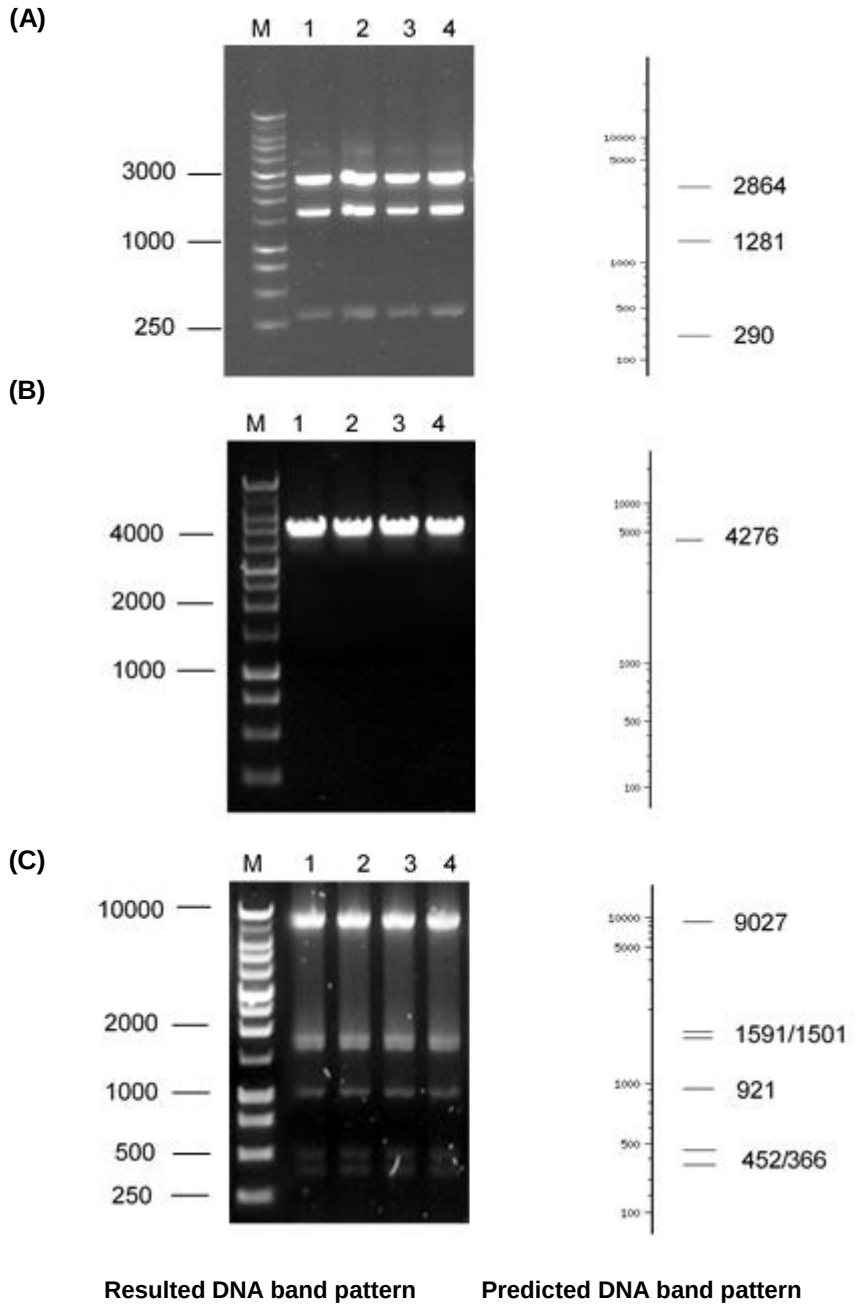


Figure 6.5 Diagnostic restriction digests of entry clones and pIPKb003 overexpression vector used to generate genetic constructs

A: Digests of *AtAOX1a* entry clone run on 0.8% agarose gel. **Lane M**: molecular weight marker in bp; **Lane 1-4**: plasmid DNA from four different single bacterial colonies carrying *AtAOX1a* entry clone cut with *BseR1*.

B: Digests of *AtNDB2* entry clone run on 0.8% agarose gel. **Lane M**: molecular weight marker in bp; **Lane 1-4**: plasmid DNA from four different single bacterial colonies carrying *AtNDB2* entry clone cut with *EcoRV*.

C: Digests of pIPKb003 empty overexpression vector. **Lane M**: molecular weight marker in bp; **Lane 1–4**: plasmid DNA from four different single bacterial colonies carrying empty pIPKb003 overexpression vector cut with *PstI*. Cut sites and lengths of expected DNA fragments were determined using NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/).

6.3.2 Identities of genetic constructs carrying Arabidopsis salt-responsive genes were confirmed by colony PCR, diagnostic restriction digestion as well as by sequencing

The identity of both genetic constructs was verified initially by colony PCR, analytical restriction digestion and finally by sequencing prior to the transformation for confirmation of fidelity. *AtAOX1a* and *AtNDB2* from Arabidopsis were cloned into pIPKb003 overexpression vector via Gateway® LR reactions. *E. coli* strain DH10B cells were transformed with plasmid constructs resulting from LR cloning, via electroporation. Transformed *E. coli* cells carrying plasmid DNA with either *AtAOX1a* or *AtNDB2* resulted in single colonies when cultured on LB selection plates supplemented with 100 µg/ml of spectinomycin. Initially, colony PCR was carried out to select bacterial colonies carrying *AtAOX1a* or *AtNDB2* gene with the relevant gene-specific primers. Colony PCR positive single colonies were selected for further verification with diagnostic restriction digestion and sequencing. Digestion of *AtNDB2* overexpression clone plasmid DNA with *PstI* resulted in 5 bands with sizes of 9027 bp, 2144 bp, 1501 bp, 921 bp, and 366 bp as determined by NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/) confirming the identity of the *AtNDB2* overexpression clone (Figure 6.8 A). Digestion of the *AtAOX1a* overexpression clone plasmid DNA with *PstI* resulted in 5 bands with sizes of 9027 bp, 1501 bp, 1460 bp, 921 bp and 366 bp as expected, verifying its identity (Figure 6.8B). Complete CDS of both genes of interest were sequenced with both vector-specific forward and reverse primers. The resulting sequences further confirmed the insertion of the correct CDS sequences of *AtAOX1a* or *AtNDB2* into the relevant overexpression clones. Overexpression clones in which the identity of the whole CDS sequence was verified by sequencing, were used to transform rice embryogenic calli through *Agrobacterium*-mediated transformation.

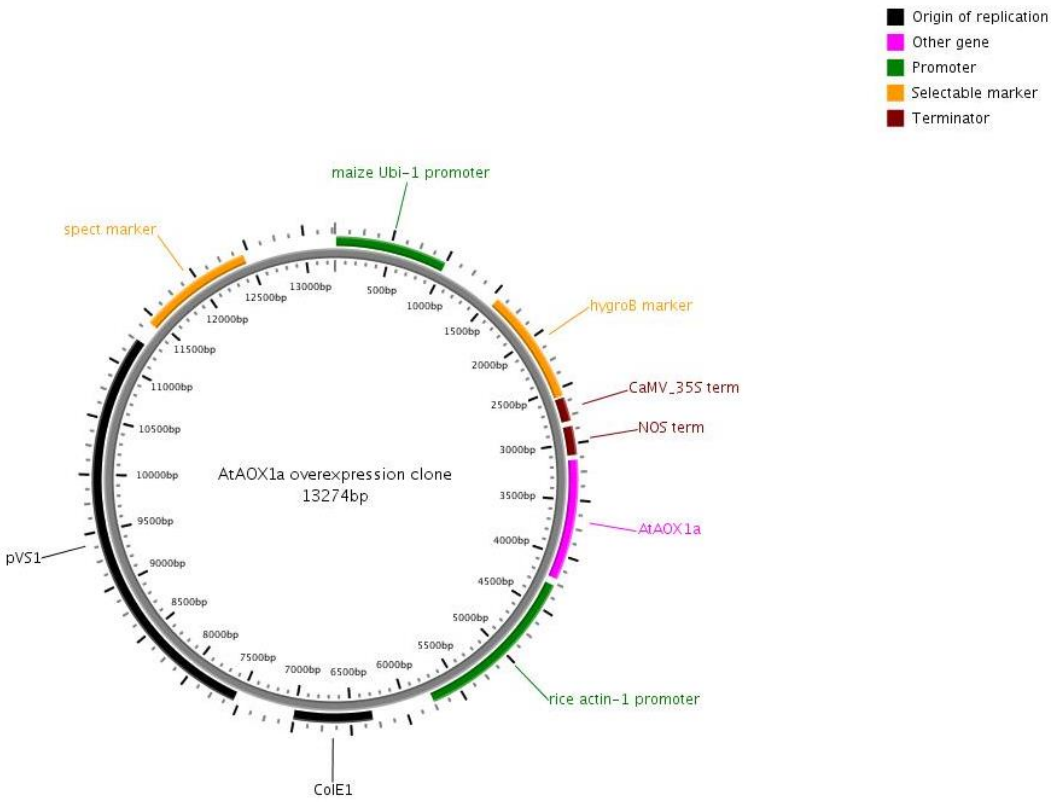


Figure 6.6 Schematic representation of *AtAOX1a* overexpression/destination clone (created with PlasMapper 2.0 (http://wishart.biology.ualberta.ca/PlasMapper/)

CDS sequence of Arabidopsis *AOX1a* gene was inserted into the pIPKb003 overexpression vector replacing the GATEWAY destination cassettes, R1 (attR1 recombination attachment site), CmR (chloramphenicol acetyltransferase gene), ccdB (negative selection marker), and R2 (attR2 recombination attachment site) sequences, during LR reaction. Identity verified *AtAOX1a* overexpression/destination clones were used for the subsequent *Agrobacterium*-mediated transformation of rice embryogenic calli.

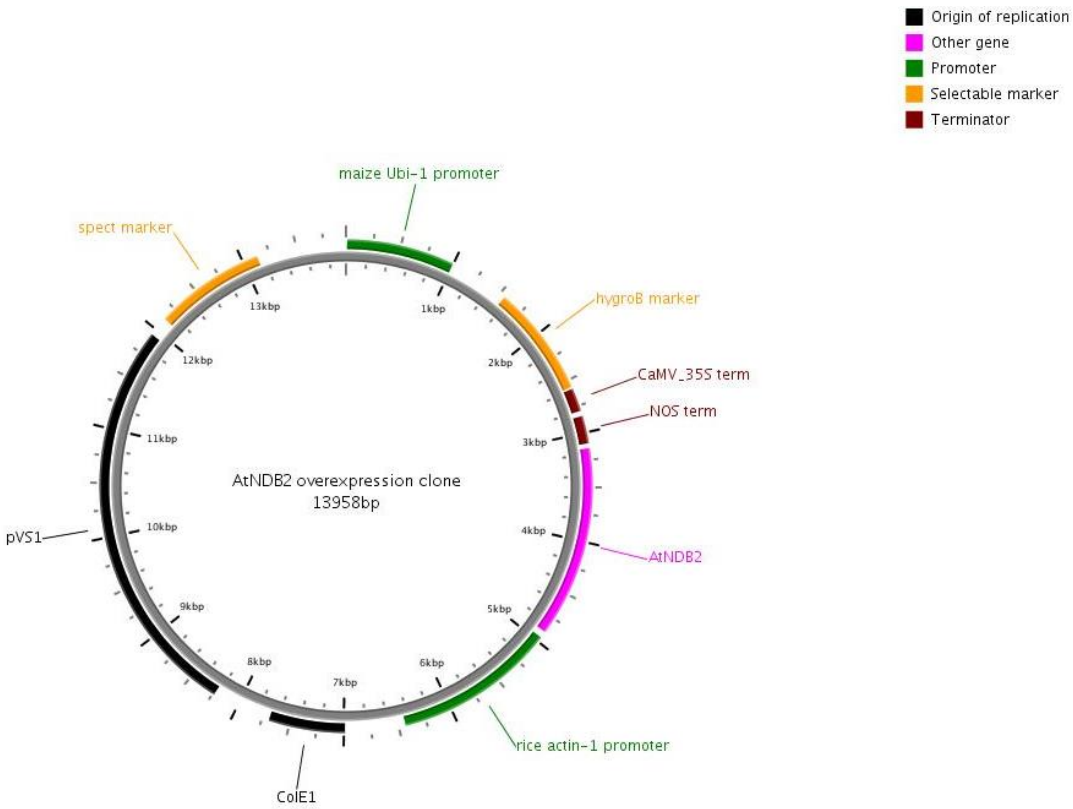


Figure 6.7 Schematic representation of *AtNDB2* overexpression/destination clone (created using PlasMapper 2.0 (http://wishart.biology.ualberta.ca/PlasMapper/)

CDS sequence of Arabidopsis *NDB2* gene was inserted into the pIPKb003 overexpression vector replacing the GATEWAY destination cassettes, R1 (attR1 recombination attachment site), CmR (chloramphenicol acetyltransferase gene), ccdB (negative selection marker), and R2 (attR2 recombination attachment site) sequences, during LR reaction. Identity verified *AtNDB2* overexpression/destination clones were used for the subsequent *Agrobacterium*-mediated transformation of rice embryogenic calli.

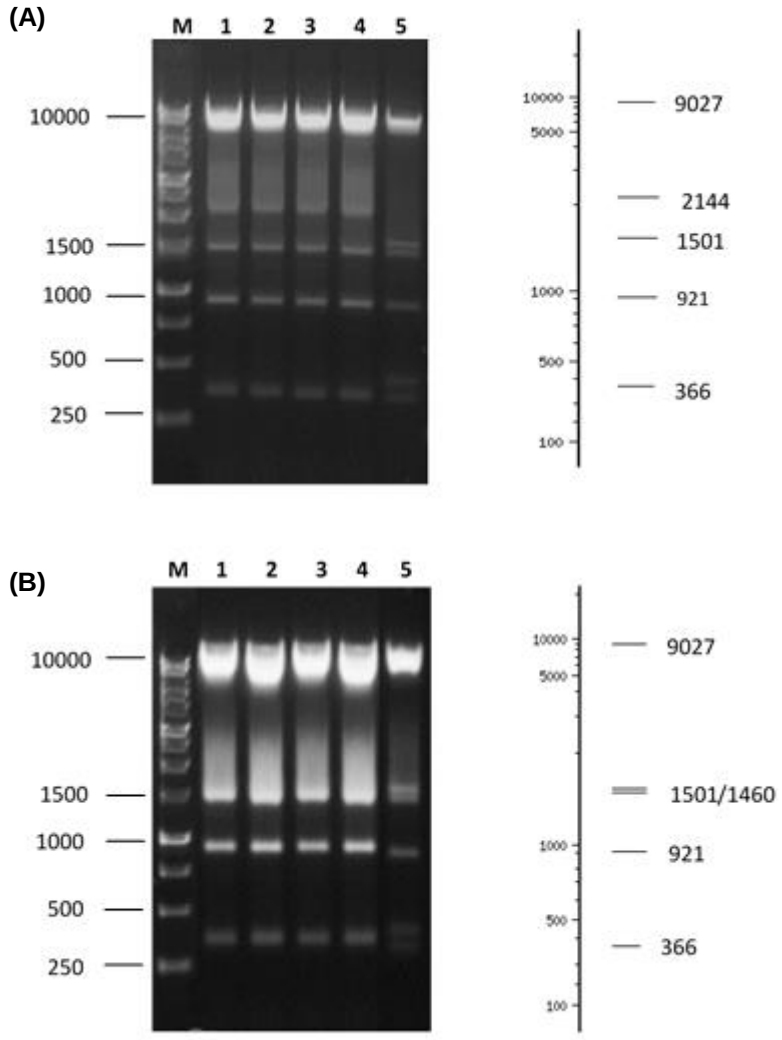


Figure 6.8 Diagnostic restriction digests to confirm the identity of genetic constructs/destination clones used in *Agrobacterium* transformation of rice

A: Digests of *AtNDB2* overexpression clone run on 0.8% agarose gel. **Lane M**: molecular weight marker in bp; **Lane 1-4**: plasmid DNA from 4 different single bacterial colonies carrying *AtNDB2* cut with *PstI*; **Lane 5:** pIPKb003 empty vector plasmid DNA cut with *PstI*

B: Digests of *AtAOX1a* overexpression clone run on 0.8% agarose gel. **M**: molecular weight marker in bp*;* **Lane 1-4**: plasmid DNA from 4 different single bacterial colonies carrying *AtAOX1a* cut with *PstI*; **Lane 5**: pIPKb003 empty vector plasmid DNA cut with *PstI*

Cut sites and length of expected DNA fragments were determined using NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/). Insertion of complete CDS sequences of *AtNDB2* or *AtAOX1a* genes into genetic constructs were further confirmed by sequencing with vector-specific forward and reverse primers. Identity verified genetic constructs were used in the *Agrobacterium*-mediated transformation of rice.

6.3.3 Transgenic rice carrying Arabidopsis salt-responsive genes were generated and confirmed by PCR

Nipponbare mature seeds inoculated on N6D medium and grown for about 2-3 weeks at 32^{o}C and continuous light showed callus induction rates of 100%. There was no contamination of *in-vitro* cultures during the process. Induced calli were transferred to fresh N6D medium after two weeks for further proliferation and these secondary calli were used for transformation. Non-transformed calli turned brown and black while transformed calli further grew into globular white and yellowish calli in the medium supplemented with hygromycin B which then regenerated into plantlets (Figure 6.9). Seven independent transgenic lines (generated from seven independently infected calli) with the *AtAOX1a* transgene and 22 independent transgenic lines (generated from 22 independently infected calli) with the *AtNDB2* transgene, were generated with 11.7% and 36.7% transformation efficiency (calculated as the number of regenerated T_0 plants recovered on hygromycin (later confirmed with PCR) and grown to maturity divided by the original number of callus infected by respective AGL1 x 100), respectively. A total of 32 putative transgenic plants carrying *AtAOX1a* and 107 putative transgenic plants carrying *AtANDB*2 were produced during the study.

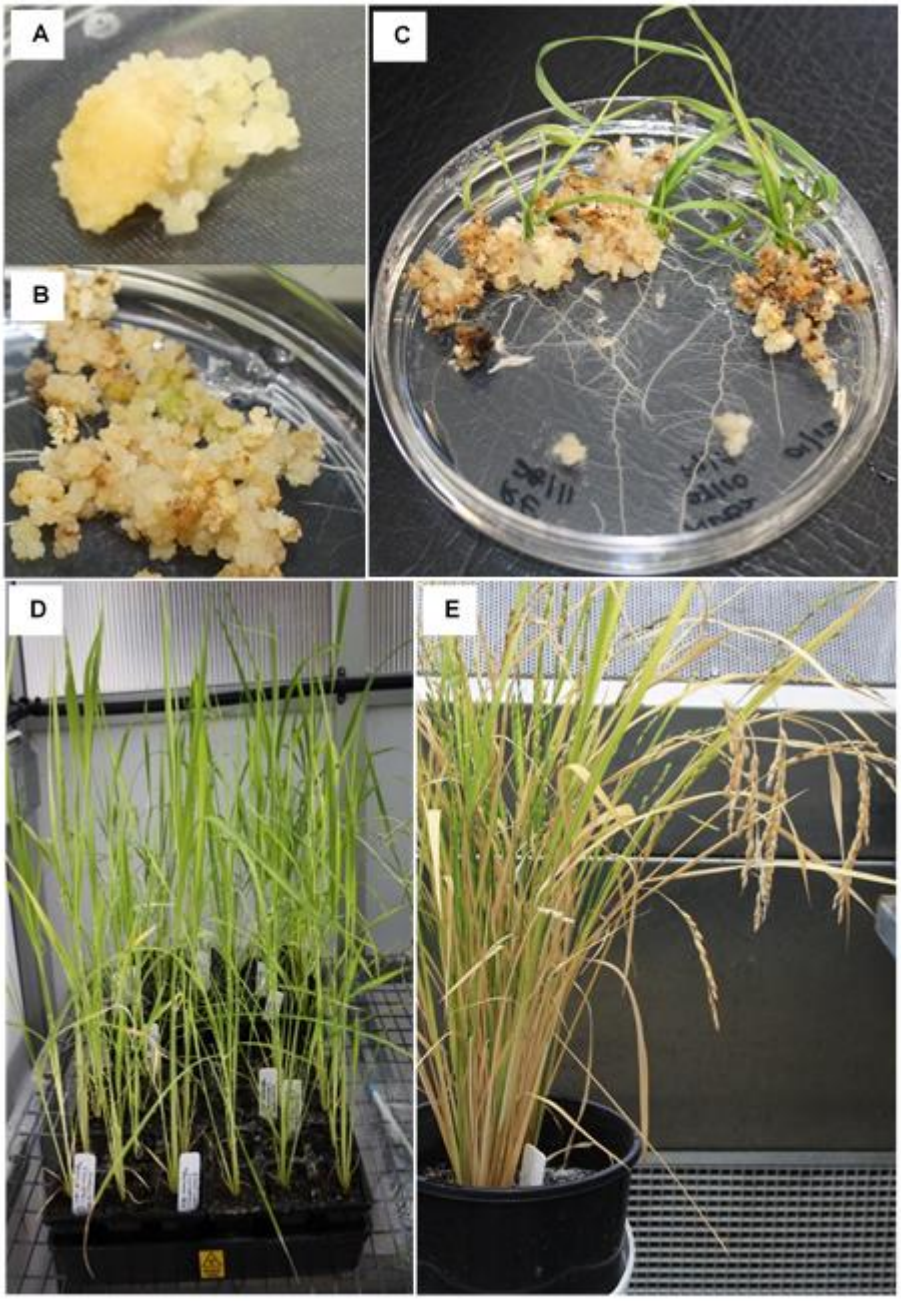


Figure 6.9 Steps in generation of transgenic rice through tissue culture

A: Callus induction from rice scutellum on N6D medium **B:** Proliferating calli growing on N6D selection medium containing 50 µg/ml of hygromycin after infection with *Agrobacterium* strain AGL1 habouring the *AtNDB2* genetic construct. **C:** Successfully transformed calli were further proliferated and formed globular white/yellowish calli and regenerated transgenic rice seedlings. Non-transformed calli were turned brown/black and failed to generate seedlings. **D:** Transgenic rice at seedling stage **E:** Transgenic rice bearing mature panicles.

6.3.3.1 Integration and expression of transgenes in T_0 plants were confirmed by PCR and RT-PCR

The integration of *AtNDB2* and *AtAOX1a* into the rice genome was confirmed by performing PCR with respective gene-specific primers (Figures 6.10A and B). The expression of *AtAOX1a* or *AtNDB2* genes in T_0 plants were examined by RT-PCR and results showed that 100% of PCR positive transgenic lines have the gene of interest expressed in both *AtAOX1a* and *AtNDB2* transgenic lines (Figures 6.11A and B). Only PCR and RT-PCR positive plants were used for further seed production

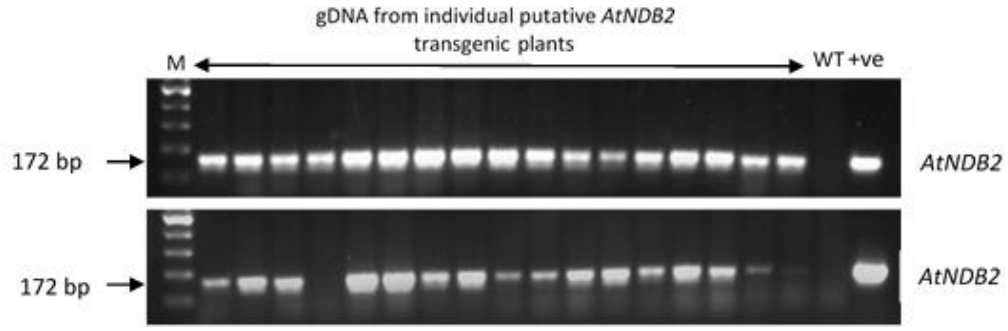


Figure 6.10A Representative gel images showing PCR analyses of T_0 plants carrying *AtNDB2* to confirm the presence of transgene

PCR was performed with gDNA isolated from leaves of T_0 plants and with *AtNDB2* gene-specific primers at 62°C annealing temperature for 37 cycles. M: 100 bp molecular weight marker; WT: gDNA from wild type Nipponbare; +ve: positive control (plasmid DNA from *AtNDB2* destination clone).

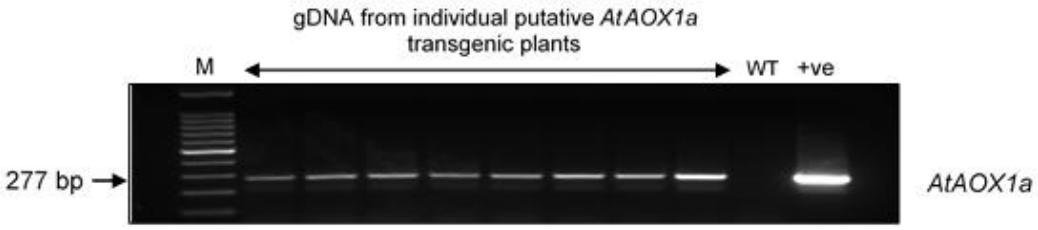


Figure 6.10B Representative gel image showing PCR analyses of T_0 plants carrying *AtAOX1a* to confirm the presence of transgene

PCR was performed with gDNA isolated from leaves of T_0 plants and with *AtAOX1a* gene-specific primers at 62°C annealing temperature for 39 cycles. M: 100 bp molecular weight marker; WT: gDNA from wild type Nipponbare; +ve: positive control (plasmid DNA from *AtAOX1a* destination clone).

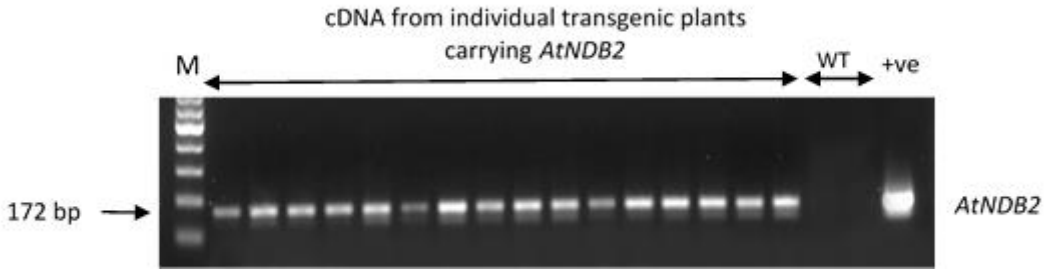


Figure 6.11A Representative gel image showing expression analyses of T_0 plants carrying *AtNDB2* to confirm the expression of transgene

The cDNAs were synthesized from total RNA extracted from leaf tissues of T_0 plants and used as templates for RT-PCR analyses. RT-PCR was performed with *AtNDB2* gene-specific primers at 62°C annealing temperature for 37 cycles. M: 100 bp molecular weight marker; WT: cDNA from wild type Nipponbare; +ve: positive control (plasmid DNA from *AtNDB2* destination clone).

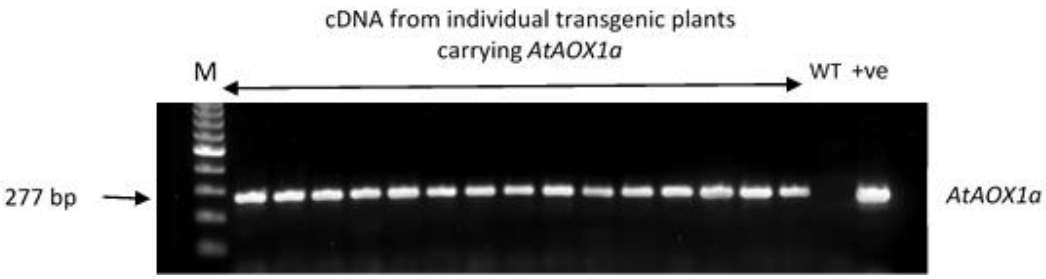


Figure 6.11B Representative gel image showing expression analyses of T_0 plants carrying *AtAOX1a* to confirm the expression of transgene

The cDNAs were synthesized from total RNA extracted from leaf tissues of T_0 plants and used as templates for RT-PCR analyses. RT-PCR was performed with *AtAOX1a* gene-specific primers at 62°C annealing temperature for 39 cycles. M: 100 bp molecular weight marker; WT: cDNA from wild type Nipponbare; +ve: positive control (plasmid DNA from *AtAOX1a* destination clone).

6.3.3.2 The majority of transgenic rice lines showed normal phenotype similar to wild type under normal growth conditions

The transgenic plants expressing Arabidopsis salt-responsive *AtAOX1a* or *AtNDB2* genes were grown under normal growth conditions in a PC2 greenhouse to reveal any differences in morphology between the plants at the seedling and the reproductive stages. Out of 22 independent lines of *AtDNB2* transgenic rice, 4 lines (~18%) were completely sterile. Almost all fertile transgenic lines showed a normal phenotype very similar to their wild type (WT) under normal growth conditions at both developmental stages, vegetative and reproductive. Seedlings from some lines showed undesirable panicle characteristics, such as less number of filled seeds per a panicle and bearing panicles with seeds at different developmental stages compared to the WT (Figure 6.12A). Out of 7 lines of *AtAOX1a* transgenic rice, 2 lines (~28%) were complete sterile and produced only empty seeds. Fertile lines of *AtAOX1a* transgenic rice didn't show any significant growth difference when compared to their WT during seedling stage but during reproductive stage, production of albino seeds in a panicle was observed in one plant from *AtAOX1a* transgenic group (Figure 6.12B). But with the maturity panicle became normal (Figure 6.12C). As observed in *AtNDB2* lines, panicles with less number of seeds and the presence of seeds with mature and immature stage in panicles were common to *AtAOX1a* transgenic lines also. Other than the above-mentioned abnormalities, the majority of plants grew without apparent phenotypic variations and produced viable seeds for further studies (Figure 6.12D).

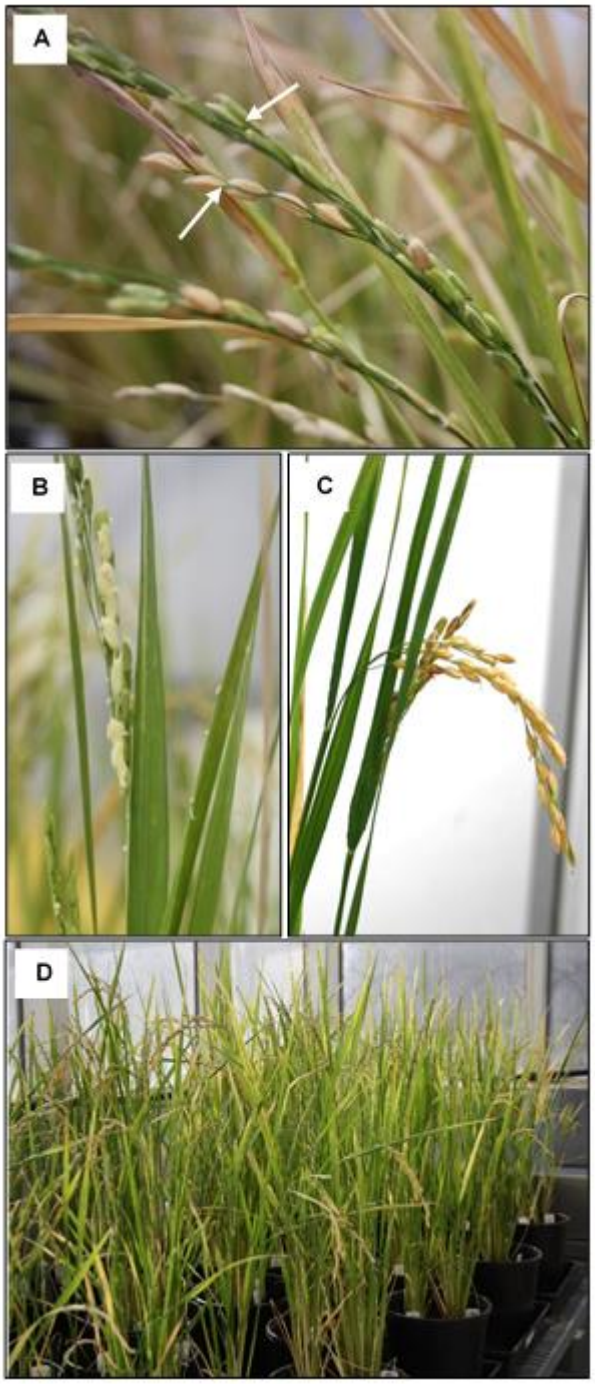


Figure 6.12 Phenotypes of transgenic rice plants transformed with Arabidopsis alternative pathway genes, *AOX1a* and *NDB2*

A: Panicles with seeds at different developmental stages, matured, browned and immature, green – observed in both types of transgenic plants **B** and **C:** Albino seeds were observed in an immature panicle from an *AtAOX1a* inserted plant and then turned into normal with maturity **D:** Majority of plants grew without showing any apparent phenotypic variations from the wild type Nipponbare and produced seeds for further studies.

6.3.4 Inheritance of transgenes into T_1 generation was confirmed by PCR analysis

The three best performed (those that produced the highest number of healthy seeds) independent lines from each transgenic group (*AtAOX1a* or *AtNDB2* inserted) were selected for further analyses. During seed germination, one *AtAOX1a* inserted line (NB_ATAOX1a_2_4 – not used for further analysis) showed 26.8% of albino plant production indicating potential somaclonal changes during tissue culture or destruction of genes related to chlorophyll biosynthesis during the insertion of the transgene into the rice genome. Other than that the growth and development of plants didn't show any distinguishable change in their phenotype under normal growth conditions compared to WT.

PCR was performed with respective gene-specific primers and gDNA from leaves from T_1 plants as template to confirm the inheritance of transgenes into T_1 plants (Figures 6.13 and 6.14) and for segregation analysis.

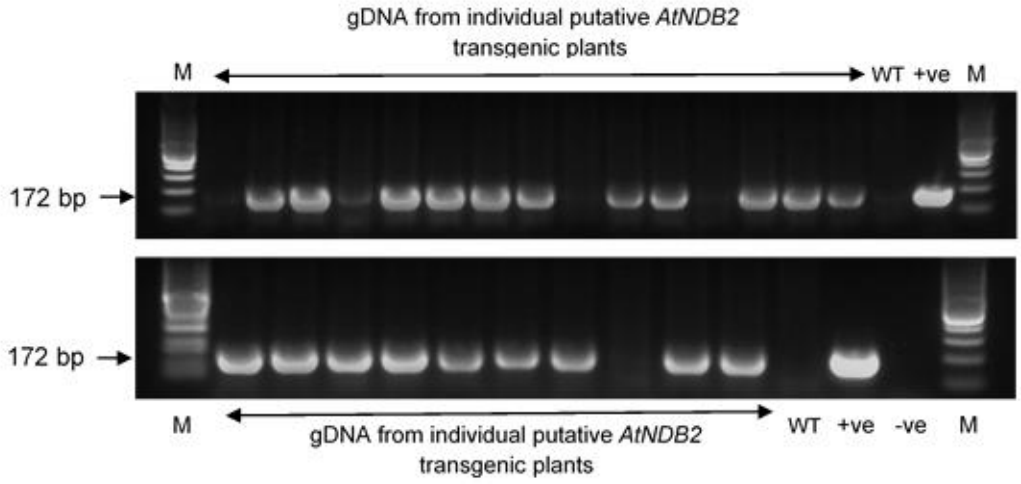


Figure 6.13 Representative gel images showing PCR analyses of T_1 plants carrying *AtNDB2* to confirm the presence of transgene

PCR was performed with gDNA isolated from leaves of T_1 plants and with *AtNDB2* gene-specific primers at 62^{o}C annealing temperature for 37 cycles. M: 100 bp molecular weight marker; WT: gDNA from wild type Nipponbare; +ve: positive control (plasmid DNA from *AtNDB2* destination clone); -ve: negative control (water); empty lanes with gDNA represent null segregants.

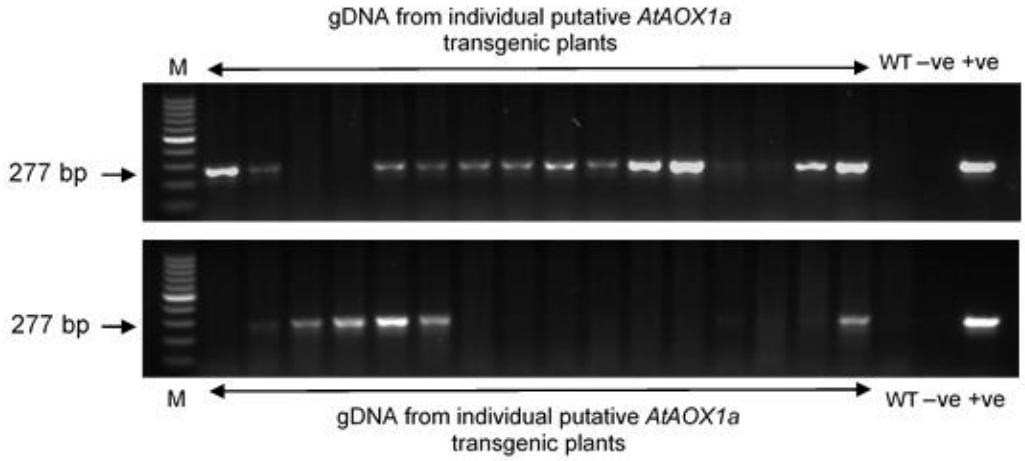


Figure 6.14 Representative gel images showing PCR analyses of T_1 plants carrying *AtAOX1a* to confirm the presence of transgene

PCR was performed with gDNA isolated from leaves of T_1 plants and with *AtAOX1a* gene-specific primers at 62°C annealing temperature for 39 cycles. M: 100 bp molecular weight marker; WT: gDNA from wild type Nipponbare; -ve: negative control (water); +ve: positive control (plasmid DNA from *AtAOX1a* destination clone); empty lanes with gDNA represent null segregants.

Table 6.1 Segregation analysis of T_1 generation of *AtNDB2* and *AtAOX1a* transgenic lines based on PCR

Transgenic line	Total no. of plants tested	No. of plants PCR +ve	No. of null-segregants	X^2 value
NB_AtNDB2_1_3	24	22	2	3.56*
NB_AtNDB2_10_2	22	19	3	1.52*
NB_AtNDB2_13_2	24	18	6	0*
NB_AtAOX1a_1_6	22	13	9	2.97*
NB_AtAOX1a_5_1	24	8	16	22.22
NB_AtAOX1a_6_1	19	5	14	24.02

* accept the Null hypothesis, data fit 3:1 segregation ratio df=1, $p<0.05$

Segregation analyses based on PCR results showed a range of segregation ratios (Table 6.1). However, the results showed that all three lines from *AtNDB2* insertions showed Mendelian segregation of 3:1 suggesting single gene insertion. Out of three lines with *AtAOX1a* insertion, only one line showed 3:1 Mendelian segregation fashion while the other two lines had segregation in T_1 generation suggesting more complex integration (Table 6.1).

6.3.5 Transgenic rice showed a tendency towards the improvement of grain yield under salinity stress

Segregating T_1 seeds produced by T_0 parent lines were used as the starting material for the evaluation studies on transgenic rice carrying *AtNDB2* or *AtAOX1a*. T_1 seeds were germinated on wet filter papers and healthy plants were transferred to soil and allowed to grow until panicle initiation stage. In the meantime, the presence of the transgene was determined by performing PCR with gDNA isolated from leaves of growing seedlings. The effect of 30 mM NaCl on further growth of transgenic plants was evaluated in terms of seed production compared to the wild type Nipponbare and the relevant null-segregants grown at the same time under the same growth conditions as the transgenic lines. Three selected lines from each transgenic rice group (transgenes carrying *AtNDB2* or *AtAOX1a*) were evaluated.

Growth and yield analysis of transgenic plants in the T_1 generation demonstrated that there was a significant reduction in the number of empty seeds produced per plant in one of transgenic lines carrying *AtAOX1a* (NB_ATAOX1a_1_6) compared to the salt-stressed WT Nipponbare. But, it was non-significant reduction compared to their null-segregants (Table 6.2). Though the transgenic line carrying *AtAOX1a* also showed an increase in number of filled seeds produced per plant compared to both controls (WT and null-segregants), it was not significant. Likewise, there was a non-significant increase of seed weight per plants compared to both controls, WT and null-segregants (Table 6.2). Though it is hard to come to a firm conclusion with the results obtained from a still segregating population, there appears to be a trend of improved seed yield

in transgenic rice lines carrying *AtAOX1a* genes under the tested salt stress compared to their controls.

There were two lines from the transgenic rice group carrying *AtNDB2* (NB_*AtNDB2*_1_3 and NB_*AtNDB2*_10_2) which recorded a significant reduction of empty seeds produced per plant compared to WT. Interestingly, one of these transgenic lines (NB_*AtNDB2*_1_3) showed this significant reduction compared to both controls, WT and null-segregants suggesting that the change is not merely because of a change which occurred during the tissue culture process but due to the inserted gene (Table 6.2). Two transgenic lines carrying the *AtNDB2* (NB_AtNDB2_1_3 and NB_AtNDB2_13_2) recorded a higher seed weight as well as higher number of filled seeds per plant compared to those of WT, while the transgenic line NB_AtNDB2_10_2 showed a lower seed weight per plant which resulted from the lower number of filled grains produced per plant compared to that of WT (Table 6.2).

Table 6.2 Growth and yield analysis of transgenic lines carrying Arabidopsis salt-responsive genes *AtAOX1a* or *AtNDB2* under 30 mM NaCl stress applied with the start of panicle initiation

Seedlings were grown under stress for one month and corresponding wild type (WT) and null-segregants (Nulls) were used as controls. Data represent means of 5-9 biological replicates ± SEM. * and ** indicate significant difference from the WT/Nulls (salt-treated) or from both salt-treated WT and Nulls respectively at p<0.05 by Student's *t*-test.

	No. of tillers/plant		No. of panicles/plant		No. of filled grains/plant		No. of empty grains/plant		grain weight/plant (g)	
	non-salt	salt	non-salt	salt	non-salt	salt	non-salt	salt	non-salt	salt
NB_AtAOX1a_1_6	12.40 ± 2.93	9.33 ± 2.09	10.60 ± 2.84	7.00 ± 1.57	66.40 ± 16.10	22.50 ± 4.26	166.20 ± 36.18	99.17 ± 15.53*	1.25 ± 0.31	0.41 ± 0.07
NB_AtAOX1a_5_1	5.50 ± 2.50	13.00 ± 1.22	5.00 ± 2.00	9.75 ± 1.25	34.00 ± 7.00	54.50 ± 13.23	74.50 ± 23.50	154.50 ± 34.65	0.87 ± 0.14	1.05 ± 0.26
NB_AtAOX1a_6_1	11.67 ± 2.33	6.50 ± 1.50	8.00 ± 1.15	6.00 ± 2.00	59.00 ± 18.72	26.50 ± 9.50	197.33 ± 47.42	120.00 ± 34.00	1.18 ± 0.40	0.47 ± 0.15
Nulls	12.67 ± 1.57	9.33 ± 1.26	9.22 ± 1.39	6.67 ± 0.87	45.67 ± 2.81	19.44 ± 5.74	178.00 ± 37.90	131.56 ± 23.90	0.79 ± 0.04	0.37 ± 0.11
WT	6.50 ± 1.32	7.80 ± 0.97	6.00 ± 1.22	7.20 ± 0.97	88.25 ± 14.94	16.60 ± 8.12	95.00 ± 19.85	150.80 ± 23.29	1.92 ± 0.23	0.28 ± 0.12
NB_AtNDB2_1_3	8.43 ± 1.34	7.71 ± 0.97	7.00 ± 0.69	6.29 ± 0.78	78.14 ± 13.96	30.43 ± 6.78	101.14 ± 14.89	106.67 ± 14.22**	1.59 ± 0.30	0.62 ± 0.14
NB_AtNDB2_10_2	12.83 ± 2.39	11.43 ± 1.59	9.50 ± 1.98	6.86 ± 1.26	32.17 ± 6.01	13.57 ± 4.77	137.20 ± 26.66	127.00 ± 17.18*	0.63 ± 0.12	0.25 ± 0.09
NB_AtNDB2_13_2	14.13 ± 1.39	24.00 ± 4.93	12.00 ± 1.25	11.25 ± 2.17	58.75 ± 12.14	48.00 ± 15.43	232.88 ± 20.81	202.25 ± 41.98	1.17 ± 0.26	0.98 ± 0.34
Nulls	17.75 ± 5.15	12.00 ± 2.97	15.50± 4.94	9.50 ±2.50	83.25 ± 28.76	46.00 ± 16.67	230.33 ± 94.12	211.33 ± 36.27	1.68 ± 0.55	0.95± 0.37
WT	6.50 ± 1.32	7.80 ± 0.97	6.00 ± 1.22	7.20 ± 0.97	88.25 ± 14.94	16.60 ± 8.12	95.00 ± 19.85	150.80 ± 23.29	1.92 ± 0.23	0.28 ± 0.12

Comparison of Na^+ and K^+ accumulation in leaves didn't show any significant differences among transgenic lines and their controls (Table 6.3). Almost similar K^+ concentrations were recorded from all transgenic lines except NB_AtNDB2_13_2 line which recorded lower concentration of 0.476 mmol/g DW. But, there was a tendency of decreased accumulation of Na^+ in transgenic lines from both transgenic groups compared to their controls.

Table 6.3 Leaf Na^+ and K^+ accumulation in seedlings grown under 30 mM NaCl stress for one month

Salt stress was applied when seedlings were at panicle initiation stage. Data represent mean ± SEM of 5-9 biological replicates.

Transgenic line	Na^+ (mmol/g DW)	K^+ (mmol/g DW)
NB_AtAOX1a_1_6	0.025 ± 0.014	0.781 ± 0.070
NB_AtAOX1a_5_1	0.029 ± 0.014	0.785 ± 0.049
NB_AtAOX1a_6_1	0.005 ± 0.001	0.702 ± 1.00
Nulls	0.042 ± 0.003	0.731 ± 0.048
WT	0.097 ± 0.048	0.613 ± 0.143
NB_AtNDB2_1_3	0.010 ± 0.001	0.813 ± 0.036
NB_AtNDB2_10_2	0.025 ± 0.016	0.818 ± 0.032
NB_AtNDB2_13_2	0.013 ± 0.005	0.476 ± 0.037
Nulls	0.143 ± 0.031	0.791 ± 0.234
WT	0.097 ± 0.048	0.613 ± 0.143

6.4 Discussion

The current study succeeded in obtaining regenerated transformed plants of rice cultivar Nipponbare carrying salt-responsive *AtAOX1a* or *AtNDB2* via *Agrobacterium*-mediated transformation using secondary calli derived from the scutellum of mature seeds. Numerous reports are available on the use of *Agrobacterium*-mediated transformation to generate transgenic rice for functional analysis of genome or crop improvement Hiei and Komari (2008) reported that the *Agrobacterium*-mediated transformation technique was used in more than 80% of studies out of 300 publications which were focused on transformation of rice. A*grobacterium*-mediated transformation has become more popular than other techniques because of the high efficiency of transformation and integration of small numbers of copies of transfer DNA (T-DNA) into the chromosome (Hiei and Komari, 2008). In the present study, transgenic lines carrying *AtAOX1a* showed lower transformation frequency of 11.7% compared to that for transgenic lines carrying *AtNDB2* (36.7%). However, both transgenic groups produced a number of independent transgenic lines (7 carrying *AtAOX1a* and 22 carrying *AtNDB2*), which ultimately produced sufficient seedlings and then seeds for subsequent studies. A range of factors contribute to the transformation efficiency of plants, including genotype, composition of the medium, gelling agents, strength of antibiotic used and exposure time of calli to the antibiotic, conditions for co-cultivation, starting material etc.(Hiei et al., 1994; Hiei et al., 1997; Opabode, 2006). However, *Agrobacterium*-mediated transformation of three different japonica varieties, Tsukinohikari, Asanohikari and Koshihikari resulted in 10-30% of transformation efficiency when scutellum derived secondary calli were used as the starting material (Hiei et al., 1994) which is comparable with my results. In contrast, Toki et al. (2006) has established an efficient *Agrobacterium*-mediated transformation system for rice cultivar Nipponbare with 95-98% transformation efficiency using scutellum derived primary calli.

The occurrence of somaclonal variation is one of the drawbacks of tissue culture techniques due to *in-vitro* culture of dedifferentiated cells in artificial culture media for prolonged periods (Hirochika et al., 1996; Toki et al., 2006). Toki et al. (2006) suggested that these genetic changes could be minimized by shortening the period of tissue culture. They established an efficient rice transformation system by early infection of rice scutellum tissue with *Agrobacterium* harbouring genes of interest, which induced rapid proliferation of calli and thereby minimized the length of tissue culture phase. During the current study, the same methodology was followed to see if transformed plants could be obtained using primary calli derived from mature seeds of Nipponbare and 5-day-old calli were infected with *Agrobacterium* harbouring genes of interest (*AtAOX1a* or *AtANDB2*). However, calli didn't grow further when transferred on to N6D selection medium supplemented with 50 µg/ml hygromycin and 150 µg/ml timentin. Therefore, the early infection of rice scutellum suggested by Toki et al. (2006) was not successful during the current study.

Detailed analysis of the cloned gene sequences by PCR, analytical restriction digests and sequencing, confirmed the amplification and recombination of the transgenes (*AtAOX1a* or *AtNDB2*) into the destination vector plasmids was complete and error free. Transgenic plant lines were successfully generated from the molecular cloning and transformation of rice callus tissue with these constructs containing *AtAOX1a* or *AtNDB2* driven by the rice actin-1 constitutive promoter. The presence and expression of transgenes in the T_0 plants was confirmed by PCR and RT-PCR with the relevant gene-specific primers. Although the regenerated T_0 plants didn't show any other apparent phenotypic abnormalities, completely sterile lines were observed in both transgenic groups. The transgenic lines carrying *AtAOX1a* produced ~28% sterile lines while the transgenic lines carrying *AtNDB2* produced ~18% sterile lines. Similarly, a variation in fertility from completely sterile to fully fertile was observed in regenerated rice plants from cultivars, Tsukinohikari, Asanohikari and Koshihikari

(Hiei et al., 1994). In contrast, transgenic rice regenerated from indica rice showed similar fertility as non-transformed plants (Rashid et al., 1996).

Although, the selection on the antibiotic containing media ensured the presence of at least one copy of the construct harbouring the transgene, molecular and segregation analysis could give the copy number of each line. The inheritance and the presence of transgenes in the T_1 generation was confirmed by PCR with gene-specific primers. The majority of transgenic lines showed a clear segregation of PCR-positive and PCR-negative plants following Mendelian segregation fashion of 3:1 suggesting single copy insertion. However, two lines carrying the *AtAOX1a* transgene didn't show simple transmission of DNA to the T_1 progeny. This pattern of segregation was also reported by Hiei et al. (1994) in their rice transformants and they suggested that chimerism in the T_0 progeny could affect the segregation ratios. Chimerism in transgenic tobacco plants generated via *Agrobacterium*-mediated transformation has also been observed (Schmülling and Schell, 1993). Therefore, the copy number estimates through segregation analysis can give unreliable estimates in transgenic lines. It is worthwhile to estimate the copy number of transgene in regenerated lines using other methods such as Southern blot or quantitative PCR on genomic DNA. This task couldn't be fulfilled during the current study due to time limitations.

Application of knowledge gained through model systems to improve crop productivity using molecular biological tools is not new to the agriculture industry. Transgenic Arabidopsis plants overexpressing *AVP1*, type 1 vacuolar H^+ pyrophosphatase (which is responsible for the activation of vacuolar Na^+/H^+ antiporters) has been shown to improve both salinity and drought tolerance compared to wild types grown under similar conditions (Gaxiola et al., 2001). Studies conducted to check whether the same strategy could apply to other plant species such as barley (Schilling et al., 2014), cotton (Pasapula et al., 2011), creeping bentgrass (*Agrostis stolonifera* L.) (Li et al.,

2010b) as well as rice (Zhao et al., 2006) have shown promising results. During the current study, an attempt was made to apply the same strategy to generate improved rice varieties with potential salt-tolerance by cloning Arabidopsis salt-responsive alternative respiratory genes. Transgenic Arabidopsis overexpressing *AtAOX1a*, which plays a regulatory role in controlling ROS production and thereby modulating the oxidative stress in cells, has been shown to reduce the accumulation of ROS in Arabidopsis plant cells and improve the biomass production compared to their controls (Smith et al., 2009). Further, altered expression of *AtNDB4* in Arabidopsis resulted in increased expression of *AtAOX1a* and *AtNDB2* when plants were exposed to salt stress and these plants showed lower ROS accumulation in cells compared to their wild types (Smith et al., 2011) confirming that *AtAOX1a* and *AtNDB2* were playing a role in controlling ROS production in salt-stressed plants.

To test whether the overexpression of these genes could improve the performances of rice under salt stress, transgenic rice lines expressing *AtAOX1a* or *AtNDB2* were generated. Preliminary performance evaluation of transgenic rice was carried out with a still-segregating T_1 population under greenhouse conditions by exposing plants to 30 mM NaCl at their panicle initiation stage. Interestingly, out of six lines tested, 3 lines (one line carrying *AtAOX1a* and 2 lines carrying *AtNDB2*) showed significant reduction of empty seed production per plant under salt stress compared to their controls. Also, there was a trend of increased production of filled seeds per plant, leading to an increase in grain yield in individual plants showing potential improvement in overall crop yield in transgenic lines compared to wild type under salt stress. However, the potential for these genes to improve the salinity tolerance in rice needs to be evaluated with a non-segregating population with the homozygote transgene.

It should be noted that so far, only transgenics overexpressing just a single gene have been generated; expression of more than one gene simultaneously would be

desirable (Gaxiola et al., 2001). A good example for this is the co-expression of *SsNHX1* (Na^+/H^+ antiporter) from *Suaeda salsa* and *AVP1* from Arabidopsis in salt-sensitive rice cultivar Zhonghua conferred higher salt-tolerance than in transgenic plants overexpressed only *SsNHX1* (Zhao et al., 2006). In Arabidopsis, when plants were exposed to salt stress, increased co-expression of *AtAOX1a* and *AtNDB2* was reported (Clifton et al., 2005; Smith et al., 2009) suggesting a co-regulation of them under abiotic stresses (Clifton et al., 2005; Elhafez et al., 2006). These findings suggest that *AtAOX1a* and *AtNDB2* could work together to create a favourable environment for cell functions under stressed conditions. Therefore, the co-expression of both *AtAOX1a* and *AtNDB2* in rice could confer higher salt-tolerance in transgenic plants than transgenic lines overexpressing *AtAOX1a* and *AtNDB2* individually. This can be achieved by crossing of the single transformants in a future study.

In summary, the current study succeeded in the development of two genetic constructs harbouring *AtAOX1a* or *AtNDB2* driven by the rice actin-1 promoter via Gateway cloning and regeneration of transgenic lines carrying *AtAOX1a* or *AtNDB2* constructs. Most of the transgenic rice lines demonstrated normal growth and development as for non-transgenic plants while a few lines showed some deviations. Preliminary studies on the performance evaluation under salt stress demonstrated that transgenic lines have improved grain yield per plant under salt stress and lower Na^+ levels in shoots compared to controls suggesting overexpression of salt-responsive *AtAOX1a* or *AtNDB2* in rice could provide a potential option for increasing grain production in cereals including rice under saline conditions.

7 General discussion

The overall aim of the study was to explore whether the modifications in the alternative pathway of plant respiration can confer tolerance to abiotic stresses in agriculturally important crops, using rice as a model cereal. A number of molecular and physiological approaches have been taken to achieve this target with significant outcomes. The main objectives of this project were: identification and characterization of alternative respiratory pathway (AP) genes in rice, screening for stress responsive genes, exploring their expression diversity in a range of rice genotypes to environmental stresses using high salinity as an example, and generation of transgenic rice overexpressing stress-responsive genes and assessing their stress tolerance and impact on seed yield.

7.1 Summary and major findings

7.1.1 Rice alternative pathway for respiration consists of AOXs and NDHs that are responsive to oxidative stress induced by the disruption of mETC

Database screening of the rice genome revealed the presence of four gene sequences homologues to Arabidopsis *AOX1*-type and six sequences homologous to NDHs. *AOX2*-type is absent in rice as reported in other monocots (Considine et al., 2002). Two rice sub-species, japonica and indica shared a very high sequence similarity as well as similar exon-intron organization, except for *NDB3*. Rice NDH homologs group into three distinct families, NDA (2 genes), NDB (3 genes) and NDC (1 gene). Out of six members, NDA2, NDB1, NDB2 are predicted to be dual targeted to mitochondria and peroxisomes while NDC1 is predicted be dual targeted to mitochondria and chloroplasts, which also has been experimentally shown by Xu et al. (2013). The unique EF-hand motif present in Arabidopsis NDB proteins is conserved in all three NDBs from indica rice group but is absent from japonica NDB3

as a result of truncation. The current study didn't investigate why/how this gene was truncated or the effect of truncation on the function of the gene. However, a detailed study of this matter could be beneficial to understand how the gene performs it proposed role without an EF-hand motif, an important part required for Ca^{2+} binding. Screening of rice databases showed that this truncated NDB3 protein is present only in japonica sub-species out of 11 rice species investigated. NDB3 from all other tested rice species shares a similar exon-intron structure to Arabidopsis, with 10 exons interrupted by 9 introns, and the resulting protein length is 580 aa. Although similar information was available on japonica NDB3 in three different databases (Gramene, Rice Genome Annotation Project, PLAZA 3), it is worthwhile to consider whether this is the real situation with japonica NDB3 or rather an error in sequence annotation.

Comparison of gene structures revealed that rice AOXs share similar exon-intron organization with Arabidopsis except *AOX1b*, which consists of three exons separated by two introns (Considine et al., 2002). It has been found that this is the predominant structure of *AOX1b* in a wide range of rice germplasm (8 species), including cultivated species as well as wild types (Figure 3.25). In contrast, *AOX1a* showed a high degree of polymorphism. Although the gene structural polymorphism in rice AOXs has been studied earlier (Considine et al., 2002; Pu et al., 2015), no work has been reported on rice NDHs until now. The current study on structural organization of rice *NDA1* revealed that three exons separated by two introns is conserved amongst rice cultivars tested (10 species), which is completely different from the Arabidopsis NDA group as well as from rice *NDA2* (Figure 3.26). An extended study could be carried out on *AOX1b* and *NDA1* from other monocots, especially on agriculturally important crop species and their relatives, to check whether they also share the same gene structure for *AOX1b* and *NDA1,* which could be important in evolutionary studies.

Although the responses of rice AOXs under different environmental stresses have been reported, less attention has been paid to NDHs. In the present study, expression patterns of AOXs as well as NDHs were investigated in response to oxidative stress created by disrupting mETC. In Arabidopsis, induction of *AOX1a* has been reported under a variety of stress treatments, and co-induction of *NDB2* was also observed in almost all cases of *AOX1a* induction (Clifton et al., 2005; Elhafez et al., 2006), suggesting that *AOX1a* and *NDB2* are likely to be co-regulated sharing common *cis*-acting regulatory elements (CAREs), which play a role during abiotic stress tolerance (Ho et al., 2008). The current results demonstrated that in rice, a number of NDHs (*NDA1*, *NDA2*, *NDB2*, *and NDB3*) are co-induced with AOXs (*AOX1a* and *AOX1b*) either in roots or roots. Analysis of promoter regions of these genes predicted that rice AOXs share a majority of the CAREs functional in Arabidopsis while NDHs share all six CAREs functional in *AtNDB2,* except *NDA1* which had only 5 of them. However, the current study didn't cover the functional analysis of these CAREs and it is certainly worthwhile to continue investigations to identify CAREs that are functioning in rice, which would be very important outcome for tracing regulatory mechanisms of rice AP genes under abiotic stress conditions.

7.1.2 Rice alternative respiratory genes are responsive to environmental stresses like high salinity and show tissue specific expression

The main objective of the experiments described in Chapter 4 was to screen for rice AP genes that are responsive to environmental stresses, taking high salinity as an example. Growth and gene expression responses were investigated in two contrasting rice cultivars exposed to salinity stress at their seedling stage. Variation in salt-tolerance responses was revealed, particularly in their ability to manage Na^+ in their tissues, which is important in understanding mechanisms of tolerance. For example, the concentration of shoot Na^+ was higher in cultivar Nipponbare compared

to Langi, but it was still capable of maintaining higher shoot growth, suggesting the restriction of Na^+ to the vacuole with minimum interruption to cellular functions or cytoplasmic osmotic adjustment.

There was an obvious tissue-specific and genotypic variation in gene expression, which paved the way to extend the research to compare gene expression in a range of rice cultivars together with variation in salt-tolerance (Chapter 5). However, no significant difference in gene expression was observed in roots or shoots of two cultivars differing in salt-tolerance. Gene expression could be studied at different developmental stages as well as at different salinity levels as the sensitivity of rice to salinity stress varies with the growth stage (Ferdose et al., 2015).

Rice AOXs also exists as homodimers as reported for other plant species (Umbach and Siedow, 1993). The reduced (active) form predominated in mitochondria from salt-stressed tissues while the oxidized (inactive) form was more abundant in non-stressed tissues, suggesting that post-translational regulation of AOX may be important in rice. There was a co-expression of rice NDHs and AOXs in response to salinity stress as observed under chemical stress (Chapter 3). The expression of these co-induced genes also showed tissue specificity and cultivar variation as seen for AOXs. Development of a polyclonal antibody to detect NDB2 is one of the important outcomes of this research. The results in current study revealed that the abundance of NDB2 protein was higher in salt-treated shoots than non-treated shoots suggesting that NDB2 might be important in salinity tolerance responses. However, in contrast to shoots, corresponding bands were not detected in salt-treated or non-treated roots, although transcriptional level responses were detected. Further investigation is needed to determine why the NDB2 protein is undetectable in roots. However, results suggest that post-translational regulation of NDB2 may be different between shoots and roots in rice. Therefore, the changes in NDB2 protein in response

to salinity stress could be studied at different time points and at different salinity levels, together with transcriptional responses, to get a clearer picture on gene responses at protein level, as gene activation may occur at earlier or later stages of the stress period and might be depend on the strength of the stress.

7.1.3 Contrasting genotypic and tissue-specific variation exists in physiological and molecular responses amongst rice cultivars in response to salinity stress

Unlike most other studies, this study evaluated the comparative salinity tolerance of 16 rice cultivars representing traditional and cultivated rice from both japonica and indica types. A significant tissue-wise and cultivar variation in terms of growth as well as tissue ion accumulation were revealed, suggesting that different mechanisms of salt-tolerance operate within the investigated germplasm groups. Although a number of tolerance mechanisms were proposed based on Na^+ and K^+ accumulation patterns in roots and shoots, further in-depth tissue analysis would be beneficial in confirming the current observations.

A group of cultivars from japonica rice showed similar shoot biomass production despite varying tissue Na^+ concentrations, showing that some cultivars can maintain shoot growth even with high tissue Na^+, suggesting the operation of tolerance mechanisms (vacuolar storage or osmotic adjustment of the cytoplasm) (Cotsaftis et al., 2011), while others maintain low Na^+ by exclusion (Figure 5.7). This suggests that tolerance mechanisms can vary even within cultivars from a particular sub-species, which strongly support the necessity of enhanced germplasm screening for tolerance mechanisms. At the same time, results clearly show that cultivars with similar genetic background generally share similar tolerance mechanisms, and biomass production also depends on the innate vigour of cultivars. It has been shown that the level of salt-tolerance of cultivar Nipponbare is greater than the well reputed traditional indica cultivar Pokkali, confirming the suitability of Nipponbare in studies related to salt stress

as well as for improving crops' tolerance to salt stress through genetic engineering.

Due to constraints in time and resources, only biomass production and Na^+ and K^+ accumulation in roots and shoots were considered in determining tolerance mechanisms. More physiological traits such as chlorophyll content, net photosynthetic rate, CO_2 assimilation rates, transpiration rate, stomatal conductance etc. could be measured along with plant survival rates to get a clearer picture of tolerance mechanisms. Also, it is recommended to assess plants at different salinity levels as well as at different growth stages, to check the consistency of results.

A strong positive correlation between the expression of *AOX1a* and *NDA2* and shoot Na^+ content and a strong negative correlation between the expression of *AOX1c* and *NDB2* and relative root growth were revealed. Apart from that, although, it is possible that some alternative pathway components play a certain role in salinity tolerance in some cultivars, the current results suggest that AP genes may not be good molecular markers at gene expression level for salt-tolerance in rice. However, transcriptome changes at different time points could be studied to capture more transcriptional level responses of AP genes as they could be active at different stages of the stress period. Further, gene expression could be assessed in a range of salinity levels and at different growth stages as gene responses might vary with the stress level as well as the developmental stage of plants, before confirming the current conclusion. In addition, the effect of other environmental stresses such as cold, drought, high temperature etc. and a combination of these stresses could also be studied, which might reveal the multi-stress responsive component of the alternative respiratory pathway. Another direction of future study is that expression of AP genes in response to salinity stress could be studied together with genes responsible for controlling Na^+ accumulation in rice, such as high affinity K^+ transporter (*HKT*) or plasma membrane Na^+/K^+ antiporter (*SOS1*).

7.1.4 Transgenic rice overexpressing *AtAOX1a* and *AtNDB2* were successfully generated

The main objective of this part of the study (Chapter 6) was to test whether the knowledge gained from the model system, Arabidopsis, could be applied to improve agriculturally important crop species. Overexpression of *AtAOX1a* in Arabidopsis improved the growth under salinity stress by reducing ROS production (Smith et al., 2009) while *AtNDB4* knock-down lines showed co-induction of *AtAOX1a* and *AtNDB2* when grown under salt stress, suggesting the two genes are playing a role in salinity tolerance in Arabidopsis (Smith et al., 2011). Transgenic rice plants overexpressing *AtAOX1a* or *AtNDB2,* driven by the rice actin-1 promoter, were successfully generated via *Agrobacterium*-mediated transformation (Chapter 6). Although, overexpression of wheat *AOX1a* in Arabidopsis has been reported for the functional analysis under low temperature (Sugie et al., 2006), cereal transformation with exogenous alternative respiratory genes has not been performed previously. Out of seven independent transgenic lines generated overexpressing *AtAOX1a*, two lines were found to be completely sterile while 4 lines out of 22 were completely sterile from the *AtNDB2* transformed lines. However, all fertile lines grew without apparent phenotypic differences to wild type Nipponbare under greenhouse conditions and produced enough seeds for subsequent analysis. Three transgenic lines from each group were evaluated for yield performances under environmentally realistic salt stress (30 mM) applied during the panicle initiation stage. Several transgenic lines performed better than wild type and segregated non-transgenic plants (null-segregants), showing a potential improvement of salinity tolerance. In particular, two lines carrying *AtNDB2* (NB_AtNDB2_1_3 and NB_AtNDB2_10_2) and one line carrying *AtAOX1a* (NB_AtAOX1a_1_6) significantly reduced the production of empty seeds compared to their controls (Chapter 6), suggesting overexpression of *AtAOX1a* and *AtNDB2* could be a potential avenue for improving rice for salinity tolerance.

Ideally, evaluation of more independently transformed lines is necessary to support this finding. Moreover, copy number estimation of these transgenic lines was merely based on Mendelian segregation and further confirmation is needed by Southern blot or qRT-PCR analysis. Further, due to time limitations, the screening was performed on a still segregating T_1 generation, which is not ideal for phenotyping as they lack transgene stability, and also the quality of seeds from T_1 plant plants was not as good as those produced from primary transgenic plants. Normally, in the literature, in most studies later generations of transgenic plants have been used for phenotyping (Zhao et al., 2006; Li et al., 2013b; Schilling et al., 2014). Therefore, it is imperative to evaluate the T_2 or T_3 generations of same transgenic rice lines generated in this project.

A number of independent transgenic lines developed in this study has yet to be characterized. Only three from each independent transgenic line overexpressing gene of interest were characterized due to time restrictions. To get reliable results as well as to identify lines with better characteristics, evaluation of more independent lines certainly would be beneficial. Although the *AtAOX1a* transformation resulted in only a few independent lines, *AtNDB2* transformation produced a number of independent transformation events, which could be supportive for a detailed study. As a ready to use overexpression vector carrying *AtAOX1a* is available, additional independent lines could be generated through repeated rounds of plant transformation.

Expression levels of exogenous and endogenous *AOX1a* and *NDB2* need to be evaluated under salt-stressed and control conditions in the transgenic plants. Changes in expression levels of both exogenous and endogenous genes could be investigated in transformed plants, under stressed and control conditions at different developmental stages as well as in a range of salinity levels, to better understand of the gene responses and their mechanisms in response to salinity stress. Further,

transgenic lines could be evaluated for other abiotic stresses such as drought, heat, nutrient deficiency and for combination of stresses, which normally plants experience in a natural environment.

7.2 Conclusions

This study has identified components of the alternative pathway of mETC in rice, explored the structural polymorphism of them, and examined their responses to chemical stress and salinity. It has also identified cultivars with varying responses to salinity and the tissue specificity and genotypic specificity in their responses. The study also demonstrated the genotypic- and tissue-specific variation in the expression of AP genes under salinity stress. Although the present study suggested that AP gene expression levels may not be good molecular markers for salt-tolerance in general in rice, as *AOX1a* showed strong positive correlation with leaf Na^+ content and ratio of Na^+/K^+, it is still possible that AP components such as AOX may play a role at protein or activity level in salt-tolerance in some cultivars. It is expected that information gathered by investigating a large range of germplasm will contribute to studies on identification of salt-tolerance mechanisms in rice. The study also has generated transgenic plants constitutively expressing Arabidopsis AP components. It is hoped that additional experiments with these plants will further elucidate the role of mitochondria in stress tolerance in this important crop plant.

Appendix

Appendix A: Pairwise alignment of AOX protein sequences from japonica and indica rice

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                    *         20          *         40          *         60
Japo_AOX1a : MSSRMAGSAILRHVGGVRLFTASATSPAAAAAAAARPFLAGGEAVPGVWGLRLMSTSSVA :  60
Indi_AOX1a : MSSRMAGSAILRHVGGVRLFTASATSPAAAAAAAARPFLAGGEAVPGVWGLRLMSTSSVA :  60
             MSSRMAGSAILRHVGGVRLFTASATSPAAAAAAAARPFLAGGEAVPGVWGLRLMSTSSVA

                    *         80          *        100          *        120
Japo_AOX1a : STEAAAKAEAKKADAEKEVVVNSYWGIEQSKKLVREDGTEWKWSCFRPWETYTADTSIDL : 120
Indi_AOX1a : STEAAAKAEAKKADAEKEVVVNSYWGIEQSKKLVREDGTEWKWSCFRPWETYTADTSIDL : 120
             STEAAAKAEAKKADAEKEVVVNSYWGIEQSKKLVREDGTEWKWSCFRPWETYTADTSIDL

                    *        140          *        160          *        180
Japo_AOX1a : TKHHVPKTLLDKIAYWTVKSLRFPTDIFFQRRYGCRAMMLETVAAVPGMVGGMLLHLRSL : 180
Indi_AOX1a : TKHHVPKTLLDKIAYWTVKSLRFPTDIFFQRRYGCRAMMLETVAAVPGMVGGMLLHLRSL : 180
             TKHHVPKTLLDKIAYWTVKSLRFPTDIFFQRRYGCRAMMLETVAAVPGMVGGMLLHLRSL

                    *        200          *        220          *        240
Japo_AOX1a : RRFEQSGGWIRTLLEEAENERMHLMTFMEVANPKWYERALVITVQGVFFNAYFLGYLLSP : 240
Indi_AOX1a : RRFEQSGGWIRTLLEEAENERMHLMTFMEVANPKWYERALVITVQGVFFNAYFLGYLLSP : 240
             RRFEQSGGWIRTLLEEAENERMHLMTFMEVANPKWYERALVITVQGVFFNAYFLGYLLSP

                    *        260          *        280          *        300
Japo_AOX1a : KFAHRVVGYLEEEAIHSYTEFLKDLEAGKIDNVPAPAIAIDYWRLPANATLKDVVTVVRA : 300
Indi_AOX1a : KFAHRVVGYLEEEAIHSYTEFLKDLEAGKIDNVPAPAIAIDYWRLPANATLKDVVTVVRA : 300
             KFAHRVVGYLEEEAIHSYTEFLKDLEAGKIDNVPAPAIAIDYWRLPANATLKDVVTVVRA

                    *        320          *
Japo_AOX1a : DEAHHRDVNHFASDIHYQGMELKQTPAPIGYH* : 332
Indi_AOX1a : DEAHHRDVNHFASDIHYQGMELKQTPAPIGYH- : 332
             DEAHHRDVNHFASDIHYQGMELKQTPAPIGYH
```

Supplementary Figure A1: Pairwise sequence alignment of full length AOX1a protein from japonica and indica rice

AOX1a from japonica rice shares 100% of amino acid residue identity with indica rice.

```
                    *         20          *         40          *         60
Japo_AOX1b : MSSRMAGATLLRHLGPRLFAAEPVYSGLAASARGVMPAAARIFPARMASTSSAGADVKEG :  60
Indi_AOX1b : MSSRMAGATLLRHLGPRLFAAEPVYSGLAASARGVMPAAARIFPARMASTSSAGADVKEG :  60
             MSSRMAGATLLRHLGPRLFAAEPVYSGLAASARGVMPAAARIFPARMASTSSAGADVKEG

                    *         80          *        100          *        120
Japo_AOX1b : AAEKLPEPAATAAAAATDPQNKKAVVSYWGIQPPKLVKEDGTEWKWLSFRPWDTYTSDTS : 120
Indi_AOX1b : AAEKLPEPAATAAAAATDPQNKKAVVSYWGIQPPKLVKEDGTEWKWLSFRPWDTYTSDTS : 120
             AAEKLPEPAATAAAAATDPQNKKAVVSYWGIQPPKLVKEDGTEWKWLSFRPWDTYTSDTS

                    *        140          *        160          *        180
Japo_AOX1b : IDVTKHHEPKGLPDKLAYWTVRSLAVPRDLFFQRRHASHALLLETVAGVPGMVGGMLLHL : 180
Indi_AOX1b : IDVTKHHEPKGLPDKLAYWTVRSLAVPRDLFFQRRHASHALLLETVAGVPGMVGGMLLHL : 180
             IDVTKHHEPKGLPDKLAYWTVRSLAVPRDLFFQRRHASHALLLETVAGVPGMVGGMLLHL

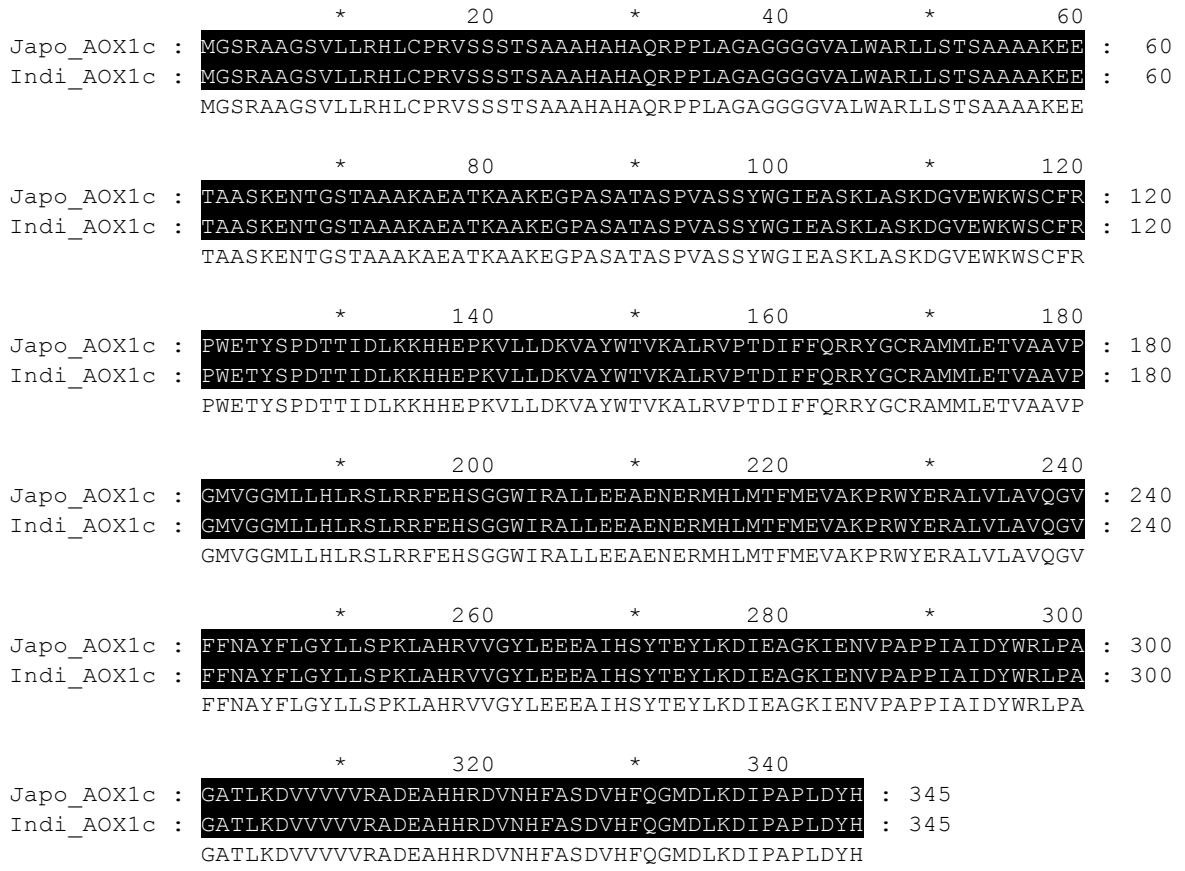
                    *        200          *        220          *        240
Japo_AOX1b : RSLRRFEQSGGWIRALLEEAENERMHLMTFLEVMQPRWWERALVLAAQGVFFNAYFVGYL : 240
Indi_AOX1b : RSLRRFEQSGGWIRALLEEAENERMHLMTFLEVMQPRWWERALVLAAQGVFFNAYFVGYL : 240
             RSLRRFEQSGGWIRALLEEAENERMHLMTFLEVMQPRWWERALVLAAQGVFFNAYFVGYL

                    *        260          *        280          *        300
Japo_AOX1b : VSPKFAHRFVGYLEEEAVSSYTEYLKDLEAGKIENTPAPAIAIDYWRLPADATLKDVVTV : 300
Indi_AOX1b : VSPKFAHRFVGYLEEEAVSSYTEYLKDLEAGKIENTPAPAIAIDYWRLPADATLKDVVTV : 300
             VSPKFAHRFVGYLEEEAVSSYTEYLKDLEAGKIENTPAPAIAIDYWRLPADATLKDVVTV

                    *        320          *
Japo_AOX1b : IRADEAHHRDLNHFASDIQQQGMKLKDTPAPIGYH : 335
Indi_AOX1b : IRADEAHHRDLNHFASDIQQQGMKLKDTPAPIGYH : 335
             IRADEAHHRDLNHFASDIQQQGMKLKDTPAPIGYH
```

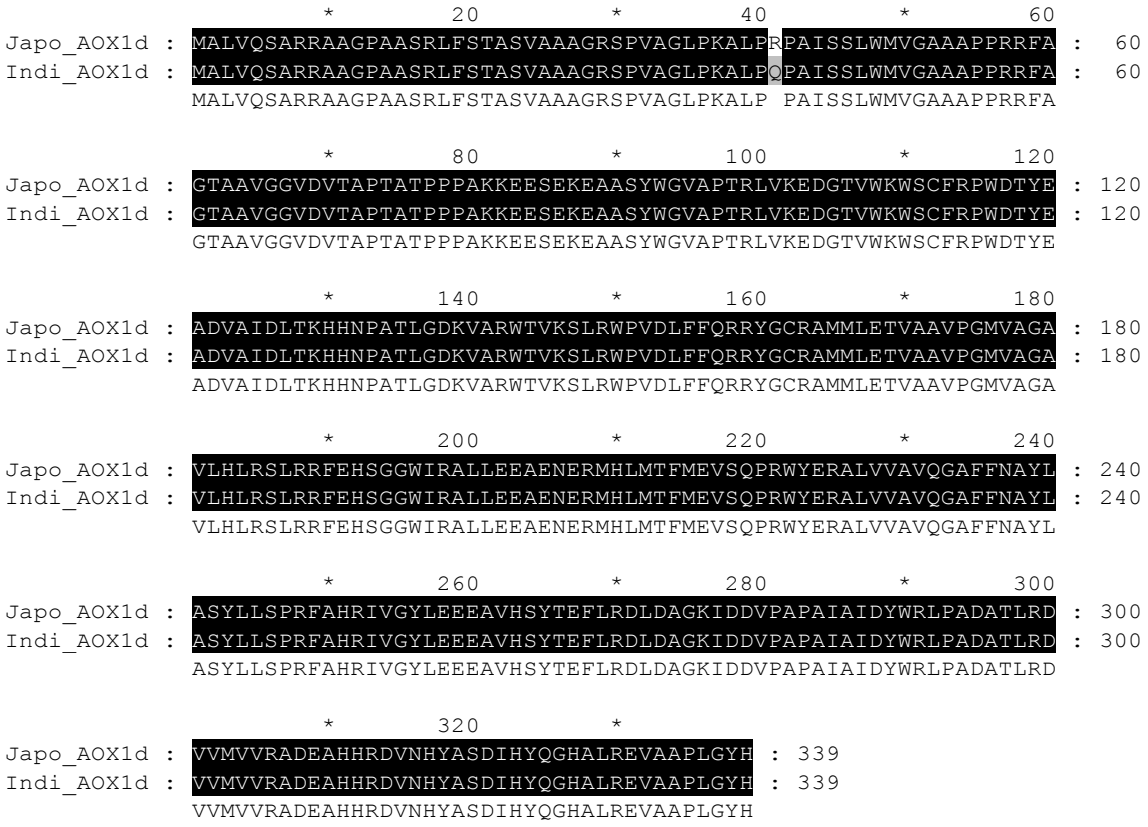
Supplementary Figure A2: Pairwise sequence alignment of full length AOX1b protein from japonica and indica rice

AOX1b from japonica rice shares 100% of amino acid residue identity with indica rice.



Supplementary Figure A3: Pairwise sequence alignment of full length AOX1c protein from japonica and indica rice

AOX1c from japonica rice shares 100% of amino acid residue identity with indica rice.



Supplementary Figure A4: Pairwise sequence alignment of full length AOX1d protein from japonica and indica rice

AOX1d from japonica rice shares 99.7% of amino acid residue identity with indica rice.

Appendix B: Pairwise alignment of NDH protein sequences from japonica and indica rice

```
                    *        20         *        40         *        60
Japon_NDA1 : MAWSRIARGSQLTQPLSRILAEGNAAATPAAYALRNAAALGQRASSASASSSFHSLALAG :  60
Indic_NDA1 : MAWSRIARGSQLTQPLSRILAEGNAAATPAAYALRNAAALGQRASSASASSSFHSLALAG :  60
             MAWSRIARGSQLTQPLSRILAEGNAAATPAAYALRNAAALGQRASSASASSSFHSLALAG

                    *        80         *       100         *       120
Japon_NDA1 : LADKYAAGAAGRLQPSRGISTTSPALRPAAEAAARVVECSDAADEAAAAAAVPDLGPTRP : 120
Indic_NDA1 : LADKYAAGAAGRLQPSRGISTTSPALRPAAEAAARVVECSDAADEAA-AAAVPDLGPTRP : 119
             LADKYAAGAAGRLQPSRGISTTSPALRPAAEAAARVVECSDAADEAA AAAVPDLGPTRP

                    *       140         *       160         *       180
Japon_NDA1 : GEKPRVVVLGTGWAACRFLKDVDTRAYDVVCISPRNHMVFTPLLASTCVGTLEFRSVVEP : 180
Indic_NDA1 : GEKPRVVVLGTGWAACRFLKDVDTRAYDVVCISPRNHMVFTPLLASTCVGTLEFRSVVEP : 179
             GEKPRVVVLGTGWAACRFLKDVDTRAYDVVCISPRNHMVFTPLLASTCVGTLEFRSVVEP

                    *       200         *       220         *       240
Japon_NDA1 : VSRIQSALATRPGSYFFLASCTGIDTGRHEVHCTAADGDGLPANPYNFKVSYDKLVIASG : 240
Indic_NDA1 : VSRIQSALATRPGSYFFLASCTGIDTGRHEVHCTAADGDGLPANPYNFKVSYDKLVIASG : 239
             VSRIQSALATRPGSYFFLASCTGIDTGRHEVHCTAADGDGLPANPYNFKVSYDKLVIASG

                    *       260         *       280         *       300
Japon_NDA1 : SEPLTFGIKGVAENAIFLREVSHAQEIRRKLLTNLMLSENPGLSEEEKKRLLHCVVVGGG : 300
Indic_NDA1 : SEPLTFGIKGVAENAIFLREVSHAQEIRRKLLTNLMLSENPGLSEEEKKRLLHCVVVGGG : 299
             SEPLTFGIKGVAENAIFLREVSHAQEIRRKLLTNLMLSENPGLSEEEKKRLLHCVVVGGG

                    *       320         *       340         *       360
Japon_NDA1 : PTGVEFSGELSDFITRDVRERYAHVKDYVKVTLIEANEILSSFDVGLRQYATDHLSKYGV : 360
Indic_NDA1 : PTGVEFSGELSDFITRDVRERYAHVKDYVKVTLIEANEILSSFDVGLRQYATDHLSKYGV : 359
             PTGVEFSGELSDFITRDVRERYAHVKDYVKVTLIEANEILSSFDVGLRQYATDHLSKYGV

                    *       380         *       400         *       420
Japon_NDA1 : NLVRGVVKEVKPREIELSDGSRVPYGVLVWSTGVGPSEFVRSLPLPKSPGGRIGVDEWLR : 420
Indic_NDA1 : NLVRGVVKEVKPREIELSDGSRVPYGVLVWSTGVGPSEFVRSLPLPKSPGGRIGVDEWLR : 419
             NLVRGVVKEVKPREIELSDGSRVPYGVLVWSTGVGPSEFVRSLPLPKSPGGRIGVDEWLR

                    *       440         *       460         *       480
Japon_NDA1 : VPSVEDVFALGDCAGFLEGTGRAVLPALAQVAEREGRYLARVMSRIAAQDGGRAGRAVGS : 480
Indic_NDA1 : VPSVEDVFALGDCAGFLEGTGRAVLPALAQVAEREGRYLARVMSRIAAQDGGRAGRAVGS : 479
             VPSVEDVFALGDCAGFLEGTGRAVLPALAQVAEREGRYLARVMSRIAAQDGGRAGRAVGS

                    *       500         *       520         *       540
Japon_NDA1 : AELGEPFVYKHIGSMASVGRYKALVDLRENKDARGVSMAGFVSWLMWRSAYLTRVVSWRN : 540
Indic_NDA1 : AELGEPFVYKHIGSMASVGRYKALVDLRENKDARGVSMAGFVSWLMWRSAYLTRVVSWRN : 539
             AELGEPFVYKHIGSMASVGRYKALVDLRENKDARGVSMAGFVSWLMWRSAYLTRVVSWRN

                    *       560
Japon_NDA1 : RFYVAVNWATTLVFGRDNTRIG : 562
Indic_NDA1 : RFYVAINWATTLVFGRDNTRIG : 561
             RFYVA6NWATTLVFGRDNTRIG
```

Supplementary Figure B1: Pairwise sequence alignment of full length NDA1 protein from japonica and indica rice

NDA1 from japonica rice shares 99.7% of amino acid residue identity with indica rice.

```
                    *         20         *         40         *         60
Japon NDA2 : MAASSLLRSLSRISRRGCVGGAGPSPFHHSRLPYSPFSTAAADAVERRGFAGLGPTAKGE :  60
Indic_NDA2 : MAASSLLRSLSRISRRGCVGGAGPSPFHHSRLPYSPFSTAAADAVERRGFAGLGPTAKGE :  60
             MAASSLLRSLSRISRRGCVGGAGPSPFHHSRLPYSPFSTAAADAVERRGFAGLGPTAKGE

                    *         80         *        100         *        120
Japon_NDA2 : KARVVVLGTGWAGSRLMKDIDTTGYEVVCVSPRNHMVFTPLLASTCVGTLEFRSVAEPLA : 120
Indic NDA2 : KARVVVLGTGWAGSRLMKDIDTTGYEGVCVSPRNHMVFTPLLASTCVGTLEFRSVAEPLA : 120
             KARVVVLGTGWAGSRLMKDIDTTGYE VCVSPRNHMVFTPLLASTCVGTLEFRSVAEPLA

                    *        140         *        160         *        180
Japon NDA2 : RIQPAVSKSPGSYFLLARCTAVDPDAHTIDCETVTEGEKDTLKPWKFKVAYDKLVFACGA : 180
Indic NDA2 : RIQPAVSKSPGSYFLLARCTAVDPDAHTIDCETVTEGEKDTLKPWKFKVAYDKLVFACGA : 180
             RIQPAVSKSPGSYFLLARCTAVDPDAHTIDCETVTEGEKDTLKPWKFKVAYDKLVFACGA

                    *        200         *        220         *        240
Japon NDA2 : EASTFGIRGVTDHAIFLREVHHAQEIRRKLLLNLMLSDVPGISEEEKRRLLHCVVVGGGP : 240
Indic NDA2 : EASTFGIRGVTDHAIFLREVHHAQEIRRKLLLNLMLSDVPGISEEEKRRLLHCVVVGGGP : 240
             EASTFGIRGVTDHAIFLREVHHAQEIRRKLLLNLMLSDVPGISEEEKRRLLHCVVVGGGP

                    *        260         *        280         *        300
Japon NDA2 : TGVEFSGELSDFIIRDVKQRYSHVKDYIHVTLIEANEILSSFDVRLRQYATNQLTKSGVR : 300
Indic NDA2 : TGVEFSGELSDFIIRDVKQRYSHVKDYIHVTLIEANEILSSFDVRLRQYATNQLTKSGVR : 300
             TGVEFSGELSDFIIRDVKQRYSHVKDYIHVTLIEANEILSSFDVRLRQYATNQLTKSGVR

                    *        320         *        340         *        360
Japon NDA2 : LVRGIVKDVQPNKLILDNGEEVPYGLLVWSTGVGPSSFVKSLPFPKSPGGRIGVDEWLRV : 360
Indic NDA2 : LVRGIVKDVQPNKLILDNGEEVPYGLLVWSTGVGPSSFVKSLPFPKSPGGRIGVDEWLRV : 360
             LVRGIVKDVQPNKLILDNGEEVPYGLLVWSTGVGPSSFVKSLPFPKSPGGRIGVDEWLRV

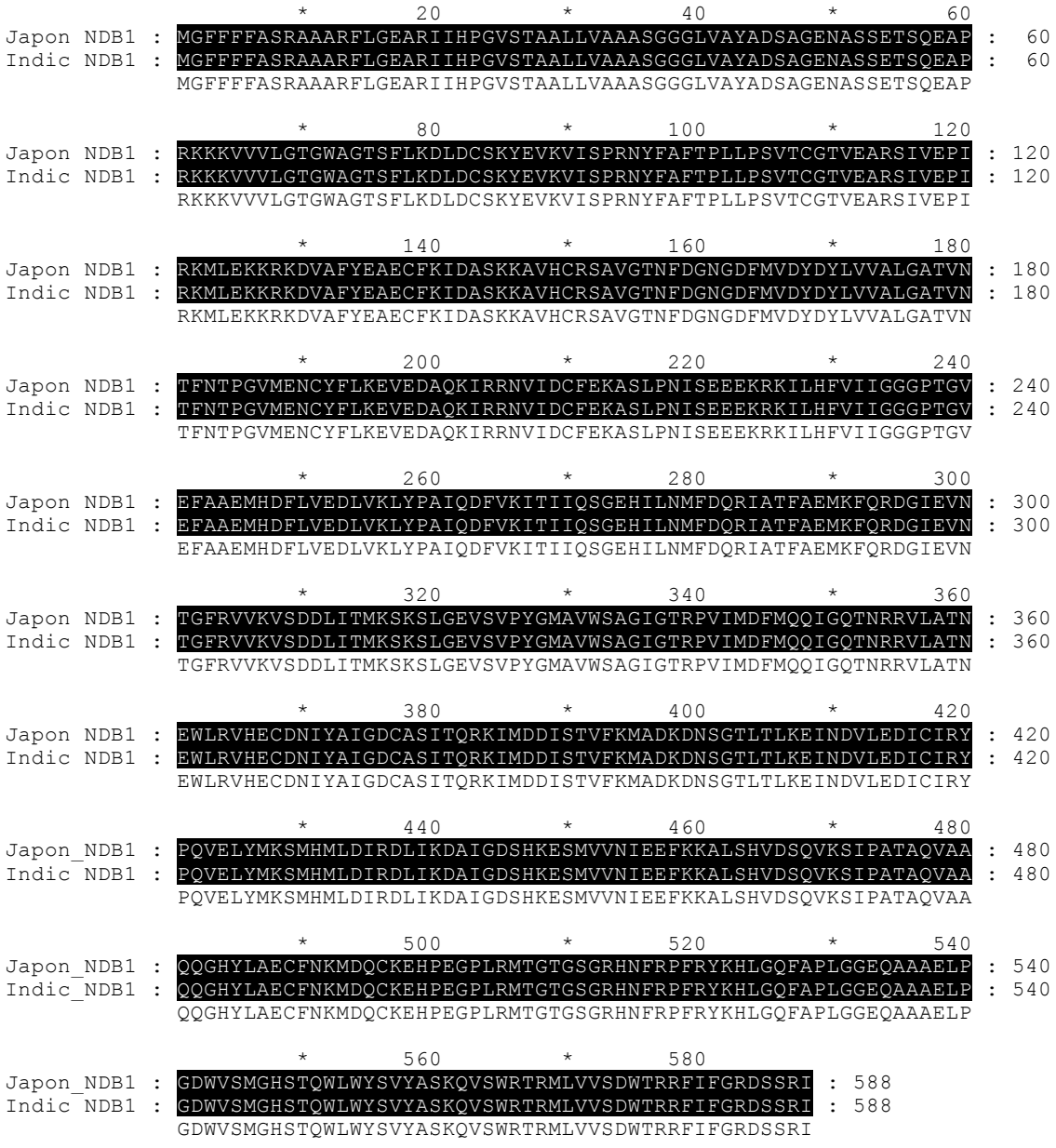
                    *        380         *        400         *        420
Japon NDA2 : PSARDVFAIGDCSGFLESTGKDVLPALAQVAERQGKYLAHLLNHVMKAGGGHANCEIDVD : 420
Indic_NDA2 : PSARDVFAIGDCSGFLESTGKDVLPALAQVAERQGKYLAHLLNHVMKAGGGHANCEIDVD : 420
             PSARDVFAIGDCSGFLESTGKDVLPALAQVAERQGKYLAHLLNHVMKAGGGHANCEIDVD

                    *        440         *        460         *        480
Japon NDA2 : LGPAFVYKHLGSMATVGRYKALVDLRQSKESKGISLAGFVSWFIWRSAYLTRVVSWRNRF : 480
Indic_NDA2 : LGPAFVYKHLGSMATVGRYKALVDLRQSK--AGISLAGFVSWFIWRSAYLTRVVSWRNRF : 478
             LGPAFVYKHLGSMATVGRYKALVDLRQSK   GISLAGFVSWFIWRSAYLTRVVSWRNRF

                    *
Japon_NDA2 : YVAINWLTTLLFGRDISRI : 499
Indic NDA2 : YVAINWLTTLLFGRDISRI : 497
             YVAINWLTTLLFGRDISRI
```

Supplementary Figure B2: Pairwise sequence alignment of full length NDA2 protein from japonica and indica rice

NDA2 from japonica rice shares 99.8% of amino acid residue identity with indica rice.



Supplementary Figure B3: Pairwise sequence alignment of full length NDB1 protein from japonica and indica rice

NDB1 from japonica rice shares 100% of amino acid residue identity with indica rice.

```
                    *         20          *         40          *         60
Japon NDB2 : MSASGGLSLVRRAVEAARRTPRWQKRLLVLTAGVGSLTYACQDNQVLQINDGTGKKRVVI :  60
Indic NDB2 : MSASGGLSLVRRAVEAARRTPRWQKRLLVLTAGVGSLTYACQDNQVLQINDGTGKKRVVI :  60
             MSASGGLSLVRRAVEAARRTPRWQKRLLVLTAGVGSLTYACQDNQVLQINDGTGKKRVVI

                    *         80          *        100          *        120
Japon NDB2 : VGTGWAGASFLRNIDTSLYDVHVVSPRNYFTFTPLLPSVTCGTVEARSIVEPIRNIVRKR : 120
Indic NDB2 : VGTGWAGASFLRNIDTSLYDVHVVSPRNYFTFTPLLPSVTCGTVEARSIVEPIRNIVRKR : 120
             VGTGWAGASFLRNIDTSLYDVHVVSPRNYFTFTPLLPSVTCGTVEARSIVEPIRNIVRKR

                    *        140          *        160          *        180
Japon NDB2 : GGAFRFWEAECYKIDPTNKKIHCRSDSDGNSEFVVDYDYLVVSVGARPNTFNTPGVVENC : 180
Indic NDB2 : GGAFRFWEAECYKIDPTNKKIHCRSDSDGNSEFVVDYDYLVVSVGARPNTFNTPGVVENC : 180
             GGAFRFWEAECYKIDPTNKKIHCRSDSDGNSEFVVDYDYLVVSVGARPNTFNTPGVVENC

                    *        200          *        220          *        240
Japon NDB2 : HFLKEVEDAQKIRKSVLKCFERASLPNLTEEERKKNLHFVVIGGGPTGVEFAAELHDFVN : 240
Indic NDB2 : HFLKEVEDAQKIRKSVLKCFERASLPNLTEEERKKNLHFVVIGGGPTGVEFAAELHDFVN : 240
             HFLKEVEDAQKIRKSVLKCFERASLPNLTEEERKKNLHFVVIGGGPTGVEFAAELHDFVN

                    *        260          *        280          *        300
Japon NDB2 : EDLAKLYPDVKKYANISVIEAGDHILTMFDKRITQFAEDKFKRTGIDLKTNFKVVKVSDK : 300
Indic NDB2 : EDLAKLYPDVKKYANISVIEAGDHILTMFDKRITQFAEDKFKRTGIDLKTNFKVVKVSDK : 300
             EDLAKLYPDVKKYANISVIEAGDHILTMFDKRITQFAEDKFKRTGIDLKTNFKVVKVSDK

                    *        320          *        340          *        360
Japon NDB2 : AITMTNSATGEIAVPYGMAVWSTGIGTRPLIMDFMKQVGQANRRVLATDEWLRVHGCDDV : 360
Indic NDB2 : AITMTNSATGEIAVPYGMAVWSTGIGTRPLIMDFMKQVGQANRRVLATDEWLRVHGCDDV : 360
             AITMTNSATGEIAVPYGMAVWSTGIGTRPLIMDFMKQVGQANRRVLATDEWLRVHGCDDV

                    *        380          *        400          *        420
Japon NDB2 : YALGDCATITQRKVMEDIASIFRVADKDNSGSLTVKKIKNVLGDIYERYPQVELYLKTNQ : 420
Indic NDB2 : YALGDCATITQRKVMEDIASIFRVADKDNSGSLTVKKIKNVLGDIYERYPQVELYLKTNQ : 420
             YALGDCATITQRKVMEDIASIFRVADKDNSGSLTVKKIKNVLGDIYERYPQVELYLKTNQ

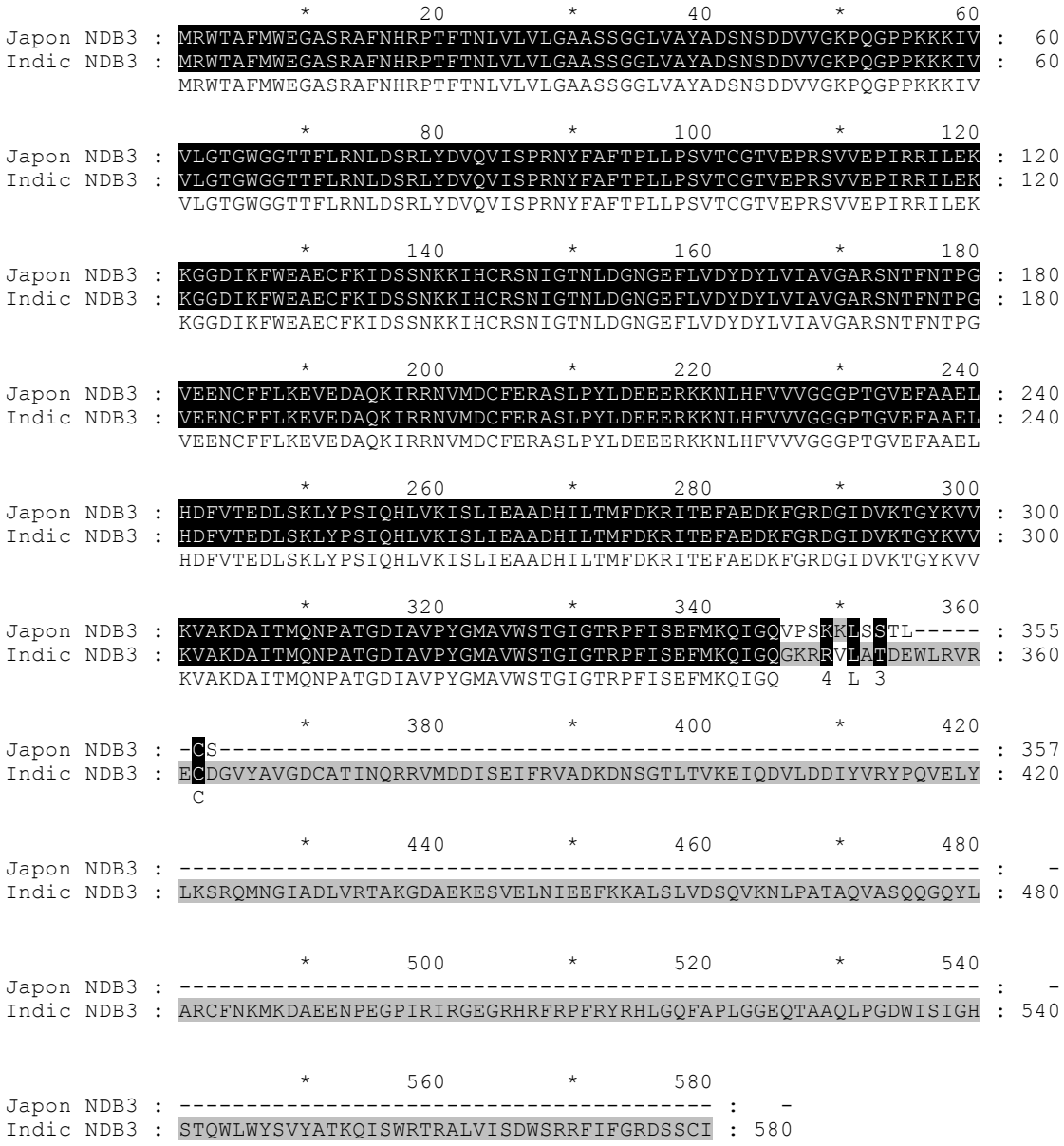
                    *        440          *        460          *        480
Japon NDB2 : MKDFHDLLKDSDGNAIKESKELNIEEFKKALARVDSQVKMLPATAQVASQEGAYLANCFN : 480
Indic NDB2 : MKDFHDLLKDSDGNAIKESKELNIEEFKKALARVDSQVKMLPATAQVASQEGAYLANCFN : 480
             MKDFHDLLKDSDGNAIKESKELNIEEFKKALARVDSQVKMLPATAQVASQEGAYLANCFN

                    *        500          *        520          *        540
Japon NDB2 : KMKYCEENPEGPLRIRGTGRHRFKPFRYRHLGQFAPLGGEQTAAQLPGDWIHVGHSTQWL : 540
Indic NDB2 : KMKYCEENPEGPLRIRGTGRHRFKPFRYRHLGQFAPLGGEQTAAQLPGDWIHVGHSTQWL : 540
             KMKYCEENPEGPLRIRGTGRHRFKPFRYRHLGQFAPLGGEQTAAQLPGDWIHVGHSTQWL

                    *        560          *
Japon NDB2 : WYSVYASKQFSWRTRMLVVSDWGRRFIYGRDSSSL : 575
Indic NDB2 : WYSVYASKQFSWRTRMLVVSDWGRRFIYGRDSSSL : 575
             WYSVYASKQFSWRTRMLVVSDWGRRFIYGRDSSSL
```

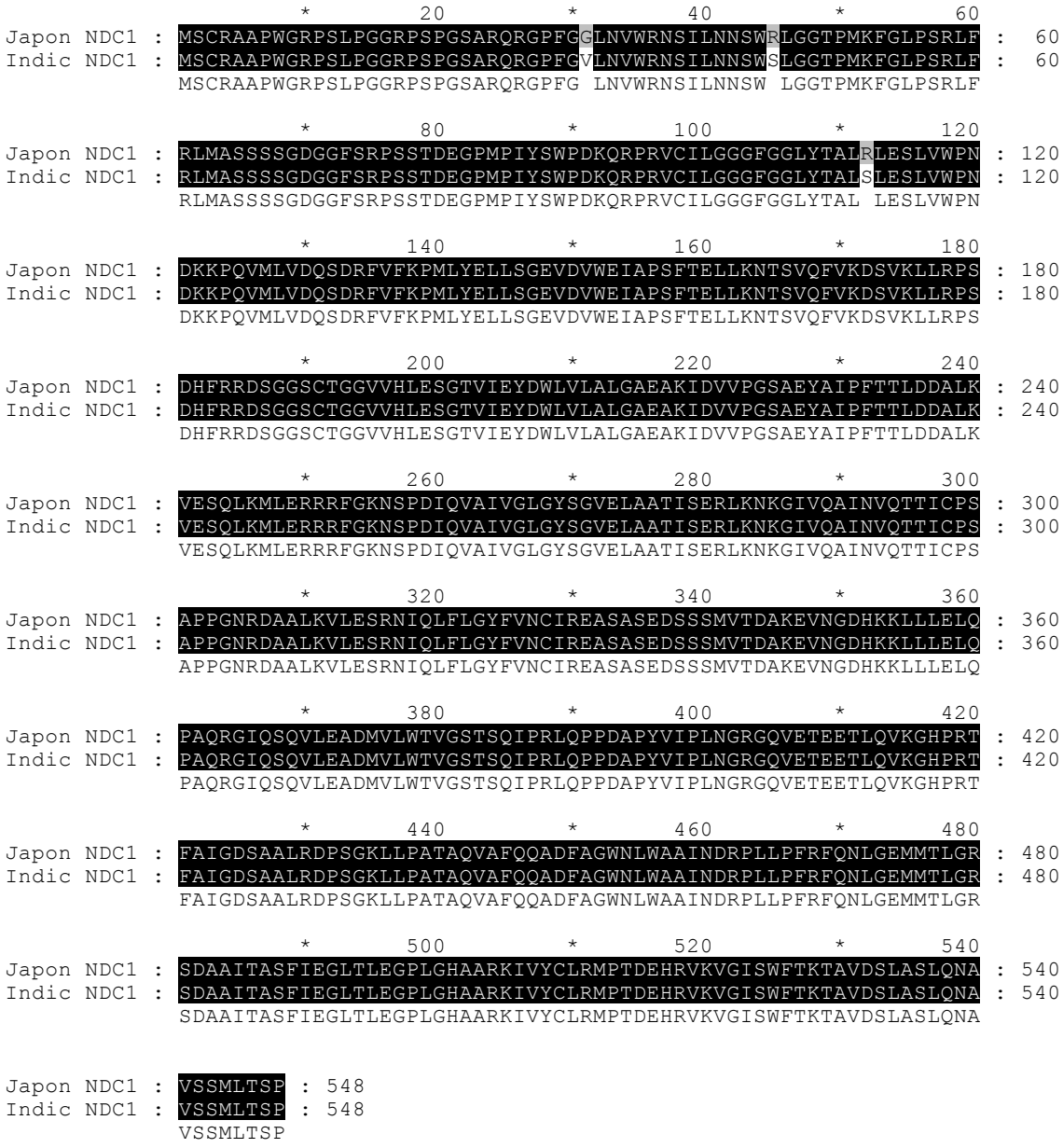
Supplementary Figure B4: Pairwise sequence alignment of full length NDB2 protein from japonica and indica rice

NDB2 from japonica rice shares 100% of amino acid residue identity with indica rice.



Supplementary Figure B5: Pairwise sequence alignment of full length NDB3 protein from japonica and indica rice

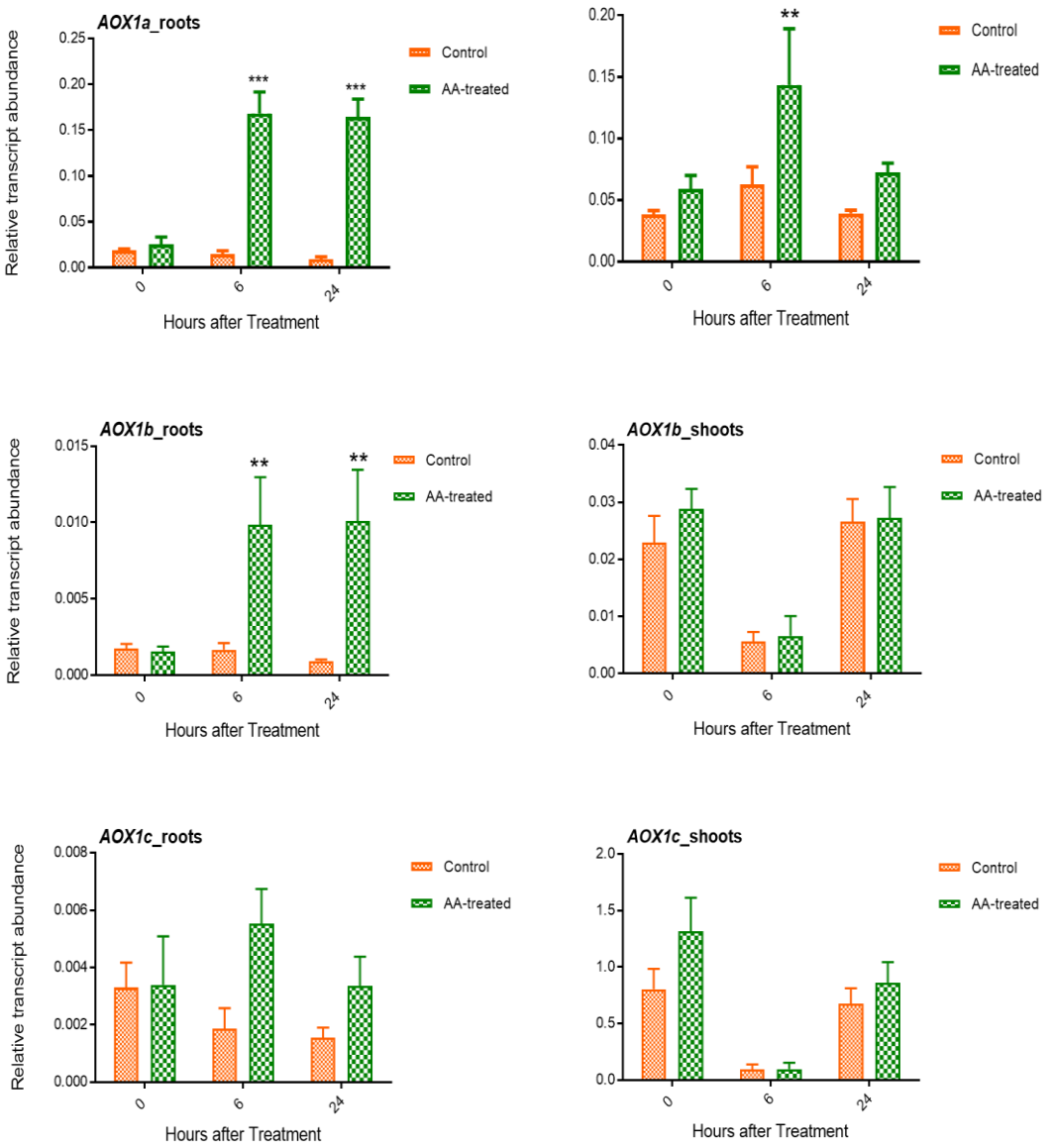
NDB3 from japonica rice shares 98.8% of amino acid residue identity with indica rice covering 353 aa. However, japonica NDB3 protein sequence (357 aa) is shorter than indica (580 aa) due to truncation.



Supplementary Figure B6: Pairwise sequence alignment of full length NDC1 protein from japonica and indica rice

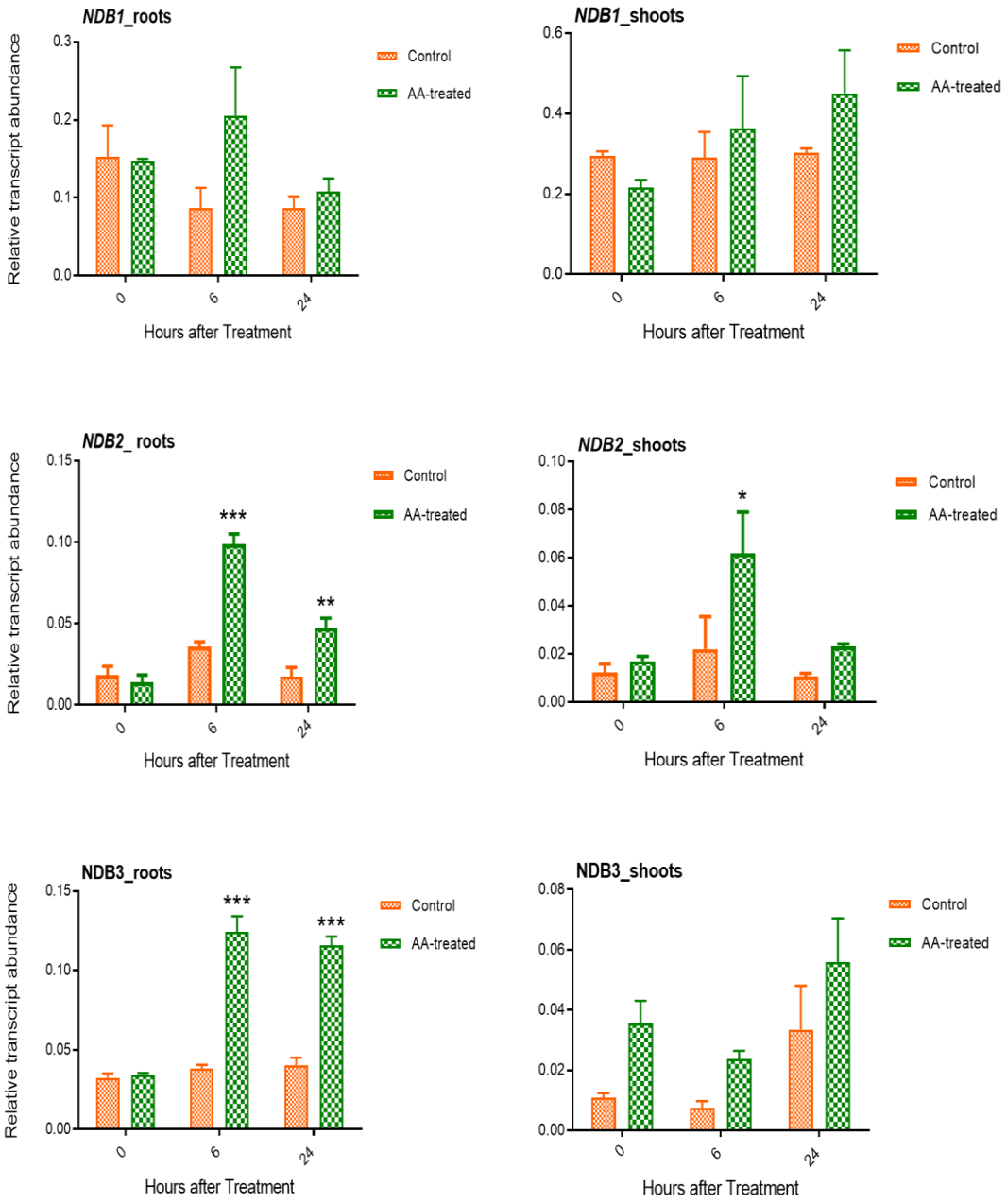
NDC1 from japonica rice shares 99.4% of amino acid residue identity with indica rice.

Appendix C: Changes in transcript abundance of alternative respiratory pathway genes in response to chemical inhibition of mETC



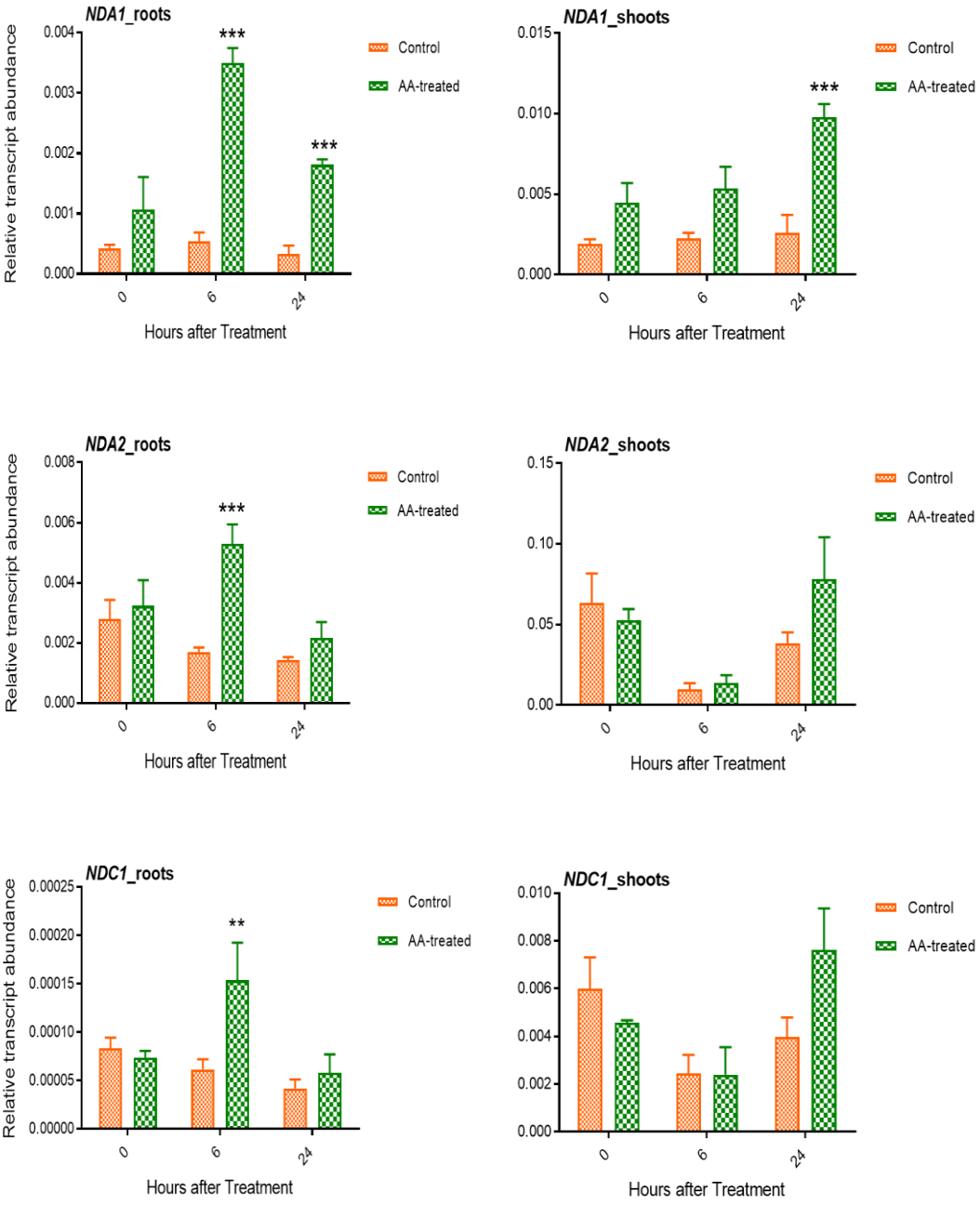
Supplementary Figure C1: Changes in transcript abundance of *AOX1a*, *AOX1b* and *AOX1c* in roots and shoots in response to antimycin A

Changes in transcript abundance of alternative respiratory genes, *AOX1a*, *AOX1b* and *AOX1c* were studies in response to the inhibition of Complex III of mETC by antimycin A (AA) and compared with their respective controls. RNA was extracted from AA-treated and non-treated 2-week old seedlings at 0, 6 and 24 hrs after the treatment and transcript abundance was assessed by qRT-PCR. Data are shown as relative transcript abundance (relative to geometric mean of three stable reference genes) of three biological replicates with + SEM. *** and ** represent the significant difference from the respective controls at $p<0.001$ and $p<0.01$ respectively according to *t*-test.



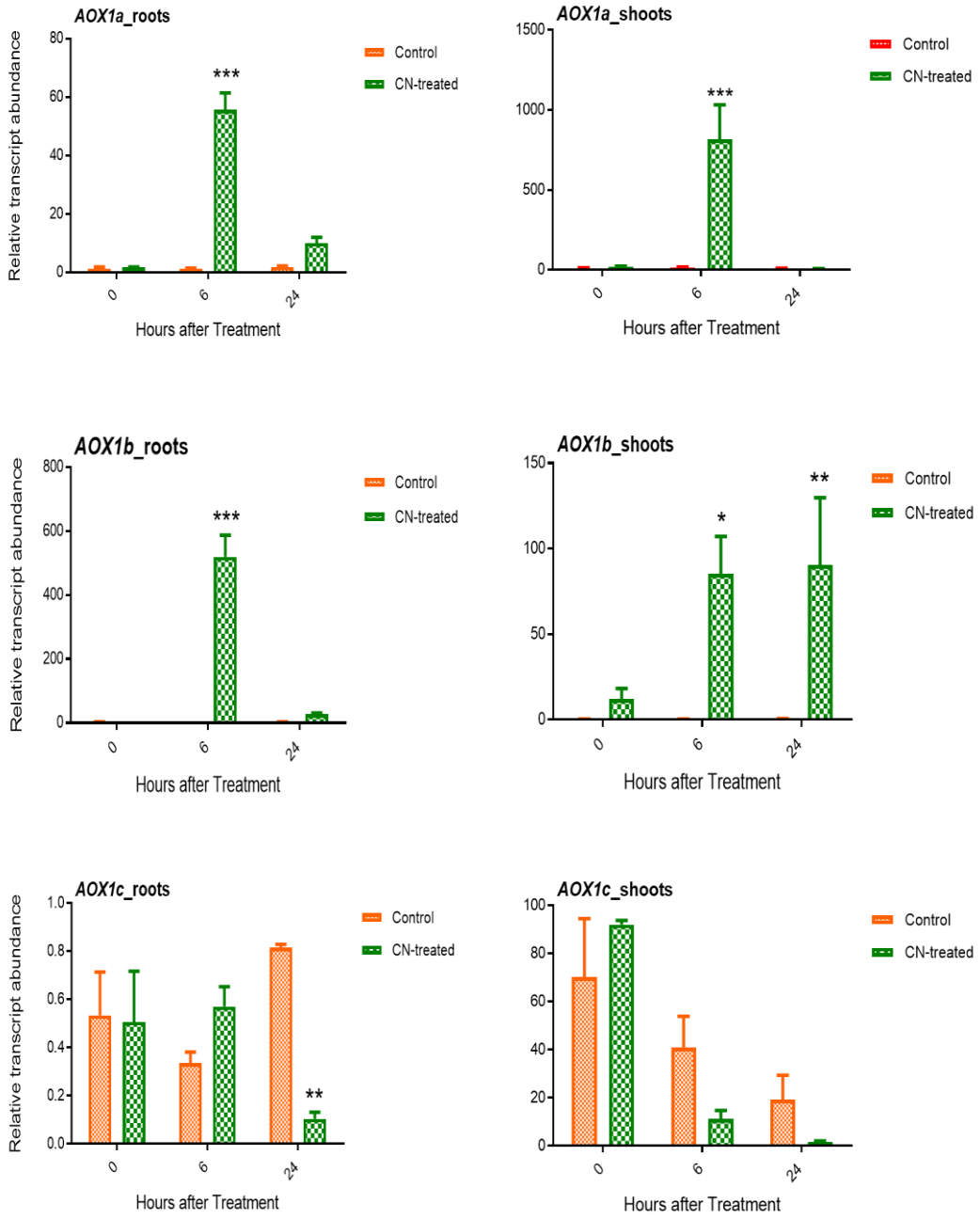
Supplementary Figure C2: Changes in transcript abundance of *NDB1*, *NDB2* and *NDB3* in roots and shoots in response to antimycin A

Changes in transcript abundance of alternative respiratory genes, *NDB1*, *NDB2* and *NDB3* were studies in response to the inhibition of Complex III of mETC by antimycin A (AA) and compared with their respective controls. RNA was extracted from AA-treated and non-treated 2-week old seedlings at 0, 6 and 24 hrs after the treatment and transcript abundance was assessed by qRT-PCR. Data are shown as relative transcript abundance (relative to geometric mean of three stable reference genes) of three biological replicates with + SEM. ***, ** and * represent the significant difference from the respective controls at $p<0.001$, $p<0.002$ and $p<0.01$ respectively according to *t*-test.



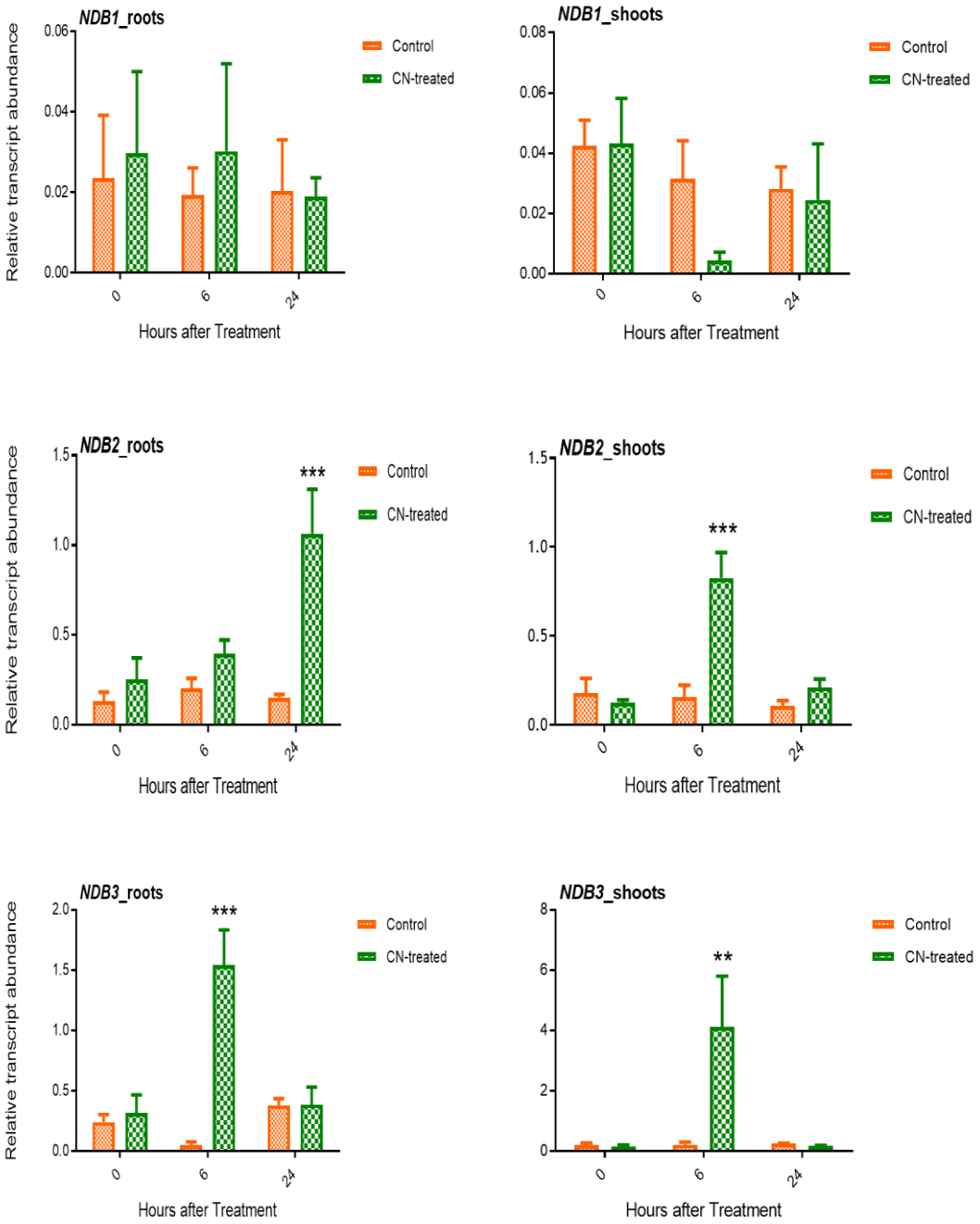
Supplementary Figure C3: Changes in transcript abundance of *NDA1*, *NDA2* and *NDC1* in roots and shoots in response to antimycin A

Changes in transcript abundance of alternative respiratory genes, *NDA1*, *NDA2* and *NDC1* were studies in response to the inhibition of Complex III of mETC by antimycin A (AA) and compared with their respective controls. RNA was extracted from AA-treated and non-treated 2-week old seedlings at 0, 6 and 24 hrs after the treatment and transcript abundance was assessed by qRT-PCR. Data are shown as relative transcript abundance (relative to geometric mean of three stable reference genes) of three biological replicates with + SEM. *** and ** represent the significant difference from the respective controls at $p<0.001$ and $p<0.003$ respectively according to *t*-test.



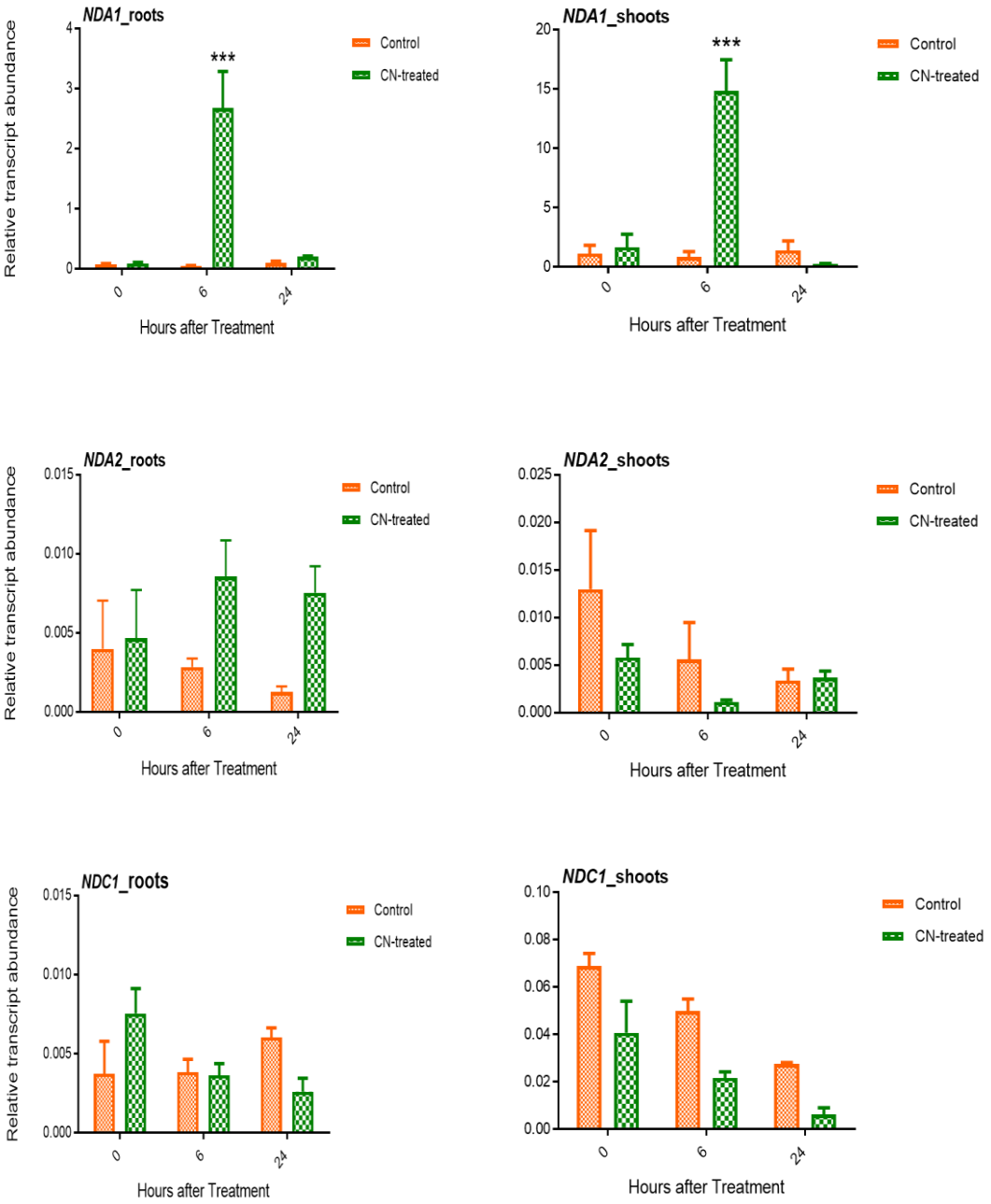
Supplementary Figure C4: Changes in transcript abundance of *AOX1a*, *AOX1b* and *AOX1c* in roots and shoots in response to KCN

Changes in transcript abundance of alternative respiratory genes, *AOX1a*, *AOX1b* and *AOX1c* were studies in response to the inhibition of Complex IV of mETC by potassium cyanide (KCN) and compared with their respective controls. RNA was extracted from CN-treated and non-treated 2-week old seedlings at 0, 6 and 24 hrs after the treatment and transcript abundance was assessed by qRT-PCR. Data are shown as relative transcript abundance (relative to geometric mean of three stable reference genes) of three biological replicates with + SEM. ***, ** and * represent the significant difference from the respective controls at p<0.001, p<0.004 and p<0.01 respectively according to *t*-test.



Supplementary Figure C5: Changes in transcript abundance of *NDB1*, *NDB2* and *NDB3* in roots and shoots in response to KCN

Changes in transcript abundance of alternative respiratory genes, *NDB1*, *NDB2* and *NDB3* were studies in response to the inhibition of Complex IV of mETC by potassium cyanide (KCN) and compared with their respective controls. RNA was extracted from CN-treated and non-treated 2-week old seedlings at 0, 6 and 24 hrs after the treatment and transcript abundance was assessed by qRT-PCR. Data are shown as relative transcript abundance (relative to geometric mean of three stable reference genes) of three biological replicates with + SEM. ***, and ** represent the significant difference from the respective controls at $p<0.001$ and $p<0.005$ respectively according to *t*-test.



Supplementary Figure C6: Changes in transcript abundance of *NDA1*, *NDA2* and *NDC1* in roots and shoots in response to KCN

Changes in transcript abundance of alternative respiratory genes, *NDA1*, *NDA2* and *NDC1* were studies in response to the inhibition of Complex IV of mETC by potassium cyanide (KCN) and compared with their respective controls. RNA was extracted from CN-treated and non-treated 2-week old seedlings at 0, 6 and 24 hrs after the treatment and transcript abundance was assessed by qRT-PCR. Data are shown as relative transcript abundance (relative to geometric mean of three stable reference genes) of three biological replicates with + SEM. *** represents the significant difference from the respective controls at $p<0.001$ according to *t*-test.

Appendix D:

Supplementary Table D1: Fresh weight and dry weight of seedlings tissues from 16 cultivars grown under control and salt-treated conditions

Rice seedlings were grown under 50 mM NaCl for 2 days followed by 120 mM NaCl for further 10 days on hydroponic culture system and plants were harvested for measurements. Data are shown as mean ± SEM of five biological replicates.

Cultivar	Control				Salt-treated			
	Shoot FW (g)	Root FW (g)	Shoot DW (g)	Root DW (g)	Shoot FW (g)	Root FW (g)	Shoot DW (g)	Root DW (g)
Sherpa	0.8032 ± 0.06	0.7029 ± 0.04	0.2214 ± 0.01	0.0819 ± 0.004	0.7991 ± 0.03	0.5380 ± 0.02	0.2038 ± 0.01	0.0762 ± 0.002
Koshihikari	0.6563 ± 0.05	0.4399 ± 0.07	0.1679 ± 0.01	0.0630 ± 0.01	0.6466 ± 0.07	0.4335 ± 0.04	0.1584 ± 0.02	0.0588 ± 0.01
Nipponbare	0.5120 ± 0.03	0.3063 ± 0.014	0.1605 ± 0.01	0.0369 ± 0.003	0.4653 ± 0.02	0.2972 ± 0.01	0.1456 ± 0.01	0.0345 ± 0.002
Reiziq	1.1732 ± 0.07	0.8195 ± 0.05	0.2563 ± 0.02	0.1029 ± 0.01	1.0179 ± 0.07	0.8117 ± 0.03	0.2477 ± 0.01	0.0960 ± 0.01
Takiminori	1.9596 ± 0.08	0.9662 ± 0.07	0.4878 ± 0.02	0.1226 ± 0.01	1.0215 ± 0.06	0.5536 ± 0.04	0.3528 ± 0.02	0.0594 ± 0.003
Topaz	0.8438 ± 0.05	0.8651 ± 0.01	0.2011 ± 0.02	0.1006 ± 0.02	0.7574 ± 0.04	0.8295 ± 0.03	0.1895 ± 0.01	0.0862 ± 0.01
Doongara	2.2070 ± 0.12	1.1570 ± 0.05	0.5249 ± 0.03	0.1419 ± 0.01	1.0434 ± 0.06	0.5845 ± 0.06	0.3307 ± 0.02	0.0576 ± 0.005
Kyeema	0.6406 ± 0.03	0.5375 ± 0.03	0.1524 ± 0.01	0.0576 ± 0.003	0.5095 ± 0.02	0.5258 ± 0.05	0.1466 ± 0.01	0.0537 ± 0.004
LTH	1.8779 ± 0.14	0.5782 ± 0.08	0.3394 ± 0.02	0.0734 ± 0.01	0.9644 ± 0.17	0.4449 ± 0.03	0.2239 ± 0.03	0.0576 ± 0.01
Milang 23	0.8959 ± 0.05	0.4205 ± 0.01	0.1809 ± 0.01	0.0449 ± 0.003	0.7321 ± 0.07	0.4097 ± 0.04	0.1657 ± 0.01	0.0411 ± 0.004
Pandang Wong-7	1.6150 ± 0.15	0.7199 ± 0.09	0.3084 ± 0.03	0.0837 ± 0.01	0.9269 ± 0.06	0.5458 ± 0.05	0.2173 ± 0.01	0.0539 ± 0.004
Tequing	1.1335 ± 0.04	0.4993 ± 0.03	0.2310 ± 0.01	0.0569 ± 0.004	0.7577 ± 0.05	0.4158 ± 0.04	0.1640 ± 0.01	0.0396 ± 0.004
IR 64	2.1857 ± 0.15	0.8449 ± 0.04	0.4167 ± 0.03	0.0933 ± 0.005	1.2313 ± 0.09	0.8079 ± 0.04	0.2831 ± 0.02	0.0709 ± 0.004
Phka Rumduol	2.4306 ± 0.08	1.0025 ± 0.02	0.4547 ± 0.01	0.1121 ± 0.003	1.4459 ± 0.11	0.6818 ± 0.06	0.3270 ± 0.02	0.0638 ± 0.004
Pokkali	3.2037 ± 0.14	1.0162 ± 0.13	0.5437 ± 0.02	0.1089 ± 0.01	1.8829 ± 0.07	0.9387 ± 0.09	0.4169 ± 0.01	0.0893 ± 0.008
Lemont	1.7720 ± 0.19	0.7171 ± 0.07	0.3440 ± 0.03	0.0735 ± 0.01	0.7987 ± 0.08	0.4989 ± 0.06	0.1873 ± 0.02	0.0421 ± 0.01

Appendix E: Media used in tissue culture and transformation of rice

- Culture media were sterilized by autoclaving at 121°C and 15 psi for 15 min.
- Stock solutions of phytohormones were filter sterilized before adding to sterilized media.

N6D medium (Callus induction & subculture medium) (Toki et al., 2006)

Composition	**mg/l**
N6 basal salt mixture with vitamins (Chu, 1978)	3990
Casamino acid (or Casein hydrolysate)	300
Glycine	2.0
myo-inositol	100
L-Proline	2878
Sucrose	30,000
pH 5.8	
Gelrite (0.4%)	4,000
Phytohormones	
2,4-D*	2.0

* 2, 4-Dichlorophenoxyacetic acid

AB medium (Toki et al., 2006)

Composition	**mg/l**
K_2HPO_4	3,000
$NaH_2PO_4.2H_2O$	1,300
NH_4Cl	1,000
$CaCl_2.2H_2O$	10
$FeSO_4.7H_2O$	2.5
KCl	150
$MgSO_4.7H_2O$	296
Glucose	5,000
PH 7.2	

AAM medium (Toki et al., 2006)

Composition	**mg/l**
Major salts	
$MgSO_4.7H_2O$	250
$CaCl_2.2H_2O$	150
$NaH_2PO_4.2H_2O$	150
KCl	3,000
Minor salts	
Fe-EDTA	40
$MnSO_4.4H_2O$	10
$ZnSO_4.7H_2O$	2.0
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.75
H_3BO_3	3.0
$Na_2MoO_4.2H_2O$	0.25
Organics	
Casamino acid	500
Glycine	7.5
L-Arginine	176.7
L-Glutamine	900
L-Aspartic acid	300
myo-inositol	100
Nicotinic acid	1.0
Pyridoxine HCL	1.0
Thiamine HCL	10.0
Sucrose	68,500
Glucose	36,000
pH 5.2	
Phytohormones	
Acetosyringone	20.0

2N6-AS medium (Co-culture medium) (Hiei et al., 1994)

N6 basal salt mixture with vitamins (Chu, 1978)	3990
Casamino acid (or Casein hydrolysate)	300
myo-inositol	100
Sucrose	30,000
Glucose	10.0g
Gelrite (0.4%)	4,000

pH 5.2

Phytohormones

2,4-D*	2.0
Acetosyringone	20.0

* 2, 4-Dichlorophenoxyacetic acid

RE III medium (Regeneration medium) (Toki et al., 2006)

Composition	**mg/l**
MS basal salt (Murashige and Skoog, 1962)	4330
MS vitamins 1000x	1mL
Casamino acid (or Casein hydrolysate)	2,000
Sucrose	30,000
Sorbitol	30,000
Gelrite (0.4%)	4,000

pH 5.8

Phytohprmones

NAA**	0.05
Kinetin	2.5

** 1-Naphthylacetic acid

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