Identification and characterization of genes from wild halophytic rice (*Porteresia coarctata),* for using in development of salt tolerant rice

Ph.D Thesis

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Identification and characterization of genes from wild halophytic rice (*Porteresia coarctata)*, for using in development of salt tolerant rice

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CERTIFICATE

This is to certify that Most. Umme Habiba has conducted her thesis work entitled "Identification and characterization of genes from wild halophytic rice (*Porteresia coarctata),* for using in development of salt tolerant rice" under my supervision for the fulfillment of the degree 'Doctor of Philosophy in Biochemistry and Molecular Biology from the University of Dhaka, Dhaka, Bangladesh. Furthermore, her works are also co-supervised by William Paul Quick, Head of C4 Lab, International Rice Research Institute, Philippines. The work or any part of the thesis has not been submitted for anywhere for any other degree.

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Dedications

To my Parents
Jasmine Reba & Abdul Halim

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This PhD journey was not just an academic pursuit — it was a life chapter filled with growth, resilience, and grace. And though the path was hard, the destination has been beautiful.

Sincerely

Most Umme Habiba

Table of Contents

List of Figures

List of Tables

Abstract

Soil salinity is a major abiotic constraint affecting rice cultivation in coastal and estuarine regions, where rising sea levels and irrigation-induced salinization increasingly threaten global food security. The halophytic wild rice species *Oryza coarctata*—the only naturally salt-tolerant species in the genus *Oryza*, which can set rice-like grains—offers a promising genetic reservoir for improving salt stress tolerance in cultivated rice (*Oryza sativa*). This study presents a comprehensive functional and molecular characterization of *O. coarctata*, along with its application in wide hybridization and genetic engineering strategies aimed at enhancing salt tolerance in rice.

Oryza coarctata exhibits exceptional salt tolerance supported by unique physiological and anatomical features. Its leaf structure includes deep adaxial invaginations, multiple vascular bundles per ridge, and salt-secreting hairs, while its roots possess a thickened exodermis, large xylem vessel, and well-developed aerenchyma—traits that aid in ionic regulation and survival in saline, waterlogged conditions. Notably, *O. coarctata* reduced electrical conductivity (EC) of saline hydroponic media by 2.77–8.51 dS/m across 100–300 mM NaCl, demonstrating a desalination ability absent in salt-sensitive rice varieties.

Co-cultivation of *O. coarctata* with BRRI Dhan28 improved the latter's growth and yield under 100 mM salt stress, increasing yields from 34% to 67%. Gas exchange measurements in *O. coarctata* showed only modest declines in photosynthesis at 100–300 mM NaCl (14–26% reduction in CO_2 assimilation), with strong light response ($r = 0.932$) and moderate J_{max} reduction (19–32%, $p < 0.05$). Unlike *O. sativa*, which fails at 80 mM salt, *O. coarctata* maintains high photosynthetic efficiency and survival under extreme salinity. These traits underscore its potential for use in salt-affected rice ecosystems through ecological facilitation and genetic improvement strategies.

To explore gene transfer from the tetraploid *O. coarctata* (4n=2x=48) into rice, a wide hybridization approach was adopted using a tetraploid *O. sativa* (var. Latisail 4n) as the maternal parent. This approach is referred to as the bulbosum technique, where chromosomal loss occurs resulting in half of the original chromosomes (2x). Despite high genomic divergence, two partial

hybrids were recovered out of 1,191 pollinated spikelets, indicating successful albeit low-frequency gene introgression. These partial hybrids exhibited intermediate phenotypes, including fibrous and tap root systems, variable seed morphology, and in some cases, the absence of leaf midribs—a signature trait of *O. coarctata*. Molecular analysis confirmed that the hybrids carried specific chromosomal segments from *O. coarctata*, particularly on chromosomes 3 and 12. Partial hybrid lines A2-06-01, A2-10-01, and B2-03-01 exhibited significantly higher chlorophyll content than diploid and tetraploid *O. sativa* following 100 mM salt stress. Lines B-02-01 and B-03-01 showed significantly greater plant height compared to *O. sativa* (2n), while A2-06-01 and A2-10-01 also demonstrated a significantly higher tiller number than *O. sativa* (2n).

A significant advancement of this work was the development of *O. coarctata*-specific SSR markers to facilitate molecular screening of hybrids lines. Initially, known SSR markers from the Gramene database showed limited polymorphism between *O. sativa* and *O. coarctata*. Subsequently, 90 markers from the *O. officinalis* genome (CC genome), developed under the OMAP project, were tested. Of these, 19 markers were optimized to uniquely detect *O. coarctata* alleles across most chromosomes (except 5 and 8). In addition, seven new SSR markers were developed from *O. coarctata* genomic sequences. These 26 markers together form a robust toolkit for identifying and validating introgressions in future breeding programs.

To directly assess the functional contribution of *O. coarctata* genes, three candidate genes—*OcAsr1* (abscisic acid stress ripening protein), *OcPVA1* (vacuolar H^+-ATPase subunit c), and *OcMT3* (metallothionein type 3)—were cloned and overexpressed in *O. sativa* using *in planta* Agrobacterium-mediated transformation method. This non-tissue culture-based transformation system enabled the successful generation of transgenic lines in the high-yielding indica background BRRI Dhan75 (for *OcAsr1*and *OcPVA1)* and BRRI Dhan67 (for *OcMT3*).

Gene expression profiling revealed distinct stress-inducible expression patterns of the genes in *O. coarctata*: *OcAsr1* peaked at 24 hours under 200 mM NaCl, *OcPVA1* showed strong and consistent expression across both 100 and 200 mM NaCl, and *OcMT3* exhibited a late response, peaking at 48 hours. Transgenic lines overexpressing *OcAsr1* (notably P_73_2, P_70_1, and P_76_2) displayed enhanced root and shoot biomass, chlorophyll retention, and a 30–50% reduction in root and shoot Na^+/K^+ ratios. These lines maintained 40–50% higher grain yield under 100 mM NaCl

and exhibited minimal yield penalty under normal conditions. *OcPVA1*-expressing lines (MUH_113, MUH_117, MUH_99) demonstrated strong early responses, enhanced membrane stability, improved ionic balance, and significant grain yield retention under salinity. These results support its role in ion transport and vacuolar compartmentalization of sodium ions. Transgenic lines expressing *OcMT3* (P-14-1, P-13-2) showed reduced oxidative damage (as measured by electrolyte leakage and histochemical staining), improved Na^+/K^+ balance, and stable yield under stress, highlighting *OcMT3*'s role in ROS detoxification and metal ion sequestration. Importantly, all three gene constructs did not compromise yield under non-saline conditions, underscoring their suitability for future breeding or biotechnological applications.

This study highlights the remarkable salt tolerance of *Oryza coarctata* and its potential to enhance salinity resilience in cultivated rice through wide hybridization and genetic engineering. Functional traits, molecular markers, and transgenic lines expressing *O. coarctata* genes demonstrated improved growth, yield, and stress tolerance under saline conditions, offering promising avenues for climate-resilient rice breeding.

Chapter one: Introduction

1.1 The consequences of climate change

Climate change is the greatest threat humanity has ever faced. People all over the world are facing the reality of climate change with many extreme weather events. This in turn is bringing about some key challenges like increasing demand for food, reduction in crop yields and increasing pressure on natural resources (FAO/climate change). Between 2000 and 2019, over 475000 people lost their lives worldwide and losses of US$ 2.56 trillion were incurred as a direct result of extreme climate-induced disasters (Global Climate Risk Index 2021).

The Covid-19 pandemic has further shown us that both risk-affectedness and vulnerability are systemic and interconnected. A recent study by the Red Cross Red Crescent Movement has shown that 90 of the 132 extreme weather events that occurred between January and September 2020, overlapped with Covid-19 pandemic. Globally, 51.6 million people had to simultaneously deal with the impacts of floods, droughts or storms whilst trying to deal with consequences of the pandemic.

The world population is growing and is expected to reach 9.7 billion in 2050 and 10.4 billion in 2100 (World Population Prospect 2022). It took until the early 1800s for the world population to reach one billion. Now we add a billion every 12-15 years. Human activities, such as poor management practices, changes in diet preferences, increasing competition for land, water and energy use, soil degradation, crop diseases are some of the factors that challenge food productivity (United Nations Convention to Combat Desertification, 2017).

Moreover, in the last decades, cultivated land has increased worldwide by about 12% at the expense of natural ecosystems. This has resulted in destruction of forests and serious impacts on wild biodiversity. Therefore, the percentages of land converted to agricultural land should be kept as low as possible (United Nations Convention to Combat Desertification, 2017). Other solutions, such as improving agricultural practices, creating new environmental policies, changes in diet, and reducing food waste, are decent but partial solutions only (Hannes Dempewolf and Jane 2014).

1.2 Food security and climate change

Food security has become a pressing challenge because of the ever-increasing human population. About 30% of the 700 million people living in extreme poverty in Asia, live in regions that are prone to abiotic stresses such as flooding, drought and excess soil salinity (Ismail 2013). These

stresses often occur in combinations of 2 or more and stress responsive pathways often show extensive cross-talk (Mittler 2006). Bangladesh's economy depends on agriculture and it has a large coastal region. Bangladesh is in 7th position among countries which will be most affected due to climate change complications according to Germanwatch.

1.3 Rice, staple food of billions

Rice is one of the world's most important staple crops, feeding more than 2.7 billion people worldwide (Muthayya et al. 2014), and is also a model for genomic research in monocots. It is a primary source of calories and nutrition for billions of people, particularly in Asia and Africa. Asia is home to rice and people in this region depend on rice economically, socially, culturally and environmentally (Huke 1982, Fuller 2011). Without Rice, sustainable development in Asia cannot be achieved. In 2021/2022 about 436 million metric tons of rice were produced only in Asia , which is roughly 90% of the total rice production. Six countries are not only the major producers, which include, China, India, Bangladesh, Indonesia, Vietnam and Philippines, but these are also the major exporters and consumers in 2022. Rice is cultivated on 150 million hectares of land, and its annual yield is close to 610 million tons all over the world (World agricultural production 2022).

Rice provides 21% of worldwide human per capita energy and 15% of per capita protein (IRRI 2002). Although rice protein ranks high in nutritional quality among cereals, its protein content is modest. Rice also provides minerals, vitamins, and fiber, although all constituents except carbohydrates are reduced by milling. While the number of people suffering from hunger globally has risen to above 783 million, an additional 1.3 billion people are affected by moderate levels of nutrition insecurity because they have inadequate access to safe and nutritious food (State of Food security and Nutrition in the World SOFI report).

1.4 Major obstacles for rice production

Due to global adverse climate changes, rice growth and productivity in recent years has been seriously affected by abiotic stresses such as salt, drought, heat, flood, and cold. Modern high-yielding improved varieties have achieved high productivity but with the simultaneous reduction of their genetic base (Fu 2015). Furthermore, these varieties were bred to emphasize reproduction rather than defense and competition mechanisms. Therefore these commercial genotypes are now

threatened by extreme conditions. To comprehensively address the problems of modern agriculture, it is therefore crucial to breed novel crop varieties resistant or tolerant to environmental stresses (Zhang, Mittal et al. 2017).

Efforts should therfore be made, to focus on traits that could be introduced to defend against key abiotic plant stresses, such as drought, salinity, and extreme temperatures. In this aspect, scientists should exploit all the available genetic diversity (Zhang, Mittal et al. 2017). Both plant breeding and domestication rely on human selection and these have led to a loss of genetic variability, because only yield traits were considered as desired traits and advanced for our purposes (Smýkal, Nelson et al. 2018).

1.4.1 Salinity

Soil salinization is a worldwide problem for agriculture. It affects 6% of total Earth's land as a result of natural accumulation over long periods of time (Rengasamy 2006). Agricultural activity contributes to secondary salinization: 2% of all dry land is becoming salinized, and more than 20% of irrigated soils are affected, mostly because of irrigation water containing small amounts of sodium chloride (Tester and Davenport 2003). Soil salinity has adverse effects on plant germination, strength, and yield (James, Blake et al. 2012). On exposure to salt stress, the ionic balance (especially Na /K ratio) and its uneven distribution is the ultimate manifestation of several physiologic processes in response to salt stress (Chen, Yan et al. 2013) This imbalance creates problems in not only in water uptake but also affects the uptake and distribution of other nutrients (Sengupta and Majumder 2010).

In Bangladesh, 1 million hectares of coastal land are currently affected by salinity, especially in the dry season. This constitutes $1/9^{th}$ of the total area suitable for cultivation. Furthermore, with the adverse effect of climate change complications, the salinity level is increasing and more areas are being affected. At present, river salinity is most severe in the southwest zone of Bangladesh (Satkhira, Khulna, Bagerhat, Barguna, Patuakhali). The soil salinity level ranges from 80-160mM, surface water salinity 150-300mM and ground water salinity 90-200 mM. The percentage of very highly saline soil has been increased from less than 1% in 1990 to 33% in 2015 which is very alarming for the agricultural production in this area (Rahman, Di et al. 2018).

The rate of salinity intrusion in coastal Bangladesh is faster than it was predicted a decade ago (Agrawala, Ota et al. 2003). Even though salinity intrusion is a slow process, but the impact is devastating. Sea level rise is likely to put the most critical threat by land submersion and salinity intrusion(Ashraf, Sarwar et al. 2002, Rashid, Hoque et al. 2004). A probable 1.6 billion tones/yr of alluvial content streams towards Bangladesh through the Ganges and the Brahmaputra. Similarly, higher rates of ice melt in the Himalayan region would cause SLR in the Bay of Bengal and its river delta (Wöppelmann and Marcos 2016, Kleinherenbrink, Riva et al. 2018, Maurer, Schaefer et al. 2019). NAPA (National Adaptation Programme of Action of Bangladesh)(Bangladesh) 2005) has warned that the impact of saline water ingression in estuary and underground water is likely to be accelerated by sea level rise, land subsidence and low flow river condition. World Bank (Bangladesh 2000) study predicted a 1 m sea level rise at the end of the century which might affect 17.5% of total land mass of the country. Miller (Miller 2004) also warned that a sea level rise of 88 cm would flood agricultural lowlands and deltas in parts of Bangladesh. Both the scenarios implied that the future sea level rise would bring further land under inundation and therefore salinity would intrude to more inlands.

1.4.2 Drought

Drought is one of the most widespread and damaging environmental stress factors in plants (Krannich, Maletzki et al. 2015). Rice is highly sensitive to drought stress because it is acclimated to either rain-fed or fully irrigated fields. The effect of drought may vary with different genotypes, development stages, and degree and duration of drought stress (Wang, Pan et al. 2011). Rice plants are highly sensitive to drought stress during vegetative stage (resulting in reduced height, tiller number, and leaf area) and at the panicle initiation and booting stages (Xinlu, Jiaguo et al. 2004, Wang, Pan et al. 2011). Drought is a critical problem for Bangladeshi agriculture, especially in the north-west region of Bangladesh (Habiba, Shaw et al. 2012). The north-west regions have been experiencing insufficient rainfall, resulting in a low groundwater table and, consequently, water shortages for agricultural and other uses (Dey, Alam et al. 2011).

1.4.3 Flooding

Flooding is a widespread environmental stress. Submergence inhibits aerobic metabolism and photosynthesis, leading to carbohydrate depletion and, depending on the stress intensity, plant

death (Bailey-Serres and Voesenek 2008). Although rice is considered a flood tolerant crop, only limited cultivars display tolerance to prolonged submergence, with most dying within 14 days of complete submergence (Niroula, Pucciariello et al. 2012).

Flooding is very common for low-lying Bangladesh (Islam, Bala et al. 2010). Three major rivers of Bangladesh: the Brahmaputra, Meghna, and Ganges. Flooding occurs along these rivers and their tributaries nearly every year during the monsoon season between June and September. Annually, almost one-third of Bangladesh is flooded by overflowing rivers induced by excess monsoon rains (Mirza 2002). Flooding is frequent in northeastern Bangladesh and along the three major rivers, the Ganges, Brahmaputra, and Meghna. Between 2014 and 2018, the flood-affected paddy rice areas accounted for 1.61–18.17% of the total paddy rice area (Singha, Dong et al. 2020).

1.4.4 Low temperature

Cold can affect growth and development of rice plants during any developmental stage, from germination to grain filling. During germination, the most common symptoms of cold temperature damage are low percentage and delayed germination (Cruz, Sperotto et al. 2013, Dametto, Sperotto et al. 2015), resulting in yield decreases up to 25% of the final yield and in increased weed competition (Fujino, Sekiguchi et al. 2004). Cold stress is one of the major environmental factors limiting the growth, productivity, and geographical distribution of crops, mostly in temperate and high altitude areas, due to the tropical origin of the rice species (Cruz, Sperotto et al. 2013, Zhang, Chen et al. 2014). Low temperature induced rice yield loss is a worldwide problem (Peyman and Hashem 2010) but it is a major constraint to rice production in mountainous regions of the tropics and in the temperate rice-growing zones of the world (Xie, Kato et al. 2012). In recent years, more than 2.0 million hectares of rice crop in northern and north-eastern parts of Bangladesh have been affected by severe cold spell causing partial to total loss (Hossain, Biswas et al. 2023).

1.5 Fighting climate change complications

Developing superior salt-tolerant crop varieties to cope with increased levels of salinity and other abiotic stresses has become mandatory now. The complexity of the salinity tolerance mechanisms and a lack of available donors for candidate genes for cultivated rice have created major challenges for developing salt-tolerant varieties (Solis, Yong et al. 2020). The method currently practiced for improving abiotic stress tolerance in rice cultivars is to explore germplasm for desirable traits

(Scafaro, Haynes et al. 2010). Recent attempts (until now limited to *O. sativa* species) have been successful, with backcrossing of *Oryza sativa* ssp. *japonica* and *indica* leading to substantial improvements in abiotic stress tolerance (Cheng, Zhuang et al. 2007). As there are about 24 known species within the *Oryza* genus, a large source of genetic material is still to be explored for the scientists. The search for stress tolerance in the ancestors of cultivated rice can bring our desired success (Solis, Yong et al. 2020).

1.6 Halophytes, a great source for salt-tolerance genes

Halophytes are considered as one of the best resource germplasms for saline agriculture. They are salt-resistant or salt-tolerant plants able to complete their life cycle in highly saline conditions. They have developed different morphological, anatomical, and physiological strategies to survive in high salt environments through evolution (Flowers and Colmer 2008, Grigore, Ivanescu et al. 2014). Halophytes are obligate and facultative based upon salt demand and tolerance for sodium salts. Halophytes that consistently require salt for their growth are referred to "obligate halophytes"(Grigore, Ivanescu et al. 2014), but some halophytes also have the ability to grow on the soil devoid of salt and are called "facultative halophytes" (Polunin 1960).

Halophytes like *Suaeda* and *Atriplex* species have been investigated to unravel the molecular mechanism of stress tolerance. *Thellungiella halophila* is one of the halophytes emerging as a model halophyte for the study of abiotic stress tolerance mechanisms (Wang, Li et al. 2004, Amtmann 2009). Among all, *Salicornia brachiata* is considered as a potential halophyte for new and useful salt-tolerant genes (Singh, Mishra et al. 2010, Udawat, Mishra et al. 2014, Udawat, Jha et al. 2016). *Cakile maritima* and *Suaeda maritima* (Megdiche, Passaquet et al. 2009, Sahu and Shaw 2009) are considered as model plants for salt-induced transcript profiling. Several known genes like antiporters (*NHX, SOS, HKT, VTPase*) (Kader, Seidel et al. 2006, Hanjagi, Sashidhar et al. 2011, Baisakh, RamanaRao et al. 2012, Kobayashi, Abe et al. 2012, Kumar, Kushwaha et al. 2012), ion channels (Cl−, Ca2+, *aquaporins*) (Kim, Waadt et al. 2007, Peng, Lin et al. 2007, Wei, Liu et al. 2013), antioxidant encoding genes (*APX, CAT, GST, BADH, SOD*) (Jia, Zhu et al. 2002, Ji, Zhu et al. 2010, Qi, Liu et al. 2010, Wutipraditkul, Boonkomrat et al. 2011, Modarresi, Nematzadeh et al. 2013, Wang, Wu et al. 2013, Wu, Wang et al. 2014, Yan, Li et al. 2016, Ali, Ali et al. 2020) have been explored for developing stress tolerance in the crop plants.

1.7 *Oryza coarctata*: a halophyte and wild relative of rice as reservoir for defense against salt stress

The only halophyte in the genus *Oryza* and known source of tolerance to salt stress among the wild relatives of rice is *Porteresia coarctata* or *Oryza coarctata* (locally called Uri dhan). *O. coarctata* can survive in upto 400mM salt stress. It can accumulate Sodium and Chloride ions in leaves and can maintain a Na:K ratio as low as 0.7 even in 25% artificial sea water after six weeks (Flowers, Flowers et al. 1990). It naturally grows along most of the coastal belt of Bangladesh, India, Pakistan and Malaysia (Latha, Salekdeh et al. 2004).

Figure 1.1 : *O. coarctata*, previously called *P. coarctata* plants and panicle.

The species can also withstand submergence with saline water for quite a long period. In deep forests of Sunderbans it is generally completely submerged by tidal water for as long as 10-11 h every day (Garg, Verma et al. 2014). It sets grains on a single unbranched panicle only once a year around October or November. The seeds get readily dehydrated, dehisce pre-maturely and do not germinate. *O. coarctata* propagates naturally through rhizomes. It acts as pioneer species in the succession process of mangrove formation along the estuaries of Bangladesh. *O. coarctata* forms a vast population as mangrove associates throughout the root matrices of tree mangroves, binds the soil and prevents the coastline from erosion (Sengupta and Majumder 2010). The leaves of *O. coarctata* are waxy unlike those of rice, and the surfaces display alternate ridges and furrows. About fifteen thousand genes have been identified by transcriptome profiling of *) O. coarctata* in 400 and 700mM salt stress by Garg et al. in 2014. These genes can be promising candidates for developing salt-tolerant rice. Being a wild rice relative it could serve as an intermediate agent for

studying the model system in monocotyledonous plants, expression and behavioral pattern during osmotic stress (Latha, Salekdeh et al. 2004).

1.8 Specific Objectives of the Study

1. Characterizing *O. coarctata* based on their phenotype and comparing those to common *O. sativa* at anatomic level.
2. Analyzing their salt excreting capability from their surrounding environment.
3. Investigating whether O. coarctata can support salt-sensitive rice varieties when grown in close proximity in salt water. .
4. Analyzing photosynthesis rate of *O. coarctata* at different levels of salt-stress to determine its photosynthetic efficiency.
5. Developing hybrid plants by wide hybridization between *O. coarctata* and *O. sativa*
6. Developing SSR markers for identification of true hybrids unique for *O. coarctata*
7. Identification of salt stress related genes from *O. coarctata* transcriptome for transformation into sensitive rice.
8. Cloning of the identified genes and transforming these into a Bangladeshi high-yielding but salt-sensitive rice variety.
9. Screening of selected transgenic lines for salt-tolerance at seedling and reproductive stages.

1.9 Uniqueness of the study

- Developing hybrids by wide hybridization between *O. coarctata* and chemically induced tetraploid *O. sativa*, for use as a salt-tolerant donor in further breeding programs with farmer preferred rice varieties.. Production of such rice would allow rice to grow at least two times per year in coastal areas and alllow the cultivation of fallow land. This is of major significance for land-strapped Bangladesh.
- So far, Indica rice has only been transformed with the *O. coarctata* gene ***Fructose 1,6-bisphosphatase (****FBPase*) for improved photosynthesis and multi-stress tolerance has been reported (Mukherjee, Mukherjee et al. 2021). The transgenic rice lines developed by this research In Bangladeshi rice genetic background will be the first to be developed from any halophytic germplasm resource.

1.10 Experiments design

Part one:

- Exploring promising characteristics of *O. coarctata.*
- Comparing root and leaf anatomy with *O. sativa*.
- Measuring desalinization capability of *O. coarctata* in Yoshida media.
- Growing salt-sensitive rice adjacent to *O. coarctata in order to investigate the effects of the latter on rice growth under saline conditions.*.

Part two:

- Wide/ forced hybridization between *O. sativa* and *O. coarctata.*
- Hand pollination of *O. sativa* with *O. coarctata*, Seed rescue and growth in media.
- Repeated pollination of putative hybrids to retain *O. coarctata* genes.
- Establishing *O. coarctata* specific SSR markers for identification of true hybrids.
- Identification of most promising hybrids and genome sequencing .

Part three:

- Identification of promising salt-responsive genes from *O. coarctata* transcriptome data.
- Analyzing their real-time expression in *O. coarctata* at different salt-concentration.
- Cloning of selected genes into plant-compatible vector.
- Transformation of high-yielding rice varieties with *O. coarctata* genes.
- Seedling and reproductive screening of transformed lines at salt-stress.
- Comparison of transgenic lines performances with respect to genes and varieties.

Chapter two: Literature Review

2.1 Overview

Development of abiotic stress tolerant especially salt-tolerant rice varieties is an urgent necessity in the context of ensuring food security. Attempts to develop salinity-tolerant rice varieties have had limited success due to the complexity of the salinity tolerance trait, high variation in the stress response and a lack of available donors for candidate genes for cultivated rice. Halophytes and wild relatives of rice can be an essential resource of salt-tolerance genes. In this chapter the current understanding of salt-tolerant rice development has been reviewed concentrating on the following areas:

1. The diversity of *Oryza* species with respect to genome type and their evolutionary relations.
2. Review on knowledge on abiotic stress tolerance in wild rice of genus *Oryza*.
3. Review on wide hybridization approaches and its limitations for using *O. coarctata*.
4. Review on candidate genes from *O. coarctata* for developing stress-tolerant plants and other promising candidates.
5. Review on available salt-tolerant rice varieties and their prospects.

2.2 The diversity and beauty in genus *Oryza*: Evolutionary relation among wild rice and common rice

Rice belongs to the genus *Oryza*, of the tribe Oryzeae, subfamily Ehrhartoideae and family Poaceae (Grass Phylogeny Working Group, 2001). The phylogeny of the *Oryza* genus spans approximately 15 million years (MY) of evolutionary history, a process that created diverse ecological adaptations (Ammiraju et al. 2010; Vaughan et al. 2003). The *Oryza* genus consists of 24 species spread worldwide (Jacquemin et al. 2013; Kellogg 2009). The *Oryza* species has 11 different genome types (AA, BB, CC, BBCC, CCDD, EE, FF, GG, KKLL, HHJJ, and HHKK)(Jacquemin et al. 2013; Lu et al. 2009; Atwell et al. 2014). The common cultivated species of rice from Asia, *O. sativa*, seems to have arisen through domestication of the wild species *Oryza rufipogon* and *Oryza nivara* (Bautista et al. 2001; Fuller et al. 2009; Sweeney et al. 2007; Xu et al. 2012; Zhu et al. 2007).

Despite there being so many accessions in germplasm banks (McCouch et al. 2012) and a further half a million landraces reported to be in existence, *O. sativa* has substantially less genetic diversity than its progenitors and congeners combined. This proportion is likely less than half the diversity found in wild relatives of *Oryza* (Xu et al. 2012; Zhu et al. 2007; Caicedo et al. 2007). *O. glaberrima*, the domesticated species of rice from Africa, has even less genetic diversity than its Asian counterpart (Li et al. 2011).

It is likely that all crop species have undergone a genetic bottleneck and subsequent loss of genetic diversity during the early stages of domestication (Tanksley and McCouch 1997), but the extent of the genetic loss is species-specific. For example, soybean (*Glycine max*) has close to 50% of the genetic diversity found in its wild progenitor, *Glycine soja* (Guo et al. 2010; Hyten et al. 2006). Maize (*Zea mays* ssp. mays) seems to have maintained a higher level of genetic diversity despite a domestication bottleneck, containing upwards of 60% of the genetic diversity found in *Zea mays ssp. parviglumis*, the wild progenitor (Eyre-Walker et al. 1998). Maize has probably maintained higher genetic diversity during domestication through outcrossing. On the other hand, rice, which is variable in its degree of self-pollination , appears to have had less introgression of DNA from wild relatives (Londo et al. 2006). The phenotypic contrast between modern and wild relatives of rice (e.g. *O. rufipogon*) would suggest that domestication arose with selection for only a few key traits, including non-shattering, absence of secondary dormancy, fewer and more upright tillers and lack of coloration (Li et al. 2006a; Sweeney and McCouch 2007; Li et al. 2006b; Huang et al.

2012; Sweeney et al. 2007; Tan et al. 2008). The use of germplasm from wild relatives to improve domesticated rice is destined to be an increasingly urgent priority because of the paucity of parental genotypes in existing commercial rice genotypes all over the world and consequential sensitivity to both biotic and abiotic stressors (Sang and Ge 2013; Tanksley and McCouch 1997; McCouch et al. 2007).

Rice gene banks around the world exhibit an extensive seed collection, covering the genetic diversity present in farmers' cultivars, landraces and wild Oryza species. The two largest gene banks are the International Rice Research Institute in the Philippines (4,370 wild species and hybrids accessions at IRRI, http://irri.org), and Oryza base in Japan (1,703 entries, http://www.shigen.nig.ac.jp) (Jacquemin et al. 2013). Moreover, the Genesys database (http://www.genesys-pgr.or) allows for searching of accessions for many species in several seed banks, being a valuable resource for germplasm distribution. A genus-wide comparative genome platform is essential to understand the genetic differences associated with abiotic factors. The sequencing of 16 *Oryza* genomes has been done with "gold standard" reference sequences available for the cultivated species *O. sativa* ssp. *japonica* and *O. glaberrima*, and for the wild species *O. barthii* and *O. brachyan* (Jacquemin et al. 2013).

2.3 Abiotic stress tolerance in wild relatives of rice for our future prospects:

2.3.1 Submergence

Genotypes from AA genome species *Oryza sativa*, *Oryza rufipogon* and *Oryza nivara* are tolerant or sensitive to submergence depending on the presence of *SUB1A-1* allele of the *SUB1A* gene. Genotypes that either have *SUB1A-2* allele or lack a *SUB1A*, carrying only *SUB1B* and *SUB1C* genes, are sensitive (Niroula et al. 2012). The tolerant *SUB1A-1* allele is derived from the *aus* subgroup of *indica* rice (Xu et al. 2006). Genotypes from CC genome species *Oryza eichingeri*, *Oryza rhizomatis* and the CCDD tetraploid species *Oryza grandiglumis* were shown to be tolerant to submergence while carrying a *SUB1A* gene-lacking *SUB1* locus, indicating the locus does not contribute to the stress tolerance in these species. Interestingly, *SUB1A* is absent in *O. grandiglumis*, indicating that a *SUB1A*-independent mechanism for a quiescent strategy is present, as observed for *O. rhizomatis* and *O. eichingeri* (Niroula et al. 2012). Genes similar to *SNORKEL1* and *SNORKEL2*, responsible for the escape strategy in deepwater- adapted *O. sativa*, as well as *O.*

rufipogon and *O. glumeapatula*, are absent in *O. grandiglumis* (Okishio et al. 2015; Hattori et al. 2009). These results indicate that *O. grandiglumis* is tolerant to both gradual and full submergence by unknown mechanisms (Okishio et al. 2014), indicating that CC genome *Oryza* species might provide new molecular mechanisms to improve cultivated rice.

Table 2.1: Tolerance to abiotic stresses found in accessions of *Oryza* species and references.

Species	**Tolerance found in accessions of *Oryza* species**
Oryza coarctata	Salt (Sengupta and Majumder, 2009), Submergence (Garg et al 2011)
Oryza eichingeri	Submergence (Niroula *et al*., 2012)
Oryza glaberrima	Salt, Drought (Ndjiondjop *et al.*, 2010, Platten *et al*., 2013)
Oryza glumaepatula	Submergence (Hattori *et al*., 2009)
Oryza grandiglumis	Submergence Okishio *et al*., 2014, 2015
Oryza meridionalis	Heat (Scafaro *et al*., 2010)
Oryza nivara	Drought (Singh *et al*., 2015)
Oryza officinalis	Drought, Heat (Early Morning Flowering) Ishimaru *et al*., 2010, Feng *et al*., 2012
Oryza rhizomatis	Submergence (Niroula *et al*., 2012)
Oryza rufipogon	Salinity, Cold, Drought, Submergence Hattori *et al*., 2009, Tian *et al*., 2011, Xiao *et al*., 2014

2.3.2 Salinity

A range of transporters involved in reducing Na accumulation in shoots and in subcellular compartmentalization was described, such as the high affinity potassium transporter (HKT), salt overly sensitive (SOS) and Na /H exchanger (NHX) gene families (Mickelbart et al. 2015). HKT

members are crucial determinants of tissue concentration of Na+. *OsHKT1;5* is the causative gene of *Saltol*, the major quantitative trait locus (QTL) for salt accumulation in *O. sativa* genotypes (Ren et al. 2005). OsHKT1;5 is a plasma membrane transporter that regulates partitioning of Na between roots and shoots by efflux of Na+ from the xylem to adjacent parenchyma cells (Hauser and Horie 2010). Four amino acid changes in *OsHKT1;5* resulted in increased Na+ efflux activity in salt tolerant *indica* cultivar Nona Bokra compared to the salt sensitive cultivar Koshihikari (Ren et al. 2005).

O. glaberrima (AA genome) genotypes, could exclude Na+ from shoots using a mechanism independent of *OsHKT1;5* (Platten *et al.*, 2013). *O. rufipogon* was shown to be salt tolerant when compared to rice sensitive cultivars (Zhou et al. 2016). Introgression lines derived from *O. rufipogon*×*O. sativa* cross revealed 15 QTLs for salinity tolerance, 13 of them derived from the *O. rufipogon* parent (Tian et al. 2011). Over-expression of *bHLH* transcription factors *OrbHLH001* and *OrbHLH2* from *O. rufipogon* resulted in Arabidopsis and *O. sativa* salt tolerant lines (Zhou et al. 2009; Li et al. 2010; Chen et al. 2013). These authors showed that *OrbHLH001* is able to positively regulate the K^+ transporter *OsAKT1*, suggesting that salt tolerance results from maintenance of K^+ homeostasis under high Na^+ conditions (Chen et al. 2013). Based on heterologous expression in Arabidopsis, *OrbHLH2* was suggested to positively regulate genes from the *CBF/DREB* pathway (Zhou et al. 2009).

2.3.3 Cold

The *japonica* cultivars of *O. sativa* are usually adapted to temperate climates, a process that was driven by domestication, while *indica* cultivars are generally tropical (Kovach et al. 2007; Hirabayashi et al. 2015). However, little is known about the molecular basis for low temperature tolerance in *O. sativa*, and even less about the variation of cold tolerance among its wild relatives. Atwell *et al.* (2014) used the distribution of each species in different climates to estimate the best candidates for stress tolerance, and *O. granulata* is suggested as a possible source for cold tolerance. *O. eichingeri* also grows in low temperature environments, and could be considered a good candidate (Atwell, Wang, and Scafaro 2014) However, screening for cold tolerance is still lacking for most *Oryza* species.

One genotype of *O. rufipogon*, named Dongxiang wild rice, is able to withstand overwintering in its natural habitat and temperature as low as 3°C for three days in laboratory conditions (Mao et al. 2015; Xiao et al. 2015). Using an experimental population derived from Dongxiang wild rice ×

anjing 11 (a cold sensitive cultivar) crosses, a *CBF3/DREB1G* gene was found to co-localize to a previously identified cold-related QTL (Xiao et al. 2015). CBF/DREBs are known regulators of cold and other abiotic stress responses (Mao et al. 2015; Mickelbart et al. 2015). Interestingly, *CBF3/DREB1G* is up-regulated as early as three hours after cold treatment in both Dongxiang wild rice, but only after 12 hours in the sensitive one. Genes known to be downstream of *CBF/DREB1* in the cold response are up-regulated accordingly in both tolerant genotypes (Xiao et al. 2015). Other QTLs unique to Dongxiang wild rice were described (Mao et al. 2015).

Recently, a SNP associated with temperate *japonica* cold tolerance was described. COLD1 (*chilling-tolerance divergence*) is a plasma membrane- and endoplasmic reticulum-localized regulator of G protein that activates Ca^{2+} influx during cold sensing. One SNP that results in an amino acid change was found to be responsible for the difference in cold tolerance. The SNP found in *japonica* is shared with accessions of *O. rufipogo*n, but not with *O. nivara* or *O. barthii* (AA genome; (Hirabayashi et al. 2015). Thus, the *COLD1* sequence found in tolerant *japonica* cultivars represents an ancient allele from *O. rufipogon* that was selected during domestication (Hirabayashi et al. 2015).

2.3.4 Drought

Rice in particular demands great amounts of water for proper development, owing to its shallow roots compared to other crops (Kondo, Murty, and Aragones 2000). QTLs for drought tolerance were identified within *O. sativa* variability, and causative genes have been cloned (Singh et al. 2015; Uga et al. 2013). DRO1 (DEEPER ROOTING 1), a previously unknown protein, is responsible for downward growth of rice roots, and introgression of *DRO1* in otherwise shallow root rice genotypes increases root angle and drought tolerance (Uga et al. 2013).

Species that are present in low moisture regions were suggested as more likely candidates for drought tolerance, namely: *O. barthii, O. australiensis, O. glaberrima, O. longistaminata* and *O. punctata* (Atwell et al 2014). At least three of these (*O. australiensis, glaberrima* and *longistaminata*), plus *O. meridionalis*, present thick leaves and high mesophyll conductance to CO_2 diffusion, indicating that they might be drought tolerant, since these traits can be associated with a higher water use efficiency (Giuliani et al. 2013; Scafaro et al. 2011). Indeed, field evaluation of *O. glaberrima* showed that some accessions could be used as donors in crossing with *O. sativa* for drought tolerance breeding (Ndjiondjop et al. 2010).

The *O. rufipogon* genotype Dongxiang was also used to breed drought tolerance in *O. sativa*. An introgressed line was shown to be more tolerant when compared to the *O. sativa* recurrent parent, with higher survival rate, along with higher proline and soluble sugar accumulation (Zhang et al. 2014). Eight accessions of *O. rufipogon* and one of *O. officinalis* have been tested for tolerance, and it was observed that accessions from tropical origin are more tolerant. Interestingly, the single *O. officinali* accession performed even better under drought conditions (Feng et al. 2012).

Another study analyzed leaf rolling score and relative water content in several *O. sativa, O. rufipogon* and *O. nivara* genotypes from India, and associated these traits with sequence diversity of *OsDREB1F*, a known drought stress-responsive transcription factor (Singh et al. 2015; Wang et al. 2008). At least five truncated versions of *OsDREB1F* were found to be associated with drought sensitivity. Interestingly, one protein variant, present in four *O. nivara* accessions, was associated with high relative water content and low leaf rolling score. The variant harbors an amino acid mutation in a putative activation domain, which, based on molecular modeling, is likely to affect the tertiary structure of the protein (Singh et al. 2015). The over-expression of *OsDREB1F* in *O. sativa* confers tolerance to drought, low temperature and salt stresses (Wang et al. 2008).

2.3.5 Heat

O. meridionalis (AA genome), is endemic to hot regions of northern Australia, and is described as heat tolerant (Scafaro et al 2010). When compared to *O. sativa, O. meridionalis* leaf elongation rate is slower under 27°C, but faster at 45°C. The photosynthesis temperature optimum of *O. meridionalis* is 3°C above that of *O. sativa*, which is accounted for by a higher RuBisCO activation state under heat stress (Scafaro et al. 2012). Proteomics analyses showed that Calvin Cycle and heat shock-related proteins increased their abundance in *O. meridionalis* leaves at high temperatures, including Rubisco activase. Thus, higher Rubisco activase accumulation is directly involved in heat tolerance of *O. meridionalis*, maintaining RuBisCO carboxylation at higher temperatures (Scafaro et al 2010).

A useful trait to avoid flowering at high temperatures is early morning flowering (EMF). The wild rice species *O. officinalis* shows EMF, which can be used to increase cultivated rice fertilization and yield by escaping heat stress (Ishimaru et al. 2010). Indeed, introgression lines were produced from *O. sativa* × *O. officinalis* crosses, and these showed increased fertility due to the shift in

anthesis timing (Ishimaru et al. 2010). QTL analyses revealed that an *O. officinalis* EMF candidate gene is located in chromosome 3 and reduces the flowering opening time by 1.5 to 2 hours in both temperate and tropical cultivars, thus demonstrating the usefulness of this trait to reduce effects of heat stress on spikelet sterility (Hirabayashi et al. 2015).

2.4 Use of *O. coarctata* as a source of salt tolerant genes and defense mechanisms

O. coarctata is adapted to high saline environments by using multiple mechanisms to cope with salt stress. Leaves of *O. coarctata* contain "salt hairs", outgrowths of the epidermis that increase their number under high salinity and secrete excessive salt (Bal and Dutt 1986; Flowers et al. 1990). Two different types of hair are actually found on the upper and lower surface of leaves in *O. coarctata*. The upper surface hairs are finger-shaped whereas the lower surface hairs are peg-like. When salt concentration in the growth media is high (300-400 mM), the salt hairs in the abaxial (lower) surface collapse and fall off from the leaf surface (Sengupta and Majumder 2009). Still, total Na+ concentration in leaves of *O. coarctata* does not increase significantly under salt stress, indicating that *O. coarctata* avoids Na+ toxicity in mesophyll cells by compartmentalization of salt in epidermal hairs, a mechanism similar to what is known for other halophyte grasses (Sengupta and Majumder 2009). In addition to its morphological adaptations, many physiological ones have been reported for *O. coarctata* including the maintenance of a low Na^+/K^+ ratio, a high photosynthetic efficiency, maintenance of relatively higher water content, and efficient functioning of vacuolar *H^+-ATPase* under salinity stress (Senthilkumar et al. 2005). Some *O. coarctata* proteins involved in ROS detoxification, photorespiration, cell wall biosynthesis, are highly upregulated under salinity stress in leaves (Garg et al. 2014; Sengupta and Majumder 2009). Much physiological and molecular research remains to be done to decipher the reasons for high salinity tolerance in this wild rice.

2.4.1 Wide hybridization between *O. sativa* and *O. coarctata* for transferring salt-tolerant phenotypes

In cereals, interspecific and intergeneric hybridizations (wide crosses) have been used as starting points to widen the genetic base of a crop and to construct stocks for genetic analysis. In bulbosum technique (Barclay 1975) after interspecific fertilization, two different parental genomes are combined within one nucleus, which, in most cases, is embedded within the maternal cytoplasm (Gernand et al. 2005). In grasses, a partial somatic elimination of chromosomes from one parental

species may occur, for example, in wide crosses of *Hordeum lechleri* × *H. vulgare*, *Avena sativa* × *Zea mays* or *Triticum aestivum* × *H. vulgare*. Complete uniparental chromosome elimination also occurs in some interspecific hybrids between closely related species *H.vulgare* or *H. parodii* × *H. bulbosum* and *H. marinum* × *H.vulgare*; as well as between remotely related parental species like wheat × *Pennisetum glaucum (Gernand et al. 2005)*. This has been referred to as the bulbosum technique.

Hybridization between *O. coarctata* and rice is very challenging because of their different ploidy levels and genomic distance. Any putative hybrids by traditional breeding will not form commercially suitable varieties so easily but can be used as donors for breeding programs for introgression of salt tolerance characteristics. However, the salt tolerant hybrids so-produced will need to be characterized with respect to chromosome and genomic content, before these can be used in breeding programs.

Attempts have been made to transfer the salt-tolerant character to cultivated rice, through wide hybridization procedures. Attempts at hybridization of *O. sativa* with *O. coarctata* resulted in production of sterile progenies (Jelodar et al. 1999). Recently however, the IRRI website has reported one fertile progeny out of 34,000 crosses between IR56 and *O. coarctata* (Jena 1994). Successful pollen growth after hybridization only occurred when *O. coarctata* was used as the female parent in wide crosses with *O. sativa* (Sarker et al. 1993). The progenies, which looked like *O. coarctata* and propagated like it through rhizomes, showed loss of the *O. sativa* chromosomes in 2-3 generations (Seraj et al. 1996).

2.4.2 Transcriptome and proteomic analysis of *O. coarctata* for promising genes and pathways

Proteomic analyses identified up-regulated proteins by Na treatment in *O. coarctata*, including transcription factors of the CBF/DREB pathway of abiotic stress response (Shinozaki and Yamaguchi-Shinozaki 2000); a cellulose synthase-like, which could help maintaining cellulose synthesis during salt stress (Endler et al. 2015); and an L-myo-inositol 1- phosphate synthase, important for inositol synthesis. Inositol derivative, pinitol, is a known osmoprotectant in many plant species, and accumulates in *O. coarctata* under high salinity (Sengupta and Majumder 2009; Sengupta et al. 2008).

The proteomic study by Sengupta and Majumdar 2009 has led to identification of 16 differentially upregulated proteins at 200mM salt-stress involved in osmolyte synthesis, photosystem functioning, RubisCO activation, cell wall synthesis and chaperone functions. Among them Ribulose bisphosphate carboxylase/oxygenase activase, Ribulose bisphosphate carboxylase large chain precursor, Glutamine synthetase, chloroplast precursor (EC 6.3.1.2), RuBisCO activase small isoform precursor, CRT/DRE-binding protein, Inositol 1-phosphate synthase, Heat shock protein 70, Alcohol dehydrogenase 1 and Sucrose synthase 2 can be great candidate genes for potential introgression into commercial rice. Root tissue specific metabolomics study showed a comparative variation in the number of metabolites like gelsemine, allantoin, benzyl alcohol, specific phospholipids, and glycerolipids, that may play a role in maintaining the superior growth of *O. coarctata* in salt (Tamanna et al. 2024).

More recently, a study evaluated *O. coarctata* transcriptomic changes under salt and submergence stresses (alone or combined, compared to control conditions), and found several transcription factors up-regulated in leaves under stress conditions, such as *NAC, WRKY* and *MYB* gene family members, indicating extensive transcriptional regulation in stress responses (Garg et al. 2014). Gene Ontology analyses showed enrichment of ABA-responsive genes under salinity stress, and of carbohydrate metabolism and anaerobic respiration genes under submergence stress. In plants under submergence stress, genes related to ethylene and gibberellin responses were also identified, along with Alcohol Dehydrogenase, a marker for anoxia stress, indicating that a SUB1A-related response might be present in *O. coarctata* (Garg et al. 2014). However, demonstration of the presence of SUB1A-like ERF transcription factors and of their role in submergence response in *O. coarctata* is lacking. Moreover, suberin and cellulose synthesis-related transcripts were identified as up-regulated in both stresses, indicating that these processes might be key for stress tolerance (Endler et al. 2015; Garg et al. 2014).

A vacuolar *NHX* homolog, *PcNHX1,* isolated from a *O. coarctata* showed diurnal expression in leaves and was found to be largely unaltered though damped by salinity, also expressed in stem and root in salt stress (Kizhakkedath et al. 2015). Cloning and characterization of *O. coarctata L-myo-inositol 1-phosphate synthase* (*INO1*) showed that enzyme activity is maintained properly even in high salt concentrations, and that its expression in plants and bacteria confers high, albeit variable, salt tolerance to these organisms (Majee *et al.* 2004; Das-Chatterjee *et al.* 2006; Sengupta and Majumder 2010). Introgression of *PcINO1* gene encoding L-myoinositol- 1-phosphate

synthase (MIPS) from *O. coarctata* in *Brassica juncea* demonstrated increased tolerance to salinity and oxidative stress with elevated level of inositol in both roots and shoots. The yield and crop quality of transgenic Brassica plants remain uncompromised and the plants were able to stably grow, set seeds and germinate in saline environments. Transgenic *Nicotiana tabacum* with *PcINO1* was able to improve the seed survival rate under salinity and dehydration. *Inositol methyl transferase 1 (PcIMT1)* implicated in the synthesis of pinitol has been cloned from *O. coarctata*, bacterially overexpressed and shown to be functional in vitro. Both transcript and proteomic analysis show the up-regulation of *PcIMT1* expression following exposure to salinity. Coordinated expression of L-*myo*-*inositol* 1-phosphate synthase (*PcINO1)* gene along with PcIMT1 indicates that in P. coarctata, accumulation of pinitol via inositol is a stress-regulated pathway (Sengupta et al. 2008). Chloroplastic fructose 1,6-bis phosphatase (PcCFR) of *O. coarctata* has a difference of five amino acid residues compared to *O. sativa*. The *PcCFR* transgenics showed better plant growth during exposure to salt stress, enhanced photosynthetic efficiency and better reactive oxygen species scavenging (Chatterjee et al 2013).

2.5 Promising genes from *O. coarctata* for cloning and developing salt-tolerant rice varieties.

2.5.1 Abscisic acid stress ripening protein (*Asr1)*

Plant cells react to mild and severe dehydration by initiating several signal transduction pathways, which results in the accumulation of different proteins, sugar molecules and lipophilic anti-oxidants. One of the major abundant proteins found under stress are members of the ancestral ASR (ABA-stress-ripening) family. Interestingly, this family of protein is conserved in the plant kingdom but lacks orthologs in *Arabidopsis*. The *asr1* cDNA was first identified in tomato (*Solanum lycopersicum*), abundant in both stressed leaves and ripe fruits (Iusem et al. 1993). Tomato *Asr* genes are short with a very simple structure: two exons separated by an intron (Gonzalez et al. 2011). In particular, the *Asr2* genomic sequence is 73 % homologous to *Asr1* (Rossi and Iusem 1994), with an AT-rich regulatory region that has been studied in depth (Rossi et al. 1998; Rossi and Iusem 1995). For example, the *Asr2* promoter has a putative ABA-response motif, and intriguingly, its 3′ untranslated region (3′ UTR) shares 92 % homology with an intron from the polygalacturonase gene, whose expression increases during fruit ripening (Rossi and Iusem 1995). A deeper examination of the cell types involved revealed *Asr1* mRNA in leaf

vascular tissue irrespective of stress (Maskin et al. 2008) and in tomato seeds (Maskin et al. 2007). *OsAsr1* is induced by ABA, a situation that in turn leads to higher tolerance to water and osmotic stress (Joo et al. 2013a).

The landscape of the *Asr* gene family in rice (*OsAsr*) is different and is composed of six members (Philippe et al. 2010). In contrast to tomato *Asr* genes, the rice genes are located on different chromosomes, except for *OsAsr3* and *OsAsr4*, which are tightly linked on chromosome 1 (Philippe et al. 2010). In case of maize (Z*ea mays*), its *Asr* gene (*ZmAsr*) family consists of nine paralogous genes, thus being larger than in tomato (four members) (Frankel et al. 2006), banana (four members) (Henry et al. 2011) and rice (six members) (Philippe et al. 2010). *OsAsr1* is expressed mostly in leaves, whereas *OsAsr3* is preferentially expressed in roots. Together, these two proteins are the most abundant ASR proteins in rice, expressed in a variety of tissues. They are induced by abiotic stress but stimulated differentially by plant hormones (ABA or GA) and by different sugars, such as sucrose and glucose (Joo et al. 2013b). Of the six rice *Asr* genes, at least *OsAsr5* is induced by gibberellins (GA), as well as by ABA, suggesting this protein is involved in both stress tolerance and plant growth particularly in leaf expansion (Takasaki et al. 2008). In the presence of Aluminium ions, all six *OsAsr* genes increase their expression level, particularly *OsAsr5*, whose induction turned out to be the highest in Al resistant cultivars (Arenhart et al. 2013b).

The only extensively studied ASR1 protein, Tomato ASR1 protein turned out to be a small, highly charged 13-kDa polypeptide rich in Gly (7 %), Ala (13 %), Glu (15 %), His (15 %), and Lys (17 %) with an isoelectric point of 7.9 (Iusem et al. 1993). It exhibits two distinct domains: a DNA-binding domain located at the carboxyterminal end, and a zinc-binding domain capable of binding two Zn atoms at the amino-terminal end (Rom et al. 2006). ASR1 has been reported to bind in vitro to chromatin in a zinc-dependent fashion (Kalifa et al. 2004a). The physical association between DNA and both ASR1 monomers and dimers was directly observed in vitro by Atomic Force Microscopy at the single molecule level (Maskin et al. 2007) and most likely involved in the binding to the short consensus DNA sequence previously determined by SELEX (Kalifa et al. 2004a).

Recombinant ASR1, is an intrinsically unfolded monomer in vitro. In the presence of Zn^{2+} ions, it undergoes a conformational transition from unfolded to folded as it gains more α-helix and β-strand domains, which implies a more highly ordered polypeptide structure (Goldgur et al. 2007).

The grape (*Vitis vinifera*) ASR ortholog named *VvMSA* was claimed to bind to the enhancer of a sugar transporter gene (*VvHT1*), although the reported regulation was not clear (Cakir et al. 2003). Therefore, a transcription factor function was later proposed (Cakir et al. 2003), as was an ASR protein-mediated crosstalk between ABA and sugars (Carrari et al. 2004).

The targets of tomato leaf ASR1 turned out to be gene encoding proteins involved in water transport like aquaporins and cell wall remodeling proteins, namely cellulose synthase and glucanases (Ricardi et al. 2014). Interestingly, ASRs (supposedly the cytosolic pool) might also act as chaperone-like proteins. For example, tomato ASR1 protected reporter enzymes against freezing or heat denaturation in vitro (Konrad and Bar-Zvi 2008). Similar results were found with ASRs from plantain (Dai et al. 2011) and lily (Hsu et al. 2011). ASR from soybean exhibited a novel biochemical function: an antioxidant activity in vitro, but no model of underlying mechanism was formulated (Li et al. 2013). On the other hand, maize *Asr* genes seem to be involved in regulating the biosynthesis of branched-chain amino acids such as Val, Leu and Ile (Virlouvet et al. 2011).

Constitutive overexpression of an additional copy of maize *Asr1* gene exhibited a higher foliar senescence ratio than wild type plants under drought conditions (Jeanneau et al. 2002). Transgenic rice plants overexpressing an extra copy of their endogenous *Asr1* showed higher tolerance to cold stress in terms of photosynthetic efficiency (Kim et al 2009). Transgenic *Arabidopsis* plants carrying and overexpressing the *Asr* gene from lily exhibit reduced sensitivity to ABA, diminished levels of dormancy and increased resistance to salt, osmotic, drought and cold stresses (Yang et al. 2005). *Arabidopsis* plants were also engineered with the plantain (*Musa paradisiaca*) *Asr* transgene, which conferred resistance to osmotic stress (Dai et al. 2011). In another study also with tobacco, plants carrying the tomato *Asr1* showed tolerance to osmotic stress (Kalifa et al. 2004b). Leaves from these plants were been found to lose less water than wild type leaves, suggesting regulation at the stomatal level. When wheat *Asr gene* was overexpressed in tobacco, plant tissues showed higher water content and higher catalase and superoxide dismutase, resulting in higher tolerance to drought (Hu et al. 2013).

2.5.2 Subunit c of *Vacuolar H^+-ATPase (Pva1)*

Tonoplast of the plant cell separates the vacuole from the cytoplasm. Specific proteins present in the tonoplast drive the transport of ions and other metabolites across it (Sze et al. 1992). The

primary active transport process in the tonoplast is the action of two H^+ translocating enzymes (Rea et al 1987), i.e. *H^+-ATPase* and *H^+-PPase*. The *vacuolar H^+-ATPase (V-ATPase)* is a universal component, and it is involved in acidification of intracellular compartments of eukaryotic cells (Bowman et al. 1988) and pumps protons from the cytoplasm to the lumen against the electrochemical gradient using energy released by ATP hydrolysis, and regulates the cytoplasmic pH (Sze et al. 1992). When the levels of Na^+ become high in the cytoplasm the stored H^+ in the vacuole (due to the activity of V-ATPase) gets exchanged by Na^+. This sequestration of Na^+ in the vacuole saves the cell cytoplasm from its toxicity.

V-ATPase is a multi-subunit complex composed of two functional domains, the peripheral V_1 cytoplasmic domain, responsible for ATP hydrolysis and the membrane integral V_o domain, involved in proton translocation (Ratajczak 2000; Steven et al. 1997). Peripheral V_1 domain consists of at least eight different subunits of molecular weight 13-70 kDa, named subunits *A-H*. Membrane integral V_o domain consists of at least five subunits of 13-70 kDa, named subunit *a-d*. The major components of *V-ATPase*, subunits *A, B* and *c* are found in all *V-ATPases* studied so far and thus considered as essential subunits. The V_o domain has been shown to contain the binding site for bafilomycin (Crider et al. 1994). The 16 kDa polypeptide subunit *c (pva1)* is particularly interesting as it is a major component of the membrane V_o domain, and it plays a major role in forming the proton conductance pathway (Crider et al. 1994; Zhang et al. 1994). The 16 kDa polypeptide is highly hydrophobic, initially cloned from bovine adrenal cells (Mandel et al. 1988) and has been widely studied in yeast (Nelson and Nelson 1989) and also in higher plants (Lai et al. 1991).

The proton conductance and bafilomycin binding by V_o domain of *V-ATPase* has been extensively studied by Zhang *et al* and Crider et al. To determine whether the V_o domain contains the information necessary to form a passive proton channel, the V_o domain subunits were dissociated from each other and separated by gel filtration. Reconstitution of the V_o subunits separately revealed that vesicles containing 17/19-kDa subunits showed dicyclohexylcarbodiimide-inhibitable passive proton transport whereas the loo-, 38-, or 100/38-kDa subunits were unable to form passive proton channels. Partial separation of the 17- and 19-kDa subunits reveals that the 17-kDa subunit alone is able to carry out proton conduction, which is enhanced in the presence of the 19-kDa subunit. These results suggest that the 17-kDa subunits form the minimal unit

necessary for passive proton transport but that the loo-, 38-, and 19-kDa subunits are able to enhance the proton conductance through reassembled V_o (Zhang *et al.* 1994; Crider *et al.* 1994).

The ability of plants to adapt and grow in a variable environment suggests that *V-ATPase* is subject to regulation by hormones and environmental factors (Sze et al. 1999), and some evidence is available for the enhancement of *V-ATPase* activity in response to stress due to chilling and salinity. *ATPase* itself undergoes changes in its fine molecular structure (Luttge and Ratajczac 1997).

The first vacuolar *H+ATPase subunit c, PcVHA-c1* was cloned from halophytic wild rice *O. coarctata, as* reported by *Senthilkumar et al*. Changes in the transcript levels for the different subunits of *V-ATPase* were reported when the plants were exposed to salinity. The *PcVHA-c1* transcript level was increased in *O. coarctata* leaf to two-fold after 24 hours and three-fold after 48 hours of 500mM salt treatment. The transcript level was increased two-fold after 5 hours in roots, indicating the response in roots was immediate and more pronounced. After salt withdrawal, the transcript level started decreasing. These results confirm that the upregulation of *PcPVA-c* in leaf and root was salt-specific (Senthilkumar et al. 2005). In *M. crystallinum*, transcript levels of *V-ATPase* subunits, particularly of *subunit c,* are markedly increased by salinity stress in mature leaves (Low et al. 1996). Coordinated upregulation of several *V-ATPase* subunits also have been reported upon imposed salt stress. For example, studies have revealed a significant increase in levels of *V-ATPase subunits A and B* in salt-treated sugar beet plants (Kirsch et al. 1996).

More than one hybridizing fragment with the full length gene probe has been found in *O. coarctata*, which indicates the possibility that a multi-gene family encodes the *subunit c V-ATPase* in *O. coarctata*. The complex genomic organization of this gene could be due to presence of more than one copy of this gene or to the tetraploid nature of *O. coarctata* (2n = 4x) (Senthilkumar *et al.* 2005). Multigene family has been reported for the *c subunit the V-ATPase* in *A. sativa* (Lai et al. 1991), *A. thaliana* (Perera et al. 1995) , *Beta vulgaris* (Kirsch et al. 1996), *K. daigremontiana (*Bartholomew et al, 1996), *M. crystallinum* (Tsiantis et al. 1996), and from *G. hirsutum* (Xiao 1995). In contrast, *S. cerevisiae* (Nelson and Nelson 1989), *Drosophila melanogaster* (Meagher et al. 1990), *Neurospora crassa* (Sista et al. 1994) all possess a single gene copy for the *V-ATPase subunit c* polypeptide. In **rice (*Oryza sativa*),** the **V-ATPase subunit c** is encoded by **3 genes (Hanitzsch et al., 2007** and **Schumacher & Krebs, 2010).**

2.5.3 Metallothionein type 3 protein (MT3)

Metallothioneins (*MT*s) are low molecular weight, cysteine-rich metal chelators. They are able to bind a variety of metal ions by the formation of mercaptide bonds between numerous Cysteine residues present in the proteins and the metal, and thus contribute to metal detoxification by buffering cytosolic metal concentration (Cobbett and Goldsbrough 2002). They are widely distributed in animals, plants, fungi as well as cyanobacteria. Plant MTs identified so far contain two Cysteine-rich domains and a large spacer region (30–50 amino acid residues, devoid of cysteine) (Freisinger 2009; Hassinen *et al*. 2011). These MTs consist of only a few histidines, Cys content varies between 10 and 17 residues and aromatic amino acids vary from none to several. Based on these, plant MTs are classified into four types, type 1 through 4 (Cobbett and Goldsbrough 2002; Hassinen et al. 2011; Palmiter 1998). Analysis of various expressed sequence tag (EST) databases shows that MTs are amongst the highly abundant transcripts in plants (Matsumura et al. 1999).

The role of plant MTs in abiotic stresses such as oxidative, dehydration, senescence as well as hormonal alterations have been established besides heavy metal tolerance (Zhigang *et al.* 2006; Yang *et al.* 2009; Xue et al. 2009). Furthermore, plant MTs are also involved in fruit development, root development and suberization (Chiang et al. 2006; Xue et al. 2009; Guo et al. 2008). The antioxidant function of MTs is attributed to the presence of a large number of cysteine residues, which besides metal binding are also capable of ROS scavenging (Hassinen et al. 2011). Recently, a type 1 MT from mustard i.e. LSC54 has been reported to be induced by ROS production (Navabpour et al. 2003). Further, LSC54 has also been documented to be related to ROS imbalance during leaf senescence. Similarly, in rice, several type 1 and type 2 MTs have been found to play a direct role in antioxidation (Zhou et al. 2005).

A type 1 MT– *OsMT1e-P* isolated from a salt tolerant genotype i.e. *O. sativa* cv. Pokkali has been reported to be induced strongly in response to salinity stress. The molecular response of rice seedlings towards salinity stress based on their subtractive transcriptome profiling have been characterized (Kumari *et al.* 2009). Ectopic expression of *OsMT1e-P* in transgenic tobacco (under the control of *35S* constitutive promoter) provides stress tolerance against salinity, drought, cold, heat and heavy metals (Cu^{2+} and Zn^{2+}). *OsMT1e-P* over expressing plants accumulate lower

amounts of ROS (H_2O_2) under salinity stress. At 100 mM salt-stress, a strong difference in seedling growth was observed between wild type and transgenic lines. Transgenic plants grew normally, flowered and produced seeds even in the presence of 200 mM NaCl, whereas wild type plants exhibited stunted growth, failed to flower and completely senesced before reaching maturity; wild type seedlings just could not grow in the presence of higher levels of salinity (300 mM NaCl), but most of the seedlings of transgenic lines were able to grow well under similar conditions. Considering all these, *OsMT1e-P* has been proposed to be an important "candidate gene" for raising 'multi-stress tolerant' crops (Kumar *et al.* 2012). Another rice *type I metallothionein*, *OsMT1a*, has been reported to provide tolerance against drought and oxidative stress besides being involved in Zn^{2+} homeostasis and its overexpression leads to higher Zn^{2+} accumulation in rice seeds (Yang et al. 2009).

A type 3 metallothionein protein, isolated from cotton, *GhMT3a*, was induced by several abiotic stress factors, including salinity, drought, and low temperature, and these induced expression patterns of *GhMT3a* could be inhibited in the presence of antioxidants. Recombinant *GhMT3a* protein showed an ability to bind metal ions and scavenge ROS in vitro. Transgenic tobacco and yeast that overexpress *GhMT3a* displayed increased tolerance to environmental stresses, indicating its role in response to abiotic stresses is by mediating the ROS balance as a ROS scavenger in plants (Xue et al. 2009). Thirteen MT genes and fifteen protein products have been found in *O. sativa* sp. Japonica (Kumar et al. 2012). In *Arabidopsis*, nine MT members have been reported (Zimeri and Dhanker *et al.* 2005). The presence of multiple *Metallothionein* genes may indicate their diverse and overlapping biological roles by the regulation of gene expression and signaling (Xue *et al.* 2009).

So far, only one *Metallothionein*, *MT* type3 has been reported from *O. coarctata*. The *PcMT3* cDNA (581 bp) encodes a protein of 64 amino acids. *PcMT3* is highly homologous (82 %) to *OsMT-I*-3a of rice, but is unique from other type 3 plant *MTs* due to the presence of an additional glycine residue in the C-terminal domain. Southern analysis suggested the presence of more than one copy of *PcMT3*-like sequences in the *O. coarctata* genome. The analysis also revealed the conservation of ten Cys residues, four at the N-terminal end and six at the C-terminus among the proteins analyzed. High levels of *PcMT3* transcript was also observed in the leaf tissues of plants exposed to heavy metals (Cd, Cu and Zn) which suggests that *PcMT3* may be involved in

maintaining the redox balance either by sequestering heavy metals or by directly scavenging deleterious oxygen radicals. The role of Metallothionein from *O. coarctata* in abiotic stress like salinity is yet to be studied (Usha et al. 2011).

2.6 Review on available salt-tolerant varieties released by BRRI and Bangladesh

Bangladesh Rice Research Institute (BRRI) has released eleven salt tolerant varieties. Among them there are five Aman, BR23, BRRI Dhan40, 41, 53 and 54. The aman genotypes are only slightly tolerant. They undergo a yield loss of >50% on exposure to 60mM salt stress. The released six Boro varieties are moderately tolerant, e.g., BRRI Dhan47, BRRI Dhan 55, BRRI Dhan 61, BRRI Dhan 67, BRRI Dhan 97 and BRRI Dhan 99. These undergo a >50% yield loss if exposed to 80 mM salt stress. Of all the varieties, only BRRI Dhan47 and BRRI Dhan67 have gained some popularity among farmers in the South during the boro season. And they can plant these if the saline levels do not go above 80mM. However the grains of BRRI dhan47 are coarse and it is prone to shattering. BRRI Dhan 67 is fine-grained and farmers are getting reasonable yields at 80mM, despite the loss in yield being up to 50%. Bangladesh Institute of Nuclear Agriculture (BINA) has released three salt-tolerant varieties named BINA Dhan-8, BINA Dhan-10 and BINA Dhan-23. BINA Dhan-23 can tolerate upto 80 mM salt; BINA Dhan-8 and BINA Dhan-10 have can tolerate up to 100 mM salt stress but with yield loss at the reproductive stage.

All of the available salt-tolerant varieties are developed by breeding approach. Traditional breeding approaches to develop salt tolerant varieties have been being applied for a long time but progress was slow due to the complexity of salt tolerance mechanism and genotype × environment interactions (Gregorio et al., 2002). Unfortunately breeding is only possible between two compatible species. Furthermore, with the adverse effect of climate change complication, the salinity level is increasing and more areas are being affected in Bangladesh. At present, river salinity is most severe in the southwest zone of Bangladesh (Satkhira, Khulna, Bagerhat, Barguna, Patuakhali). The soil salinity level ranges from 80-160mM, surface water salinity 150-300mM and ground water salinity 90-200mM (Rahman *et al*. 2017). The percentage of very high saline soil has increased from less than 1% in 1990 to 33% in 2015 which is very alarming for the agriculture growth in this area (Rahman *et al*. 2018).

Chapter three:

Promising characteristics of *Oryza coarctata*

3.1 Overview

In order to address modern agricultural problems, it is crucial to develop novel crop varieties resistant or tolerant to environmental stresses. For this purpose, scientists are exploiting all the available genetic diversity for key abiotic stresses, such as salinity, drought and extreme temperatures. *Oryza coarctata*, being the only halophytic members of genus *Oryza*, has been considered one of the most promising gene sources for several characteristics. In this chapter, we have explored some of the key phenotypes of *O. coarctata* which are unique compared to common high yielding rice varieties.

O. coarctata has special salt-hairs on both of its leaf surfaces, which can accumulate salt in their structures during salt-stress. The root and leaf structures of O. coarctata were analyzed anatomically and compared with common rice. We have also compared the leaf structure at different levels of salt-stress (100mM -400mM) to investigate their response to salt.

One of the key characteristics of *O. coarctata* is its maintenance of its internal low Na/K ratio despite the high environmental salinity levels. Moreover it can maintain this crucial ionic ratio despite its ability to desalinate the surrounding water or soil. We have analyzed and compared the desalinizing ability of *O. coarctata* with naturally salt-tolerant (Pokkali) and salt-sensitive but high-yielding rice varieties. Furthermore, we have used the desalinizing ability of *O. coarctata* to help grow a salt-sensitive, high yielding rice variety by pairing these with each other such that their respective soils are connected through the same nutrient solution.

Another key characteristic of *O. coarctata* is a better CO_2 fixation rate and efficient biomass production compared to *O. sativa*. As rice is a C_3 plant, we have investigated the photosynthesis type and rate of *O. coarctata* at different levels of salt-stress to find out whether it has any C_4-like characteristics which can be incorporated in *O. sativa*.

3.2 Method and materials

3.2.1 Collection of *O. coarctata* from coastal area of Bangladesh

We have collected *O. coarctata* plants from six regions of three districts, Khulna, Noakhali and Chittagong.

3.2.2 Establishing *O. coarctata* in University of Dhaka and International Rice Research Institute (IRRI)

The collected nine accessions of *O. coarctata* from six different regions used in this research were cultured in soil for 1-2 years in the net house of Plant Biotechnology Lab, University of Dhaka. For establishment of *O. coarctata* in hydroponic system Yoshida culture solution (Appendix 1) and Artificial Sea Water (ASW) (Appendix 2) both were used.

For establishment in IRRI, all nine *O. coarctata* rhizomes were sent to C4 lab, IRRI, Philippines by standard procedure. We were able to regrow only one accession (collected from Sonadia North) in the IRRI net house.

3.2.3 Anatomical features (leaves and roots) of *O.coarctata, O. sativa (*Latisail 2n, Latisail 4n, and high-yielding variety BRRI Dhan28)

Fresh materials were placed in water. Then free-hand sectioning was done. Flexible structures, such as leaves and roots require some support during sectioning. So, a young potato was used in this experiment. The material was inserted into the cut end of a young potato. The material and the surrounding potato tissue were then sliced thinly at the same time. Sections of dry material were first soaked in water to soften it and remove air from the cells. Then the section was placed on a clean slide in a drop of water and glycerin.

Leaf cross sections were viewed under Olympus Microscope Digital Camera Model DP21 (10× magnification) and root cross-sections were viewed under blue excitation U-MNB2 (excitation BP 460–490 nm, barrier filter IF 520, dichromatic mirror 500) using an Olympus BX51 photomicroscope and photographed with an Olympus DP70 camera (10× magnification).

The leaf bundle sheath structure of *O. coarctata* at different salt-stress has been photographed at C4 lab, IRRI with Olympus BX51 photomicroscope.

3.2.4 Analyzing desalinization ability of *O. coarctata* and comparing with naturally salt-tolerant and sensitive rice

O. coarctata has a remarkable ability of soil desalination like other halophytes which are thought to help other sensitive plants growing easily in the salinity prone areas when they are planted side by one another. Desalination experiment was done to understand whether *O. coarctata* can desalinate soil or not and to what extent compared to other salt sensitive (BRRI Dhan28) and salt tolerant (Pokkali) rice plants.

For this experiment, one-month old young *O. coarctata* was taken from soil, the soil was washed off under tap water and the roots placed in a hydroponic system in netted styrofoam. Three weeks after germination of BRRI Dhan28 and Pokkali, salt stress of 100, 200 and 300 mM were applied to all the plants. The process started with 2 dS/m (~20 mM) NaCl salt per day and gradually increased to reach 100, 200 and 300mM. Then, EC was checked every day up to 30 days to determine whether *O. coarctata* could desalinate water or not. All the varieties were also grown in hydroponic systems with no salt as control.

For each of the stresses applied, three replicate floaters were used where each floater contained three plants of the same age. In addition, 2 replicates of each conditions also used to check any fluctuation of EC due to environmental cues.

3.2.5 Reproductive screening of salt-sensitive rice variety using desalination ability of *O. coarctata*

After analyzing the desalination capability of *O. coarctata,* an experiment was designed to observe the growth and yield pattern of a high-yielding but salt-sensitive rice variety, BRRI Dhan28 while growing in a paired state with *O. coarctata*. The aim was to analyze the effect of desalination on the growth and yield pattern of a sensitive variety which could have profound utility in rice production in the coastal areas of Bangladesh.

At first, fresh and young *O. coarctata* plants were cleaned and established in the hydroponic system. Then those plants were transferred to the soil containing pots. Those plastic pots were specially designed for this experiment, to contain small holes (90-100) throughout the pots. The pots containing soil were the placed inside a large bowl containing water. The holes helped the plants growing within a single bowl to be in the same physical environment with each other. The

O. coarctata was kept in this soil system until it was established whichh required about a month . Meanwhile, BRRI Dhan28 seeds were germinated and grown in a hydroponics system for about a month and transferred to soil in the pots with holes. One-month old BRRI Dhan28 plants were transferred inside the bowl adjacent to the *O. coarctata* pots.

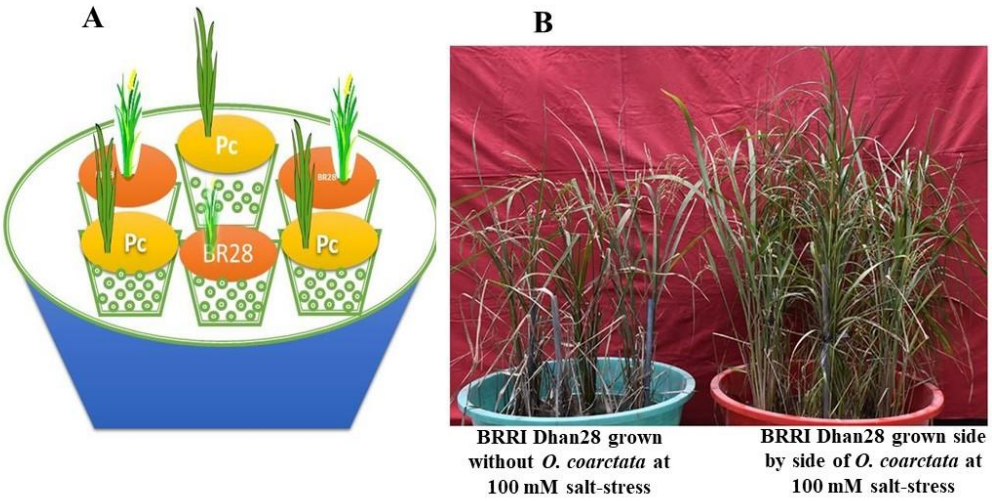


Figure 3.1: A) Experiment set up for desalinization test of *O. coarctata (Pc)* with sensitive rice variety BRRI Dhan28 (BR28). B) The large bowl contained water. Inside this, there were six pots filled with soil, three containing *O. coarctata* and three containing BRRI Dhan28. The control bowl contains six BRRI Dhan28 pots. Each of the pots contain 90-100 holes for up taking salt-solution from the large bowls.

Just before the booting stage, the water from the bowls was drained and replaced by 100mM and 150mM salt (NaCl) solution and the specific water level was maintained throughout their reproductive stage. After the seeds matured, they were harvested very carefully. We have analyzed yield data and percent fertility of BRRI Dhan28.

3.2.6 Leaf gas exchange experiment for analyzing CO_2 fixation ability of *O. coarctata* at different salt concentration

Salt stress hampers plant photosynthesis leading to death. But *O. coarctata* is an extreme halophyte and can happily grow in 400-500mM salt. This experiment aims to determine the CO_2 fixation rate of *O. coarctata* at 0, 100, 200 and 300mM salt-stress.

containing new fresh soil. Then pots are placed in a big bowl containing Yoshida solution and kept there for 10-12 days for their establishment in the new pots. After that, 100, 200 and 300mM salt were added to each bowl at a time. Two weeks after application of salt, CO_2 assimilation data was taken. Leaf gas exchange measurements were carried out using a portable open photosynthesis

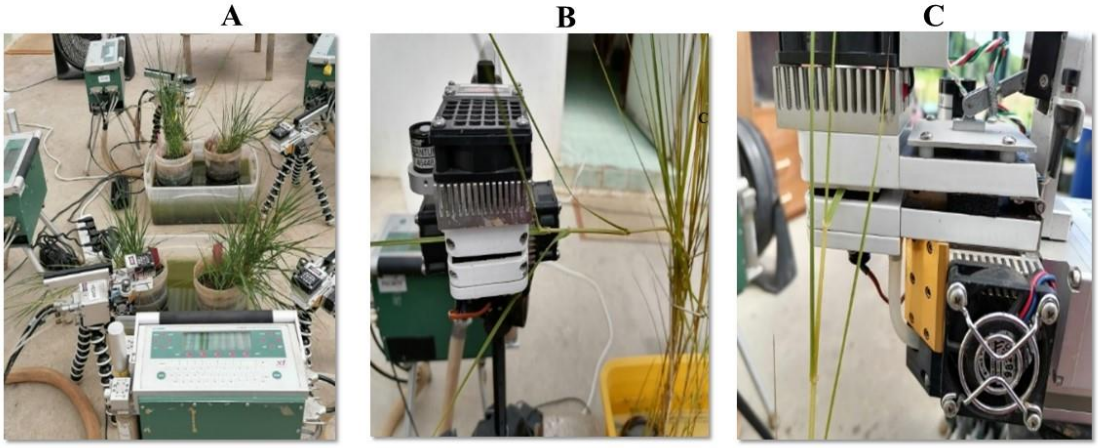


Figure 3.2: Leaf gas exchange experiment for analyzing photosynthetic ability of *O. coarctata* at 0, 100, 200 and 300 mM salt concentration. A) LI-COR instrument set up and experiment plants; B & C) The leaf used for measurement of CO_2 assimilation rate is placed inside the chamber.

system (LI-6400XT, LI-COR,Lincoln, USA). Measurements were conducted on the last fully expanded leaf (LFEL) attached to the main stem. In order to measure the responses of CO_2 assimilation rate (*A*) intercellular CO_2 was increased (*C*i) by raising the LI-6400XT leaf chamber [CO_2] in 10 steps (i.e.50, 100, 150, 200, 250, 325, 400, 650, 1200, and 1500 µl l−1) with 2 and 3 min as the minimum and maximum waiting times during each step change, respectively. The initial slope (IS) of each *A*-*C*i curve was estimated by fitting a linear model to the initial 3–4 linear data points. The maximum CO_2-saturated rate of each *A*-*C*i curve at each leaf temperature was considered as the CO_2-saturated rate (CSR).

Result: Promising phenotypes of *O. coarctata*

3.3 Results

3.3.1 Collecting *O. coarctata* from different coastal regions of Bangladesh and establishing them in University of Dhaka and IRRI

Oryza coarctata is endemic to the coastal region of Bangladesh, India, Pakistan and Malaysia. We had collected *O. coarctata* from nine different areas. The *O. coarctata* plants looked phenotypically different from each other depending on the area from which it was collected. We selected four areas from the Khulna region, three from the Noakhali region and two from Chittagong. Their accessions and area names are given in table 3.1.

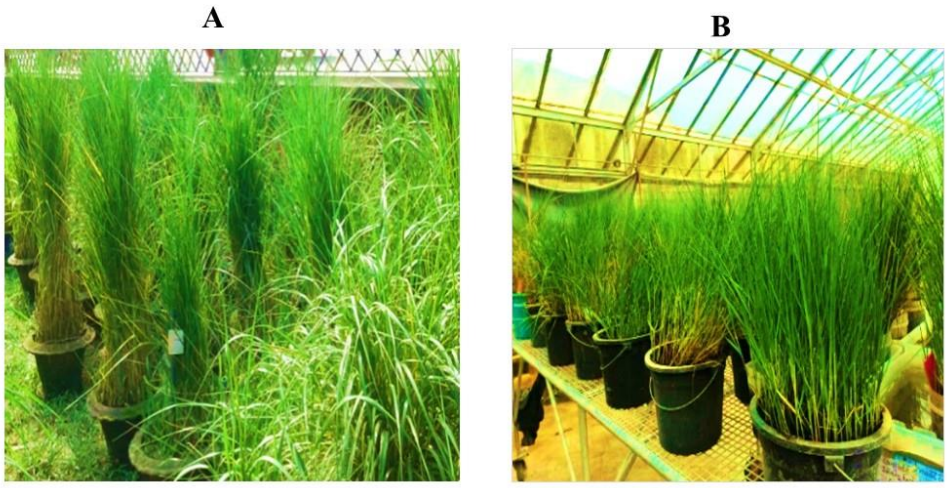


Figure 3.3: *O. coarctata* plants established in net house of (A) University of Dhaka and (B)International Rice Research Institute.

O. coarctata are medium in height (60-120cm). They look like grass, but in favorable growing condition they may grow taller. They have thick rhizomes and they propagate throw these rhizomes. They enable the plant to form new shoots and roots from nodes along the rhizome, leading to the development of new plants. They have succulent and waxy leaves. The leaves of *O. coarctata* are narrow, elongated and taper to a pointed tip, resembling the shape of a lance head. The leaves have parallel venation, which means the veins run parallel to each other from the base to the tip of the leaf. They can set only few rice like grains which normally do not germinate.

Table 3.1: Accession and area of collected *O. coarctata* from coastal region of Bangladesh for present study.

Serial	Accession	Region of Collection	Area of collection
01	KKRC1.1	Khulna(1)	Koyra
02	KMGC1.2	Khulna(1)	Munshiganj
03	KNBC1.3	Khulna(1)	Noabeki
04	KSKC1.4	Khulna(1)	Satkhira
05	NSNC2.1	Naokhali(2)	Sonadia North
06	NSSC2.2	Naokhali(2)	Sonadia south
07	NSGC2.3	Naokhali(2)	Sonagazi
08	CBKC3.1	Chittagong(3)	Bakkhali
09	CTNC3.2	Chittagong(3)	Teknaf

3.3.2 Analysis of anatomical features of leaves and roots of *O. coarctata* and comparison with *O. sativa* 2n (var Latisail), *O. sativa* 4n (var Latisail) and high yielding but salt-sensitive BRRI Dhan28

3.3.2.1 Leaf anatomy

Leaf and root anatomy are of paramount importance in the field of plant science for several reasons. The study of leaf anatomy provides valuable insights into the structure and function of leaves, which are critical for a plant's growth, development, and overall performance.

In the leaves of *O. coarctata*, the adaxial leaf surface shows deep invaginations with distinct ridges and furrows. The ridges show the presence of two vascular bundles each. The major vascular bundle in each ridge occurs abaxially, while the minor vascular bundle is oriented towards the adaxial leaf surface, below a patch of sclerenchymatous cells (SC). A layer of colourless bundle sheath cells (BSC) surrounds the vascular bundles. Epidermal cells (EC) in the ridges show numerous papillaceous (P) outgrowths.

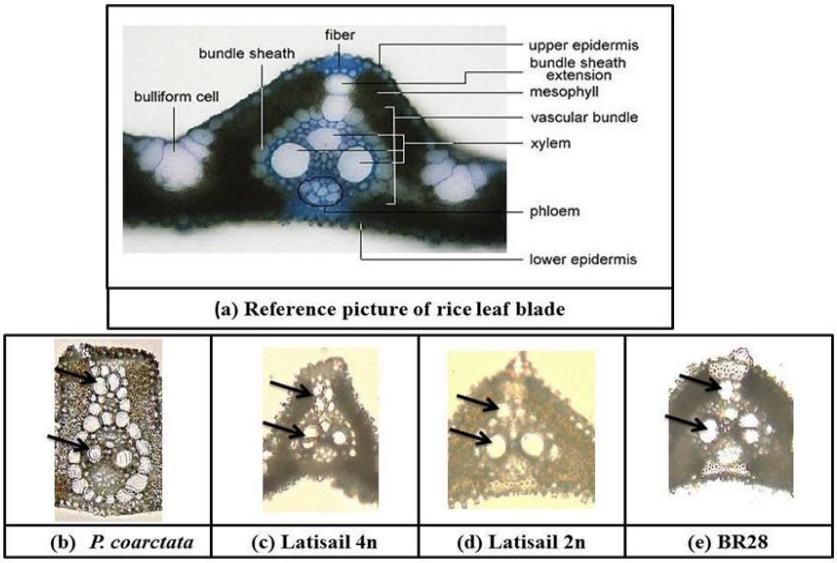


Figure 3.4: Anatomical characteristics of leaves of *O . coarctata* and other rice cultivars. **(a)** Reference picture of rice leaf blade indicating its different parts. **(b)** *O. coarctata* had square shape leaf blade with three xylem cells in the vascular bundle but more cells in the bundle sheath (extension). **(c)** Latisail (4n)(*O. sativa*) had four xylem cells in vascular bundle and also increased number of cells in bundle sheath extension. **(d)** Latisail (2n))(*O. sativa*) had three xylem cells in vascular bundle like reference picture. **(e)** BRRI Dhan28, salt-sensitive but high-yielding, had four xylem cells in vascular bundle.

The anatomy of the leaf blade of *O. coarctata* was different from other rice cultivars (Figure 3.4). The leaf blade of *O. coarctata* was square in appearance but in the other rice cultivars it was pyramidal in shape. In *O. coarctata* there was extensive bundle sheath extension. *O. coarctata* has three xylem cells, so was for Lat2n and Lat4n, but the sensitive BRRI Dhan28 has four xylem cells.

3.3.2.2 Root anatomy

Plant root anatomy is vital for understanding plant growth, nutrient and water uptake, adaptations to different environments and interactions with soil microorganisms. The anatomy of root structure of *O. coarctata* was also different from other rice cultivars (Figure 3.5). It has one xylem in the middle of root but in the other rice cultivars three or four xylem present in the center of root. It was also noticed that *O. coarctata* had thicker exodermis and bigger air sacs than other rice cultivars.

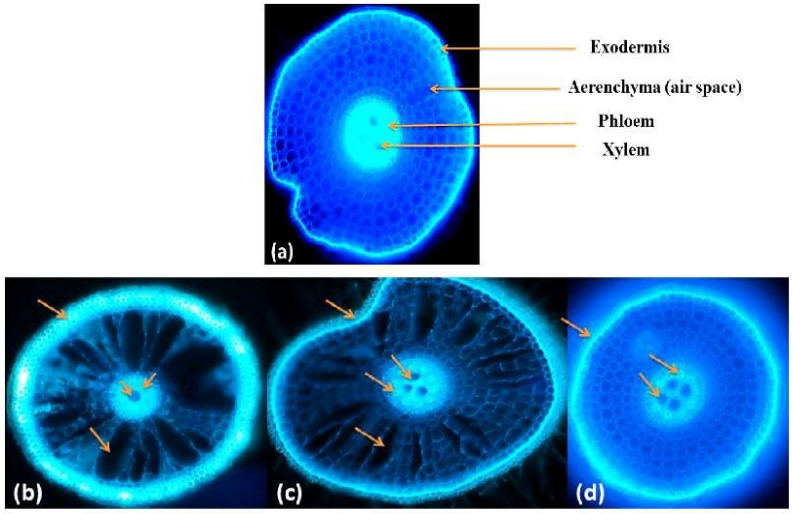


Figure 3.5: Transverse section of roots of *O. coarctata* and other rice cultivars. **(a)** BRRI Dhan28 had three xylem, presence of several phloem and thinner exodermis; **(b)** *O. coarctata* had one xylem, presence of phloem and thicker exodermis and bigger air sacs; **(c)** Latisail (4n) indicating that it had three xylem, presence of phloem and thinner exodermis and bigger air sacs. **(d)** Latisail (2n) had four xylem, presence of phloem and thinner exodermis.

3.3.3 Analyzing desalinization ability of *O. coarctata* and comparing with naturally salt-tolerant rice Pokkali and salt-sensitive but high-yielding rice BRRI Dhan28

O. coarctata plants were stabilized in soil for about a month and then established in the hydroponic system containing Yoshida culture solution. Desalination is a process that extracts mineral components from saline water. The desalinizing capability of *O. coarctata* compared to other salt-tolerant rice Pokkali and salt-sensitive but high-yielding rice BRRI Dhan28 plants (Figure 3.6).

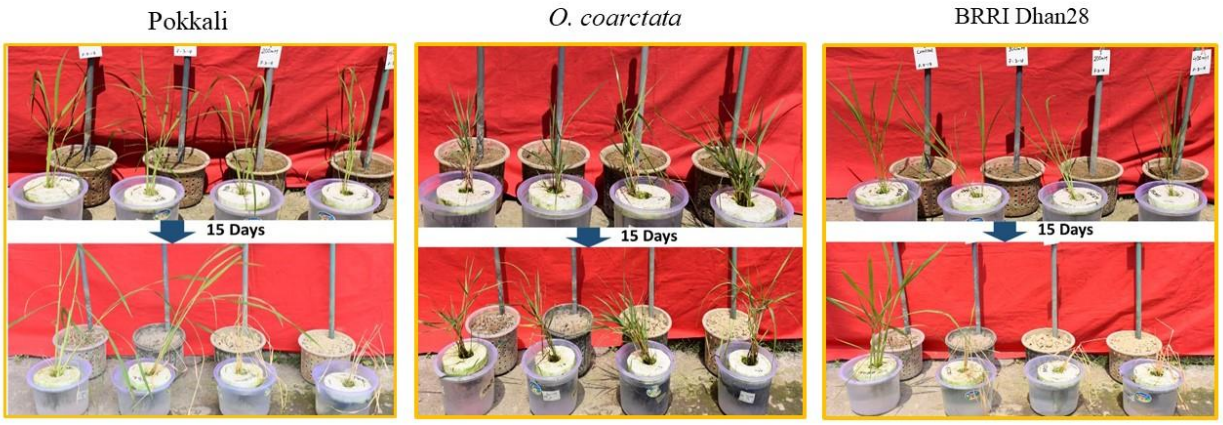


Figure 3.6: Desalinization experiment of (A)salt-tolerant Pokkali, (B) *O. coarctata*, and (C) salt-sensitive BRRI Dhan28. After 15 days of salt-stress only *O. coarctata* under all salt stress and Pokkali in 100mM salt stress were still surviving. Salt-sensitive BRRI Dhan28 died after 10 days and 8 days at 200 and 300 mM salt-stress respectively.

When 100, 200 and 300mM salt concentration were applied, the decline of electrical conductivity (EC) for *O. coarctata* was found to be 2.77, 5.37, and 8.51 dS/m respectively after one month and all the plants were still surviving till the end of the experiment. For BRRI Dhan28, the reduction was 0.97, 1.17, and 2.13 dS/m respectively after 10, 8 and 8 days at 100, 200 and 300mM salt stress. For Pokkali, EC reduction was found to be 2.5, 1.47 and 2.40 dS/m respectively after 30, 10 and 10 days (Figure 3.8). The EC of the control and blank remained more or less constant throughout the period.

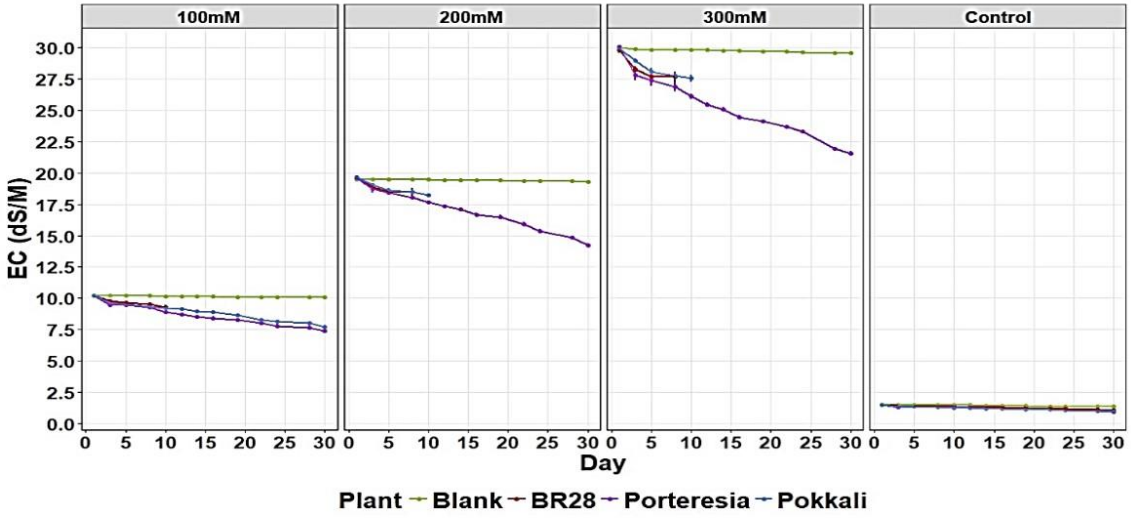


Figure 3.7: Decrease in Electrical conductivity from hydroponics containing *O. coarctata*, BRRI Dhan28(BR28) and Pokkali over a period of 30 days.

For *O. coarctata*, the average decrease rate of 0.0923, 0.179, and 0.2837 dS/m per day at 100, 200, and 300 mM salt respectively clearly demonstrated that the desalinization rate is increased with the increment of salt concentration in the hydroponic system. Pokkali and BRRI Dhan28 also shown desalinizing rate similar to *O.coarctata* but the survival rate is very low. Pokkali survived for 10 days at 200 and 300mM and for 30 days under 100mM salt stress. BRRI Dhan28 survived for 10 days at 100mM salt stress but only 8 days in 200 and 300mM salt stress (Figure 3.7). The desalination rate in Pokkali and BRRI Dhan28 is also higher at higher salinities but they could not survive long.

3.3.4 Reproductive screening of salt-sensitive variety BRRI Dhan28 using desalination ability of *O. coarctata*

This experiment was designed to observe the growth and yield pattern of a sensitive rice variety, BRRI Dhan28 while growing in presence of *O. coarctata*. *O. coarctata* and BRRI Dhan28 grown for about one month in hydroponics system, then transferred to soil in pots. The pots were placed in a large bowl coning salt solution. The plants were treated with 100mM and 150mM salt solution (NaCl). The plants were grown in this system till seed maturation of BRRI Dhan28.

In case of 100 mM salt stress, BRRI Dhan28 has showed significant increase in yield when grown side by side of *O. coarctata*. In 100mM salt-stress, yield of BRRI Dhan28 reduced to only 34% compared to no stress condition, but it has been increased to 67% when grown side by side of *O. coarctata*. In 150 mM stress condition, plant didn't do great but better than those without *O. coarctata*. Analyzing the reproductive data of yield and growth of sensitive rice variety BRRI Dhan28, it was found that while *O. coarctata* was present within a perfect distance in the media (100 mM salt stress), BRRI Dhan28 plants were benefited in their growth and development as well as in yields. Though while in the close proximity, *O. coarctata* probably harm the growth as it possessed a complex root system with vigorous growth.

Figure 3.8: Panicles of BRRI Dhan28 after reproductive screening of the variety using desalination ability for better yield. A) BRRI Dhan28 in control condition had shown yield of 5.528 g/plant; B) In 100mM salt-stress, without *O. coarctata* had shown yield of only 1.81 g/plant; C) In 100mM salt-stress, in presence of *O. coarctata* had shown yield of 3.73 g/plant.

Fertility is another important parameter. The percent fertility of the plants are also calculated and as predicted, the plants grown by side of *O. coarctata* did far better than those grown without *P. coarctata* (figure 3.10).

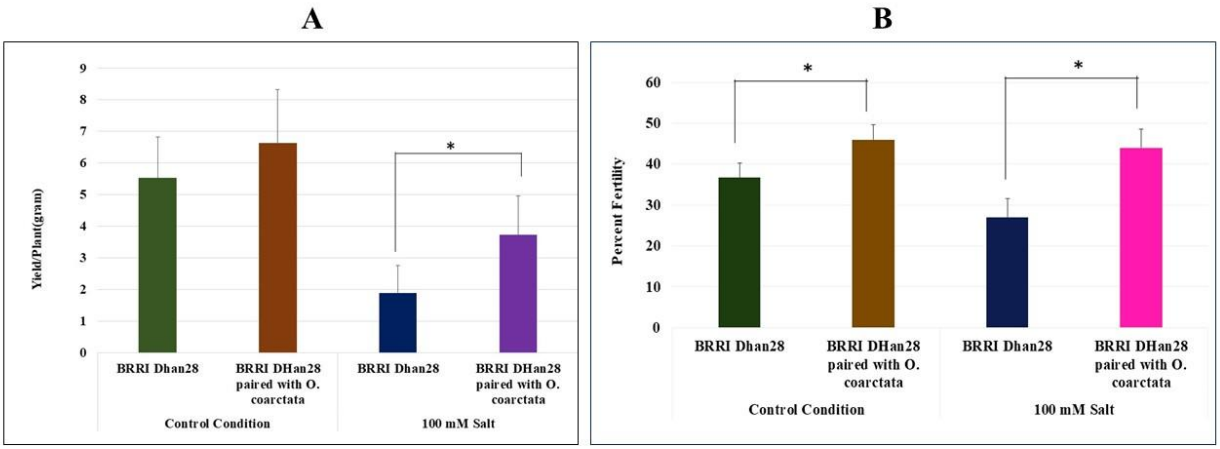


Figure 3.9: A) Yield per plant of BRRI Dhan28 in control condition and 100mM salt-stress grown with and without *O. coarctata. Due to 100mM alt-stress,* BRRI Dhan28 yield reduced to only 34%, but in presence of *O. coarctata* , the yield in increase to 67% *;* B) In 100mM salt-stress. Fertility is reduced to 18%, but the presence of *O. coarctata* side by side of BRRI Dhan28 helped to increase in fertility 72%. Moreover, in control condition the presence of *O. coarctata* has helped to increase in fertility by 24%.

The fertility of BRRI Dhan28 is decreased to 72% due to 100mM salt-stress. But when they are grown side by side of *O. coarctata*, their fertility is increased by 21.49% than in control condition. Furthermore, in control condition, the fertility of BRRI DHan28 is increased by 24% in presence of *O. coarctata*, which also prove their role in facilitating plant growth.

3.3.5 Characterization of *Oryza coarctata* based on their CO_2 fixation performances in salt-stress

O. coarctata has been analyzed for its photosynthetic performance at different salt concentrations. Analyses of A/Ci curves and light curve of photosynthesis have been very useful for for predicting photosynthetic responses to global change. Another important fact to consider is CO_2 compensation point, which differs in C_3 and C_4 plants. C_4 plants have a higher CO_2 assimilation rate and lower compensation point because of having higher CO_2 fixation system. In cultivated rice, photosynthetic activities are negatively correlated with the magnitude and duration of salinity stress (Yeo et al. 1985; Radanielson et al. 2018).

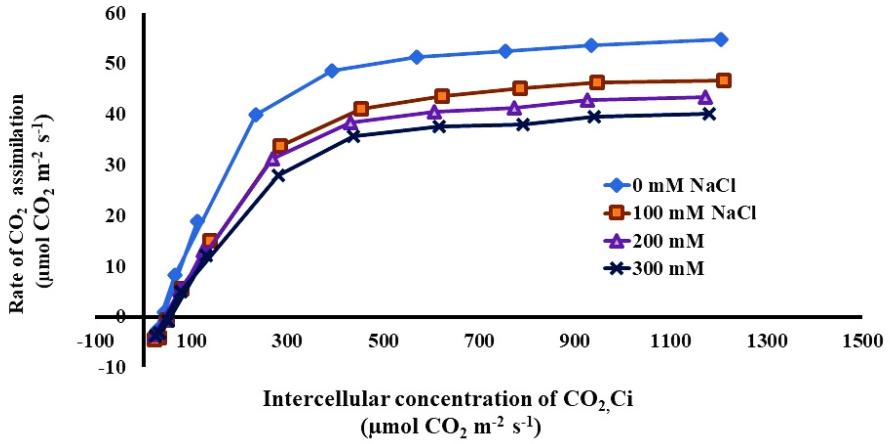


Figure 3.10A: A/Ci curve, showing comparative CO_2 assimilation rate at control, 100 mM salt, 200 mM salt and 300 mM salt stress

At 100 mM salt stress, maximum CO_2 assimilation rate (A_{max}) was 14% lower, at 200 mm salt 21% and at 300 mm salt 26% lower than the control condition (54.86 µmol m-2 s-1) (P value 0.008, 0.0002 , 0.008 respectively), whereas *O. sativa* dies only at 80 mM salt-stress (figure 3.10A). The compensation points (minimum CO_2 concentration needed for fixation by cell) are 38.4, 51.7, 48.7 and 52.1 µmol m-2 s-1 at 0,100, 200 and 300mM salt respectively (figure 3.10B).The compensation points similar to C_3 plants.

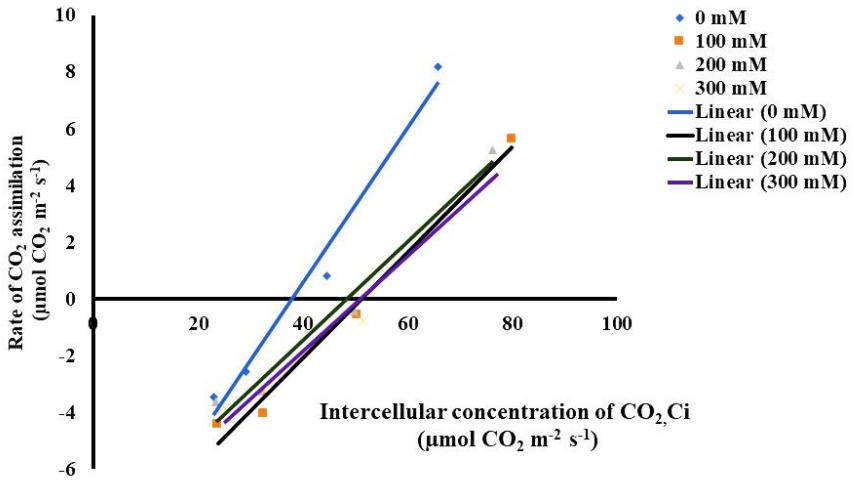


Figure 3.10B: Compensation point of *O. coarctata* at different salt-stress determined from A/Ci curve.

The light response curve of *O. coarctata* at control condition, 0 mM salt, 100 mM salt and 300 mM salt (figure 3.10C) shows that CO_2 assimilation rate is very strongly correlated with light (Correlation coefficient = 0.932). The curve shows maximum CO_2 assimilation rate is significantly reduced at 200 and 300 mM salt stress. At control condition, *O. coarctata* shows Jmax of 42.45 µmol m-2 s-1. But the rate is decreased by 19% at 100 mM salt, 31% at 200 mM salt and 32% at 300 mM salt stress (P value 0.034, 0.0009, 0.006 respectively).

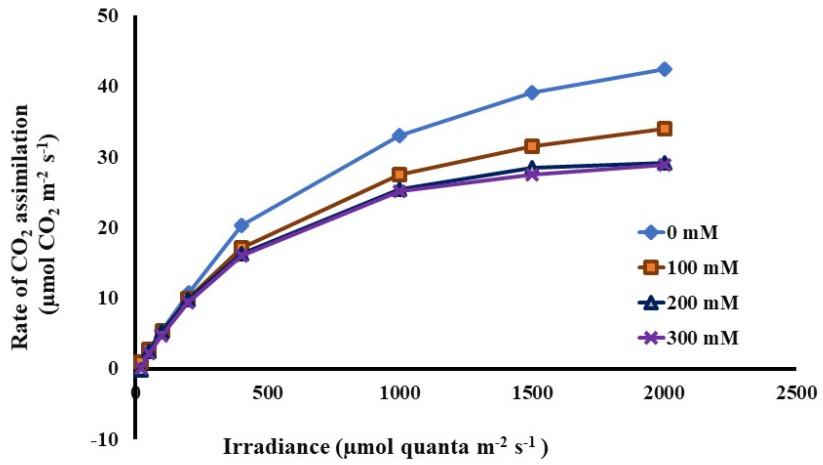


Figure 3.10C: Light response curve of *O. coarctata* at control , 100, 200 and 300mM salt stress. The photosynthesis rate is decreased with increased salt concentration.

Gas exchange analysis shows that *O. coarctata* is almost doubly photosynthetically active than normal rice at control condition. Even at 300 mM salt stress, its photosynthesis rate is decreased only by 26%. Considering A/Ci curve, light curve and photosynthesis rate, it can be said that, *O. coarctata* has a very active photosynthetic system, highly salt tolerant and grows happily in higher salt concentration like 300 mM salt stress.

3.4 Discussion

Oryza coarctata (Porteresia coarctata), the only halophyte in the Oryza genus, is a valuable genetic resource for salt tolerance in rice. It can survive up to 400 mM salt stress and maintain a low Na:K ratio (as low as 0.7) even in 25% artificial seawater (Flowers et al., 1990). O. coarctata naturally withstands prolonged submergence in saline tidal waters, particularly in the Sunderbans (Garg et al., 2014). Transcriptome analyses have identified approximately 15,000 genes responsive to high salt stress (Garg et al., 2014), providing potential candidates for developing salt-tolerant rice. Its unique physiological and genetic features make it a promising model for studying salinity tolerance mechanisms in monocot plants (Latha et al., 2004).

In this study, a series of experiments were conducted to functionally characterize *Oryza coarctata*, the only halophytic member of the genus *Oryza*, focusing on its potential as a genetic resource for improving rice salt tolerance. The results highlight multiple unique physiological and anatomical traits of *O. coarctata* that contribute to its exceptional adaptation to saline environments. These findings provide new insights into plant resilience under salt stress and suggest promising applications in future rice improvement programs.

Anatomical Adaptations and Salt Management

Anatomical analyses revealed significant differences between *O. coarctata* and cultivated rice varieties. The unique leaf structure of *O. coarctata*, characterized by deep adaxial invaginations, multiple vascular bundles per ridge, extensive bundle sheath extensions, and papillose epidermal outgrowths, suggests adaptations for salt secretion, mechanical support, and ion regulation. The square-shaped leaf cross-section and presence of specialized salt hairs likely contribute to minimizing salt toxicity through salt sequestration and excretion mechanisms, a feature absent in typical *O. sativa* cultivars.

The root anatomy further supports this adaptation. *O. coarctata* roots exhibited a single large xylem vessel, thickened exodermis, and prominent aerenchyma, promoting both salt exclusion and efficient oxygen transport in saline and waterlogged conditions. These structural features likely

reduce salt uptake while maintaining water and nutrient transport, allowing survival in high-salinity environments where conventional rice cannot persist.

Desalination Ability: A Novel Mechanism of Salt Stress Alleviation

A remarkable finding of this study is the desalination ability of *O. coarctata*. When grown in a hydroponic system, *O. coarctata* significantly reduced the electrical conductivity (EC) of the surrounding medium, indicating active removal or sequestration of salts from the environment. The rate of desalinization increased proportionally with the external salt concentration, demonstrating a robust physiological response to salinity.

This trait was superior in *O. coarctata* compared to both Pokkali (a salt-tolerant landrace) and BRRI Dhan28 (a high-yielding but salt-sensitive variety). While Pokkali showed some desalinization ability, it could not survive prolonged exposure to high salinity. BRRI Dhan28 exhibited minimal desalinization capacity and poor survival at salinities beyond 100 mM. In contrast, *O. coarctata* survived and continued desalination even at 300 mM NaCl, suggesting that it actively mitigates salt stress not only for itself but also for neighboring plants.

Facilitation of Growth and Yield in Salt-Sensitive Rice

A co-cultivation experiment further validated the ecological and agricultural importance of *O. coarctata*'s desalinization capacity (figure 3.1). BRRI Dhan28 plants grown side by side with *O. coarctata* exhibited significant improvements in growth, yield, and fertility under salt stress, particularly at 100 mM NaCl. Yield reduction in BRRI Dhan28 was mitigated from 66% loss (when grown alone) to only 33% loss when grown with *O. coarctata*. Fertility also improved substantially in the co-cultivation system, even under non-saline conditions, suggesting additional rhizospheric or microenvironmental benefits provided by *O. coarctata* (figure 3.8 & 3.9).

However, it was noted that close proximity may lead to competition due to *O. coarctata*'s vigorous root and rhizome system. Therefore, spatial arrangement must be optimized in potential agronomic applications to balance facilitation and competition.

Photosynthetic Performance and Salt Resilience

Photosynthetic performance analysis revealed that *O. coarctata* possesses exceptionally high CO_2 assimilation capacity compared to cultivated rice. Even at 300 mM NaCl, *O. coarctata* maintained 74% of its maximum photosynthetic rate, whereas *O. sativa* cannot survive beyond 80 mM salinity.

Although the CO_2 compensation points of *O. coarctata* remained similar to those of C_3 plants, the significantly higher Amax and Jmax values, coupled with minimal reduction under salt stress, highlight its superior photosynthetic efficiency. The strong correlation between light intensity and CO_2 fixation also underscores *O. coarctata*'s efficient light utilization, even under adverse conditions. This trait could be exploited for improving photosynthetic performance in rice under stress. Considering A/Ci curve, light curve and photosynthesis rate, it can be said that, *O. coarctata* has a very active photosynthetic system, highly salt tolerant and grows happily in higher salt concentration like 300 mM salt stress (figure 3.10A, 3.10B & 3.10C). It can be proposed that, *O. coarctata* has some C_4-like characteristics, most probably it uses C_3-C_4 intermediate carbon metabolism. C_4 plants-like characteristics should be explored in future like leaf anatomy and C_4 specific proteins specially enzymes of photosynthetic pathways. Candidate pathways and genes can be studied and used for developing new crop varieties.

3.5 Implications and Future Prospects

The comprehensive analysis of *O. coarctata* in this study reveals its multi-layered stress tolerance mechanisms, including:

- Structural adaptations for salt exclusion and water conservation
- Physiological capacity for active desalinization of the environment
- High photosynthetic efficiency under salinity stress
- Potential for facilitative interaction with salt-sensitive rice varieties

These findings open new avenues for using *O. coarctata* as a gene source for transgenic or marker-assisted breeding approaches. Genes related to its salt hairs, ion transport, CO_2 fixation efficiency, or root structure could be targeted for crop improvement. Additionally, the ecological model of co-cultivation could be developed for salinity management in rice fields, although further research is needed to refine intercropping strategies.

3.6 Conclusion

Overall, the characterization of *Oryza coarctata* presented in this thesis establishes it as a promising genetic and ecological resource for improving rice resilience to salinity stress. Its ability to not only tolerate but actively transform saline environments represents a unique trait in the genus *Oryza*. Future work should focus on genomic analysis, gene isolation, and field trials to translate these findings into sustainable agricultural practices.

Chapter four: Wide hybridization between *O. coarctata* and induced tetraploid *O. sativa* var. Latishail

4.1 Overview

Oryza coarctata is a halophyte (Bal and Dut, 1986) native to coastal saline areas of Bangladesh, India and Pakistan. It is a perennial species with 2n = 4x = 48 chromosomes (Richharia and Roy, 1965) and is distinctly different from *O. sativa* by the anatomy and morphology of the leaf and embryo (Duistermaat, 1987).

Here, we have attempted wide hybridization between *Oryza sativa* and *Oryza coarctata* by *Hordeum bulbosum* (L.) method (Devaux 2003). In this method some of the genes/portion of genes from one species are transferred to another species and retained by chromatin introgression but due to genetic incompatibility, the uniparental chromosomes might be lost (Gernand et al. 2005). Another special attribute of our hybridization technique is using an *O. sativa* whose genome has been doubled by colchicine treatment. The developed putative hybrid plants have been already screened for salinity tolerance. We have been able to establish some partial hybrids which carry small parts of specific chromosomes in their genome. We have confirmed the hybrid status by genome sequencing. We have also established 21 *O. coarctata* genome specific SSR markers which can be used for molecular identification of any future hybrid plants developed using *O. coarctata*. Parts of this research have already been published in 2018 and 2023.

Complete understanding of the salt-tolerance mechanism which allows *O. coarctata* to complete its life-cycle and set rice-like grains in sea water may take many years. This opportunity will allow the development of truly characterized hybrids which can be used as salt-tolerant donors in further breeding programs with farmer preferred rice varieties.

Methods and Materials

4.2 Methods and materials

4.2.1 Wide hybridization between *O. coarctata* and *O. sativa* (var. Latishail 4n)

The materials used in hybridization comprised of the rice cultivar Latisail (2n = 4x = 48) and wild rice, *Oryza coarctata* (2n = 48). Two accessions of *O. coarctata* one from Bakkhali (accession CBKC3.1) and another from Teknaf (accession CTNC3.2) were used in the hybridization.

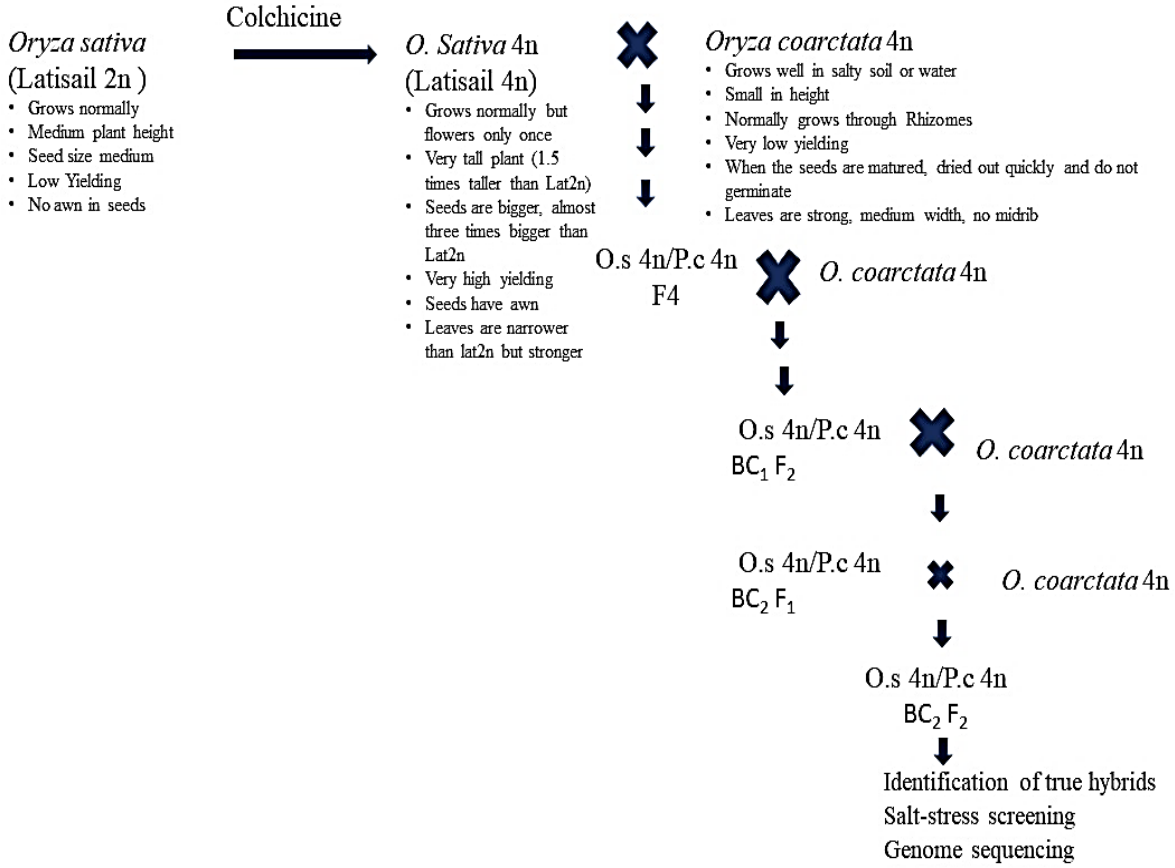
Latisail (4x) and *O. coarctata* were grown and prior to flowering, the panicles of the donor *O. coarctata* were cut in the morning (between 11 a.m. and 2 p.m.) and dipped in water for 3 hours. Emasculation of the recipient Latisail (4x) panicles was accomplished at the same time and covered with oil paper to prevent external pollination. Pollen from O. coarctata were collected in a Petri plate and dusted onto the stigma of the recipient plant by a small brush. The panicles were then covered again. Gibberellic acid (75 ppm) was sprayed every 24 hrs after crossing for 6 consecutive days. This was done particularly to avoid spikelet shattering in the maternal rice parent, Latisail (4x).

Figure 4.1: Wide hybridization between *O. coarctata* and *O. sativa* (var. Latishail 4n) A. *O. coarctata* plants grown in University of Dhaka net house; B) Mother plant induced tetraploid O. sativa (var. Latishail 4n) ; C) Collected pollen from *O. coarctata*; D) Pollen brushed into mother plant; E) Embryo formation after hybridization; F) Germination of Embryo in MS media.

Immature seeds were harvested at 10-12 days while some were allowed to completely mature and seeds collected at day 20-21 from crosses after pollinations. After harvesting, seeds were sterilized and germinated in a semi-solid MS (1/4th strength) medium. The hybrids germinated in more than 14 days and took more than 4 - 6 weeks to grow to about 10 - 12 cm.

In the bulbossom method, hybrids can retain part of the chromosome of the donor in their genome. We attempted backcrossing with *O. coarctata* to insert more and more genes from *O. coarctata*. Finally we had around 80 plants which were crossed three times with *O. coarctata*, always using it as the male pollen donor.

Figure 4.2: Hybridization events flowchart



4.2.2 Selection of promising putative hybrids

Phenotypic screening for salinity tolerance of the putative hybrids at the seedling stage was done by the method described by (Amin et al. 2012). Screening was done on the three times crossed F_2 population (Os4n/Pc4n BC_2F_1). The seedlings were allowed to grow in Yoshida (Yoshida et al. 1976) culture solution until they reached the four-leaf stage (14 - 18 days after germination). NaCl stress was applied gradually starting from 5 to 10 dS/m at 24 hourly increments of 2 dS/m. An increase of leaf number and length was measured after an interval of 4 days up to 16 days or until 90% of the leaves of the sensitive control were damaged. The tolerance related traits (Leaf damage score or LDS, chlorophyll content, plant height and tiller) of all stressed plants were then recorded. The level of salinity tolerance was evaluated mainly based on the value of LDS, which is based on the percentage of the leaf damage. The plants were scored according to the protocol mentioned by (Amin et al. 2012).

All statistical analyses were done using Data Analysis ToolPak of Microsoft Office Excel 2007 and R package. The F test was performed to verify equal variance of the independent set of samples and the test and ANOVA was performed based on the result assuming equal variance or unequal variance as applicable to compare significant differences ($p > 0.05$) between the stressed and control hybrid plants along with Latisail (4x).

4.2.3 Developing *O. coarctata* genome specific marker

To characterize putative hybrids there are several ways like karyotyping, chromosome-specific genetic marker and phenotyping. *O. coarctata* genome sequence was recently published, but the genetic markers have not yet been optimized. We have tried about 200 SSR marker from the Gramenae database and *Oryza rhizomatis* genome for SSR markers which will likely be specific for *O. coarctata.*

After analyzing all the SSR markers, we have optimized 21 SSR markers which have unique allele size in *O. coarctata*. We have optimized each SSR marker Polymerase reaction, amplified the *O. coarctata* and *O. sativa 2n* and *O. sativa* 4n allele, and then determined their size in agarose gel.

Leaf tissues were collected at the screen house and DNA was prepared by the modified CTAB method or a simple DNA preparation method (Kim et al. 2016). PCR was conducted with the newly developed markers with the following thermal cycles: 94 °C for 3 m, 35 cycles of 30 s at

95 °C, 30 s at 55 °C, and 50 s at 72 °C, and final extension at 72 °C for 5 m. The PCR products were analyzed in 2% agarose gel by electrophoresis.

The DNA bands were analyzed by Gel analyzer software (GelAnalyzer 23.1.1 (available at www.gelanalyzer.com) by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, CSc).

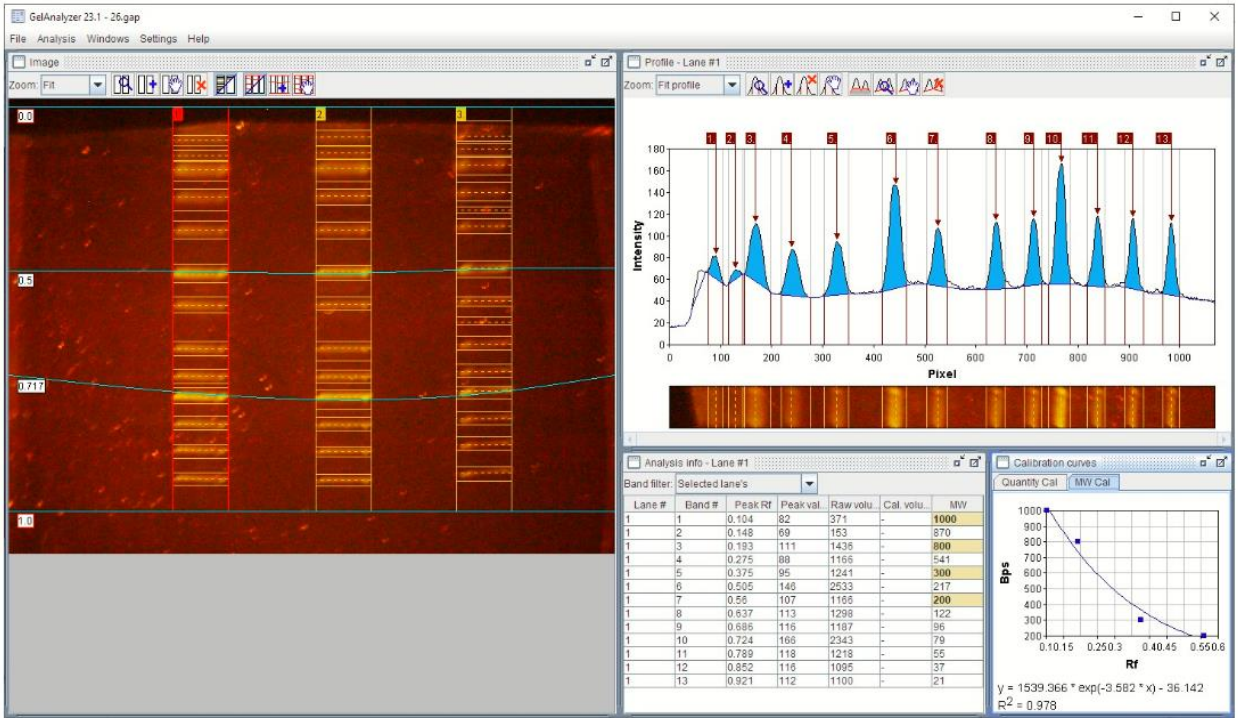


Figure 4.3: Gel analyzer main window. The size of unknown band in agarose gel found for *O. sativa* (2n and 4n) and *O. coarctata* has been determined by Gel analyzer software.

4.2.4 Genome sequencing of most promising hybrids

Two most promising hybrid plants were genome sequenced by Illumina and short DNA introgression from *O. coarctata* were observed. The genome was sequenced by the courtesy of Rod wing, Arizona state University, USA.

Results

4.3 Results

4.3.1 Developing hybrid plants

A total of 1191 spikelets were pollinated in the wide crosses. Only two putative hybrid plants were able to survive. Then F_1 populations from these putative hybrid plants were grown and selected plants were again crossed with O. coarctata. The same process was repeated 2 more times. After three rounds of crossing, promising hybrid pants were selected for salt-stress screening. After that two most promising plants were genome sequenced to find introgression from *O. coarctata*.

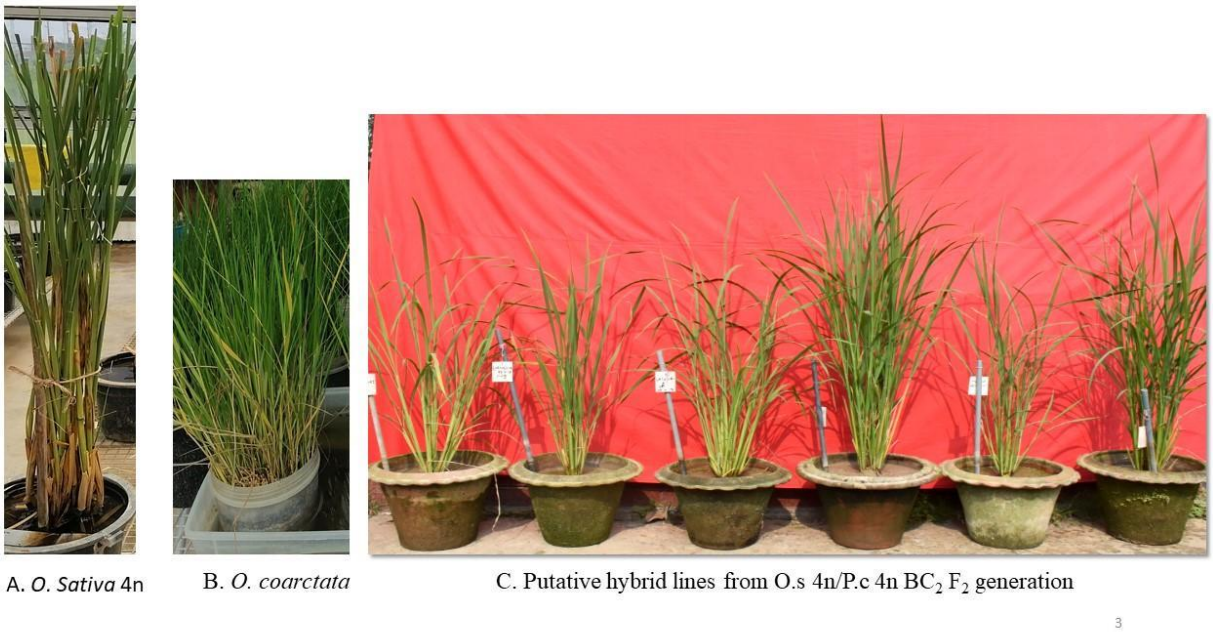


Figure 4.4 : A) O. sativa 94n) var. Latiahail, the maternal parent plant used in wide hybridization; B) O. coarctata, used as the paternal parent ; C) Six putative Os(4n)X *O. coarctata* hybrid plants from BC_1F_2 generation

4.3.2 Characterizing hybrid plants based on their phenotypes

Rice plants form fibrous root systems consists of an ephemeral seminal root, nodal roots, and their lateral roots. On the other hand, *O. coarctata* has complex rhizome system instead of single root. In case of putative hybrid plants we have found both fibrous root and tap root (Figure 4.5). Taproot contain a single and thick primary root, secondary roots grow from the sides of the main root. In contrast, fibrous roots contain numerous small roots that branch out. Tap root can secure plant system better than fibrous root.

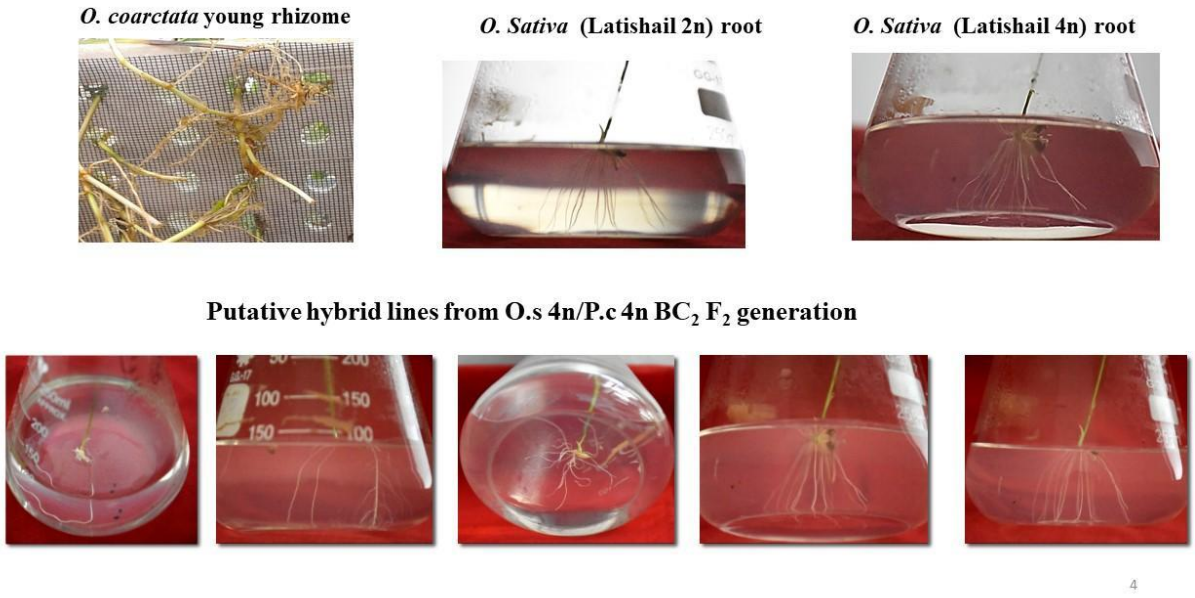


Figure 4.5 : Putative hybrids of *O.coarctata* X O. sativa (4n). Immature seeds are collected and germinated in MS media. Some putative hybrid plants shown fibrous root, whearas some have shown tap root.

O. coarctata have a distinct leaf structure. It will also be useful if we can transfer some the key leaf characteristics into hybrids. After two times backcross to *O. coarctata* we found a hybrid plant whose leaf has no midrib just like *O. coarctata*.

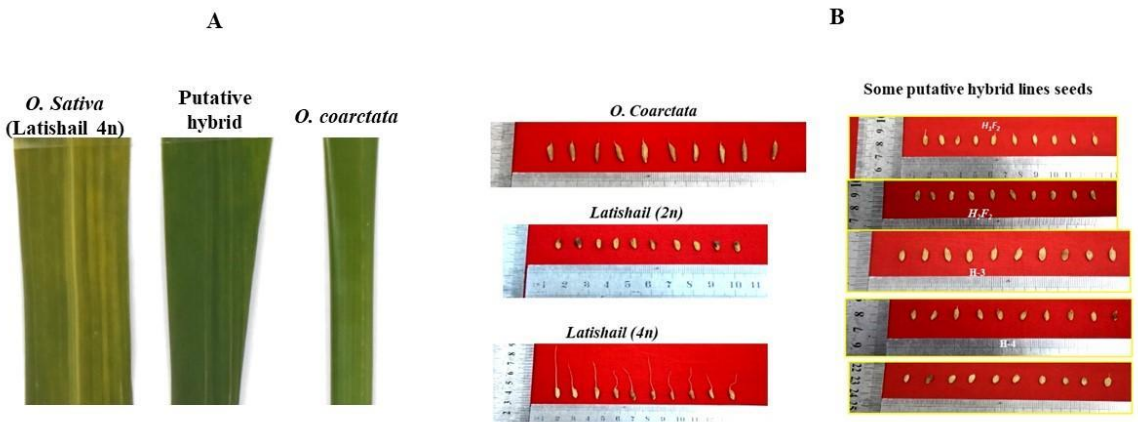


Figure 4.6 : A) Leaf of putative hybrids of *O.coarctata* X *O. sativa* (4n). One of the putative hybrids (from BC_2F_2) have shown *O. coarctata* leaf like feature. The putative hybrid leaf had no midrib. B) Seeds of *O. coarctata*, *O. sativa* (Latishail 2n), *O. sativa* (Latishai 4n), putative hybrids three times crossed to *O. coarctata*, BC_2F_2 generation.

4.3.3 Developing *O. coarctata* genome specific primer for identification of true hybrids

As there were no established SSR markers specific for the *O. coarctata* genome, we had to first develop some markers unique for this wild rice. At first, we tried the SSR markers of *O. sativa* from the Gramene database but almost all markers showed the same sized band both for *O. sativa* and *O. coarctata*. Therefore, we tried the SSR markers optimized for another wild rice *O. rhizomatis*. The primer sequences have been taken from publication done by KK Jena group at IRRI (Hechanova, Prusty et al. 2018). We have amplified ninety markers, 7-8 markers representing each chromosome. SSR markers were originally designed by Sherry et al. (ref) using the bacterial artificial chromosome (BAC) end sequences of *O. officinalis* having CC genome produced by the *Oryza* Map Alignment Project (OMAP) of Arizona Genomic Institute, USA. The InDel regions between *O. sativa* and *O. officinalis* were selected to design markers.

Each of the markers was amplified for *O. sativa* (var. Latishail 2n), *O. sativa* (var. Latishail 4n) and *O. coarctata*. The marker size was determined by Gel analyzer software. Finally we could establish nineteen SSR markers with a unique size for the *O. coarctata* genome, representing each chromosome except chromosome 5 and 8. Seven more markers established from *O. coarctata* DNA sequences are only present in *O. coarctata*, not in *O. sativa*. The representative chromosome

number and their allele size in *O. sativa* (2n and 4n) and *O. coarctata* is shown in Table 4.1 and 4.2. In each case. We have used *Osactin1*(324bp) primer as PCR positive control.

Table 4.1: Name and product size of markers present only *in O. coarctata*, but absent in *O. sativa*.

Chromosome	Name of marker	Amplicon size in *O. coarctata* (base pair)
03	PcLH 03001	521
	PcLH 03002	501
	PcLH 03004	361
	PcLH 03006	647,205
	PcLH 03007	358
12	PcLH 12002	384
	PcLH 12005	445

Table 4.2: SSR markers optimized for *O. coarctata* from *O. officinalis* genome.

Chromosome	Name of SSR	Amplicon size in Latishail (Os2n) bp	Amplicon size in Latishail 4n(Os4n) bp	Amplicon size in *O. coarctata* bp
1	ORH 01001	216	217	270,234
	ORH 01002	274	278	243
2	ORH 02001	256	258	284
	ORH 02003	279	279	221
	ORH 02008	178	179	149
3	ORH 03004	256	258	233

	ORH 03005	220	222	204
	ORH 03009	305	304	259
	ORH 03011	249	253	286
4	ORH 04003	301	311	266
	ORH 04006	458	461	496
6	ORH 06003	219	215	145
7	ORH 07007	183	184	198
9	ORH 09007	222	227	163
10	ORH 10004	429	423	483
11	ORH 11006	226	231	336, 240, 204
12	ORH 12003	330	332	274
	ORH 12007	213	214	244

4.3.4 Agarose gel electrophoresis of selected SSR markers and promising putative hybrids

We have tested all the promising putative hybrids with the established SSR markers. Few lines from BC_2F_2 have shown presence of two markers, PcLH03001and PcLH12005, representing chromosome 3 and 12 respectively. The agarose gel for each SSR marker optimized as a unique marker for *O. coarctata* showing alleles from *O. sativa* 2n, *O. sativa* 4n and *O. coarctata* has been presented below.

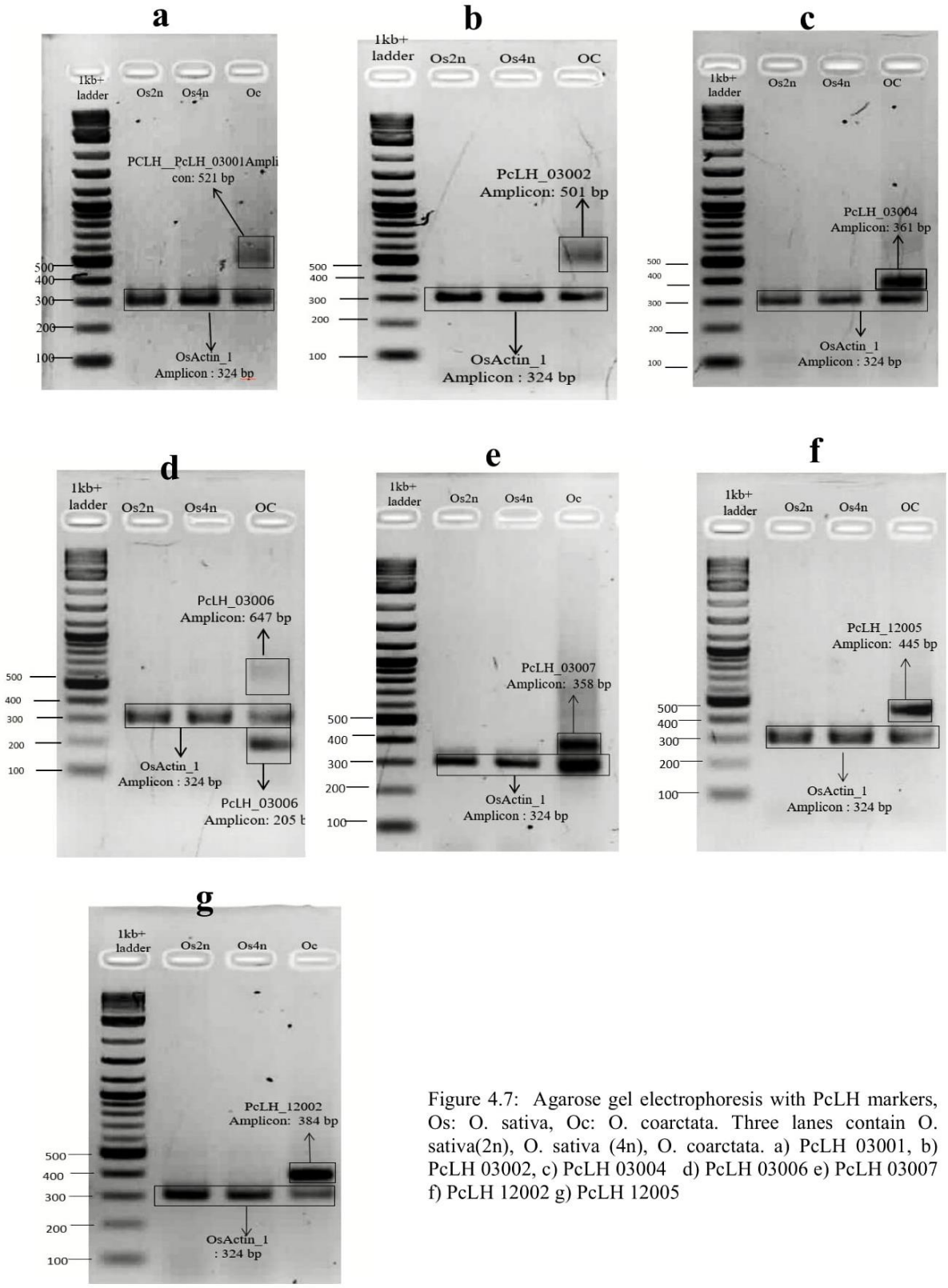


Figure 4.7: Agarose gel electrophoresis with PcLH markers, Os: O. sativa, Oc: O. coarctata. Three lanes contain O. sativa(2n), O. sativa (4n), O. coarctata. a) PcLH 03001, b) PcLH 03002, c) PcLH 03004 d) PcLH 03006 e) PcLH 03007 f) PcLH 12002 g) PcLH 12005

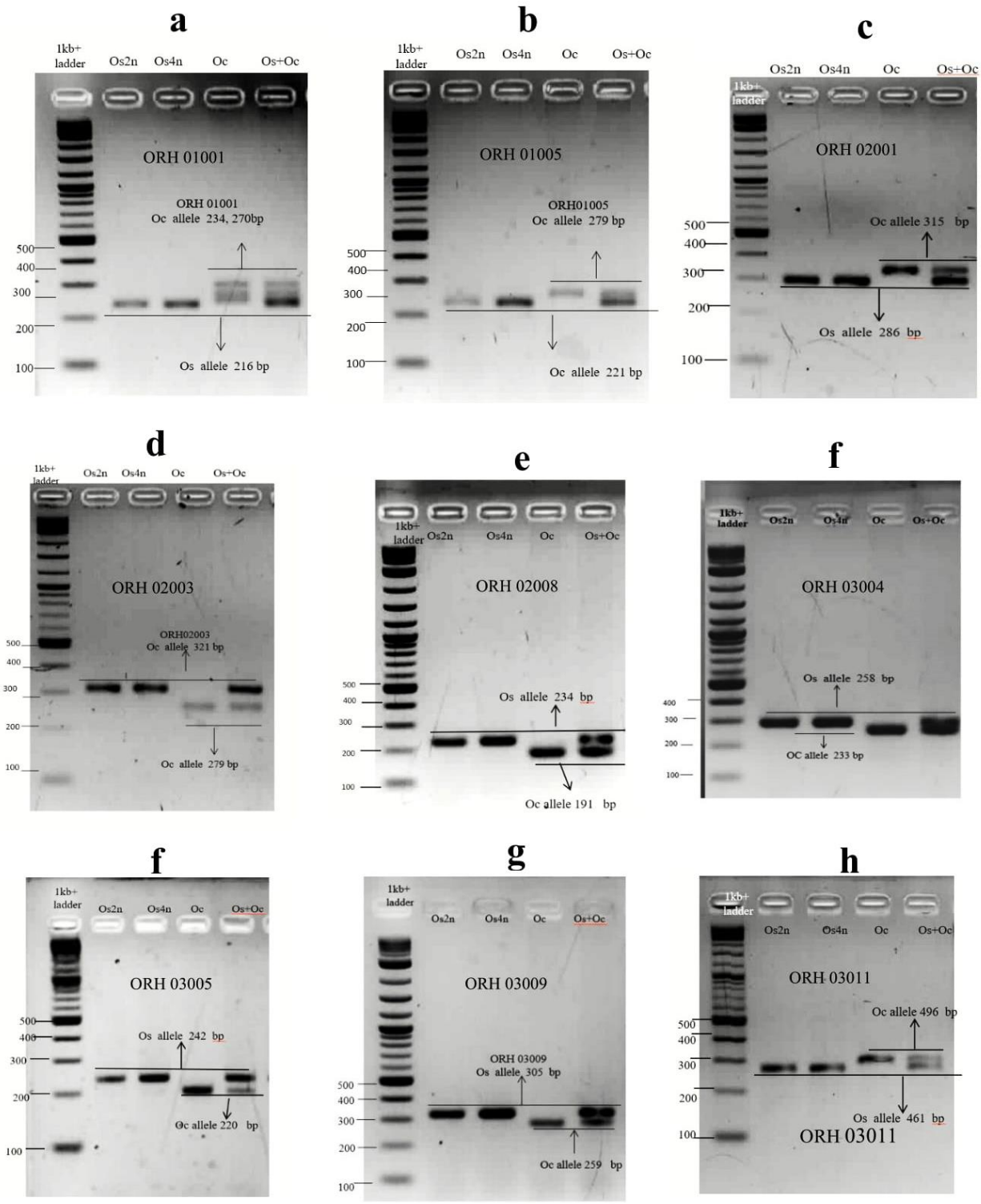


Figure 4.8 : Agarose gel electrophoresis with ORH markers from chr1, 2 &3; Os: O. sativa, Oc: O. coarctata. Three lanes contain *O. sativa(2n), O. sativa (4n), O. coarctata.* a) ORH 01001, b) ORH 01005, c) ORH 02001, d) ORH 02003, e) ORH 02008, f) ORH 03004, g) ORH 03005, h) ORH 03009, i) ORH 03011.

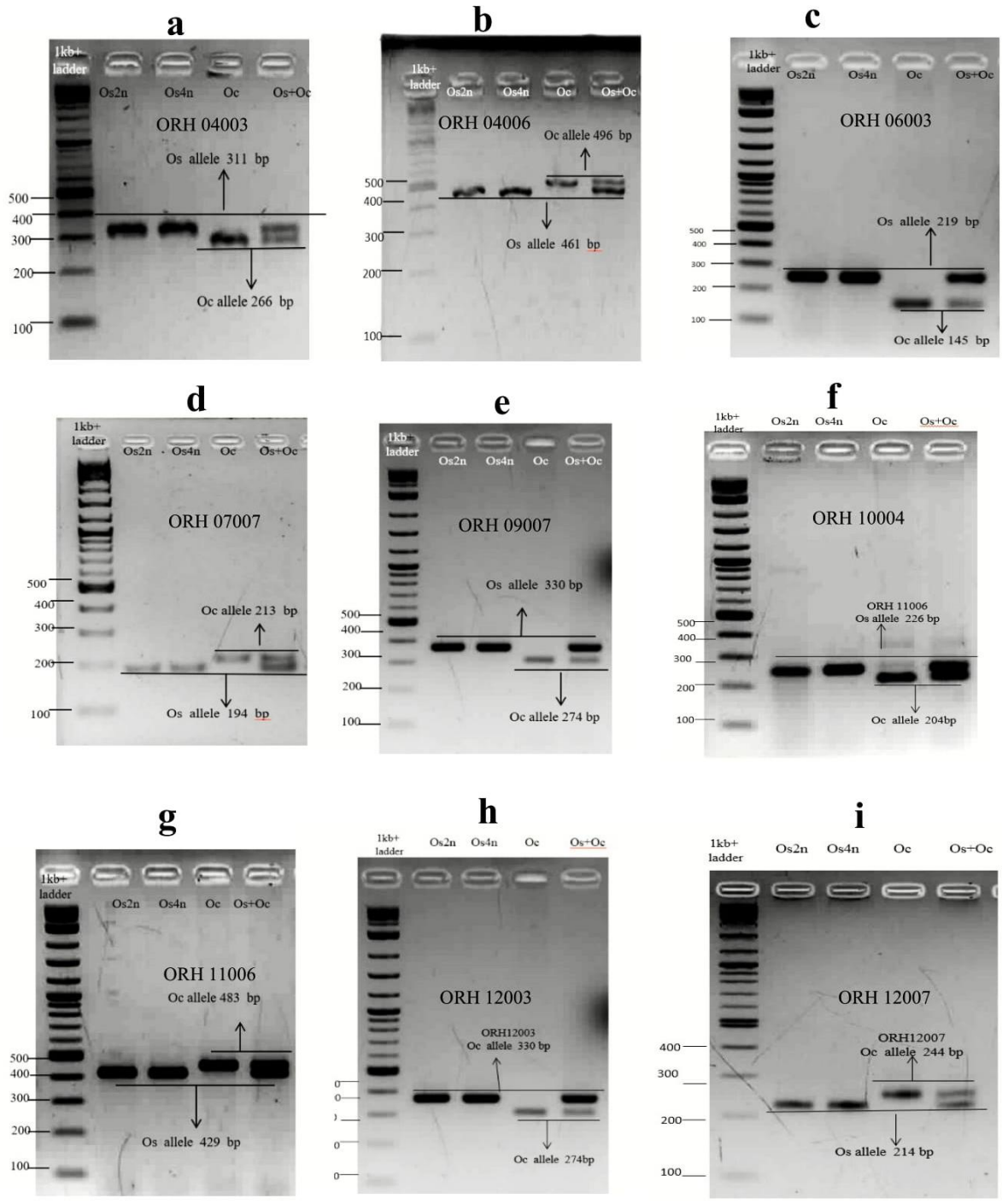


Figure 4.9: Agarose gel electrophoresis with ORH markers from chr4,6,7,9,10,11, &12; Os: *O. sativa*, Oc: *O. coarctata*. Three lanes contain *O. sativa(2n), O. sativa (4n), O. coarctata.* a) ORH 04003, b) ORH 04006, c) ORH 06003, d) ORH 07007, e) ORH 09007, f) ORH 10004, g) ORH 11006, h) ORH 12003, i) ORH 12007.

4.3.5 Identification of positive hybrids by SSR marker

We found a few hybrid lines showing the same band size as *O. coarctata* for marker PcLH03006 and PcLH12005. Unfortunately, the developed hybrid didn't show any of the ORH marker alleles specific for *O. coarctata*.

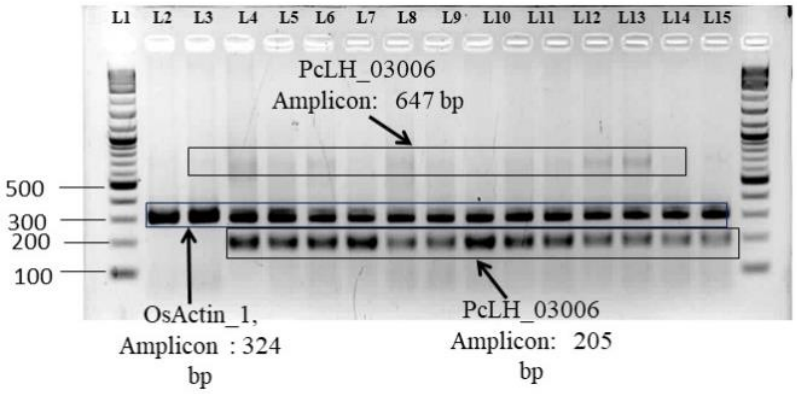


Figure 4.10: *O. coarctata* chr 3 specific marker PcLH_03006 (band size 205 and 647bp) amplification in hybrid lines. L1=1Kb+ ladder, L2= Os2n, L3=Os4n, L4= Oc, L5-L15= hybrid lines. OsActin_1 has been used as housekeeping gene for all plants.

Twelve hybrid lines have been found positive for PcLH03006 marker (Chr 3) (Figure 4.10) and eleven hybrid lines positive for PcLH12005 (Chr 12) (Figure 4.11). `

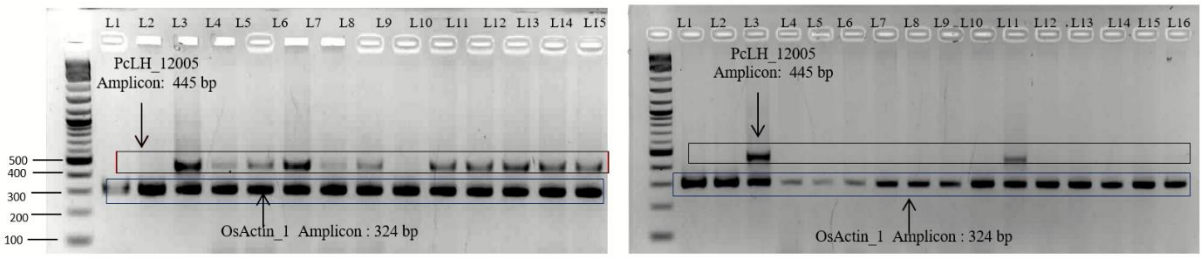


Figure 4.11: A) *O. coarctata* chr 12 specific marker PcLH_12005 (band size 445bp) amplification in hybrid lines. L1=1Kb+ ladder, L2= Os2n, L3=Os4n, L4= Oc, L5-L14= hybrid lines. *OsActin*_1 has been used as housekeeping gene for all plants.

4.3.6 Salt-stress screening of hybrid lines having introgression from *O. coarctata*

The hybrid lines which have introgressions from *O. coarctata* (Positive for PcLH03006 and PcLH12005 marker) were screened for salt-tolerance at seedling stage to check if they have acquired any salt-tolerant characteristics from *O. coarctata*. After 100mM salt-stress of two weeks, we selected some promising hybrid lines by analyzing their chlorophyll content, plant height and tiller number.

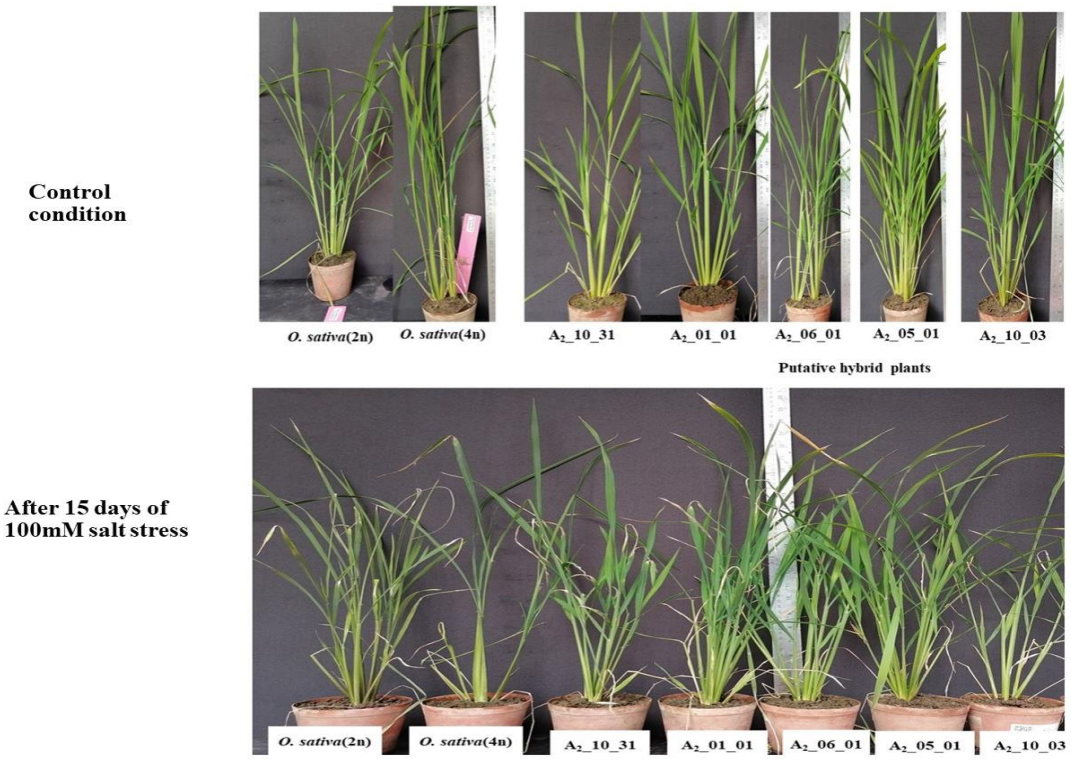


Figure 4.12: *O. sativa* (2n), *O. sativa* (4n) and some of the putative Os4nXOc4n hybrid plants in control condition and after 15 days of 100mM NaCl stress. In case of *O. sativa* (2n), 60-70% leaves became yellowish and gradually after salt-stress. The tiller number drastically reduced in *O. sativa* (4n) and leaves became wilted. Whereas selected hybrid lines have shown healthier and greener leaves than both *O. sativa* (2n) and *O. sativa* (4n).

The selected partial hybrid lines have shown better salt-tolerance than *O. sativa*(2n) and *O. sativa*(4n). For *O. coarctata*, 100mM salt stress is a rather favorable condition for better growth as a halophyte. It has shown 12.6% increase in plant height and almost no effect on plant health. *O. sativa*(2n) have shown 47.6% reduction in chlorophyll content, 40% reduction in plant height and 25% reduction in tiller number. *O. sativa*(4n) have shown 43% reduction in chlorophyll

content, 37% reduction in plant height and 20% reduction in tiller number. Partial hybrid lines A2-06-01, A2-10-01 and B2-03-01 have shown significantly higher chlorophyll content than *O. sativa*(2n) and *O. sativa*(4n) after salt-stress (figure 4.13A). Partial hybrid lines B-02-01 and B-03-01 have shown significantly higher height than *O. sativa*(2n) (figure 4.13B). Partial hybrid lines A2-06-01 and A2-10-01 have shown significantly higher tiller number than *O. sativa*(2n) (figure 4.13C).

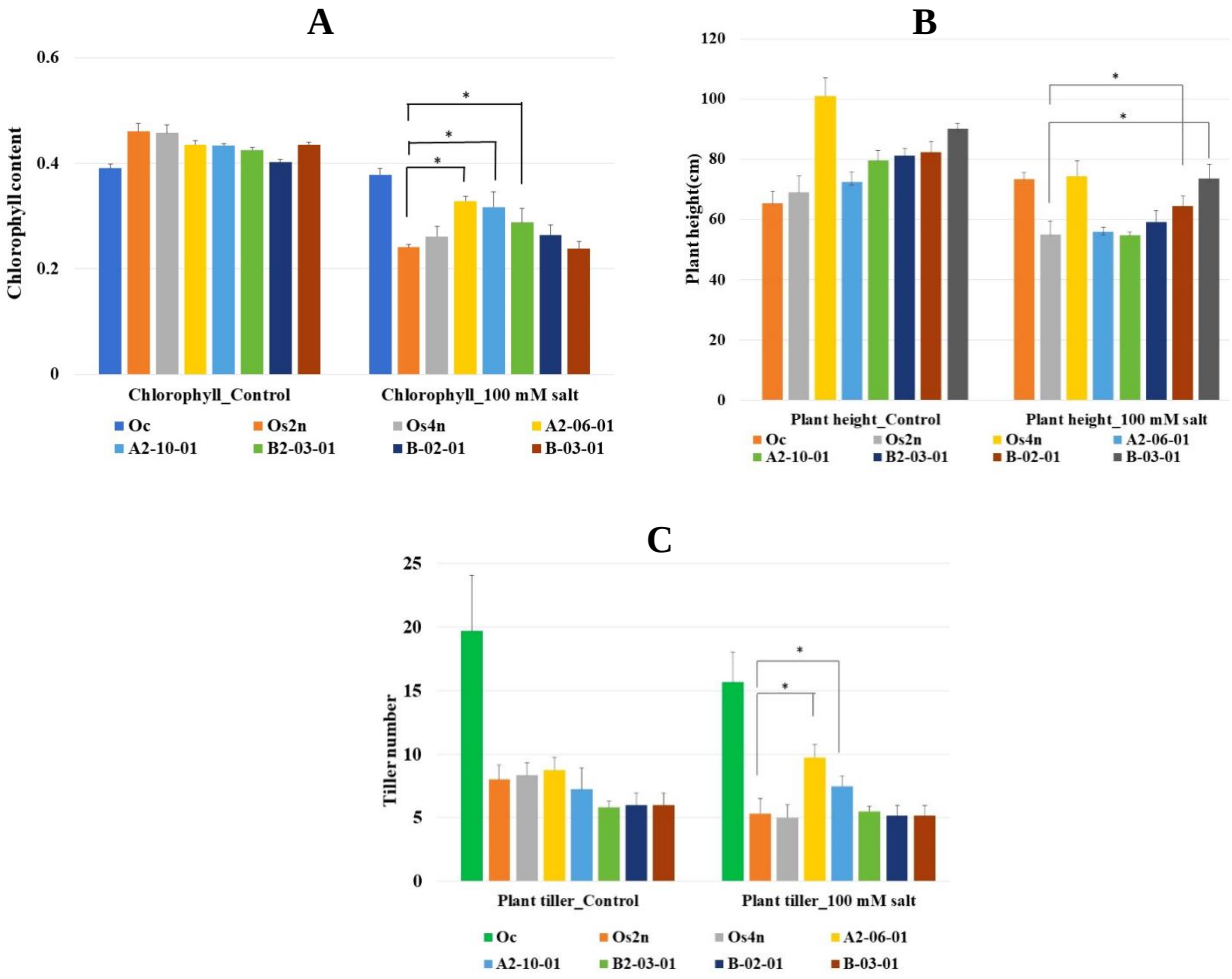


Figure 4.13: 100mM salt-stress screening of *O. coarctata* (Oc), *O. sativa* 2n(Os2n) and *O. sativa* 4n(Os4n) and selected hybrid plants. A) Chlorophyll content of Oc, Os2n, Os4n and hybrid plants in control condition and two weeks of 100mM salt treatment. Os2n and 4n plants chlorophyll content is greatly affected by salt stress (47.6% and 43% reduction respectively), Oc plants had shown almost no change in salt environment (only 3% reduction), and hybrid line A2-06_01, A2-06_01 and B2-03-01 have shown significantly higher chlorophyll content than Os2n and Os4n in 100mM salt-stress. B) Plant height of Oc, Os2n, Os4n and hybrid plants in control condition and two weeks of 100mM salt treatment. Oc plants have shown 12.6% increase in height, whereas Os2n and Os4n have shown 40% and 37% decrease respectively, hybrid line B-03-01 have shown significantly better height than Os2n in salt-stress. c) Tiller number of of Oc, Os2n, Os4n and hybrid plants in control condition and two weeks of 100mM salt treatment. Hybrid line A2-06-01 have shown significantly higher tiller number than Os2n and Os4n in salt-stress.

4.3.7 Genome sequencing of best two hybrid lines:

After considering all the data, we have selected two promising lines, A2-06-01 and A2-10-01 which are better than *O. sativa* in 100mM salt-stress and genome sequenced to confirm the genomic introgression from *O. coarctata.*

The hybrid genome sequence variants were called against the pan genome; *IRGSP*, *O. coarctata* and *O. australiensis* reference genomes were used to build the pangenome graph using *minigraph* (Li, H., Feng, X. & Chu, C. The design and construction of reference pangenome graphs with minigraph. *Genome Biol* 21, 265. 2020). The hybrids were found to cluster with the *Oryza sativa* Indica group.

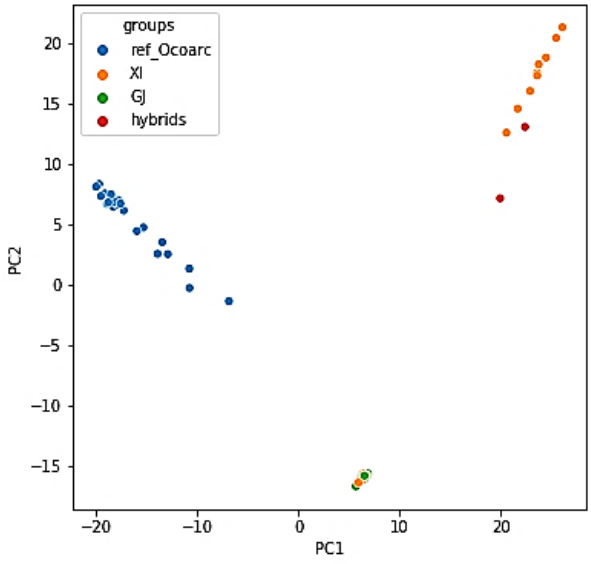


Figure 4.14: Minigraph to map the hybrid sequence read *O. coarctata* samples, indica samples (XI), japonica samples(GJ) against the pan genome. Each bubble represents a structural variation (the unshared portion among the genomes being compared). On a genomic scale, the hybrid plants are virtually identical to the members of the Indica subgroup. (Proved by Alice et al.)

4.3.8 Looking for unique chromosomal segments acquired from Oc in the partial hybrids

Reads From two hybrids line were mapped to the *Oryza* pan genome, then unmapped reads were extracted and mapped to the *O. coarctata* genome directly. If chromosomal segments that are unique to *O. coarctata* were transferred to the hybrid in any of the crosses, reads would be mapped across a stretch of a few kilobases. Four strong signals observed using The Integrative Genomics Viewer (IGV). Two of these regions are in chromosome 03, other two in chromosome 10 and 12. The details of these regions are described in table 4.3.

Table 4.3: Position , size and blast result of four strong signals found in putative hybrids.

Chromosomal segments showing strong IGV signals	Size of the segment(kb)	Most identical sequence found in database	Query coverage	Percent Identity	E-value
Region 1: ChrK03:2,931,00 0-2,953,000	22kb	GQ203301.1 O. coarctata clone OC_Ba202M20	92%	82.8%	0.0
Region 2: ChrK03:25,427,000-25,449,000	22kb	GQ203301.1 O. coarctata clone OC_Ba202M20	73%	82.6%	0.0
		FJ032635.1 OC clone a0226NO1	61%	77.1%	0.0
Region 3: ChrK10:12,389,000-12,411,000	22kb	MN542932.1 Oc keratin associated protein 5-4, tyrosin protein kinase domain	100%	94.51%	0.0
		AP014963.1 O. sativa Japonica group DNA chromosome 7, cultivar Nipponbare	99%	76.96%	8e-156
Region 4: ChrK12:1,941,000-1,963,00	22kb	GQ203301.1 O. coarctata clone OC_Ba202M20	92%	85.09%	0.0

Based on the span of coverage, BLAST searches of signals 1,2 and 4 against the nr database suggest that these are uniquely *Oc* sequences. Results showing the signals and the BLAST results have been presented below (figure 4.15 & 4.16).

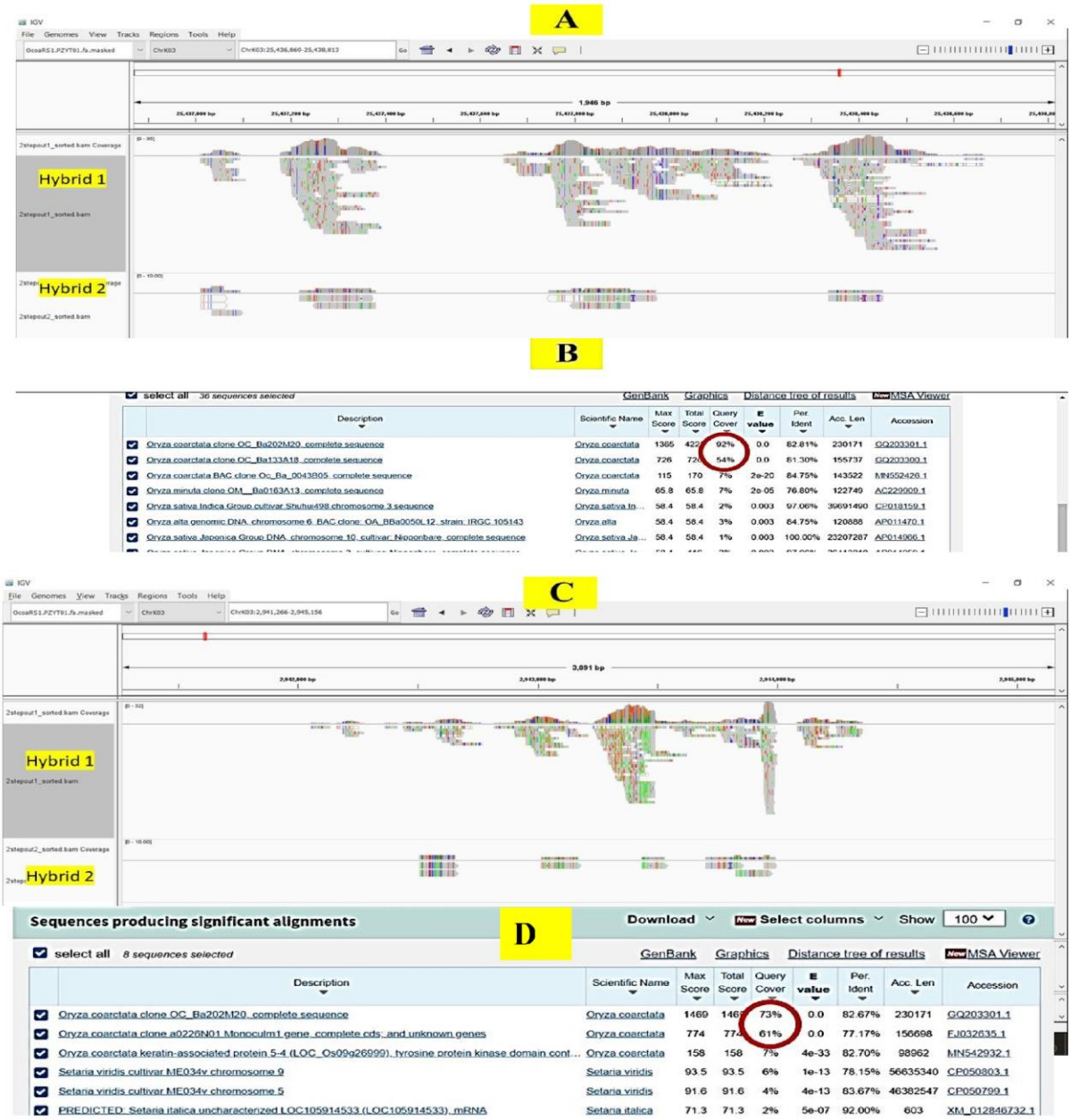


Figure 4.15: A & B) IGV Signal and blast result of Region 1: ChrK03:2,931,00 0-2,953,000, aligned with *O. coarctata* genome sequence. No significant identity with *O. sativa*. C & D) IGV Signal and blast result of Region 2: ChrK03:25,427,000-25,449,000, aligned with *O. coarctata* genome sequence. No significant identity with *O. sativa*

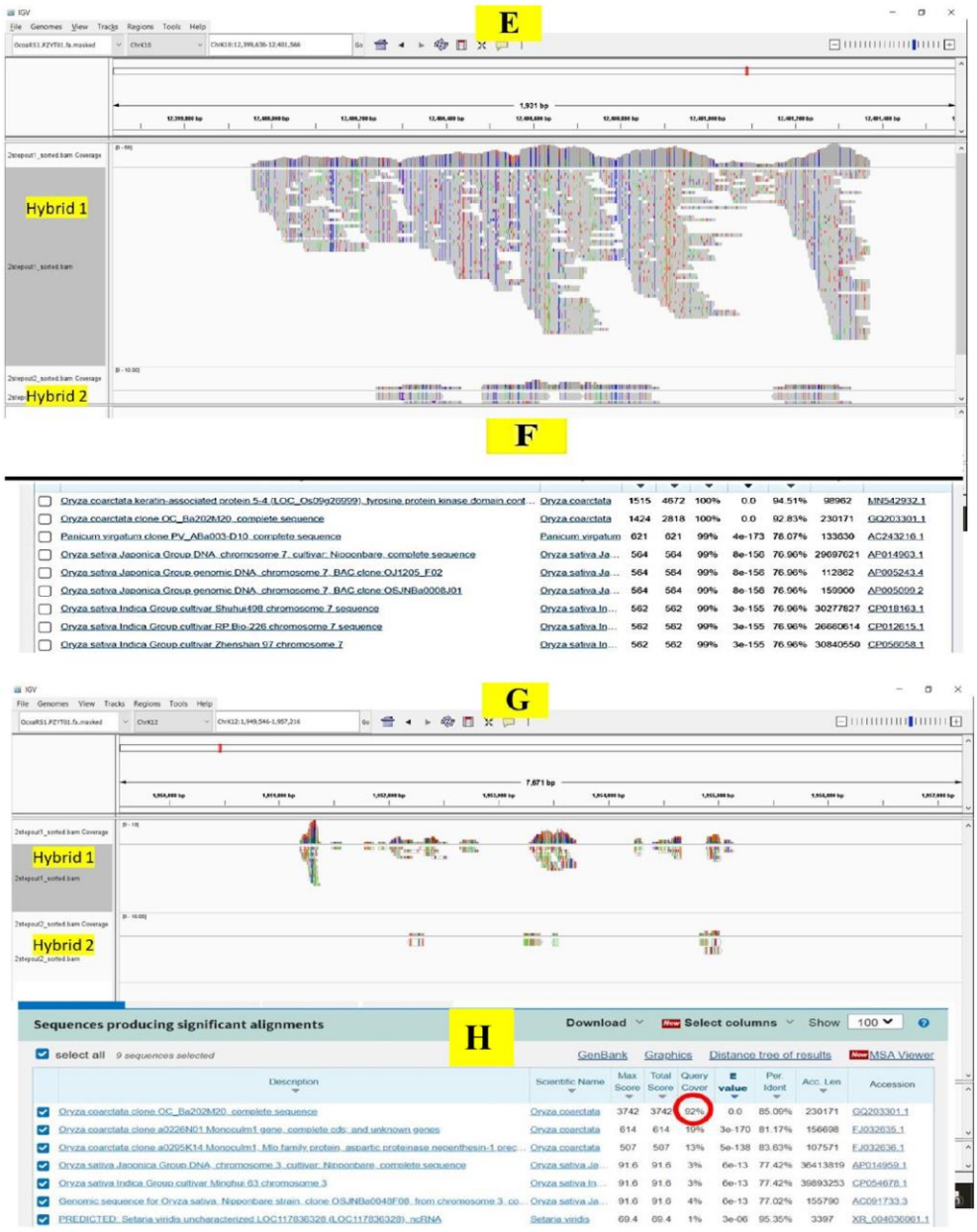


Figure 4.16: E & F) IGV Signal and blast result of Region 3: ChrK10:12,389,000-12,411,000 aligned with *O. coarctata* genome sequence. This region have shown 78% identity with 99% query coverage with part of *O. sativa* chromosome 07. G & H) IGV Signal and blast result of Region 4: ChrK12:1,941,000-1,963,00aligned with *O. coarctata* genome sequence. No significant identity with *O. sativa*

It was previously observed (*Alice et al.*) that mother variety *O. sativa* (var. Latisail 4n) belongs to the Indica subgroup. To confirm that the regions were not from mother *O. sativa* 4n, the sequence reads further aligned against Shuhui498 and Zhenshan97 that were best related to our isolated segments based on the BLAST results, and raw data from over 120 varieties from the Indica subgroup from the 3,000-rice genome dataset. No reads from Shuhui498 and Zhenshan97 or the ~120 *Indica* plants could be mapped to our four regions of interest. Therefore, we have found four uniquely mapped regions in these two partial hybrids which are present in *O. coarctata* but not in *O. sativa*.

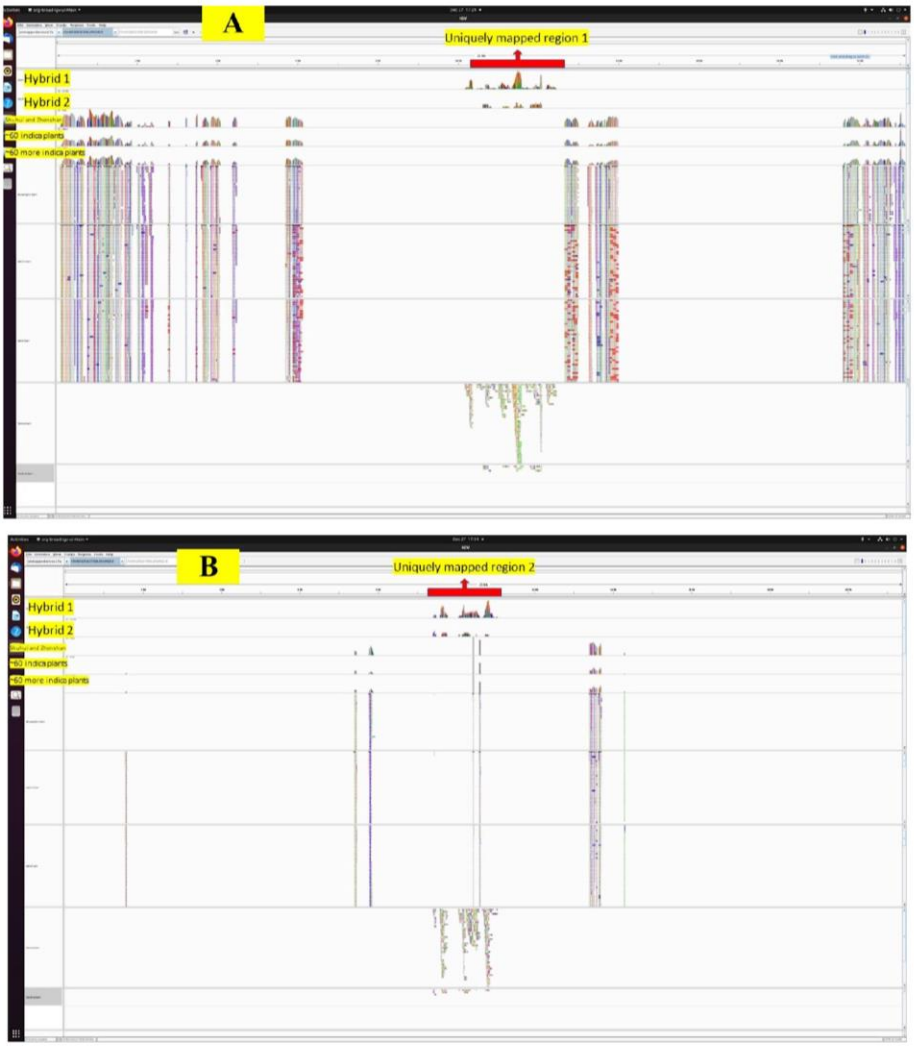


Figure 4.17: Region 1(figure A) and 2 (figure B) aligned to Shuhui498 and Zhenshan97 that were best related to our isolated segments based on the BLAST results, and raw data from over 120 varieties from the Indica subgroup from the 3,000-rice genome dataset.

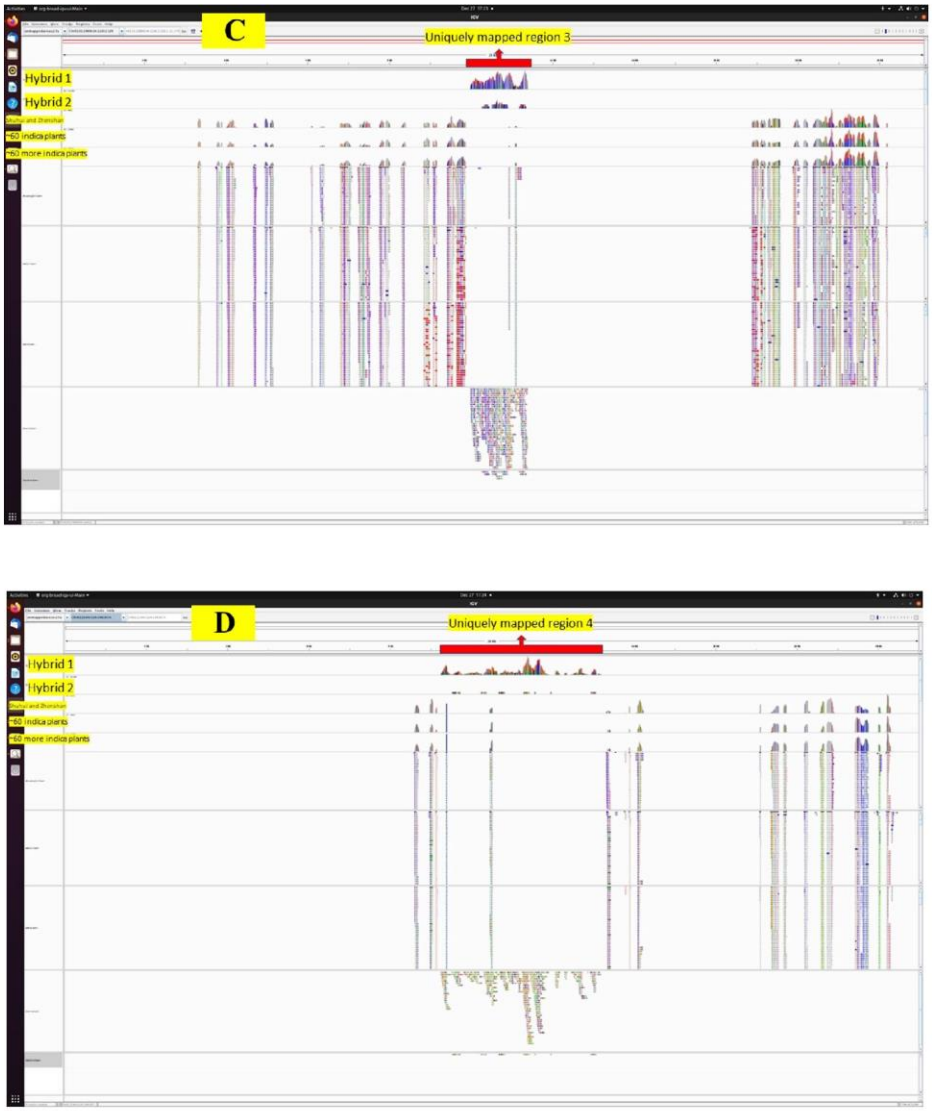


Figure 4.18: Region 3(figure C) and 4 (figure D) aligned to Shuhui498 and Zhenshan97 that were best related to our isolated segments based on the BLAST results, and raw data from over 120 varieties from the Indica subgroup from the 3,000-rice genome dataset.

4.4 Discussion

The development of salt-tolerant rice cultivars is a critical challenge for global food security, especially given the increasing salinization of arable land. Wild relatives of cultivated crops, such as *Oryza coarctata*, which naturally thrive in saline coastal habitats, offer valuable genetic resources for stress tolerance improvement. However, the substantial genomic and ploidy differences between *O. coarctata* (2n = 4x = 48) and cultivated *O. sativa* (2n = 24 or 4x = 48 in this study) have historically hindered conventional breeding efforts to exploit this wild species (Jelodar et al. 1999, Jena 1994, Seraj et al. 1996).

This study employed a strategic approach combining the bulbosum wide hybridization method with colchicine-induced genome doubling (Blakeslee et al. 1937) of the maternal *O. sativa* (var. Latisail 4n) to overcome these reproductive barriers. This innovative combination facilitated the creation of partial hybrids that incorporate genomic segments from *O. coarctata*, as demonstrated by molecular and genomic analyses. Two putative hybrids of *O. coarctata* have been already reported by our team (Tasmia et al 2017).

Challenges and Success of Wide Hybridization

Wide hybridization between *Oryza sativa* (4n) and *Oryza coarctata* is complicated by significant genomic divergence and ploidy differences. The low frequency of hybrid plant survival (2 hybrids from 1,191 pollinated spikelets) highlights the reproductive barriers and genomic incompatibilities typical of such crosses. Nonetheless, the application of the bulbosum technique(Barclay 1975, Gernand et al. 2005) coupled with colchicine-induced genome doubling of the maternal parent effectively overcame some of these barriers. This approach enabled the creation of partial hybrids that retained donor chromosomal fragments, demonstrating that even in highly divergent crosses, gene introgression is achievable.

Morphological and Phenotypic Characteristics of Hybrids

The partial hybrids exhibited few intermediate morphological traits between the two parental species, mostly Latishail (O. sativa 2n). The presence of both fibrous and tap root systems in hybrids suggests a combination of root architectures, which may confer improved anchorage and

water uptake under saline conditions—an important adaptive trait for salt tolerance (Aroca et al. 2012, Bao et al 2014). Seed morphology of the hybrids was notably variable and intermediate, further indicating partial genomic mixing. The discovery of hybrid plants with leaf characteristics such as the absence of midribs, a hallmark of *O. coarctata*, signifies phenotypic introgression of key structural traits potentially linked to salt tolerance.

Molecular Confirmation of Introgression

Molecular analysis using *O. coarctata*-specific SSR markers identified small chromosomal segments from the wild donor retained in several hybrid lines, specifically on chromosomes 3 and 12. The development of these markers fills a critical gap, providing a reliable means to distinguish true hybrids from progenies lacking introgressed DNA.

Enhanced Salt Tolerance in Hybrid Lines

Salt stress screening at 100 mM NaCl demonstrated that certain partial hybrid lines, especially A2-06-01 and A2-10-01, exhibited significantly better tolerance than both diploid and tetraploid *O. sativa*. These hybrids maintained higher chlorophyll content, greater plant height, and increased tiller numbers under stress, indicating enhanced photosynthetic capacity, growth vigor, and reproductive potential. This improvement closely aligns with the halophytic adaptations of *O. coarctata*, validating that the transferred chromosomal segments likely carry functional salt tolerance genes.

Developing *O. coarctata* genome specific SSR markers

After developing some putative hybrids, it was necessary to identify true hybrids, However, there were no established *O. coarctata* specific SSR markers. We first tested the *O. coarctata* and *O. sativa* by SSR markers from Gramene database, but almost all the markers showed were identical in size. Then we tried the SSR marker designed from cc genome of *O. officinalis* by the *Oryza* Map Alignment Project (OMAP) of Arizona Genomic Institute, USA. KK Jena used them for designing SSR marker for *O. rhizomatis*. Among 90 markers we have optimized 19 markers specific for *O. coarctata* which have unique allele size, representing each chromosome except chromosome 5 and 8. Seven more markers established from *O. coarctata* DNA sequences are only

present in *O. coarctata*, not in *O. sativa*. The representative chromosome number and their allele size in *O. sativa* (2n and 4n) and *O. coarctata* is shown in Table 4.1 and 4.2.. These 26 markers can be used to identify future hybrids developed using *O. coarctata*. We have found only two markers in our partial hybrid lines.

Genomic Insights from Whole-Genome Sequencing

Whole-genome sequencing of the top-performing hybrids confirmed four unique introgressed regions from *O. coarctata*, absent from the maternal *O. sativa* 4n genome and other Indica varieties. The localization of these regions on chromosomes 3, 10, and 12. Studies should be conducted to test if these chromosome segments are related with abiotic stress responses.

Implications for Rice Breeding

The generation of salt-tolerant partial hybrids offers a promising new genetic resource for rice improvement. By combining the salinity resilience of *O. coarctata* with the agronomic traits of cultivated rice, these hybrids can be used as bridging variety for the development of higher salt-tolerant rice varieties. The use of molecular markers facilitates marker-assisted selection, allowing breeders to track and stabilize desired introgressions while minimizing unwanted linkage drag.

4.5 Limitations and Future Challenges

While encouraging, the limited number of surviving hybrids and partial nature of introgressions highlight persistent challenges. Fertility issues, genomic instability, and potential incompatibilities may limit commercial use unless addressed through backcrossing, cytogenetic analysis, or modern biotechnological tools such as genome editing. Moreover, the salt tolerance observed at the seedling stage requires validation at later growth stages and under field conditions to confirm agronomic utility.

4.6 Conclusion

To further advance the development of salt-tolerant rice, future research should focus on precisely mapping the introgressed *Oryza coarctata* genomic segments to identify candidate genes responsible for salinity tolerance. Functional validation of these genes using gene editing and

transgenic methods will clarify their roles in stress response. Stabilizing the introgressions through backcrossing with elite *O. sativa* cultivars is essential to combine salt tolerance with favorable agronomic traits. Additionally, comprehensive field testing under varied salinity conditions will confirm the hybrids' adaptability and yield stability. Integrating omics approaches will deepen understanding of underlying tolerance mechanisms, while addressing fertility barriers through cytogenetics and breeding will enhance hybrid viability. Finally, expanding wide hybridization efforts to other wild rice relatives will broaden the genetic resources available for breeding resilient rice varieties.

Chapter five: Cloning and transformation

5.1 Overview

Salinity is a major constraint to rice production, especially in coastal regions, where soil salinization severely reduces crop yield. Traditional breeding for salt tolerance in rice has achieved limited success due to the complex polygenic nature of the trait and yield penalties in tolerant varieties. This study explores a transgenic approach using stress-responsive genes from the halophytic wild rice species *Oryza coarctata*, known for its exceptional salt resilience.

Three candidate genes were selected for cloning and functional analysis: *OcAsr1* (ABA-stress-ripening protein), *OcPVA1* (vacuolar H^+-ATPase subunit c), and *OcMT3* (metallothionein type 3). These genes were introduced into the salt-sensitive but high-yielding rice cultivar BRRI Dhan75 (for *OcAsr1* and *OcPVA1*) and medium salt-tolerant at seedling stage , BRRI Dhan67 (for OcMT3) via an in planta Agrobacterium-mediated transformation method. Stable integration and expression were confirmed through PCR, sequencing, and qRT-PCR.

All the transgenic lines has been screened at both seedling and reproductive stage at 100-120mM salt-stress. Physiological and biochemical analysis like Biomass, chlorophyll content, electrolyte leakage has been performed at 100mM salt-stress for the transgenic lines. Then agronomic performance like yield, Na^+-K^+ has been evaluated at reproductive stage in salt stress.

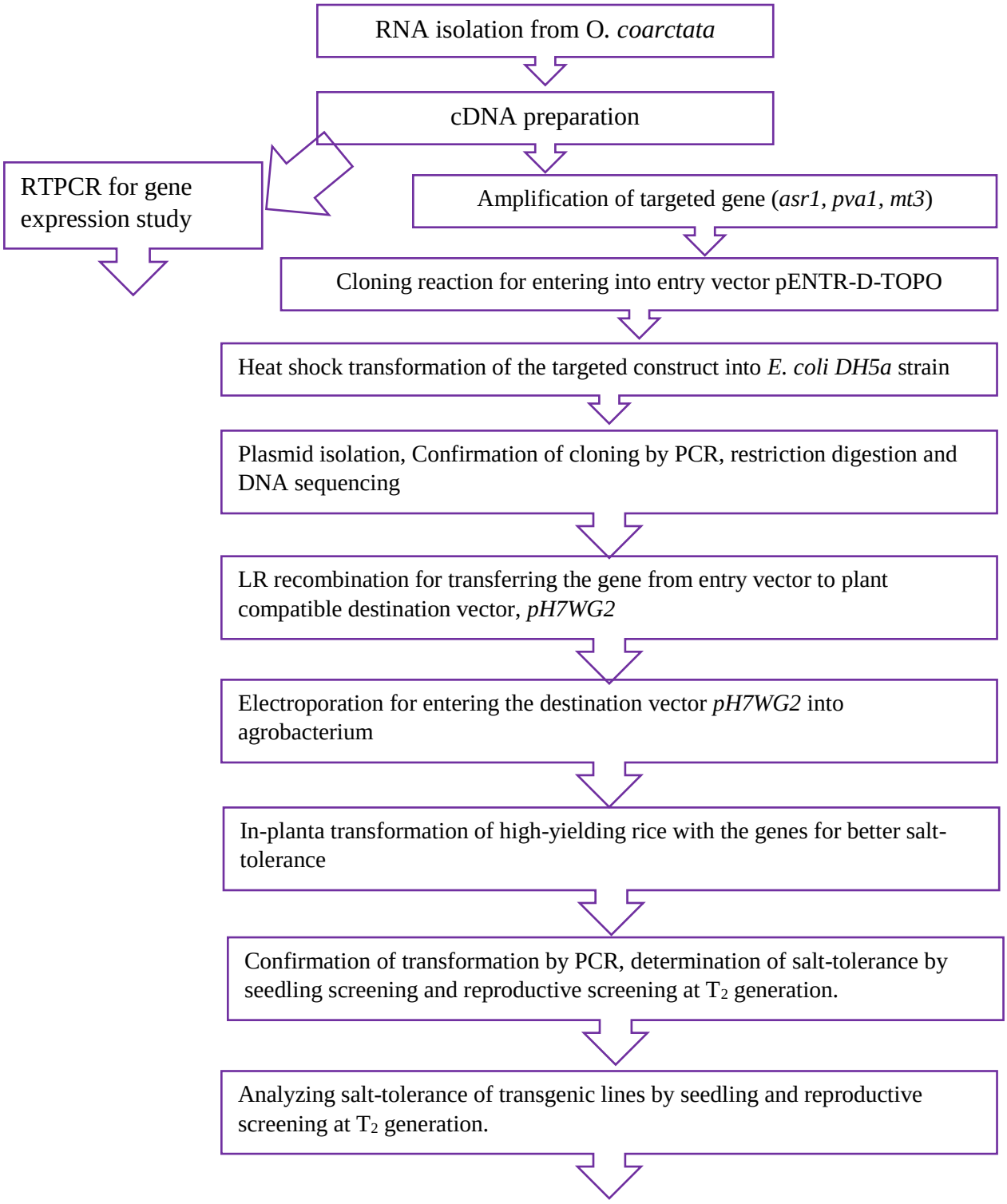
5.2 Method and Materials

A comparative analysis of the *Oryza coarctata* genes with their *Oryza sativa* homologs revealed varying levels of sequence similarity and domain conservation. *OcAsr1* showed only 50% identity with *OsAsr1*, with the ABA_WDS domain located in a different region, suggesting significant divergence. In contrast, *OcPVA1* was highly conserved, sharing over 91% identity with both *japonica* and *indica* subspecies and containing two key ATPase-related domains. *OcMT3* showed moderate similarity to *OsMT-I-3a* (83%) and *OsMT-I-3b* (75%), and also shared 50% identity with a protein from *Oryza meyeriana*. These results highlight both conserved and divergent features that may influence stress adaptation.

Table 5.1 : Comparative Analysis of Target Genes from *Oryza coarctata* with *Oryza sativa*

Target gene from *O. coarctata*	Comparison with *O. sativa*
Abscisic acid stress ripening protein (Asr1)	• Contain *ABA_WDS* domain (AA 50-126), responsive to Water deficit stress and Abscisic acid • Only 50% identical to *OsAsr1* • No similar proteins at100% identity for this isoform • *OsAsr1* also contain *ABA_WDS* domain but in different location (IndicaAA23-93; Japonica AA26-103), also no significant homology in sequence
Subunit c of Vacuolar H^+-ATPase (PVA1)	• *OcPVA1* shares 88% query coverage and 91.27% sequence identity with the *PVA1* gene from *Oryza sativa* ssp. *japonica*. Similarly, it exhibited 91.15% identity with the *indica* subspecies. • Contains two conserved domains, V_ATP_synt_C and ATP-synt_V0_c_ATP6C_rpt2
Metallothionein type 3 protein (MT3)	• 83 % identity to *OsMT-I-3a* and 75 % identity to *OsMT-I-3b* genes of rice • No similar proteins at 100% identity for this isoform • 50% identical to a uncharacterized protein of *Oryza meyeriana var. granulata*

Workflow for method and materials



5.2.1 RNA isolation and cDNA preparation for gene expression analysis

5.2.1.1 RNA isolation from plant tissue

Three genes, *Asr1*, *Pva1* and *MT3* The primary purpose of the experiment was to observe the expression level of three target genes in *O. coarctata* using quantitative Real-Time PCR technique. Therefore, the plants were subjected to 100 and 200 mM salt stress for 6 and 24 h. For the *MT3* gene, the stress was extended to 48 hours. After the stress treatments, leaf samples were collected for extracting RNA. Leaf samples were also taken from the plant without salt stress as control. Three replicates of leaf samples were used for each condition.

Table 5.1A: Salt Stress treatment tonditions for qRT-PCR expression analysis of OcASR1, OcPVA1, and OcMT3 in *Oryza coarctata*

Gene	Salt Concentration (mM NaCl)	Stress Duration (hours)	Sample Type	Biological Replicates
OcAsr1	100, 200	0, 6, 24	Leaf	3
OcPva1	100, 200	0, 6, 24	Leaf	3
OcMT3	100, 200	0, 6, 24, 48	Leaf	3

Total RNA was extracted from the shoot and root of *O. coarctata* using the Trizol reagent (Ambion, Invitrogen) following the manufacturer's protocol.

a. First frozen plant tissue was ground in liquid nitrogen using mortar and pestle.1 ml of TRIZOL was added per 50-100 mg of ground tissue and homogenized thoroughly.
b. The homogenized samples were incubated at room temperature for at least 5 minutes and sometimes more (until all samples were homogenized).
c. The tubes were centrifuged at 12000 × g for 10 minutes at 4ºC temperature. The supernatant was transferred to a clean tube and 0.2 ml of Chloroform (without IAA) was added for each 1 ml of TRIZOL used initially. The tubes were shaken vigorously by hand or vortex for 15 seconds and incubated at room temperature for 2 to 3 minutes.
d. The tubes were centrifuged at 12000 × g for 10 minutes at 4ºC temperature.
e. The aqueous phase was transferred to a clean tube. It was about 60% of the initial volume of TRIZOL reagent added to the sample.

f. 0.25 ml of Isopropanol and then 0.25 ml of 2M NaCl (DEPC treated) per 1 ml of TRIZOL used initially. The contents of the tubes were mixed by inversion and incubated for 10 minutes at room temperature. The incubation period can be stretched up to 1 hr.
g. The tubes were centrifuged at 12000 × g for 10 minutes at 4ºC temperature. The supernatants were removed and the pellets were washed with 75% ethanol (made in DEPC water).
h. 1 ml of 75% ethanol was added for each ml of TRIZOL used initially. The tubes were vortexed well. The tubes were centrifuged at 7500 × g for 5 minutes or at 12000 × g for 2 minutes at 4ºC temperature.
i. The supernatants were removed and the pellets were dried briefly. Drying the pellets completely should be avoided. ~100 µl of DEPC-treated water was added for 100 to 500 mg of tissue.
j. The tubes were vortexed for 10 to 30 seconds and incubated at 60ºC for 5 minutes. The tubes were then vortexed again and the pellets were broken with the pipette tip when necessary.
k. The pellet appeared gelatinous and transparent if RNA was still not dissolved. In that case the tubes were incubated again at 60ºC for 5 minutes and vortexed again.
l. If the transparent gelatinous pellet was gone but white 'flake' were still present, the tubes were centrifuged for 5 minutes at 12000 × g and the supernatant (RNA) was transferred to a new tube. The RNA quality was checked using 0.5 µg per lane on a 1% agarose gel in 1 × TAE.

5.2.1.2 cDNA preparation

First-strand cDNA was synthesized from 1.5 ug of total RNA using the Invitrogen Superscript III reverse transcription (RT)-PCR following the manufacturer's protocol (Invitrogen, USA).

For cDNA synthesis, 10µl of PCR amplification reaction was done. For this two different master mixture had to be prepared.

After the preparation of first master mixture, 2µl of RNA for each sample was added to it. Then denaturation was done at 65ºC for 5 minutes. Then the second master mixture was prepared. After the first denaturation, the second master mixture was added and then for amplification reaction, it was kept at 50°C for 50 minutes and at 85˚C for 5 minutes. Then the PCR reaction was paused to

add 0.5µl of RNase H. After that the reaction was resumed to keep at 37°C for 20 minutes. When it was over, the concentration of cDNA was measured using NanoDrop and then finally, the sample cDNA was stored at -20°C.

Table 5.1B: Composition of master mixture for cDNA preparation.

Composition of master mixture no.1 for cDNA synthesis		Composition of master mixture no. 2 for cDNA synthesis	
Component	Amount for one reaction	Component	Amount for one reaction
Oligo dT	0.5 µl	RT buffer	1.0 µl
10mM dNTP	0.5 µl	25mM MgCl2	2.0 µl
DEPC treated water	2.0 µl	DTT (1,4-Dithiothreitol)	1.0 µl
		RNase out	0.5 µl
		SSIIIRT (Superscript III Reverse transcriptase)	0.5 µl

5.2.1.3 Designing of Primers

Three genes, *Asr1*, *Pva1* and *MT3* The primary purpose of the experiment was to observe the expression level of three target genes in *O. coarctata* using quantitative Real-Time PCR technique. For designing the primers first internal and non-conserved sequences of the genes were taken. Primers were designed in Primer 3 website.

Table 5.1C: Selected genes, their size and protein size

Name of the gene	**Genbank ID**	**CDS Size**	**Protein Size**
Abscisic Acid Stress Ripening Protein (Asr1)	KM349310.1	414bp	137 amino acid
Vacuolar H+ATPase Subunit c (Pva1)	AF286464.1	834 bp	165 amino acid
Metallothionein Type 3 (Mt3)	AF257465.1	581 bp	64 amino acid

AF257465.1 581 bp 64 amino acid

Quantitative Real-time PCR was performed in a 10 ul reaction using SYBR Green (Bio-Rad, USA) with gene-specific primer (Table 5.1) in CFX96TM Real-Time PCR detection system (Bio-Rad, USA). PCR efficiency (95–105 %) was verified. Amplification specificity was validated by melt curve analysis at the end of each PCR cycle. Relative transcript abundance was calculated using

Table 5.1D: List of gene specific primers used in quantitative Real-Time gene expression analysis.

Name of the primers	Forward Primer Sequences (Forward and Reverse)	Length	GC content(%)	T_m (^{0}C)
PBT_U6_snoRNA	5'-TACAGATAAGATTAGCATGGCCCC-3'	24	50	66.3
	5'-GGACCATTTCTCGATTTGTACGTG-3'	24	45.8	64.7
PBT_Pc_RT_ASR	5'-CAGGACGAGTACGAGAGCTACAAC-3'	24	54.2	68.1
	5'-CGCTCTTGTGGTCCTTCTTCTT-3'	22	54.5	67.1
PBT_Pc_RT_PVA	5'-GTCATCATCAGTACCGGGATTAAC-3'	24	45.8	64.7
	5'-ACAATGAGACCATACAGAGCAAGAG-3'	25	48	66.2
PBT_Pc_RT_MT3	5'-AAGTCTCAGTGCGTGAAGAA-3'	20	50	60.3
	5'-ACTAAGGTAGGTGCATGACG-3'	20	50	60.4

the comparative cycle threshold method described by Chen et al. (2014). snoRNA gene was used as the normalization control. Expression of the targeted genes were performed at 25 cycles using genes internal primers.

Table 5.1E: Composition of master mixture for RTPCR Reaction.

RTPCR Master mix components	**Amount for one reaction**
RT Enzyme mix	0.5 µl
Primer forward	0.4 µl
Primer reverse	0.4 µl
Template RNA	1.0 µl
Nuclease free Water	2.7 µl

5.2.1.4 Quantification of Relative Gene Expression by qRT-PCR

a. Ct Value Acquisition: The threshold cycle (Ct) values for each gene were obtained from the qRT-PCR output. Each sample was run in technical triplicates to ensure accuracy.

b. Normalization to Reference Gene: For each sample, the Ct value of the target gene was normalized to that of a constitutively expressed reference (housekeeping) snoRNA, to account for differences in cDNA input.

This gave the ΔCt:
ΔCt = Ct_target gene − Ct_reference gene

c. ΔΔCt Calculation: The ΔCt of the treated or transgenic sample was then normalized to the ΔCt of the control (untreated or wild-type) sample to obtain ΔΔCt:
ΔΔCt = ΔCt_treated − ΔCt_control

d. Fold Change Calculation

The fold change in gene expression was calculated using the formula:
Fold change = 2^(−ΔΔCt)
A fold change greater than 1 indicates upregulation, whereas a value less than 1 indicates downregulation relative to the control.

e. Data Representation

Final results were expressed as relative fold changes compared to the control sample. Standard error was calculated from replicate measurements, and statistical significance was evaluated as described in the statistical analysis section.

5.2.2 Cloning of target genes (*OcAsr1, OcPva1, OcMt3*)

For cloning, we have used pENTR/D-TOPO vector as entry vector and pH7WG2 as expression vector.

The forward primer was designed adding CACC overhang to ensure compatibility with pENTR/D-TOPO vector PCR conditions were, initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing temperature for 1.0 min, extension at 72°C for 1.0 min with a final extension at 72°C for 10 minutes. Final PCR amplicons were gel extracted and quantified through nanodrop. Cloning reaction into pENTR/D-TOPO vector (Invitrogen) was then initiated following manufacturer's protocol (Publication part number 25-0434, Invitrogen).

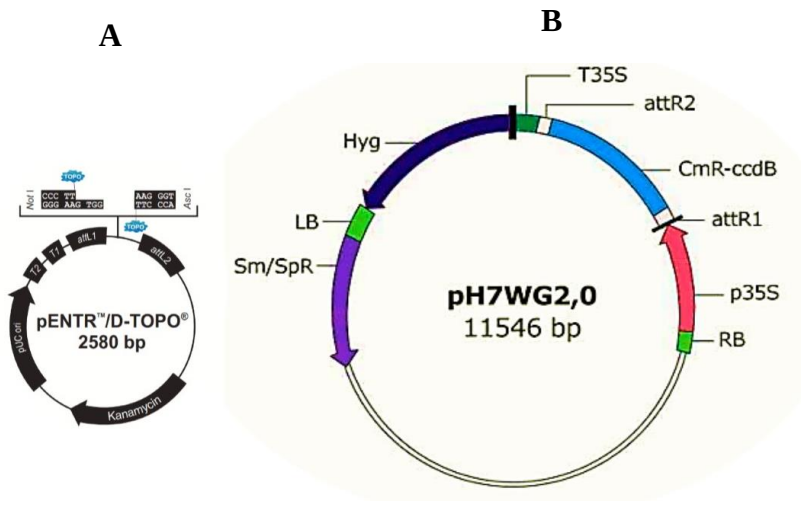


Figure 5.1: A) pENTR™/D-TOPO® (2,580 bp), a high-copy entry vector with attL1 and attL2 recombination sites flanking the gene of interest. Directional TOPO® cloning is enabled by a 5′ CACC overhang. The vector carries a kanamycin resistance gene (KanR) for bacterial selection and a pUC origin for replication in *E. coli*. B) pH7WG2,0 (11,546 bp) , a plant binary vector containing attR1 and attR2 sites flanking a ccdB-Cm^R negative selection cassette, which is replaced upon recombination with the entry clone. The CaMV 35S promoter drives constitutive gene expression, and the 35S terminator ensures proper transcript termination. The vector carries a hygromycin resistance gene (Hyg^R) for selection in plants, and spectinomycin/streptomycin resistance (Sm/SpR) for bacterial selection. The left and right border (LB and RB) sequences facilitate T-DNA integration via *Agrobacterium*-mediated transformation.

The pENTR plasmid construct was transformed into E. coli DH5α competent cells through heat shock using standard protocols (Sambrook et al. 1989). Successful cloning was then confirmed by PCR, restriction digestion of the isolated plasmid appropriate restriction enzymes for each gene (NEBr inc) individually followed by a final confirmation by direct sequencing of the vector and gene specific primers.

Table 5.2: Primer used for gene cloning

Primer Name	Sequence (5'→3')	GC Content (%)	Tm (°C)
PBT_Pc_ASR1	CACCATGGCGGAGGAGAAGCACCACCA	64.3	64
	TCAGCCGAAGAGGTGGTGCTTCTTCTC	61.5	65
PBT_Pc_PVA1	CACCATGTCGTCGGTTTTCAGCGGCGAT	63.3	64
	TTAATCTGCACGGGATTGACCAGCACG	57.7	63
PBT_Pc_-MT3	CACCCTTAAGCGAAAGCAGC	55.0	64
	TCACACAAATACACGCTGCATT	43.5	62

Successful pENTR/D-TOPO cloning allowed recombining the desired sequence of target gene into a destination vector by using the Gateway® LR recombination reaction (Invitrogen) (figure 5.1). The LR reaction was carried out following the manufacturer's protocol. Positive colonies were screened out by gene specific primers. Finally, Agrobacterium tumefaciens (LBA4404) was electroporated with the constructed pH7WG2 containing target gene separately using standard protocols (Sambrook et al. 1989). Positive colonies were authenticated by PCR reactions with gene specific primers (Table 5.2).

5.2.3 In-planta transformation of high-yielding rich with target gene

Plant material

BRRIdhan75 and BRRI Dhan67 by Bangladesh Rice Research Institute (BRRI) was used in this study. BD75 is a farmer-popular high-yielding, medium duration-Boro and Aus season variety. The average production capacity of this variety is about 7.0 tons/hectare.

BRRI Dhan67 is medium salt-tolerant rice, can tolerate 120-140mM salt stress at seedling stage up to 3 weeks according to BRRI. It is a Boro season variety, lifespan 140-250 days and yield 3.8-7.4 tons/hectare depending on the level of soil salinity.

Transformation procedure:

The mature rice seeds were sterilized with 99% ethanol for 3 mins. The ethanol was then poured off and washed in 30% chlorox with 1 drop of tween 20 for 5 mins. After that the seeds were washed with ddH_2O five times. The seeds were placed on filter paper and soaked with ddH_2O. The seeds were incubated at 37°C for 2 days. During soaking, water was replaced once. After 2 days of soaking, the embryo region of the seeds turned white (figure 5.2).

Transformed Agrobacterium strain (LBA4404) target gene was cultured and prepared for transformation following the standard protocol discussed in Lin et al. (2009) with some

modifications. Here, bacterial cells were first inoculated into liquid YM medium and incubated at 28 C overnight, then centrifuged and resuspended into bacterial re-suspension medium (Sucrose, glucose, AB buffer and AB salt) instead of 1/2 MS medium. Acetosyringone was added to both liquid YM medium and bacterial re-suspension medium instead of ½ MS medium for improving the transformation efficiency.

Antibiotic selection was done with streptomycin (20 mg/l) and spectinomycin (20 mg/l). Finally, bacterial density was measured at OD600 and fixed at 0.6.

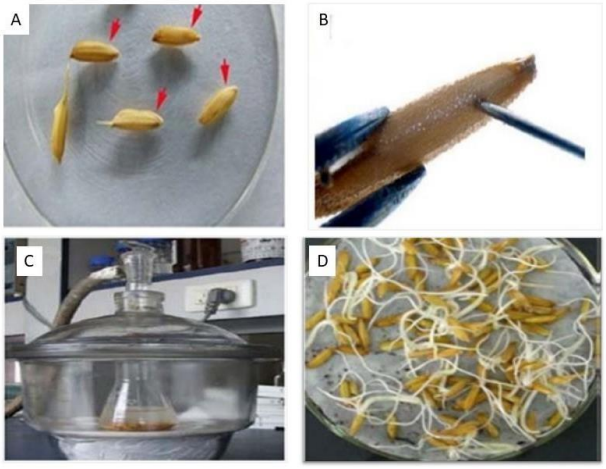


Figure 5.2 In-planta transformation procedure step by step. A) Embryo region of seed turns white after germination ;B) Inoculation of a seed with a needle; C) An illustration showing the direction of piercing; D) seeds soaked in the Agrobacterium inoculum and drawn vacuum for infiltration (Lin, Zhou et al. 2009).

The embryo of seeds was then pierced once by a needle. The depth of piercing was about 1 mm. Before piercing, the needle had been dipped in the Agrobacterium inoculum. In order to inoculate Agrobacterium into the embryonic apical meristem and not to seriously damage the embryo, the needle was pierced the side of the plumule which lies beneath the husk where a shoot would later emerge. The pierced seeds were then placed in a conical flask and soaked in the Agrobacterium inoculum. The conical flask with the seeds was placed into a bell jar (D). The air in the bell jar was drawn out by a vacuum pump at a pressure of 80 kPa for 15 min. Then, the vacuum was released for 2 min and pumped again for 3 min. The inoculated seeds were transferred onto Petri-disk containing wet filter papers and incubated in the dark at 28°C for 6-7 days. When the seedlings turns into green, these were transferred to hydroponic solution. After 2-3 days later, the hydroponic

pots were transferred to net-house. When the seedlings were enough mature, then transferred to soil.

5.2.4 Confirmation of the transformation of T_0 transformants:

5.2.4.1 Leaf disk senescence test

Leaf disks were excised from healthy and fully expandedrice leaves of similar age from both WT BR55 and BR55_SNAC1 transgenic lines at T2 and T3 stages. The disks were floated in a 20 mL solution containing 0, 100, 200 mM NaCl for 3 days. The treatment was carried out at 25 _C (Sahoo et al. 2012). After the treatment, the leaf pieces were blotted with tissue paper and weighed before measuring chlorophyll.

5.2.4.2 DNA isolation and PCR analysis

Genomic DNA was isolated at T0 generation from the upper leaves (flag leaves and the second leaves immediately below the panicles) of the transformants which plants have shown better salt tolerance in leaf disk senescence assay. DNA was isolated using CTAB method (Doyle and Doyle 1987). The CTAB method is a less expensive procedure and it is characterized by high yields of DNA from a small amount of tissue. The main drawbacks of this procedure are less pure DNA is obtained, time consuming and laborious.

a. 0.01 g-1.0 g of leaf tissue was ground to a very fine powder in a mortar containing liquid nitrogen. The powdered tissue was transferred directly into the screw capped tube containing 5 ml of preheated (65°C) CTAB buffer and 12μL of beta mercapto ethanol and was swirled gently to mix. [CTAB (Cetyl trimethylammonium bromide) is a detergent as well as a compound similar to an anion-binding reagent. Any negatively charged molecule may become bound with CTAB. It also precipitates some negatively charged proteins and polysaccharides and beta mercapto ethanol was added to bind and precipitate polyphenolics.]
b. The sample was incubated at 65°C in water bath continued for 30 minutes with occasional vigorous shaking with 5 minutes interval. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added, and was gently mixed. [Phenol was used to precipitate proteins and for purification].
c. The tube was centrifuged at 6000 ×g for 15 minutes to resolve phases. The aqueous phase was transferred to a fresh tube.2/3 volume of ice cold isopropanol was added, mixed gently

and then kept overnight at 4°Cto precipitate DNA. [Isopropanol increases the concentration of DNA].

d. Tube was then centrifuged at 6000 ×g for 15 minutes to collect the precipitate.
e. The supernatant was discarded and the tube was washed with 70% ice cold ethanol.
f. After that, the tube was centrifuged at 6000 ×g for 10 minutes and the supernatant was discarded and pellets were allowed to dry completely.
g. TE buffer was added according to the concentration of precipitate and the precipitate was dissolved by gentle inversion.
h. RNase A (100 µg/mL) was added and the tube was kept in 37°C for 30 to 40 minutes. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and was shaken.
i. The tube was centrifuged at 6000 ×g for 15 minutes. Aqueous phase was taken into 1.5 mL eppendorf tubes and then equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added.
j. The sample was centrifuged at 15000 ×g for 15 minutes. The supernatant was transferred into fresh tube and equal volume of phenol: chloroform:IAA (25:24:1) was added. The sample was shaken and centrifuged at 15000 ×g for 10 minutes.
k. The aqueous phase was transferred into fresh tube. One tenth volume of 3M Na-acetate (pH 5.2) and double volume of 99% ice cold ethanol was added and was shaken. The DNA should be observable at this step. [Precipitation of the protein is aided by the addition of salts such as sodium acetate.]
l. The tube was kept for overnight incubation at -20°C.The tube was centrifuged at 15000 ×g for 15 minutes. 1 ml of 70% ice cold ethanol was added to wash any salt.
m. The tube was centrifuged for 5 minutes at 15000 ×g and then the supernatant was discarded and the pellet was allowed to dry completely.
n. Finally, the PCR graded TE buffer was added according to the concentration of the pellet (Doyle 1991).

5.2.4.3 Preparation of PCR reaction

To prepare the sample, genomic DNA, 20% DMSO and nuclease free water were dispensed in the labeled PCR tubes as follows in Table: 5.3A. The DNA, DMSO and water mixture was then denatured at 95ºC for 5 minutes and instantly transferred into ice. After through mixing and momentary spin 6.4

µl of above master mixture was dispensed to thin walled PCR tubes containing genomic DNA, 20 % DMSO and nuclease free water. The volume was made upto 15 µl by adding varying amounts of sterilized nuclease free water. Taq DNA polymerase was added just before the start of the reaction. Finally, the tubes were subjected to momentary spin and transferred to thermocycler (GeneAtlas of Astec and nexus gradient of eppendorf) for the amplification reaction.

PCR was carried out at 95^0C for 5 min followed by 35 cycles of 1 min denaturation at 95^0C, 1 min annealing at specific Tm (Table for details, same primers used for cloning) and 1 min extension at 72^0C, then a final extension of 10 min at 72^0 C. In addition, gene-specific (*asr1, pva1, mt3*) primers were designed (Table for details). Amplification of target gene was carried out at both T_1 and T_2 generation. PCR program was carried out as follows: initial denaturation at 95^0C for 5 min, followed by 35 cycles of 1 min at 95^0C, 1.0 min at specific Tm of the primers and 1.0 min at 72^0 C, then a final extension of 10 min at 72^0C. The composition of master mix is given in Table 5.3B.

Table 5.3 A: Preparation of sample, control & positive control tubes with DNA, DMSO and ddH2O

Tube usage	**DNA (50ng/ µl)**	**DMSO (20%)**	**ddH20 (adjustable)**	**Total volume**
Sample tube	2.0µl	3.0µl	5.0µl	10µl
Control tube	0.0µl	2.0µl	8.0µl	10µl
Positive control	2.0µl	3.0µl	5.0µl	10µl

Table 5.3B: Composition of PCR master mix

Composition of PCR master mixture **Components**	**1 reaction (µL)**
PCR buffer (10X)	1.5
dNTPs (1 mM)	1.5
MgCl2 (25 mM)	1.0
Forward primer (50ng/ µL)	0.5
Reverse primer (50ng/ µL)	0.5
Taq DNA polymerase (10X diluted)	0.5
Total	

5.2.4.4 Real time PCR for transgenics for analyzing their gene expression

The fold change in gene expression in transgenic lines at T2 generation has been done following the method described in 5.2.1.

5.2.4.5 Semi-quantitative PCR (Semi q-PCR) for Transgenic Lines

Total RNA was extracted from the leaf tissues of transgenic plants following the method described in Section 5.2.1.1. First-strand cDNA was synthesized from the isolated RNA using a standard reverse transcription protocol.

PCR amplification was carried out using gene-specific primers designed for OcMT3, which were previously optimized for RT-PCR analysis. The PCR products were separated on an agarose gel to confirm amplification.

The intensity of the DNA bands was measured using ImageJ software to evaluate relative gene expression levels among the transgenic lines.

5.2.5 Salinity screening of putative hybrids at seedling stage

5.2.5.1 Physiological screening

1. Seeds are germinated normally in water.
2. The seedlings were allowed to grow in Yoshida (Yoshida et al. 1976) culture solution until they reached the four-leaf stage (14–18 days after germination).
3. Then, gradually NaCl stress was applied starting from 6 dS/m (deciSiemens per meter which is the unit of electrical conductivity (EC) of a solution; 6 dS/m = 60 mM) to 10 dS/m at 24-hourly increments of 2 dS/m.
4. When 90 % of the leaves of the Wild-type control are damaged, the tolerance-related traits (LDS, chlorophyll content and electrolyte leakage) of all control and stressed plants were then measured.
5. The plants were scored according to the protocol mentioned by Amin et al.(2012).
6. The chlorophyll content and electrolyte leakage of the stressed and control transgenic shoots as well as WT were measured at this stage (Cao et al. 2007; Farkhondeh et al. 2012; Ogawa et al. 1969)

5.2.5.2 Determining the chlorophyll concentration

Fresh leaves from control and stress seedlings were cut into pieces and 100 mg of fresh tissues was put into a bottle containing 12 ml of 80 % acetone. After 48 h, absorbance of leaf tissues

extract was measured at wavelength 663 and 645 nm. The total amount of chlorophyll was calculated following the protocol by Yoshida et al. (1976) and Chutia and Borah (2012).

A = ECd

A is proportional to C (because E and d is constant)

Here,

A = observed absorbance

E = a proportionality constant (extinction coefficient) (= 36 mL / cm)

C = chlorophyll concentration (mg / mL)

d = distance of the light path (= 1 cm)

Reduction of chlorophyll content was determined using following formula:

Chlorophyll reduction= (Control-Stress)/Control x 100

5.2.5.3 **Measurement of Relative Electrolyte Leakage**

Relative electrolyte leakage was measured following the method described by Cao et al. (2007).The leaf segments from the seedlings were taken into bottle containing deionized water and kept in shaker for 2 hours. The conductivities (C1) of the obtained solutions were then determined. Then the leaf segments in deionized water were autoclaved. After being thoroughly cooled to room temperature, the conductivities (C2) of the resulting solutions were determined. The values of C1 to C2 (C1/C2) were calculated and used to evaluate the relative electrolyte leakage. Each data point represents average from three independent experiments. The data were subjected to statistical analysis using t test.

5.2.6 Salinity tolerance at reproductive stage

5.2.6.1 Yield data

Seeds are germinated in clean Petri dish covered with fresh filter paper. After 2-3 days seedlings were transferred to hydroponic solution. When plant age 20-25 days, plants are transferred to soil in pots. The pots are taken in a large bowl, added hydroponic solution submerging the pots. Each pot contains one plant. Each bowl therefore contains wild type and the three transgenic/experimental plants. Just before booting stage, we had applied 100mM salt-stress in the bowl at once. The water level is marked. The EC and pH of the solution is maintained throughout the reproductive stage. Then while the seeds were matured, harvested very carefully. We have calculated the yield data.

5.2.6.2 Measurement of Na^+, K^+ content

Sodium and potassium concentrations in transgenic and wild-type shoot and root at 0 and 12 dS/m were measured. Plants were washed in flowing tap water for 30 s and oven-dried. The plants from each biological replicates were pooled, ground and analyzed by a flame photometer 410 (Sherwood, UK) after 48 h of extraction with 1 N HCl following the procedure described by Yoshida et al. (1976). Concentrations were calculated as percent of dry weight. Then sodium to potassium ratio was determined.

5.2.6.3 ROS measurement at salt-stress

NBT assay provides a rapid and semi-quantitative method to evaluate oxidative stress levels in plants exposed to salinity and can be used to screen for stress tolerance in different genotypes or treatments.

To perform the test, freshly excised leaf samples from both control and salt-treated plants are immersed in a 0.1% (w/v) 50mL NBT solution prepared in 10 mM potassium phosphate buffer (pH 7.8), often supplemented with 10 mM sodium azide to inhibit endogenous peroxidase activity and enhance specificity (Fryer et al., 2002). The samples are vacuum-infiltrated (optional) and incubated in the dark for 1–2 hours at room temperature. After incubation, leaves are boiled in 95–100% ethanol to decolorize chlorophyll, allowing clearer visualization of the blue formazan deposits.

The intensity of blue staining correlates with the accumulation of superoxide radicals. Under salt stress, susceptible rice lines typically exhibit extensive blue coloration, indicating high levels of ROS accumulation and oxidative damage. In contrast, transgenic or salt-tolerant lines often show reduced blue staining, suggesting enhanced ROS detoxification capacity or lower ROS generation (Kwon et al., 2002).

Results

5.3 Results of *Abscisic acid stress & ripening (OcAsr1)* gene cloning and transformation

5.3.1 Gene expression analysis of *OcAsr1, OcPVA1,* and *OcMT3* at salt stress in *O. coarctata*

To investigate the temporal expression patterns of stress-responsive genes under salt stress, the relative expression levels of *OcAsr1, OcPVA1*, and *OcMT3* were analyzed under 100 mM and 200 mM NaCl treatments at different time points (Figure 5.3). The *OcAsr1* gene exhibited a modest induction at 100 mM NaCl after 24 hours (~1.3-fold), while a significant upregulation (~2.8-fold) was observed at 200 mM after 24 hours, indicating a dose-dependent and delayed response (Figure 5.3A). In contrast, *OcPVA1* displayed strong and consistent induction under both salt concentrations at 24 hours, with expression levels reaching approximately 2.7- to 3.2-fold, suggesting its broader role in early salt stress adaptation (Figure 5.3B). Interestingly, *OcMT3* showed minimal expression at earlier time points (6 h and 24 h), but was significantly upregulated at 48 hours under both 100 mM (~2.4-fold) and 200 mM (~2.7-fold) salt treatments, highlighting its potential function in long-term or late-phase stress responses (Figure 5.3C). Collectively, these findings suggest distinct regulatory dynamics among the three genes, with *OcAsr1* and *OcMT3* responding primarily under higher and prolonged stress, while *OcPVA1* may act as a more immediate and general responder to salt stress.

Table 5.4: Comparative gene expression study of *OcAsr1, OcPVA1* and *OcMT3*

Gene	Peak Time Point	Peak Condition	Max. Fold Change	Response Pattern	Significance
OcAsr1	24 hours	200 mM NaCl	~2.8×	Late response, dose-dependent	Significant at 200 mM, 24h
OcPVA1	24 hours	Both 100 & 200 mM NaCl	~3.2×	Strong, consistent upregulation	Significant at 24h for both
OcMT3	48 hours	200 mM NaCl	~2.7×	Very late response, stronger at higher salt	Significant at 48h for both

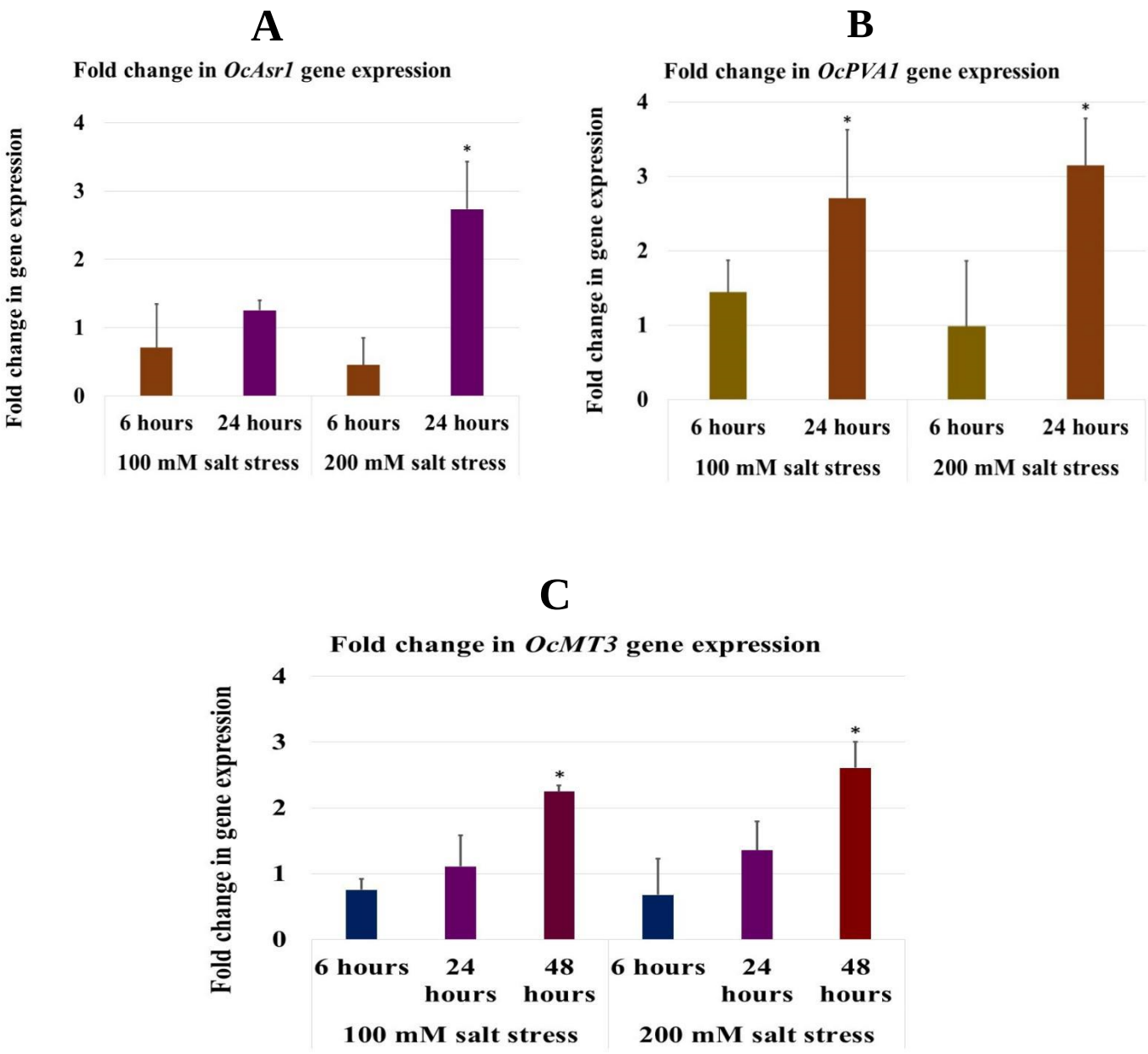


Figure 5.3: Gene expression study in *O. coarctata* at 100mM and 200mM salt-stress. A) *OcAsr1* gene expression at 6 & 24 hrs in 100 & 200mM salt-stress, *OcAsr1* has been found to be highly expressed at 24 hrs after 200mM NaCl stress. B) *OcPVA1* found to be highly expressed at 24 hrs after both 100mM and 200mM NaCl stress. C) *OcMT3* has been found to be expressed at 24 hrs after both 100mM and 200mM NaCl stress. The highest expression found after 48 hrs in both stress condition.

5.3.2 Cloning of Abscisic acid, stress & ripening (*OcAsr1)* gene

The Abscisic acid stress ripening protein gene (*OcAsr1*) was successfully cloned from *Oryza coarctata*. Initial cloning was performed into the entry vector pENTR, and the presence of the gene was confirmed through gene-specific PCR (figure 5.4A), restriction enzyme digestion, and DNA sequencing. Following confirmation in the entry vector, the gene was subsequently transferred to the plant expression vector *pH7WG2.0* using Gateway recombination technology. Successful recombination and correct orientation of the insert were again validated by restriction digestion (figure 5.4C) and sequencing, confirming the integrity of the cloned *OcAsr1* gene construct for downstream transformation studies.

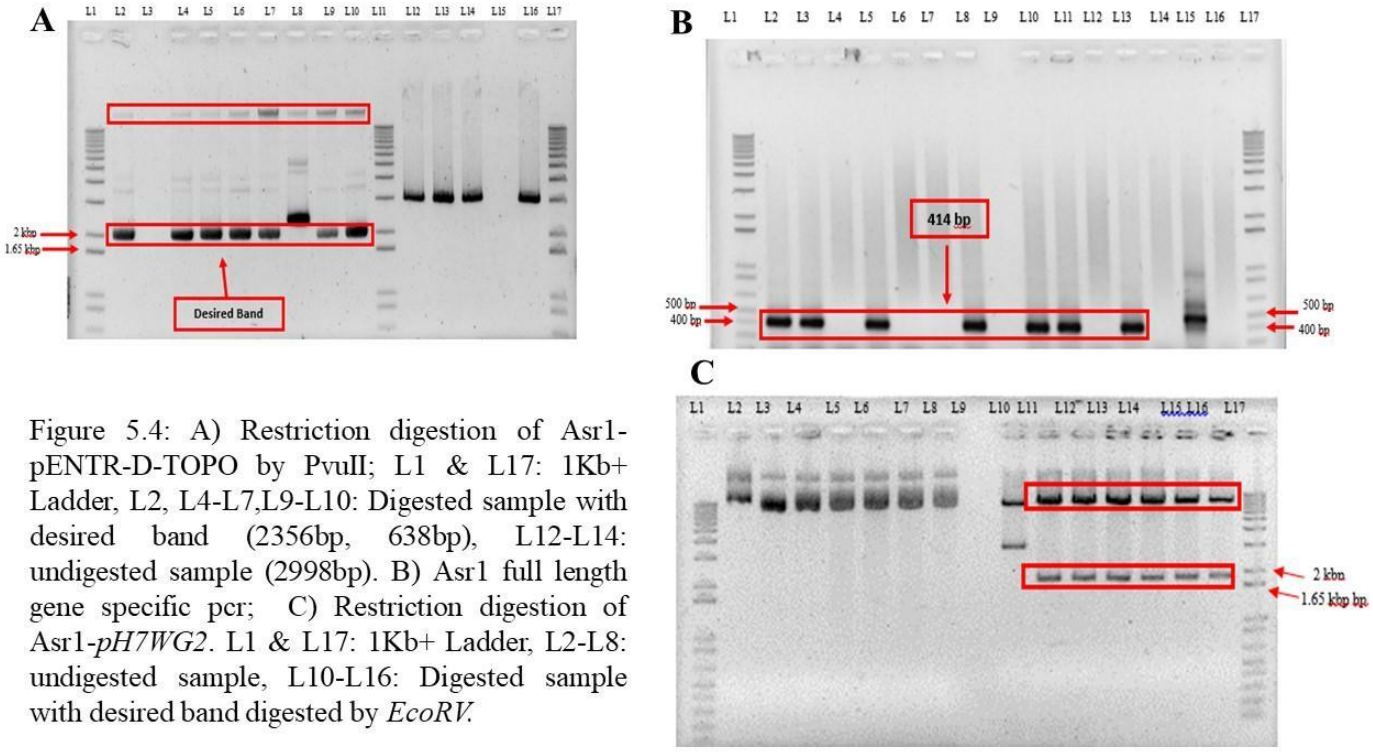


Figure 5.4: A) Restriction digestion of Asr1-pENTR-D-TOPO by PvuII; L1 & L17: 1Kb+ Ladder, L2, L4-L7,L9-L10: Digested sample with desired band (2356bp, 638bp), L12-L14: undigested sample (2998bp). B) Asr1 full length gene specific pcr; C) Restriction digestion of Asr1-*pH7WG2*. L1 & L17: 1Kb+ Ladder, L2-L8: undigested sample, L10-L16: Digested sample with desired band digested by *EcoRV.*

5.3.3 Transformation of high yielding rice variety with *OcAsr1*

The *OcAsr1* gene was introduced into the salt-sensitive rice variety BRRI Dhan75 (BR75) using *in planta* transformation method. Germinating seeds were directly infected with *Agrobacterium*

containing the *OcAsr1* construct. Fifteen plants out of 40 transformants survived after transferring to soil in the net house. Similar survival rates were obtained in repeated infections. Transformed plants were allowed to grow, mature, flower and set seeds. Following transformation, putative transgenic plants at the T_0 stage were initially screened using the leaf disk senescence assay**.** Plants showing delayed senescence compared to the wild type were selected as potential transformants. The presence of the *OcAsr1* transgene in these selected plants was subsequently confirmed by PCR amplification using gene-specific primers (figure 5.5 B), validating the successful integration of the gene into the rice genome.

5.3.4 Generation advancement to T_2 and selection of the best transformed plants (*OcAsr1)*

Five plants (P_65_1, P_70_1, P_73_2, P_76_2, P_77_2) showing positive results in both leaf disk senescence assay and PCR at T_0 were chosen for generation advancement. Further analyses were conducted in the succeeding T_1 and T_2 generations. T_1 seeds were collected only from those panicles whose flag leaves were positive in the PCR test. Genetic segregation was calculated based on the hygromycin assay at the seedling stage of T_1. Transgenic line P_65_1, P_70_1 and P_73_2 followed the Mendelian inheritance law (table 5.5).

Table 5.5: Segregation analysis of transgenic (resistant) and non-transgenic (susceptible) seedlings in the T_1 plants

Hygromycin resistant plant ID at To generation (*OcAsr1*)	Number of resistant seedlings (T1)	Number of susceptible seedlings (T1)	Chi-square test or v2 test = (observed-expected)2 /(expected)	P value	Mendelian inheritance (3:1 segregation ratio)
P_65_1	25	18	1.96	0.16151332	Follwed
P_70_1	22	15	2.227273	0.135593	Follwed
P_73_2	29	19	3.448276	0.06331779	Follwed
P_76_2	24	14	4.166667	0.04122683	Did not follow
P_77_2	27	14	6.259259	0.01235459	Did not follow

5.3.5 Effect of salt (NaCl) stress in *OcAsr1* transgenic lines at seedling stage

To evaluate the physiological performance of the *OcAsr1*-expressing transgenic rice lines under salt stress, several growth and stress indicators were assessed at the seedling stage under 100 mM NaCl treatment. The parameters measured included root length, shoot length, root fresh weight, shoot fresh weight, chlorophyll content, and electrolyte leakage, and were compared with those of the non-transformed BRRI Dhan75 control plants. Under salt stress, transgenic lines exhibited significantly greater root and shoot growth, along with increased biomass accumulation, compared to the wild-type controls. Chlorophyll content was also better retained in the transgenic plants, indicating improved photosynthetic efficiency under saline conditions. Furthermore, electrolyte leakage**,** a key indicator of membrane stability, was substantially lower in the transformed lines, suggesting enhanced cellular integrity during salt stress. These findings collectively support the role of *Ocasr1* in conferring salinity tolerance through improved growth maintenance and membrane stability under adverse conditions.

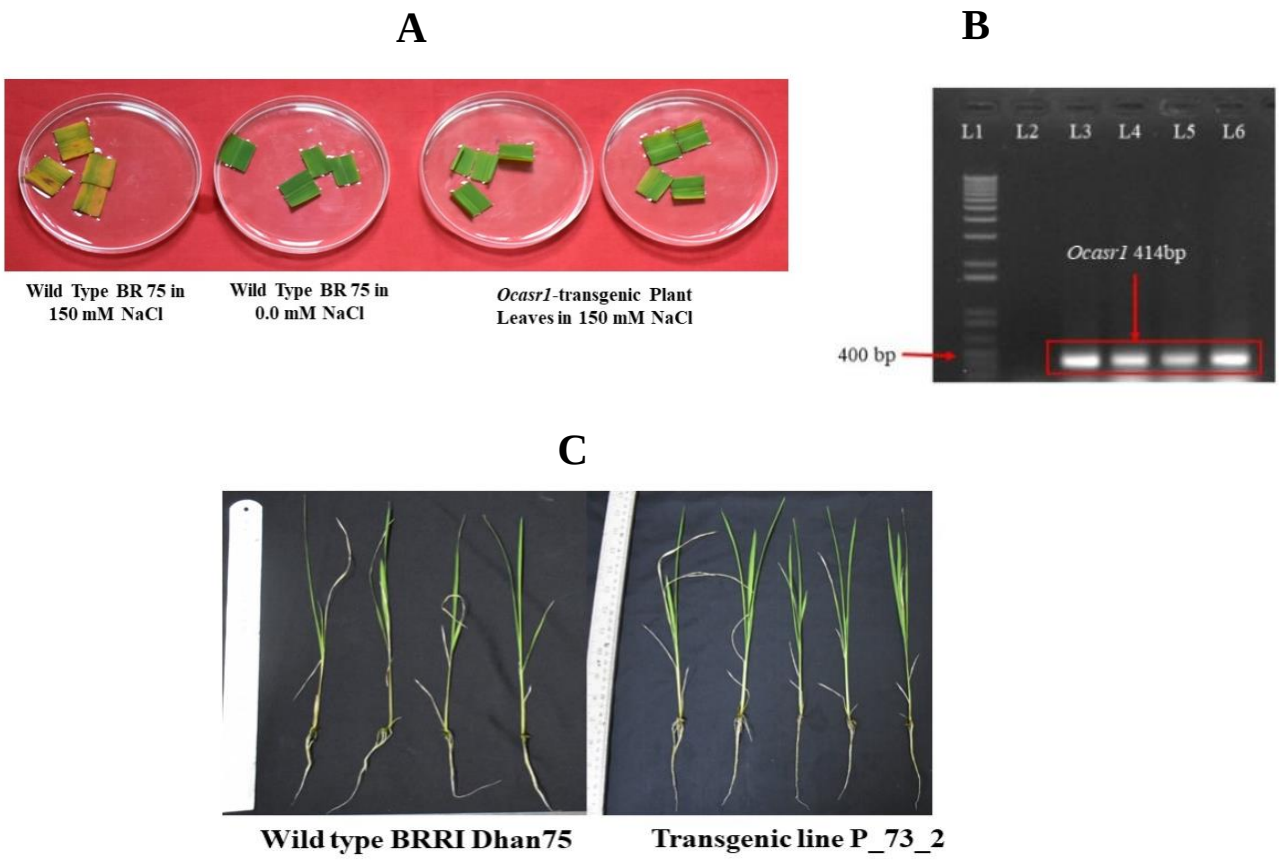


Figure 5.5: Seedling stage screening of *OcAsr1*-BRRIDhan75 at 100mM salt stress for15 days A) Leaf disk senescence assay of Wild type BRRI Dhan75 and transgenic lines at control condition and 100mM NaCl; B) Presence of transgene confirmed by *Asr1* gene specific PCR in T_0 generation C) *Asr1*Transformed seedling of P_73_2 line after 100mM salt-stress after seven days.

To evaluate the functional role of the *OcAsr1* gene in conferring salt tolerance, chlorophyll content was measured in both wild-type (WT_BR75) and transgenic rice lines expressing *OcAsr1* under control and 100 mM NaCl conditions. Under control conditions, all genotypes maintained similar levels of chlorophyll, ranging between 0.32 to 0.38 mg/ml, indicating no growth disadvantage or phenotypic difference among the transgenic lines in the absence of stress. However, under salt stress, a significant reduction in chlorophyll content was observed in the wild-type plants, with levels dropping to approximately 0.16 mg/ml. In contrast, several transgenic lines—particularly P_65_1, P_70_1 and P_76_2 lines retained substantially higher chlorophyll content under the same stress, with P_70_1 showing the highest retention, nearly 0.27 mg/ml. Statistical analysis

confirmed that the differences between these transgenic lines and the wild-type were significant ($p < 0.05$, $p < 0.01$), suggesting an enhanced photosynthetic stability under saline conditions. These findings indicate that *OcAsr1* may play a protective role in chlorophyll preservation, thereby contributing to improved salt tolerance in rice at the seedling stage (figure 5.6A).

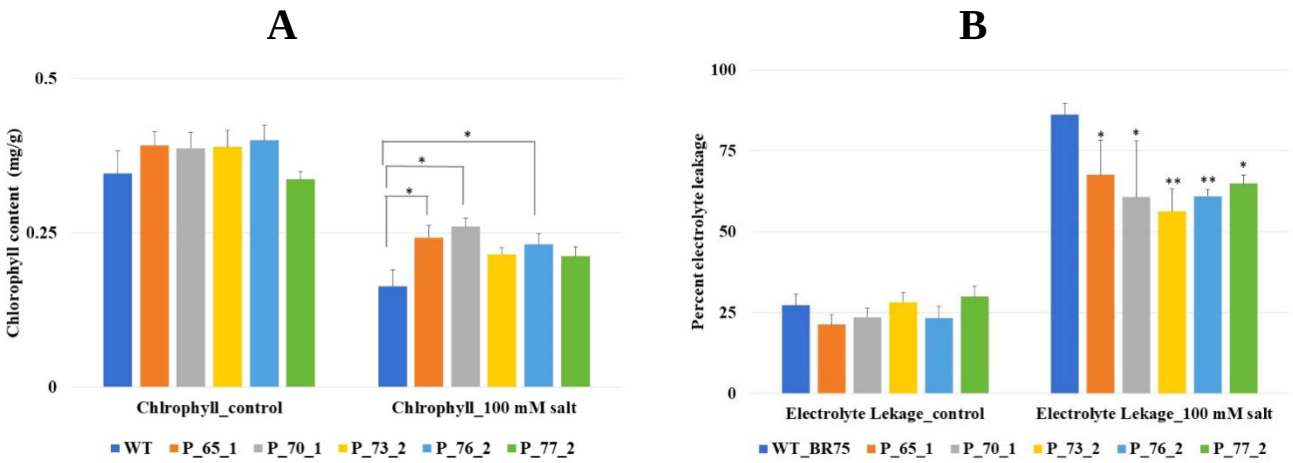


Figure 5.6: Seedling stage screening of Ocasr1-BRRIDhan75 at 100mM salt stress for15 days A) Chlorophyll content of Wild type BRRI Dhan75 and transgenic BRRI Dhan75 at control condition and 100mM NaCl, three lines have shown significantly (p-value 0.05) better performance than Wild type BRRI Dhan75. B) percent Electrolyte leakage, five lines showed significantly better performance.

Electrolyte leakage was measured as an indicator of membrane integrity under salt stress in both transgenic and wild-type BRRI Dhan75 plants. Under control conditions, all lines exhibited relatively low leakage, ranging between 22% and 32%, with no significant differences observed among the genotypes. However, upon exposure to 100 mM NaCl, the non-transformed BRRI Dhan75 (WT_BR75) showed a dramatic increase in electrolyte leakage, reaching nearly 85%, indicating severe membrane damage. In contrast, the transgenic lines demonstrated significantly lower levels of leakage under the same conditions. Among them, lines P_70_1, P_73_2, and P_76_2 showed the most pronounced reductions, with leakage values around 55–60%, significantly lower than the wild type ($p < 0.01$). The other transgenic lines also exhibited moderate reductions ($p < 0.05$). These results suggest that *OcAsr1* expression enhances membrane stability under salt stress, possibly by mitigating oxidative damage or maintaining osmotic balance (figure 5.6B).

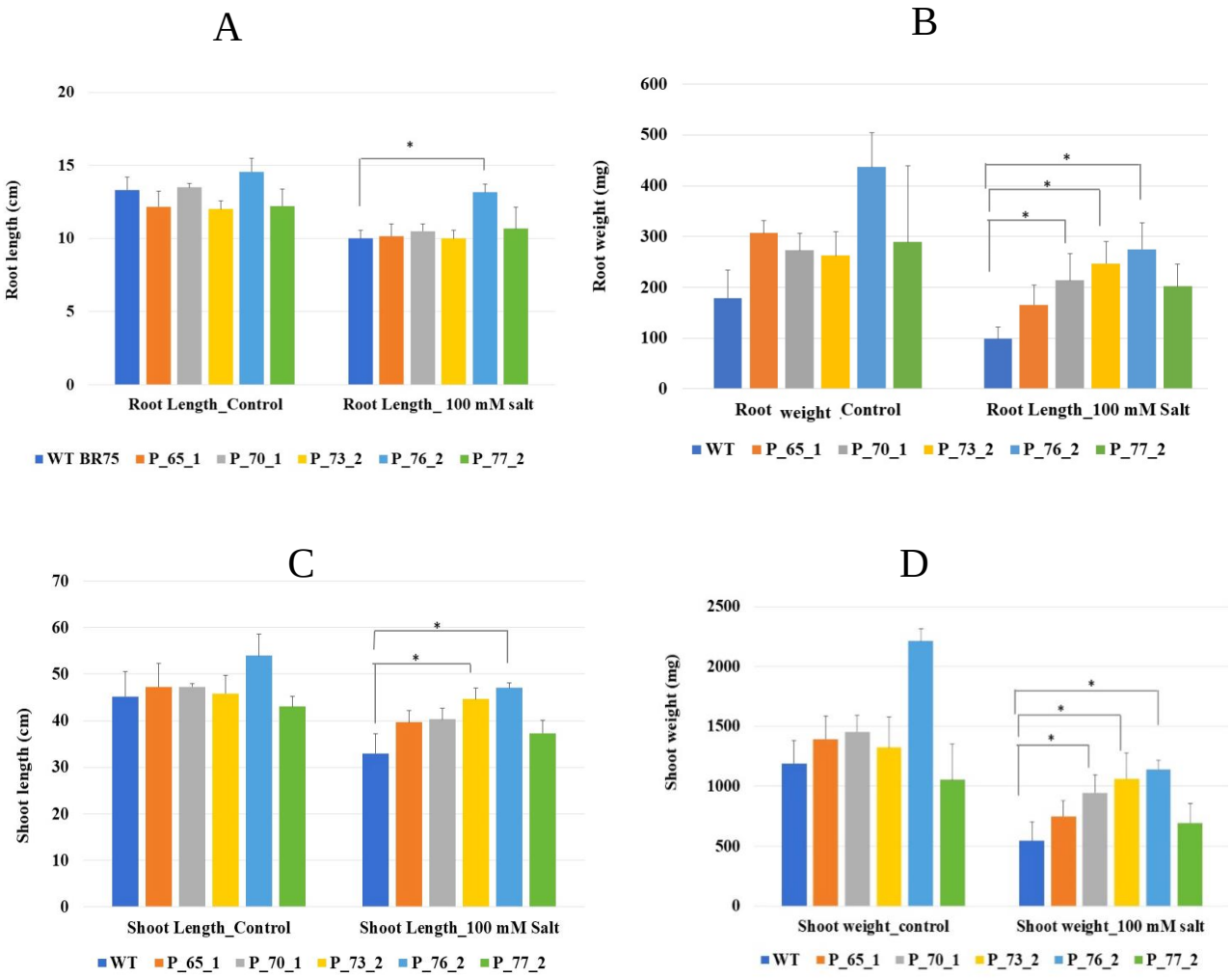


Figure 5.7: Seedling stage screening of *OcAsr1*-BRRIDhan75 at 100mM salt stress for15 days compared to control condition; A) Root length; B) Root weight; C) Shoot length & D) Shoot weight.

At seedling stage, other important parameters like root length, root weight, shoot length and shoot parameters also have been considered (figure 5.7). Three lines (P_70-1, P_73_2 & P_76_2) have shown significantly higher root weight and shoot weight compared to wild type at 100 mM salt stress. Two lines, P_73_2 & P_76_2 have shown significantly higher shoot length. Lastly, line P_76_2 have shown significantly higher root length than wild type. line P_76_2 have shown greater performance than wild type both in control and stress condition.

5.3.6 Effect of salt (NaCl) stress at reproductive stage in *OcAsr1*-transgenic lines

In this study, a comprehensive reproductive screening was conducted to evaluate the performance of *Asr1* gene-transformed rice lines relative to the wild-type cultivar BRRI Dhan75. The analysis focused on key agronomic and physiological parameters, including yield-related traits, as well as the accumulation of sodium (Na^+) and potassium (K^+) ions in both root and shoot tissues. This comparative assessment was aimed at determining the impact of *Asr1* overexpression on reproductive development and ion homeostasis under 100mM NaCl stress condition.

Figure 5.8: Transgenic lines of *OcAsr1*-BRRIDhan75 at reproductive stage

Under both control and salt stress conditions, grain yield was assessed in *Asr1*-transformed rice lines relative to the wild-type cultivar BRRI Dhan75 (WTBR75) (figure 5.9). Under non-stress conditions, all transgenic lines exhibited comparable or slightly enhanced yield performance compared to the wild type, with no statistically significant differences observed. However, exposure to 100 mM NaCl resulted in a marked decline in yield in WTBR75, highlighting its sensitivity to salinity. In contrast, several *Asr1*-overexpressing lines, particularly P_65_1, P_73_2, P_76_2, and P_77_2, maintained significantly higher yields under salt stress. These lines demonstrated improved reproductive resilience, with yields that were significantly greater than that of WTBR75 ($p < 0.05$), as indicated by the statistical analysis. This suggests that *Asr1* overexpression confers a protective effect on reproductive development under salinity, potentially through enhanced stress tolerance mechanisms. The observed yield stability under saline conditions (figure 5.9) underscores the functional relevance of *Asr1* in maintaining agronomic performance under abiotic stress.

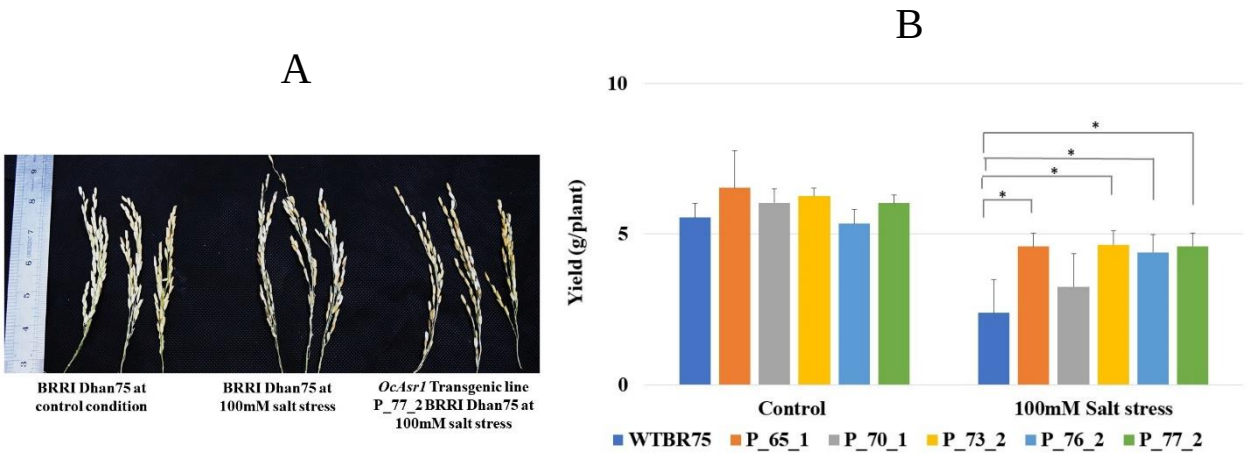


Figure 5.9: Reproductive screening of transgenic lines of *OcAsr1*-BRRIDhan75 at 100 mM salt stress. A) Panicles of wild type BRRIDhan75 and transgenic line P_77_2 at control condition and salt-stress; Transgenic line have shown healthier and better panicles than wild type. B) Yield data of wild type and selected transgenic lines at control and salt stress. Four lines have shown significantly higher yield than wild type after salt-stress.

To further investigate the role of *Asr1* overexpression in ion homeostasis under saline conditions, the Na^+/K^+ ratio in root tissues was measured in both transgenic and wild-type lines (figure 5.10). Under control conditions, all genotypes, including WTBR75 and the *Asr1*-transformed lines, exhibited low and comparable Na^+/K^+ ratios, indicative of balanced ion uptake in the absence of stress. However, upon exposure to 100 mM NaCl, a substantial increase in the root Na^+/K^+ ratio was observed in the wild-type line, reflecting a disruption in ion homeostasis and a pronounced accumulation of sodium ions relative to potassium. In contrast, the *Asr1*-overexpressing lines maintained significantly lower Na^+/K^+ ratios under salt stress, suggesting an enhanced ability to exclude Na^+ or retain K^+ in root tissues. Among these, lines P_73_2, P_70_1, and P_76_2 demonstrated the most effective ion regulation, with root Na^+/K^+ ratios nearly 30–50% lower than that of WTBR75. These findings support the hypothesis that *Asr1* plays a critical role in modulating root ion homeostasis under saline conditions, potentially contributing to the improved yield performance observed in these lines (figure 5.10A).

To complement the root ion analysis, Na^+/K^+ ratios were also evaluated in shoot tissues to assess the extent of ionic stress experienced in the aerial parts of the plants. Under control conditions, all

genotypes including WTBR75 and the *Asr1*-transformed lines maintained low and comparable Na^+/K^+ ratios, indicating well-regulated ion balance in the absence of salinity. However, when subjected to 100 mM NaCl, a substantial increase in the shoot Na^+/K^+ ratio was observed in the wild-type line, reaching values exceeding 4.5, which reflects severe ionic imbalance and excessive sodium accumulation. In contrast, all *Asr1*-overexpressing lines exhibited significantly lower shoot Na^+/K^+ ratios under salt stress, with reductions ranging from 20% to 40% relative to WTBR75 figure 5.10B).

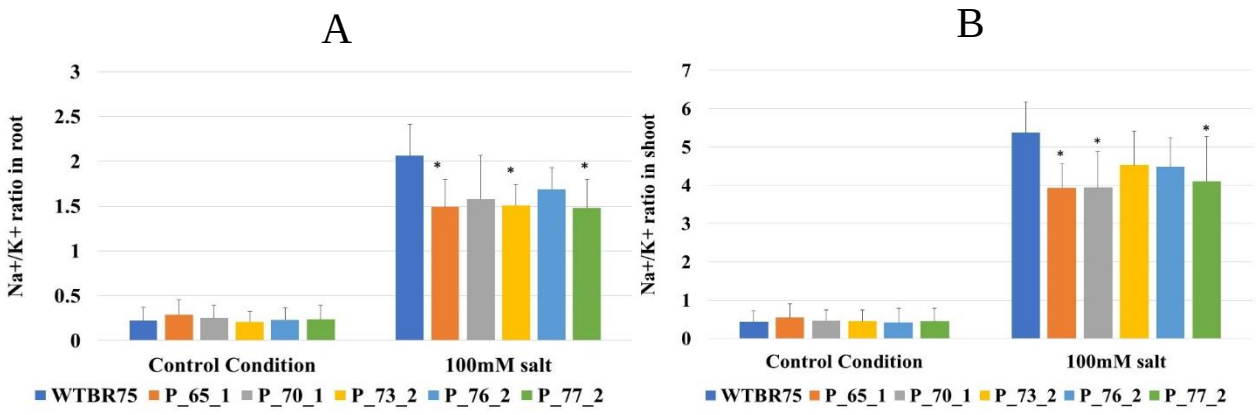


Figure 5.10: Root and shoot Na^+/K^+ ratios in transgenic rice lines at the reproductive stage under 100 mM salt stress. (A) Root Na^+/K^+ ratio in control and salt-treated plants. Transgenic lines P_65_1, P_73_2, and P_77_2 showed a significantly lower Na^+/K^+ ratio in roots under salt stress, indicating enhanced root ion selectivity. (B) Shoot Na^+/K^+ ratio in control and salt-treated plants. Lines P_65_1, P_70_1, and P_77_2 maintained a significantly reduced Na^+/K^+ ratio in shoots under salt stress, suggesting restricted Na^+ translocation to aerial parts. Error bars represent standard error (SE). Different letters indicate statistically significant differences at $p < 0.05$.

This reduction suggests improved exclusion or compartmentalization of sodium and/or enhanced potassium retention in the shoots of transgenic plants. Notably, lines P_73_2 and P_77_2 displayed the most favorable shoot ion profiles under stress conditions. These results reinforce the conclusion that *Asr1* overexpression contributes to more effective ion homeostasis, not only in roots but also in shoots, thereby mitigating the deleterious effects of salt stress on plant physiological integrity and reproductive performance.

5.3.7 Real time gene expression analysis in transgenic lines (*OcAsr1)*

To validate the overexpression of the *Asr1* gene in the best transgenic rice lines (P_70_1, P_73_2 and P_76_2), qRT-PCR analysis was performed using RNA extracted from leaf tissues at the reproductive stage. The housekeeping gene SnoRNA1 was used as an internal control, and relative expression levels were quantified using the 2^–ΔΔCt method. All three transgenic lines demonstrated significantly elevated *Asr1* transcript levels compared to WTBR75 and control condition, confirming successful transgene expression. Among the lines analyzed, P_70_1, P_73_2 and P_76_2 showed the highest *Asr1* expression, which positively correlated with their improved reproductive yield and ion balance under salt stress (figure 5.11). These results suggest a functional role of *Asr1* expression level in conferring enhanced salt tolerance in rice.

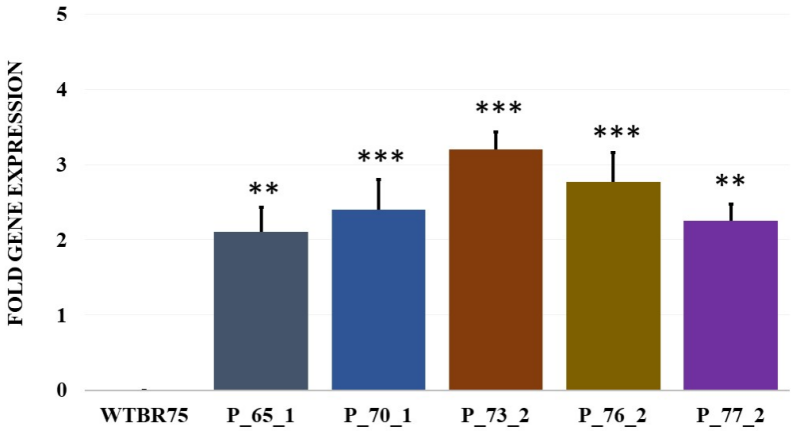


Figure 5.11: Fold gene expression analysis by real-time pcr of wild type BRRI Dhan75 and Asr1- transgenic lines under salt stress. The selected five transgenic lines have shown significantly higher gene expression analysis in salt stress compared to control condition. wild type BRRI Dhan75 lacks the gene hence no expression found. Transgenic lines P_73_2, P_76_2 & P_70_1 have shown highest expression levels.

5.3.8 Performance of Transgenic Rice Lines Under Salt Stress (*OcAsr1)*

The transgenic rice lines exhibited diverse responses to salt stress (Table 5.5). P_73_2 emerged as the best overall performer, showing high biomass, low electrolyte leakage, very high grain yield,

and very low Na^+/K^+ ratios in both root and shoot, indicating superior ionic balance and stress tolerance. P_70_1 and P_76_2 also performed well, maintaining high growth, low electrolyte leakage, and high yield, with favorable Na^+/K^+ ratios. In contrast, P_77_2 and P_65_1 displayed moderate stress responses with relatively higher electrolyte leakage and Na^+/K^+ levels, resulting in good to very good overall performance. These results highlight the potential of the selected lines, particularly P_73_2, for salt-tolerant rice improvement.

Table 5.6: Performance of Transgenic Rice Lines Under Salt Stress (*OcAsr1)*

Line ID	Growth & Biomass	Chlorophyll Retention	Electrolyte Leakage	Grain Yield under Salt	Na^+/K^+ (Root/Shoot)	Overall Performance
P_70_1	High	Moderate	Low	High	Low / Low	Excellent
P_73_2	High	Moderate	Low	Very High	Very Low / Very Low	**Best Overall Performer**
P_76_2	High	Moderate	Low	Very High	Low / Moderate	**Top Performer**
P_77_2	Moderate	Moderate	Moderate	High	Moderate / Very Low	Very Good
P_65_1	Moderate	Moderate	Moderate	High	Moderate / Moderate	Good

5.3.9 Major agronomic traits under control condition (*OcAsr1)*

Under control conditions, key agronomic traits were compared between transgenic rice lines (P_65_1, P_70_1, P_73_2, P_76_2, and P_77_2) and the non-transgenic BRRI Dhan75. Parameters assessed included plant height, tiller number, panicle number and length, spikelet fertility, and grain yield.

Plant height remained largely unaffected by transgene expression, with BRRI Dhan75 showing the tallest plants (111.3 ± 5.0 cm), while transgenic lines displayed similar or slightly reduced heights. Tiller number was highest in BRRI Dhan75 and P_70_1 (13 ± 3 and 13 ± 4), indicating good vegetative vigor in some transgenic lines. Panicle number and length were reduced in P_76_2 and P_77_2, possibly affecting yield potential.

Spikelet fertility was comparable across lines, with P_65_1 and P_70_1 slightly exceeding the control. Notably, P_65_1 showed the highest yield (6.52 ± 2.1 g/plant), followed by P_73_2 and

P_70_1, surpassing BRRI Dhan75 (5.53 ± 0.83 g/plant). These findings suggest that certain transgenic lines, particularly P_65_1, exhibit enhanced yield potential under non-stress conditions without growth penalties.

Table 5.7: Major agronomic traits of wild type and *Asr1* transgenic lines under control condition (*OcAsr1)*

Name of Plant	Plant Height (cm)	Number of Tillers	Number of Panicles	Panicle Length (cm)	Spikelet Fertility (%)	Yield (g/plant)
BRRI Dhan75	111.3 ± 5.0	13 ± 3	11.0 ± 2	19.0 ± 3.5	61.7 ± 6.9	5.53 ± 0.83
P_65_1	109.0 ± 4.0	12 ± 2	11.0 ± 3	18.5 ± 2.5	63.9 ± 5.5	**6.52 ± 2.10**
P_70_1	109.5 ± 4.5	13 ± 4	10.0 ± 3	18.5 ± 3.5	62.9 ± 5.4	6.02 ± 0.40
P_73_2	105.5 ± 5.5	11 ± 3	9.0 ± 2	17.0 ± 4.5	61.8 ± 6.4	6.26 ± 0.45
P_76_2	107.5 ± 4.6	9 ± 3	7.0 ± 3	16.5 ± 1.5	56.5 ± 6.5	5.33 ± 0.83
P_77_2	106.5 ± 3.5	9 ± 2	8.0 ± 2	16.5 ± 2.5	62.6 ± 6.8	6.02 ± 0.45

In conclusion, the evaluation under control conditions suggests that the introduction of the transgene does not compromise, and in some cases enhances, key agronomic traits such as yield and fertility. Lines such as P_65_1 and P_73_2 emerge as promising candidates for further evaluation under stress conditions, especially considering their stable growth and reproductive performance both under normal conditions as well as 100mM stress. These results set a baseline for assessing wider performance under salt stress, which is a primary focus of this study.

5.4 Results of Subunit c of Vacuolar H+-ATPase(*Pva1)* gene cloning and transformation

5.4.1 Cloning of Subunit c of Vacuolar H+-ATPase(*Pva1)* gene

The Vacolar H+ ATPase subunit c (*OcPVA1*) gene was successfully isolated from *Oryza coarctata* and cloned into the entry vector pENTR™ as part of a Gateway® cloning strategy. The presence and correct insertion of the gene within the entry vector was confirmed through gene-specific PCR analysis (Figure 5.12A), restriction enzyme digestion**,** and Sanger sequencing. Following confirmation, the *OcPVA1* gene was recombined into the binary plant expression vector pH7WG2.0 via Gateway® LR recombination technology**.** Successful recombination, as well as the proper orientation and integrity of the inserted gene, were further verified by restriction digestion (Figure 5.12B) and sequencing analysis**.** These steps ensured that a fully validated *OcPVA1* gene construct was obtained for use in subsequent Agrobacterium-mediated transformation and functional characterization in transgenic rice lines.

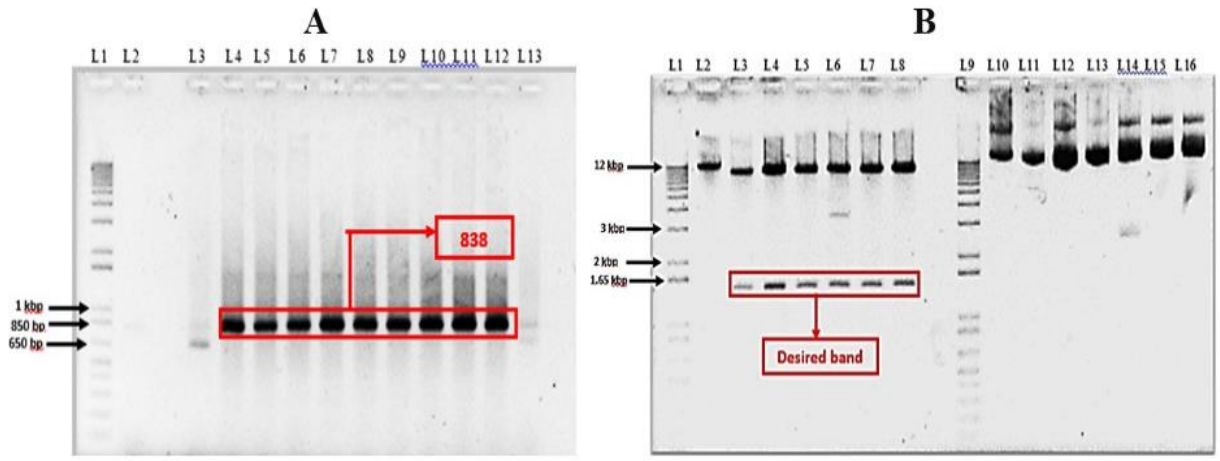


Figure 5.12: A) Lysate PCR of *OcPVA1* after LR recombination between pENTR-D-TOPO and pH7WG2. L1 : 1Kb+ Ladder,L4-L14: Desired band of pva1, 838 bp C) Restriction digestion of *OcPVA1* -pH7WG2 by BamHI. L1 & L9: 1Kb+ ladder, L2-L8: Digested sample, L10-L16: Undigested sample.

5.4.2 Transformation of High-Yielding Rice Variety with *OcPVA1*

The *OcPVA1* gene was introduced into the salt-sensitive, high-yielding rice variety BRRI Dhan75 (BR75) using the *in planta* (tissue-culture independent transformation) Agrobacterium-mediated transformation method. Germinating seeds were directly infected with *Agrobacterium tumefaciens*

harboring the *OcPVA1* construct. Following infection, the seeds were transferred to soil under controlled net house conditions. Out of 50 treated seedlings, 20 plants survived, indicating a survival rate of 40%. Similar survival frequencies were observed in independent transformation batches, confirming reproducibility of the method.

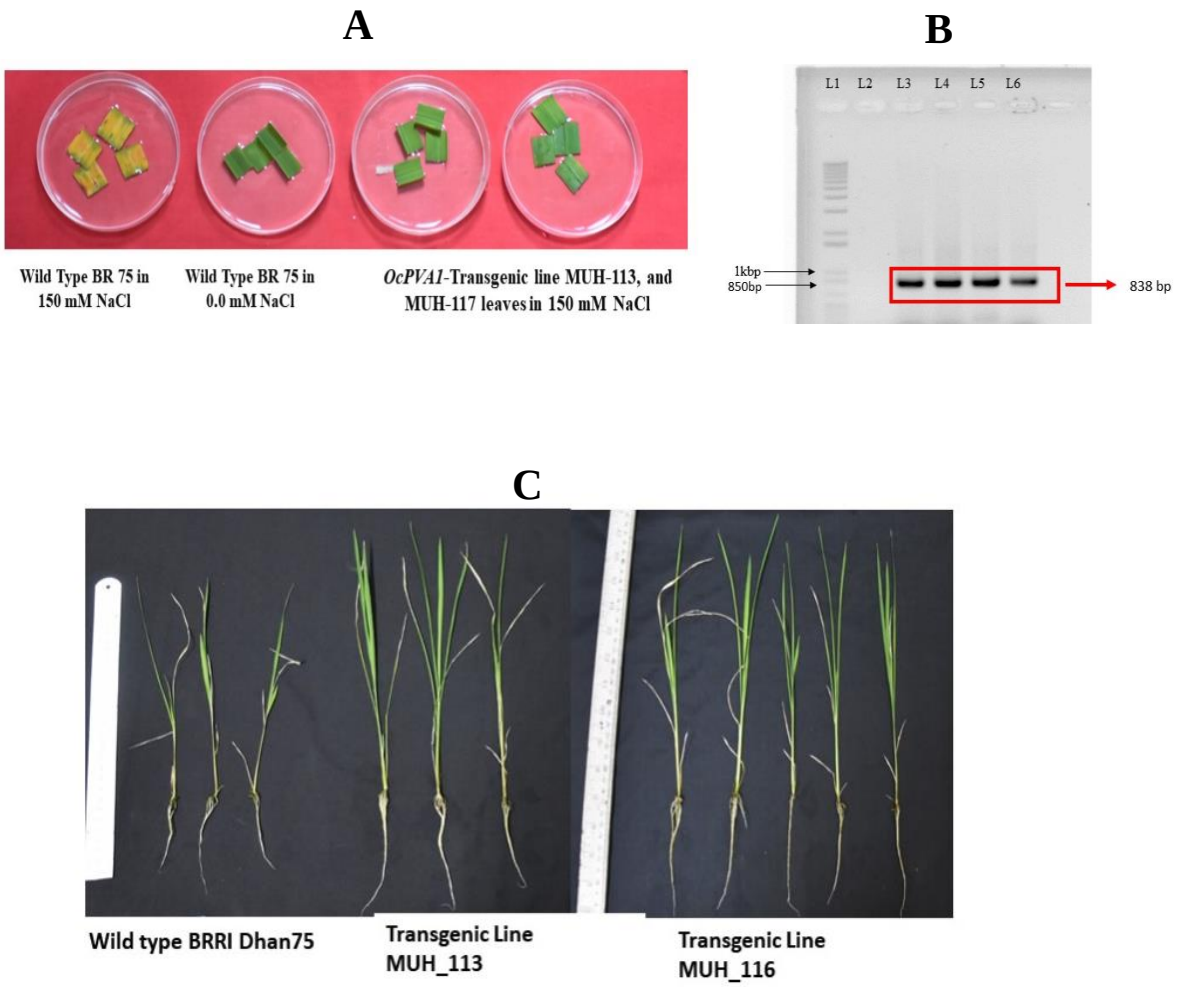


Figure 5.13: Seedling stage screening of *OcPVA1*-BRRIDhan75 at 100mM salt stress for14 days A) Leaf disk senescence assay of Wild type BRRI Dhan75 and transgenic lines at control condition and 100mM NaCl; B) Presence of transgene confirmed by *OcPVA1* gene specific PCR (838bp) in T_0 generation C) Wild type BRRI Dhan75 compared to *OcPVA1* transformed seedling of MUH-113 and MUH-116 line after 100mM salt-stress.

The putative transformants were allowed to grow to maturity, undergo flowering, and set seeds. At the T_0 stage, preliminary screening of putative transgenic plants was performed using the leaf disk senescence assay under salt stress conditions (Figure 5.13A). Plants that exhibited delayed senescence compared to wild-type BR75 were identified as potential transformants (Figure 5.12C). To confirm the presence of the *OcPVA1* transgene, PCR amplification was performed using gene-specific primers. Amplification of the expected fragment (Figure 5.13B) validated the successful integration of the *OcPVA1* gene into the rice genome.

5.4.3 Advancement to the T_2 generation and selection of promising *OcPVA1*-transgenic lines

Based on the results of the leaf disk senescence assay and PCR screening at the T_0 stage, six promising lines (MUH-99,112,113,116,117 & 118) were selected for generation advancement. These lines exhibited both enhanced stress tolerance and were confirmed for transgene presence. For the T_1 generation, seeds were collected only from panicles whose corresponding flag leaves tested PCR-positive, ensuring selection of true transformants. Plants from these lines were grown and subjected to further screening to evaluate the stability of transgene inheritance and phenotypic consistency. Subsequent selection and performance analysis were continued in the T_2 generation, focusing on physiological and molecular responses under salt stress to identify the most effective *OcPVA1*-expressing lines. Genetic segregation was calculated based on the hygromycin assay at the seedling stage of T_1. Transgenic line MUH_99, MUH_113, MUH_116 and MUH_118 followed the Mendelian inheritance law (table 5.7).

Table 5.8: Segregation of transgenic (resistant) and non-transgenic (susceptible) seedlings in the T_1 plants (*OcPVA1)*.

Hygromycin resistant plant ID at To generation (*OcPVA1*)	Number of resistant seedlings (T1)	Number of susceptible seedlings (T1)	Chi-square test or v2 test = (observed-expected)2 /(expected)	P value	Mendelian inheritance (3:1 segregation ratio)
MUH_99	27	19	2.37037	0.123658	Followed
MUH_112	30	17	5.633333	0.017622	Did not follow
MUH_113	23	16	2.130435	0.1444	Followed
MUH_116	26	17	3.115385	0.077556	Followed
MUH_117	21	12	3.857143	0.049535	Did not follow
MUH_118	28	20	2.285714	0.13057	Followed

5.4.4 Effect of salt (NaCl) stress in *OcPVA1* transgenic lines at seedling stage

To assess the physiological role of *OcPVA1* in enhancing salt stress tolerance in rice, two key parameters—chlorophyll content and electrolyte leakage—were measured in wild-type BRRI Dhan75 and selected *OcPVA1*-expressing transgenic lines following 14 days of exposure to 100 mM NaCl. Chlorophyll content was assessed in wild-type BRRI Dhan75 (WT_BR75) and

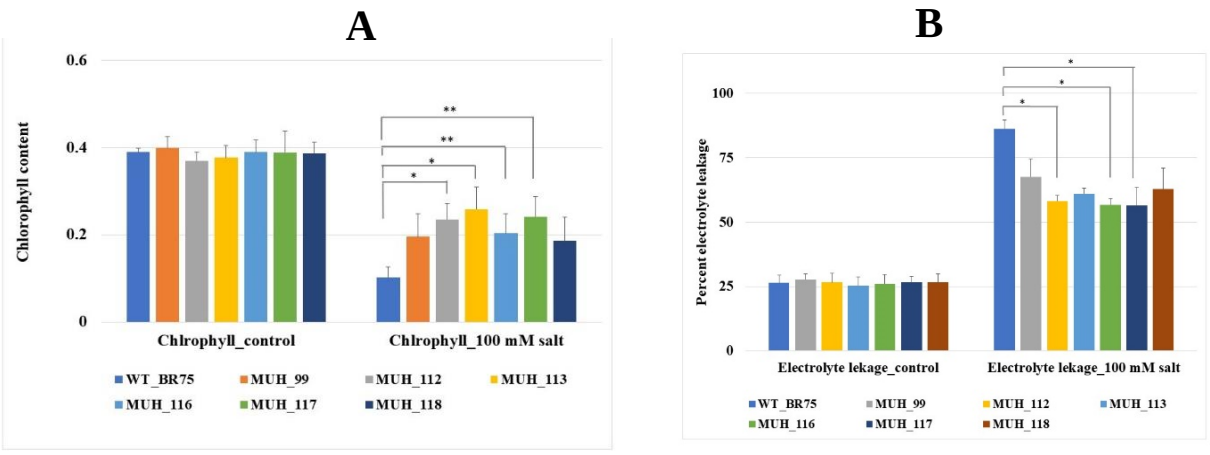


Figure 5.14. Physiological responses of *OcPVA1* transgenic BRRI Dhan75 seedlings under 100 mM NaCl stress for 14 days. (A) Chlorophyll content**:** Transgenic lines MUH_112, MUH_113, MUH_116, and MUH_117 retained significantly higher chlorophyll levels than wild-type BR75 under salt stress ($p < 0.05$), indicating improved photosynthetic stability. (B) Electrolyte leakage: Lines MUH_112, MUH_116**,** and MUH_117 showed significantly lower leakage, reflecting better membrane integrity under stress.

OcPVA1 transgenic lines under control and 100 mM NaCl conditions (Figure 5.14A).

Under control conditions, all genotypes showed similar chlorophyll levels (~0.37–0.41 mg/ml), indicating no effect of the transgene on baseline chlorophyll content. Under salt stress, WT_BR75 exhibited a significant reduction in chlorophyll content (<0.15 mg/ml), reflecting stress-induced degradation. In contrast, transgenic lines MUH-99, MUH-112, MUH-116, and MUH-117

maintained significantly higher chlorophyll levels ($p < 0.05$, $p < 0.01$), with MUH-112 and MUH-116 performing best (~0.27–0.29 mg/ml). These results suggest that *OcPVA1* enhances chlorophyll retention under salt stress, likely contributing to improved photosynthetic stability and stress tolerance in the transgenic lines.

Electrolyte leakage was measured in wild-type BRRI Dhan75 (WT_BR75) and *OcPVA1*-expressing transgenic rice lines under both control and 100 mM NaCl conditions to assess the integrity of cell membranes under salt stress (Figure 5.14B).

Under control conditions, all genotypes—including WT and transgenic lines—exhibited similar levels of electrolyte leakage (~25%), indicating intact membrane systems and comparable baseline physiological states. However, exposure to 100 mM NaCl stress led to a significant increase in electrolyte leakage in WT_BR75, reaching nearly 85%, reflecting substantial membrane damage due to salt-induced oxidative and osmotic stress. In contrast, several *OcPVA1* transgenic lines showed markedly lower leakage, indicating improved membrane stability under stress conditions. Specifically, MUH_112, MUH_113, MUH_116, and MUH_117 demonstrated significantly lower electrolyte leakage ($p < 0.05$), ranging between 55–65%, compared to the wild type. Among them, MUH_112, MUH_116, and MUH_117 consistently exhibited better performance, suggesting enhanced ability to maintain membrane integrity, likely due to *OcPVA1*-mediated stress protection.

At seedling stage, other important parameters like root length, root weight, shoot length and shoot parameters also have been considered (figure 5.15). Three lines (P_70-1, P_73_2 & P_76_2) have shown significantly higher root weight and shoot weight compared to wild type at 100 mM salt stress. Two lines, P_73_2 & P_76_2 have shown significantly higher shoot length. Lastly, line P_76_2 have shown significantly higher root length than wild type. line P_76_2 have shown greater performance than wild type both in control and stress condition.

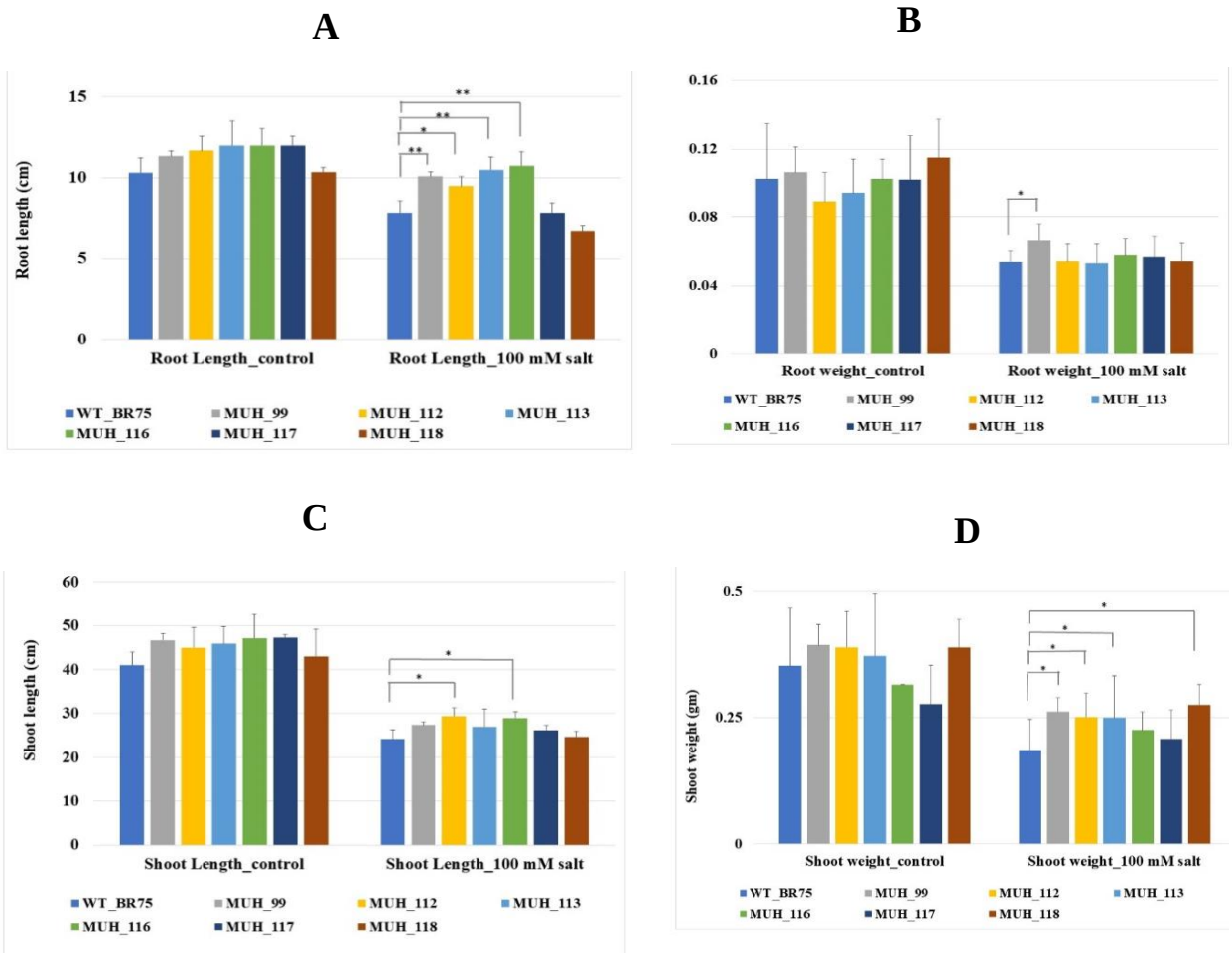


Figure 5.15: Seedling stage screening of *OcPVA1*-BRRIDhan75 at 100mM salt stress for14 days compared to control condition; A) Root length; B) Root weight; C) Shoot length & D) Shoot weight.

5.4.5 Effect of salt (NaCl) stress in *OcPVA1*- transgenic lines at reproductive stage

Grain yield was assessed in *OcPVA1*-expressing BRRI Dhan75 transgenic lines and compared with the wild-type cultivar BRRI Dhan75 (WT_BR75) under both control and salinity stress conditions (Figure 5.16). Under control conditions, all transgenic lines produced grain yields that were comparable to, or slightly higher than, the wild type, with no statistically significant differences observed. This indicates that *OcPVA1* expression does not negatively affect yield potential under normal growing conditions.

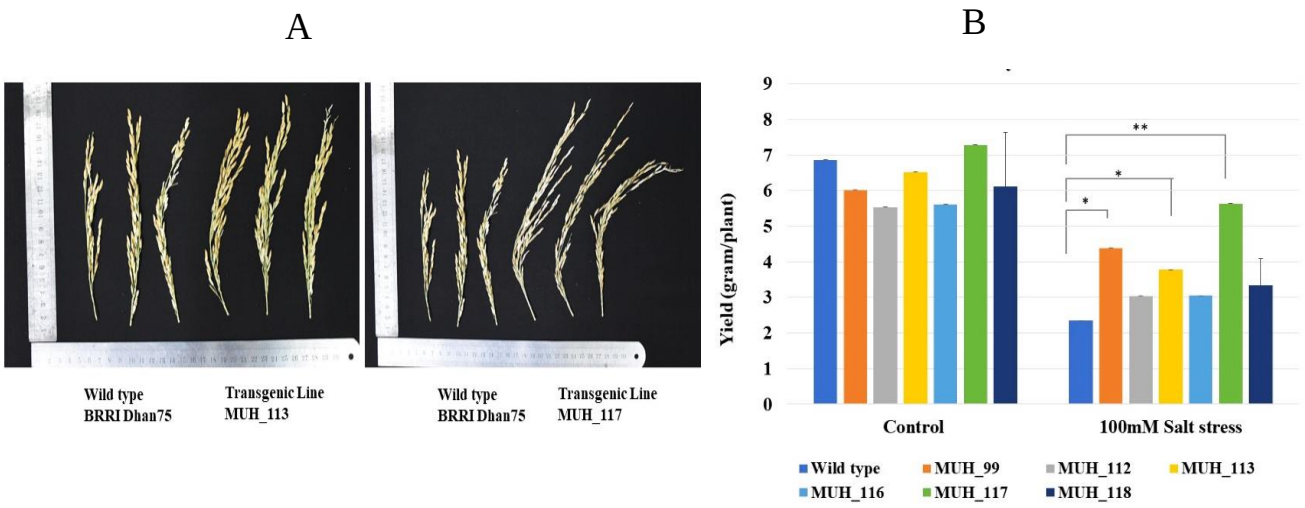


Figure 5.16: Reproductive screening of transgenic lines of *OcPVA1*-BRRIDhan75 at 100 mM salt stress. A) Panicles of wild type BRRIDhan75 and transgenic MUH_113 & MUH_117 at control condition and salt-stress; Transgenic line have shown healthier and better panicles than wild type. B) Yield data of wild type and selected transgenic lines at control and salt stress. Three lines, MUH_99, 113 & 117 have shown significantly higher yield than wild type after salt-stress.

However, under 100 mM NaCl stress, a substantial decline in grain yield was recorded in WT_BR75, highlighting its susceptibility to salinity during the reproductive phase. In contrast, several *OcPVA1*-overexpressing lines—particularly MUH_99, MUH_113, and MUH_117, maintained significantly higher yields compared to the wild type ($p < 0.05$). These lines demonstrated enhanced reproductive resilience, suggesting that *OcPVA1* plays a role in protecting yield components under salt stress. These findings support the conclusion that *OcPVA1* overexpression contributes to improved yield stability under saline conditions, likely through its involvement in stress mitigation pathways that preserve reproductive development.

5.4.6 Ionic Homeostasis in Roots and Shoots under Salt Stress *(OcPVA1)*

To investigate the role of *OcPVA1* in ionic regulation, Na^+/K^+ ratios were measured in both root and shoot tissues of wild-type BRRI Dhan75 and transgenic lines under control and salt stress

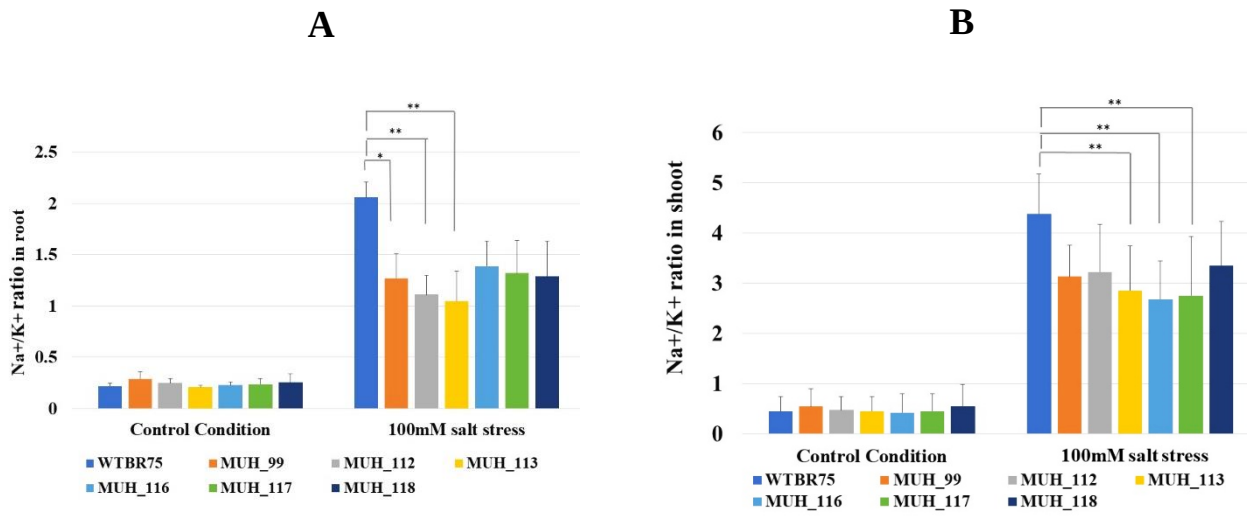


Figure 5.17: Na^+/K^+ ratios were measured in root and shoot tissues of wild-type BRRI Dhan75 (WTBR75) and transgenic lines under control and 100mM NaCl salt stress conditions. A) Under salt stress, WTBR75 showed a sharp increase in Na^+/K^+ ratio, indicating poor ionic regulation. In contrast, transgenic lines MUH_99, MUH_112**,** and MUH_113 maintained significantly lower ratios ($p < 0.05$ or $p < 0.01$), reflecting enhanced sodium exclusion and potassium retention in root tissues. B) In case of shoot, WTBR75 exhibited a high Na^+/K^+ ratio under stress, indicating significant sodium accumulation in shoots. Transgenic lines MUH_113, MUH_116, and MUH_117 maintained significantly lower shoot Na^+/K^+ ratios **(**$p < 0.01$), suggesting better ionic balance and reduced translocation of Na^+ to aerial parts under salinity.

conditions. The Na^+/K^+ ratio in root tissues was measured in *OcPVA1*-expressing transgenic BRRI Dhan75 lines and compared with the wild-type (WT_BR75) under both control and 100 mM NaCl stress conditions to evaluate ionic homeostasis (Figure 5.17A). Under control conditions, all genotypes—including WT and transgenic lines—exhibited similarly low Na^+/K^+ ratios (~0.2–0.3), indicating balanced ion uptake and homeostasis in the absence of salt stress. In contrast, under 100 mM NaCl stress, a significant increase in the Na^+/K^+ ratio was observed in WT_BR75, reaching above 2.0, which reflects excessive sodium accumulation and impaired potassium retention. However, transgenic lines—particularly MUH_99, MUH_112, and MUH_113—maintained significantly lower Na^+/K^+ ratios ($p < 0.05$ or $p < 0.01$), ranging between 1.1–1.4. This indicates an improved ability to restrict Na^+ uptake and/or retain K^+ in roots under saline conditions.

To evaluate ionic homeostasis in aerial tissues, the Na^+/K^+ ratio was measured in shoot samples of wild-type BRRI Dhan75 and *OcPVA1*-expressing transgenic lines under both control and 100 mM NaCl conditions (Figure 5.17B).

Under control conditions, all genotypes exhibited similarly low Na^+/K^+ ratios (~0.3–0.5), indicating effective ion regulation in the absence of stress and no basal difference between wild-type and transgenic plants. Following exposure to 100 mM NaCl, WT_BR75 exhibited a pronounced increase in shoot Na^+/K^+ ratio, reaching approximately 4.5, indicating excessive sodium accumulation and impaired potassium retention in the shoots. In contrast, transgenic lines—particularly MUH_99, MUH_112, and MUH_113—maintained significantly lower Na^+/K^+ ratios ($p < 0.01$), ranging between 2.8–3.2. These results suggest that *OcPVA1* overexpression enhances the plant's ability to restrict Na^+ translocation to shoots and/or retain K^+ under salt stress. Combined with root data, this result underscores the role of *OcPVA1* in promoting systemic ionic balance, likely contributing to improved physiological performance and stress tolerance in transgenic rice lines.

5.4.7 Major agronomic traits of wild type BRRI Dhan75 and transgenic lines under control condition *(OcPVA1)*.

Under control conditions**,** key agronomic traits were compared between transgenic rice lines and the non-transgenic BRRI Dhan75. Parameters assessed included plant height, tiller number, panicle number and length, spikelet fertility, and grain yield.

Agronomic traits including plant height, tiller number, panicle attributes, spikelet fertility, and grain yield were evaluated in *OcPVA1*-expressing BRRI Dhan75 lines under (Table 5.X). The wild-type (WT_BR75) showed yield of 6.87 g/plant and spikelet fertility of 63.7%. All transgenic lines demonstrated comparable morphological traits, indicating that *OcPVA1* expression did not adversely affect growth. Among the transgenic lines, MUH_117 showed superior performance, with the highest yield (7.29 g/plant), spikelet fertility (65.4%), and panicle number (13), exceeding that of the wild type. Other lines such as MUH_99 and MUH_113 also maintained acceptable yield levels (6.02 g and 6.52 g/plant, respectively).

Table 5.9. Evaluation of *OcPVA1* transgenic rice lines based on physiological and agronomic traits under control condition

Name of Plant	Plant Height (cm)	Number of Tillers	Number of Panicles	Panicle Length (cm)	Spikelet Fertility (%)	Yield (g/plant)
BRRI Dhan75	108.3 ± 4.0	15 ± 2	11.0 ± 2	19.0 ± 4.5	63.7 ± 6.9	6.867333 ± 0.41
MUH_99	108.0 ± 4.0	13 ± 2	11.0 ± 2	17.5 ± 3.6	62.9 ± 4.9	6.019667 ± 0.45
MUH_112	107.5 ± 4.5	11 ± 3	9.0 ± 3	16.5 ± 2.8	62.9 ± 6.3	5.535333 ± 0.83
MUH_113	109.5 ± 5.5	12 ± 2	10.0 ± 2	18.0 ± 3.6	62.7 ± 5.8	6.524 ± 2.15
MUH_116	106.5 ± 4.6	10 ± 4	9.0 ± 3	17.5 ± 4.5	57.5 ± 5.6	5.623667 ± 2.61
MUH_117	108.5 ± 3.5	14 ± 4	13.0 ± 4	19.5 ± 5.5	65.4 ± 5.4	7.291667 ± 0.81

In conclusion, the evaluation under control conditions suggests that the introduction of the transgene does not compromise, and in some cases enhances, key agronomic traits such as yield and fertility. Lines such as MUH_117 and 113 emerge as promising candidates for further evaluation under stress conditions, especially considering their stable growth and reproductive performance. These results set a baseline for assessing performance under salt stress, which is a primary focus of this study.

5.4.8 Top-performing transgenic tines exhibit superior salt tolerance and yield stability *(OcPVA1)*

Among the transgenic lines evaluated, MUH_117**,** MUH_113, and MUH_99 demonstrated the most promising performance under salt stress. MUH_117 was identified as the best overall performer, exhibiting very high chlorophyll retention, minimal electrolyte leakage, the highest grain yield, and stable Na^+/K^+ homeostasis**.** MUH_113 and MUH_99 also showed excellent performance, maintaining high growth and biomass, low Na^+/K^+ ratios in both root and shoot, and significantly improved grain yield compared to the other lines. These results highlight their potential for developing salt-tolerant rice cultivars.

Table 5.10. Evaluation of *OcPVA1* transgenic rice lines based on physiological and agronomic traits under salt stress

Line ID	Growth & Biomass	Chlorophyll Retention	Electrolyte Leakage	Grain Yield under Salt	Na^+/K^+ (Root / Shoot)	Overall Performance
MUH_99	High	Moderate	Low	High	Low / Low	Excellent
MUH_112	Moderate	High	Very Low	Moderate	Low / Low	Very Good
MUH_113	High	High	Moderate	High	Low / Low	Excellent
MUH_116	Moderate	High	Very Low	Moderate	Moderate / Moderate	Good
MUH_117	High	Very High	Very Low	**Very High**	Moderate / Moderate	**Best Overall Performer**
MUH_118	Low	Low	High	Low	**Very Low / High**	Fair

5.4.9 Real time gene expression analysis in transgenic lines *(OcPVA1)*.

To confirm the overexpression of the *OcPVA1* gene in the best-performing transgenic rice lines **(**MUH_99**,** MUH_113**,** and MUH_117**),** quantitative real-time PCR (qRT-PCR) was performed using total RNA extracted from leaf tissues. The SnoRNA1 gene served as an internal control for normalization, and relative expression levels were calculated using the 2^–ΔΔCt method. Results revealed that all three transgenic lines exhibited significantly higher *OcPVA1* transcript accumulation compared to the wild-type BRRI Dhan75 (WTBR75)**,** which lacks the transgene and showed no detectable expression. Among the transgenic lines**,** MUH_113 demonstrated the highest *OcPVA1* expression level, which corresponded with its superior reproductive yield and improved ion homeostasis under salt stress conditions **(**Figure 5.18**).** These findings confirm the successful expression of the *OcPVA1* transgene and its potential role in enhancing salt stress tolerance.

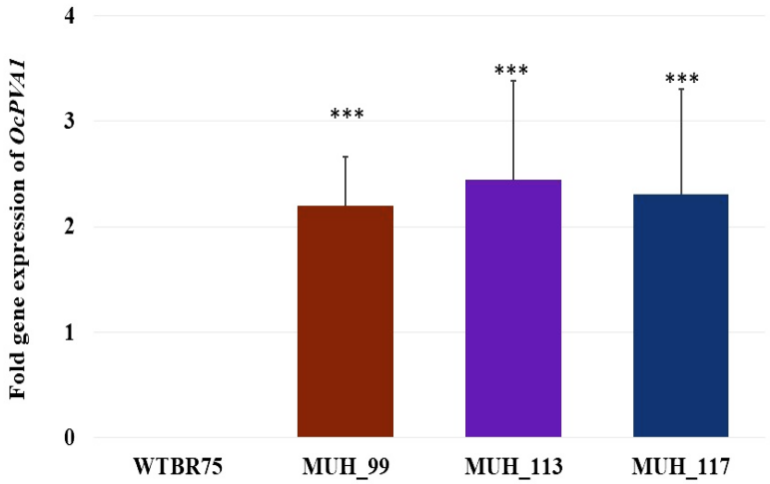


Figure 5.18: Fold-change in gene expression of *OcPVA1* in transgenic rice lines and wild-type BRRI Dhan75 under salt stress, analyzed by real-time PCR. Three selected transgenic lines MUH_99, MUH_113, and MUH_117 exhibited significantly higher *OcPVA1* expression under salt stress compared to the control (non-stressed) condition. In contrast, the wild-type BRRI Dhan75(WTBR75) showed no detectable expression of *OcPVA1*, as it lacks the transgene. Data represent mean values with standard error bars, confirming the enhanced transcriptional response of the selected transgenic lines to salt stress.

5.5 Results of Metallothionein type 3 gene cloning and transformation

5.5.1 Cloning of Metallothionein 3 (*OcMT3)* gene

The Metallothionein 3 (*OcMT3*) gene was successfully isolated from *Oryza coarctata* and cloned into the entry vector pENTR™ using the Gateway® cloning system**.** The correct insertion of *OcMT3* into the entry vector was confirmed through gene-specific PCR analysis (figure 5.19A), restriction enzyme digestion**,** and Sanger sequencing**.** After validation, the *OcMT3* gene was recombined into the binary plant expression vector pH7WG2.0 via Gateway® LR recombination technology**.** The successful transfer, correct orientation, and structural integrity of the insert were

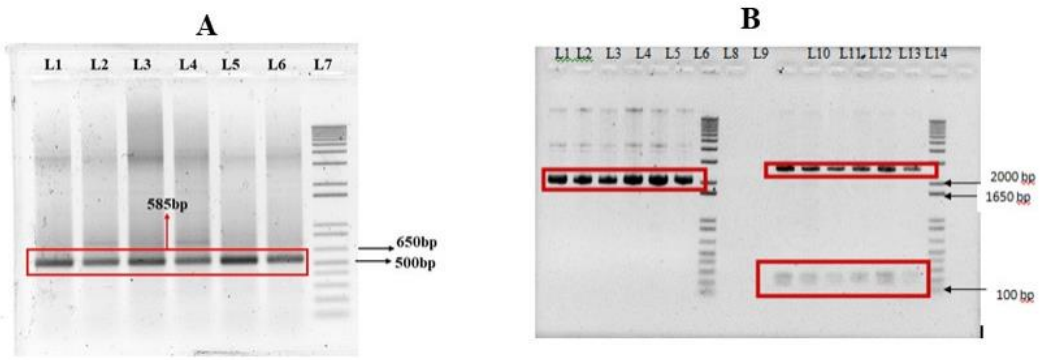


Figure 5.19: A) *OcMT3* Gene specific Lysate PCR ; L1-L6: desired band at 585 bp, L7: 1Kb+ ladder; B) Restriction digestion of MT3-pENTR-D-TOPO by Eco RI & Pvu I, desired band of 2626bp, 263bp & 279bp ; L1-L5: Uncut plasmid construct (3168 bp) L6 & L14: 1Kb+ ladder.

further confirmed by sanger sequencing (Sequence given in supplementary)**.**

For cloning we had designed the experiment using the sequence available in GenBank. After sequencing, we found that the targeted *OcMT3* has additional nine nucleotides. For confirmation, we sequenced a total of 8 different samples from different colonies. We have deposited the sequence in NCBI, GenBank ID: OP382350 (figure 5.20).

Score	Expect	Identities	Gaps	Strand	Frame
913 bits(494)	0.0()	527/541(97%)	9/541(1%)	Plus/Plus	

Features:

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Query  124  CTTAAGCGAAAGCAGCAGCTAGCAGCACAAGGAATTCATCGCTCGCTTCAGCTAATCTCT  183
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Sbjct  1    CTTAAGCGAAAGCAGCAGCTAGCAGCACAAGGAATTCATCGCTCGCTTCAGCTAATCTCT  60

Query  184  TCTTCGATCATGTCGGACAAGTGCGGCAACTGCGACTGCGCTGACAAGTCTCAGTGCGTG  243
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Sbjct  61   TCTTCGATCATGTCGGACAAGTGCGGCAACTGCGACTGCGCTGACAAGTCTCAGTGCGTG  120

Query  244  AAGAAGGGAAACAGCTATGGCGTCGTGCTAGTCGACACGGAGAAGAGCCACTTGGAGGAG  303
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Query  304  ATCGCCGCCGCCGGCGCTGAGAACGACGGGTGCAAGTGCGGCTCCAGCTGCTCCTGCGGC  363
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Query  664  A  664
            |
Sbjct  532  A  532
```

Figure 5.20: BLAST alignment between the OcMT3 sequence from the database and the *OcMT3* sequence isolated in the present study. The alignment shows 9 nucleotide variations, consistently observed across all 8 sequenced clones. These differences likely represent natural polymorphisms within Oryza coarctata populations, reflecting genetic diversity in the species.

5.5.2 Transformation of High-Yielding Rice Variety with Metallothionein 3 (*OcMT3)*

The *OcMT3* gene was introduced into the medium salt-tolerant, high-yielding rice variety BRRI Dhan67 (BR67) using the in planta Agrobacterium-mediated transformation method. Out of 35 treated seedlings, 16 plants survived, indicating a survival rate of 46%.

The putative transformants were allowed to grow to maturity, undergo flowering, and set seeds. At the T_0 stage, preliminary screening of putative transgenic plants was performed using the leaf disk senescence assay under salt stress conditions (figure 5.21A). To confirm the presence of the *OcMT3* transgene, PCR amplification was performed using gene-specific primers. Amplification of the expected fragment (figure 5.21B) validated the successful integration of the *OcMT3* gene into the rice genome.

5.5.3 Advancement to the T_1 generation and selection of promising transgenic lines (*OcMT3*)

Based on the results of the leaf disk senescence assay and PCR screening at the T_0 stage, four promising lines (P-13-2, P-14-1, P-28-2 and P-34-1) were selected for generation advancement. These lines exhibited both enhanced stress tolerance and confirmed transgene presence. For the T_1 generation, seeds were collected only from panicles whose corresponding flag leaves tested PCR-positive, ensuring selection of true transformants. Genetic segregation was calculated based on the hygromycin assay at the seedling stage of T_1. Transgenic line P-13-2, P-14-1 and P-28-2 followed the Mendelian inheritance law (table 5.9).

Table 5.11: Segregation of transgenic (resistant) and non-transgenic (susceptible) seedlings in the T_1 plants (*OcMT3*).

Hygromycin resistant plant ID at To generation (*OcMT3*)	Number of resistant seedlings (T1)	Number of susceptible seedlings (T1)	Chi-square test or v2 test = (observed-expected)2 /(expected)	P value	Mendelian inheritance (3:1 segregation ratio)
P-13-2	25	18	1.96	0.16151332	Followed
P-14-1	22	15	2.227273	0.135593	Followed
P-28-2	29	20	2.793103	0.09467072	Followed
P-34-1	24	14	4.166667	0.04122683	Did not follow

5.5.4 Effect of salt (NaCl) stress in *OcMT3* transgenic lines at seedling stage

To assess the physiological role of *OcMT3* in enhancing salt stress tolerance in rice at seedling stage, two key parameters, chlorophyll content and electrolyte leakage were measured in wild-type BRRI Dhan67 and selected *OcMT3*expressing transgenic lines following 15 days of exposure to 120 mM NaCl (figure 5.21).

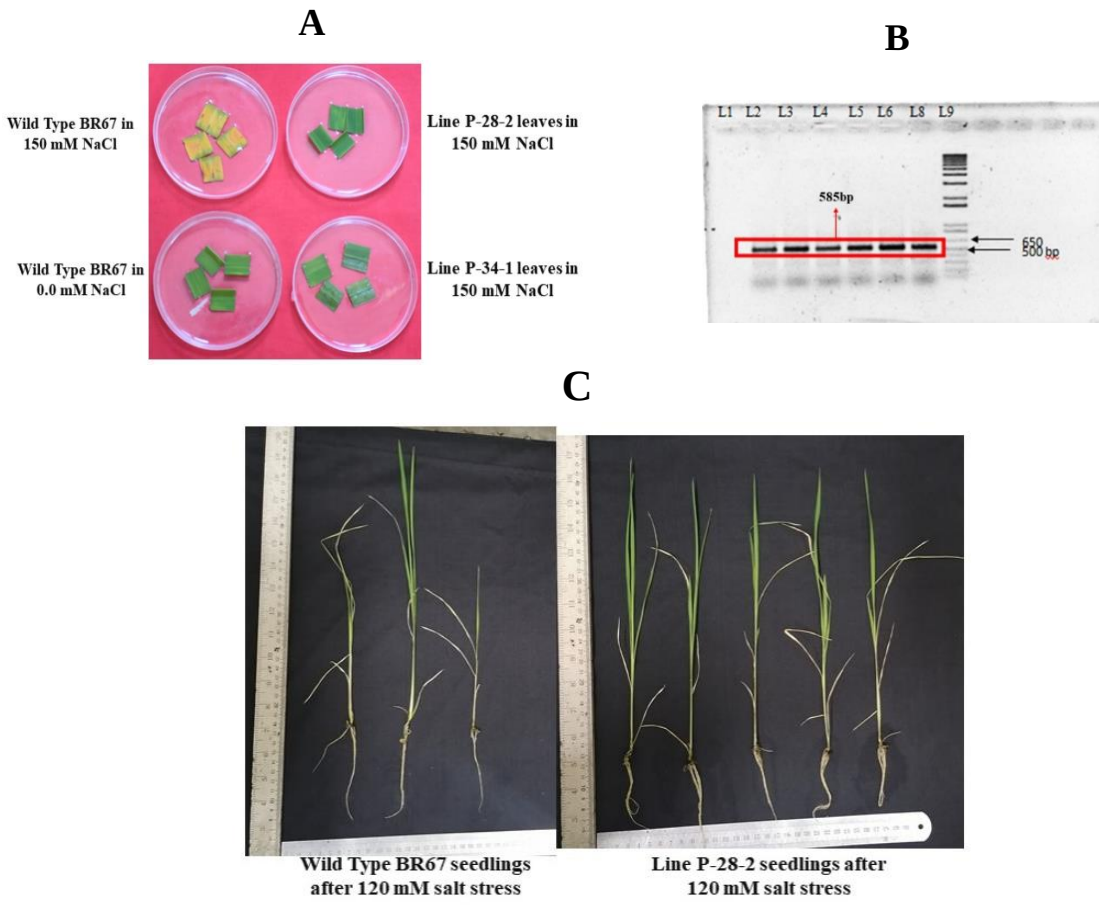

Figure 5.21: Seedling stage screening of *OcMT3*-BRRI Dhan67(BR67) at 120mM salt stress for15 days A) Leaf disk senescence assay of Wild type BR67 and transgenic lines at control condition and 120mM NaCl; B) Presence of transgene confirmed by *MT3* gene specific PCR in T_0 generation C) *MT3* Transformed seedling of P_28_2 line after 120mM salt-stress after 15 days.

Chlorophyll content was assessed in wild-type BRRI Dhan67 (WT_BR67) and *OcMT3*transgenic lines under control and 100 mM NaCl conditions (Figure 5.22A). Under control conditions,, all genotypes maintained similar levels of chlorophyll, ranging between 0.43 to 0.48 mg/ml,

indicating no growth disadvantage or phenotypic difference among the transgenic lines in the absence of stress. However, under salt stress, a significant reduction in chlorophyll content was observed in the wild-type plants, with levels dropping to approximately 0.19 mg/ml. In contrast, several transgenic lines—particularly P-13-2, P-14-1 & P-34-1 lines retained substantially higher chlorophyll content under the same stress, with P_14_1 showing the highest retention, nearly 0.34 mg/ml. Statistical analysis confirmed that the differences between these transgenic lines and the wild-type were significant ($p < 0.05$**,** $p < 0.01$), suggesting an enhanced photosynthetic stability under saline conditions (figure 5.22A).

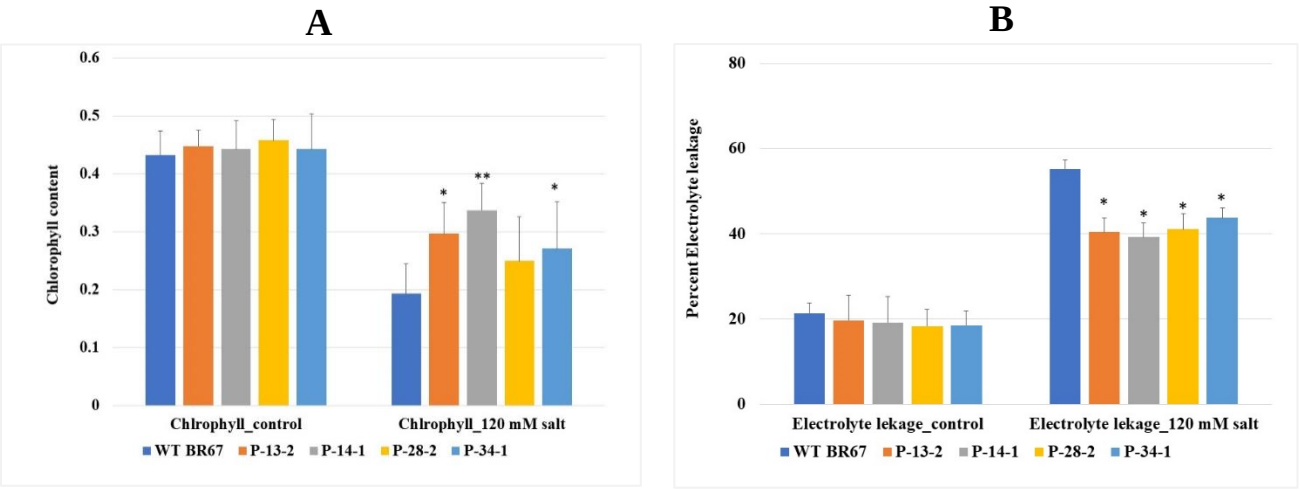

Figure 5.22: Seedling stage screening of *OcMt3*-BRRIDhan67 at 120mM salt stress for15 days A) Chlorophyll content of Wild type BRRI Dhan67 and transgenic lines at control condition and 120mM NaCl, three lines (P-13-2, P-14-1 & P-34-1) have shown significantly (p-value 0.05, 0.01) better performance than Wild type. B) percent Electrolyte leakage, all four lines showed significantly better performance than Wild type.

Electrolyte leakage was measured as an indicator of membrane integrity under salt stress. Under control conditions, all lines exhibited relatively low leakage, ranging between 18% and 22%, with no significant differences observed among the genotypes. However, upon exposure to 120 mM NaCl, the non-transformed BRRI Dhan67 (WT_BR67) showed an increase in electrolyte leakage, reaching nearly 55%, indicating severe membrane damage. In contrast, the transgenic lines demonstrated significantly lower levels of leakage under the same conditions with leakage values around 40–43%, significantly lower than the wild type ($p < 0.05$) (figure 5.22B).

At seedling stage, other important parameters like root length, root weight, shoot length and shoot parameters also have been considered (figure 5.23). Two lines (P_28_2 & P_34_2) have shown significantly higher root length compared to wild type at 120 mM salt stress. Two lines, P_13_2 & P_34_2 have shown significantly higher shoot weight.

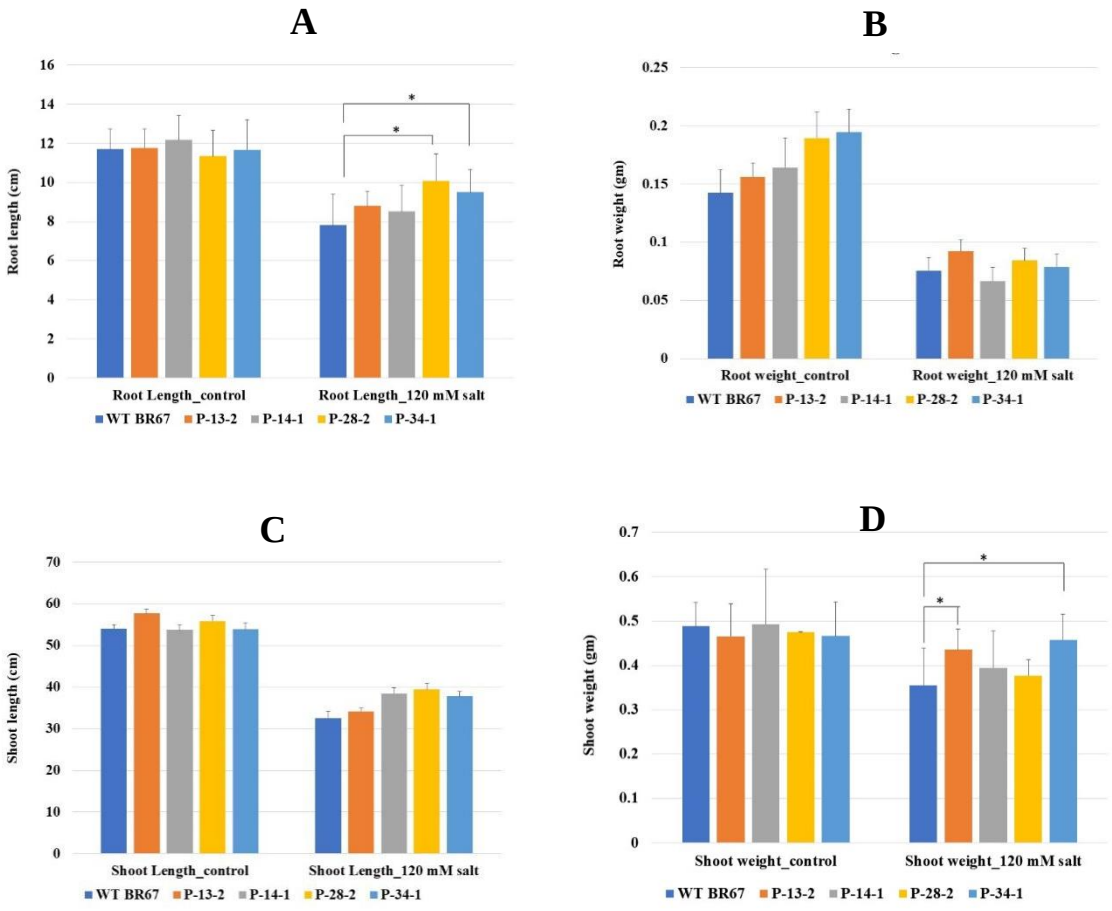

Figure 5.23: Seedling stage screening of *OcMT3*-BRRIDhan67 at 120mM salt stress for15 days compared to control condition; A) Root length; B) Root weight; C) Shoot length & D) Shoot weight.

5.5.5 Effect of salt (NaCl) stress *OcMT3* transgenic lines at reproductive stage

At reproductive stage, the analysis focused on key agronomic and physiological parameters, including yield-related traits, as well as the accumulation of sodium (Na^+) and potassium (K^+) ions in both root and shoot tissues. This comparative assessment was aimed at determining the impact

of *OcMT3* overexpression on reproductive development and ion homeostasis under 120mM NaCl stress condition.

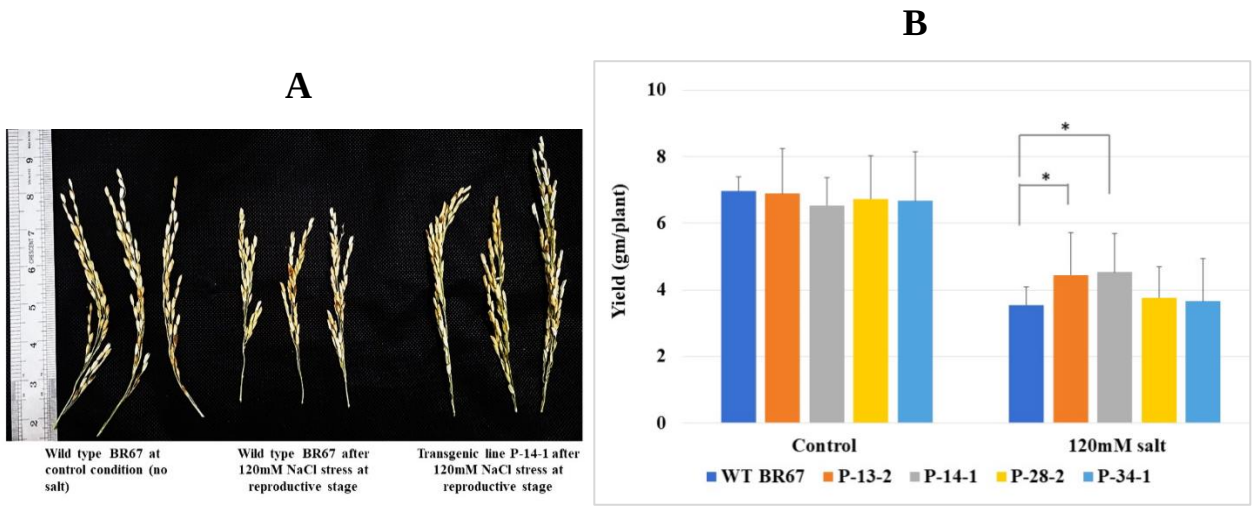

Figure 5.24: Reproductive screening of transgenic lines of *OcMT3*-BRRIDhan67 at 120 mM salt stress. A) Panicles of wild type BRRIDhan67 and transgenic line P_77_2 at control condition and salt-stress; Transgenic line have shown healthier and better panicles than wild type. B) Yield data of wild type and selected transgenic lines at control and salt stress. Lines P-13-2 & P-14-1 have shown significantly higher yield than wild type after salt-stress.

Under both control and salt stress conditions, grain yield was assessed in *OcMT3*-transformed rice lines relative to the wild-type cultivar BRRI Dhan67 (WTBR67) (figure 5.24). Under non-stress conditions, all transgenic lines exhibited almost same yield performance compared to the wild type, with no statistically significant differences observed. However, exposure to 120 mM NaCl resulted in a decline in yield in WTBR67, highlighting its sensitivity to salinity at reproductive stage. In contrast, transgenic line P-13-2 & P-14-1 maintained significantly higher yields under salt stress. Other two lines showed slightly better performance than wild type.

5.5.6 Ionic Homeostasis in Roots and Shoots under Salt Stress (*OcMT3*)

To further investigate the role of *MT3* overexpression in ion homeostasis under saline conditions, the Na^+/K^+ ratio in root tissues was measured in both transgenic and wild-type lines (figure 5.10). Under control conditions, all genotypes, exhibited low and comparable Na^+/K^+ ratios, indicative

of balanced ion uptake in the absence of stress. However, upon exposure to 120 mM NaCl, a substantial increase in the root Na^+/K^+ ratio was observed in the wild-type line, reflecting a disruption in ion homeostasis and a pronounced accumulation of sodium ions relative to potassium. In contrast, the *OcMT3*-overexpressing lines maintained lower Na^+/K^+ ratios under salt stress, suggesting an enhanced ability to exclude Na^+ or retain K^+ in root tissues. Among these, line P-13-1, and P-28-2 showed a significantly lower Na^+/K^+ ratio in roots (figure 5.25A).

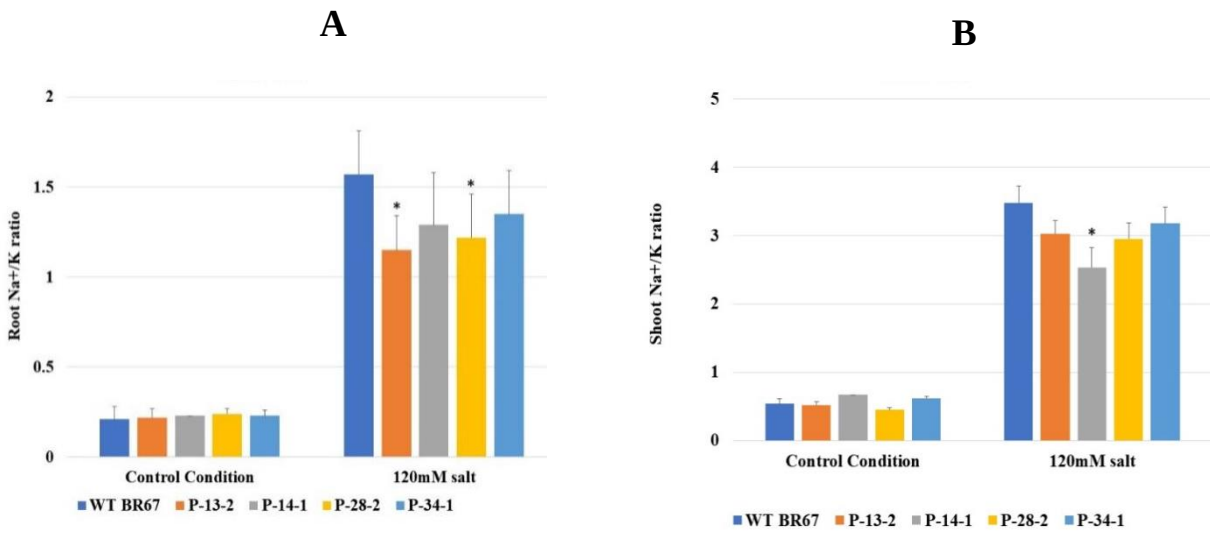

Figure 5.25: Root and shoot Na^+/K^+ ratios in transgenic rice lines at the reproductive stage under 120 mM salt stress. **(A)** Root Na^+/K^+ ratio in control and salt-treated plants. Transgenic lines P-13-1, and P-28-2 showed a significantly lower Na^+/K^+ ratio in roots under salt stress, indicating enhanced root ion selectivity. **(B)** Shoot Na^+/K^+ ratio in control and salt-treated plants. Line P-14-1, maintained a significantly reduced Na^+/K^+ ratio in shoots under salt stress, suggesting restricted Na^+ translocation to aerial parts. Error bars represent standard error (SE). Different letters indicate statistically significant differences at $p < 0.05$.

In case of shoot, under control conditions, all genotypes exhibited low and comparable shoot Na^+/K^+ ratios, indicating normal ionic homeostasis without stress. However, under salt stress, the wild-type plants displayed a substantial increase in shoot Na^+/K^+ ratio (~3.8), reflecting a typical salt-sensitive phenotype with high Na^+ accumulation and impaired K^+ retention. In contrast, the transgenic lines showed significantly lower Na^+/K^+ ratios under salt stress, with P-14-1 exhibiting the most pronounced reduction (~2.6), representing approximately a 31.6% decrease compared to

the wild type**.** Other transgenic lines (P-13-2, P-28-2, and P-34-1) also demonstrated reductions ranging from 15.8% to 21% (figure 5.25B)**.** These results suggest that the *OcMT3* gene enhances ionic regulation by limiting Na^+ translocation to shoots or improving K^+ conservation mechanisms, thereby reducing the ionic toxicity commonly associated with salinity stress. This improvement in shoot ion homeostasis highlights the potential role of *OcMT3* in conferring salt tolerance at the whole-plant level and supports its functional relevance in stress mitigation strategies.

5.5.7 Nitroblue Tetrazolium (NBT) test for histochemical detection of superoxide radicals under Salt Stress

Salt stress induces oxidative damage in plants by promoting the overproduction of reactive oxygen species (ROS), particularly superoxide radicals (O_2^-). The Nitroblue Tetrazolium (NBT) assay is a well-established histochemical method for the in situ detection of superoxide radicals in plant tissues (Jabs et al., 1996). In this assay, superoxide radicals react with NBT, resulting in the formation of an insoluble blue formazan precipitate, which serves as a visible indicator of oxidative stress.

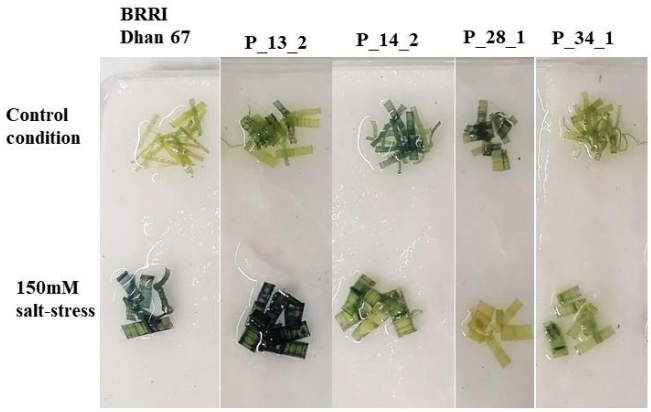

Figure 5.26: ROS detection in 150mM salt stress by NBT staining. P_34_1, P_28_1, and P_14_2 *OcMT3*-transgenic lines showed lesser staining in leaf sections meaning less ROS accumulation in 150mM salt-stress.

5.5.8 Major agronomic traits of wild type BRRI Dhan67 and transgenic lines under control condition (*OcMT3*).

To evaluate the potential impact of *OcMT3* gene expression on rice plant growth and development, major agronomic traits of the transgenic lines were assessed under control (non-stress) conditions. The measured traits included plant height, tiller number, panicle number, panicle length, spikelet fertility, and grain yield per plant. The results demonstrated that the transgenic lines maintained normal growth and development comparable to the wild-type BRRI Dhan67. Plant height ranged from 105.5 cm (P-28-2) to 109.5 cm (P-14-1)**,** showing a slight, non-significant increase compared to the wild type **(**101.3 cm**).** The number of tillers varied between 10 and 13, and the number of panicles ranged from 8 to 11**,** with no substantial differences detected between transgenic lines and the control (table 5.10).

Table 5.12: Major agronomic traits of wild type and *OcMT3* transgenic lines under control condition

Name of Plant	Plant Height (cm)	Number of Tillers	Number of Panicles	Panicle Length (cm)	Spikelet Fertility (%)	Yield (g/plant)
BRRI Dhan67	101.3 ± 5.0	12 ± 3	10.0 ± 2	18.0 ± 4.5	65.7 ± 7.3	6.97 ± 0.41
P-13-2	109.0 ± 4.0	11 ± 2	11.0 ± 2	19.5 ± 1.5	68.9 ± 3.5	**6.89 ± 1.35**
P-14-1	109.5 ± 4.5	12 ± 4	10.0 ± 3	18.5 ± 2.7	63.5 ± 4.4	6.53 ± 0.83
P-28-2	105.5 ± 5.5	13 ± 3	8.0 ± 2	19.0 ± 3.4	64.2 ± 2.4	6.72 ± 1.29
P-34-1	107.5 ± 4.6	10 ± 3	8.0 ± 3	18.5 ± 1.7	64.5 ± 4.5	6.67 ± 1.48

Panicle length remained consistent across all lines, averaging 18–19.5 cm, indicating stable reproductive development. Spikelet fertility showed minor variation, with transgenic lines displaying fertility rates between 63.5% and 68.9%, similar to the wild-type **(65.7%).** Grain yield per plant in the transgenic lines ranged from 6.53 g to 6.89 g, closely matching the wild-type yield of 6.97 g. These findings confirm that the introduction and expression of the *OcMT3* gene do not negatively affect key agronomic traits under normal growth conditions, ensuring that the transgene does not impose growth penalties in the absence of stress (table 5.10).

5.5.9 Top-performing transgenic lines exhibit superior salt tolerance and yield stability (*OcMT3*).

The comparative analysis of transgenic rice lines under salt stress revealed significant differences in physiological and agronomic performance. The wild-type (WT BR67) exhibited medium salt sensitivity, as indicated by reduced growth and biomass, drastic chlorophyll loss, high electrolyte leakage, and a strong reduction in grain yield. In contrast, transgenic line P-14-1 demonstrated superior salt tolerance, maintaining growth and biomass with minimal yield reduction. It showed the highest chlorophyll retention (~22% loss)**,** lowest electrolyte leakage**,** and the best Na^+/K^+ ratio (1.30 / 2.6)**,** suggesting effective ionic homeostasis under stress conditions. Lines P-13-2 and P-28-2 also maintained growth and had improved ion balance compared to the WT, though their chlorophyll retention and membrane stability were moderately affected. P-34-1 showed moderate tolerance, with some stress symptoms evident in ion regulation and yield parameters. Overall, the transgenic lines, particularly P-14-1**,** exhibited enhanced physiological stability and ion regulation mechanisms under salt stress, supporting the hypothesis that the introduced gene confers improved salt tolerance (Table 5.11). These findings highlight the potential of the selected transgenic lines for future breeding and development of salt-tolerant rice cultivars.

Table 5.13: Performance of *OcMT3* Transgenic Rice Lines Under Salt Stress

Line ID	Growth & Biomass	Chlorophyll Retention	Electrolyte Leakage	Grain Yield under Salt	Na^+/K^+ Ratio (Root/Shoot)	Overall Performance
WT BR67	Reduced	Low (Severe loss)	High (Severe damage)	Strong reduction	**1.55 / 3.8** (High)	Salt Sensitive
P-13-2	Maintained	Moderate (~28% loss)	Moderate	Mild reduction	**1.15 / 3.0** (Improved)	Improved Tolerance
P-14-1	Maintained	High (~22% loss) **(**Best**)**	Low (Most stable)	Least affected	**1.30 / 2.6** (**Best**)	**Highly Tolerant**
P-28-2	Maintained	Moderate (~28% loss)	Moderate	Mild reduction	**1.20 / 3.0** (Improved)	Improved Tolerance
P-34-1	Maintained	Moderate (~25% loss)	Moderate	Mild reduction	**1.35 / 3.2** (Moderate)	Moderate Tolerance

5.5.10 Semi quantitative gene expression analysis in *OcMT3* transgenic lines

To validate the overexpression of the *MT3* gene in the transgenic rice lines RT-PCR analysis was performed using RNA extracted from leaf tissues at the seedling stage. The housekeeping gene U6_Sno RNA was used as an internal control, and relative expression levels were quantified using ImageJ software (figure 5.27A).

The expression analysis of *OcMT3* revealed distinct expression patterns under control and salt stress conditions. Under normal conditions, *O. coarctata* exhibited a moderate basal level of

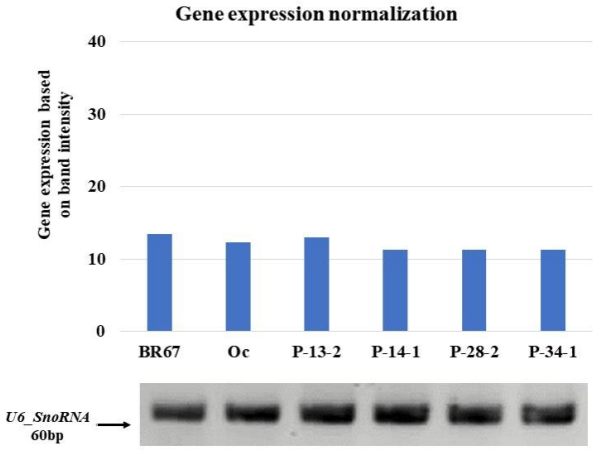

Figure 5.27A: Expression of *U6_SnoRNA* in *Oryza coarctata* and transgenic rice lines as internal control. All the lines showed similar level of expression.

OcMT3 expression. The transgenic rice lines (P-13-2, P-14-1, P-28-2, P-34-1) displayed variable

basal expression of *OcMT3*, suggesting successful gene introgression but differential promoter activity or expression regulation. After 48 hours of 100 mM NaCl treatment, a significant induction of *OcMT3* expression was observed in *O. coarctata*, indicating its natural adaptive mechanism for coping with salt stress. Similarly, the transgenic lines, particularly P-13-2 and P-14-1, showed

substantial upregulation of *OcMT3* under salt stress (figure 5.27B). This suggests that these lines have successfully acquired the stress-responsive expression of *OcMT3*, which may contribute to enhanced salt tolerance. Overall, these results support the role of *OcMT3* as a stress-inducible gene, and highlight P-13-2 and P-14-1 as promising candidates for developing salt-tolerant rice varieties through transgenic approaches.

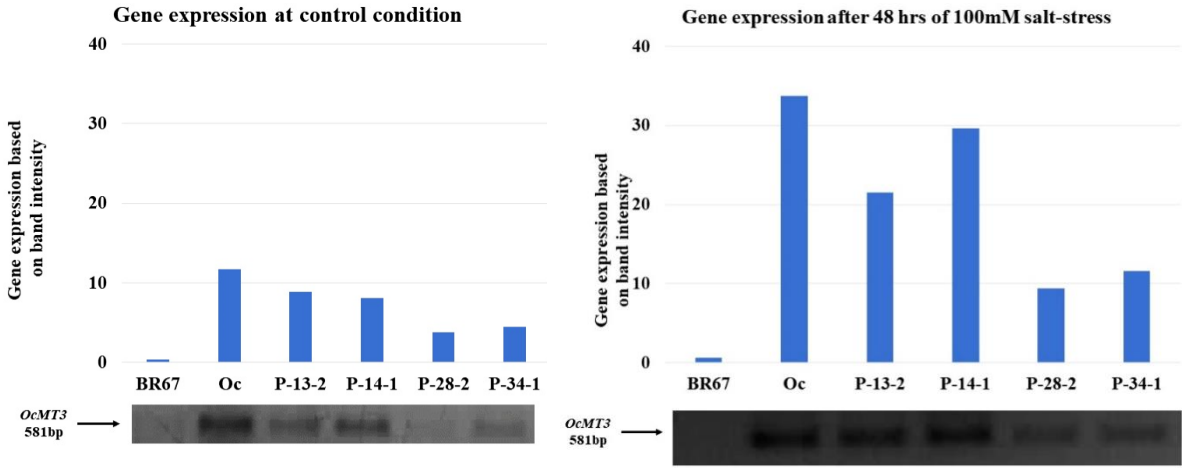

Figure 5.27B: Expression of *OcMT3* in Oryza coarctata and transgenic rice lines under control and salt stress conditions. Semi-quantitative RT-PCR analysis of *OcMT3* (581 bp) was performed in BR67**,** O. coarctata (Oc)**,** and transgenic lines **(**P-13-2, P-14-1, P-28-2, P-34-1**).** The left panel shows expression under control conditions; the right panel shows expression after 48 hours of 100 mM NaCl treatment**.**

5.6 Discussion

Salinity is one of the most critical abiotic stresses limiting rice productivity worldwide, particularly in coastal and estuarine regions where soil salinization is prevalent (Munns & Tester, 2008). Traditional breeding approaches for salt tolerance in rice have met with limited success due to the polygenic nature of salt tolerance and yield penalties often associated with tolerant varieties (Gregorio et al., 2002). In this context, the introgression of stress-responsive genes from halophytic wild relatives such as *Oryza coarctata* offers a promising alternative to enhance salt tolerance (Krishnamurthy et al., 2016). More recently, a transcriptomic analysis of *Oryza coarctata* under salt and submergence stress, revealed significant changes in gene expression profiles.

This study focused on cloning and functional characterization of three genes from *O. coarctata*-Abscisic acid stress ripening protein(*OcAsr1)*, *Subunit c of vacuolar H^+-ATPase (OcPVA1)*, and *Metallothionein type 3 (OcMT3)*, to evaluate their roles in conferring salt tolerance in transgenic rice lines. Because high-yielding rice cultivars often exhibit poor tissue culture response, an in *planta* Agrobacterium-mediated transformation method was employed to generate transgenic lines. The first *in planta* method in rice was established by Supartana et al. (2005) for japonica cultivars and later developed by Lin et al. (2009) for indica ones, Molecular confirmation through PCR, restriction digestion, and sequencing verified stable transgene integration. Subsequent quantitative RT-PCR analysis confirmed strong transgene expression in selected lines, validating successful transformation.

Temporal gene expression patterns in *Oryza coarctata*

Gene expression profiling under salt stress revealed distinct temporal patterns. *OcAsr1* showed a **late, dose-dependent response,** peaking at 24 hours with a ~2.8-fold increase under 200 mM NaCl, suggesting a role in the mid-to-late phases of stress response. This is consistent with ASR gene family members known to participate in osmotic adjustment and stress protection during prolonged exposure (González & Iusem, 2014; Padmanabhan et al., 2020). Conversely, *OcPVA1* displayed **robust and consistent upregulation** at 24 hours in both 100 and 200 mM NaCl treatments, with the highest fold change (~3.2×) among the tested genes. Lastly, *OcMT3* responded **very late,** peaking at 48 hours with a ~2.7-fold increase, particularly under high salt concentration. All genes demonstrated statistically significant induction under their respective peak conditions, underscoring their roles in salinity-responsive gene regulation.

***OcAsr1* gene integration confers enhanced salt tolerance in transgenic rice**

One of the most abundant protein families expressed under abiotic stress conditions is the ASR (ABA-stress-ripening) family**,** which plays a key role in stress adaptation. Interestingly, ASR proteins are widely conserved across the plant kingdom but are notably absent in *Arabidopsis thaliana* (Iusem et al., 1993; Frankel et al., 2006). The first *Asr1* cDNA was identified in tomato (*Solanum lycopersicum*), where it was found to accumulate in both stressed leaves and ripe fruits (Iusem et al., 1993). In rice, the *OsAsr* gene family is more complex, comprising six distinct members (Philippe et al., 2010). Unlike tomato, where ASR genes are clustered, rice ASR genes

are distributed across different chromosomes, with the exception of *OsAsr3* and *OsAsr4*, which are tightly linked on chromosome 1 (Philippe et al., 2010). This structural diversity suggests possible functional specialization of ASR proteins in rice.

In this study, the *OcAsr1*-overexpressing transgenic rice lines seedling stage displayed significantly improved physiological performance under 100 mM NaCl stress. These lines accumulated greater root and shoot biomass, indicating enhanced growth despite the saline environment. Notably, lines P_70_1 and P_76_2 retained higher chlorophyll levels compared to the wild type, suggesting better preservation of photosynthetic activity under stress. Chlorophyll retention under salinity is a well-documented marker of stress tolerance, as it reflects delayed senescence and sustained energy production (Ashraf and Harris, 2013; Siddiqui et al., 2015). In addition, the transgenic lines exhibited a 22–35% reduction in electrolyte leakage, highlighting improved membrane integrity and reduced cellular damage caused by salt-induced oxidative stress. Lower electrolyte leakage has been widely recognized as an indicator of enhanced cellular stability under abiotic stress conditions (Blum and Ebercon, 1981; Farooq et al., 2015). These findings suggest that *OcAsr1* plays a protective role in transgenic rice by promoting osmoprotection, maintaining membrane stability, and supporting photosynthetic function under salt stress, aligning with the known functions of ASR proteins in other crops (González & Iusem, 2014; Padmanabhan et al., 2020).

Salt stress during the reproductive stage commonly reduces grain yield and spikelet fertility in rice, leading to yield loss (Zeng et al., 2002). In this study, *OcAsr1* transgenic lines maintained significantly higher yields under 100 mM NaCl**,** with P_73_2, P_76_2, and P_70_1 retaining 40–50% more yield than the wild type. Ion analysis showed that these lines had 30–50% lower root Na^+/K^+ ratios and 20–40% lower shoot Na^+/K^+ ratios, suggesting efficient sodium exclusion and better potassium retention. These results support the role of *OcAsr1* in regulating osmolyte balance and ion transport, contributing to improved salt tolerance at both vegetative and reproductive stages (Kumar et al., 2013; Gupta and Huang, 2014).

Importantly, transgenic lines exhibited no growth penalties in the absence of salinity stress. Some lines, such as P_65_1 and P_73_2, displayed comparable or even enhanced yield traits including

plant height, tiller number, panicle fertility, and grain yield, suggesting the potential of *OcAsr1* to stabilize yield across varying environments (Zhao et al., 2011).

A comprehensive evaluation of physiological, biochemical, and agronomic parameters identified P_73_2 as the top-performing transgenic line, followed by P_70_1 and P_76_2**.** These lines demonstrated superior performance through high biomass accumulation**,** reduced electrolyte leakage**,** better chlorophyll retention under salt stress, and improved Na^+/K^+ balance in both roots and shoots. Importantly, they maintained stable or enhanced grain yield under both normal and saline conditions. This combination of traits makes them promising candidates for field trials and potential use in breeding programs aimed at improving rice cultivation in salt-affected areas (Munns and Tester, 2008).

OcPva1 transgenic rice lines exhibit superior salt stress resilience

The vacuolar H^+-ATPase subunit c gene *PcVHA-c1* from halophytic *Oryza coarctata* is upregulated by salt stress, with transcript levels rising two-fold in roots within 5 hours and up to three-fold in leaves after 48 hours of 500 mM salt treatment (Senthilkumar et al., 2005). This increase is salt-specific, as transcript levels decline after salt removal. Similar salt-induced upregulation of V-ATPase subunits, especially subunit c, has been observed in *Mesembryanthemum crystallinum* (Low et al., 1996) and sugar beet (Kirsch et al., 1996).

Introduction of *OcPVA1* into the high-yielding but salt-sensitive rice variety BRRI Dhan75 resulted in transgenic lines with significantly improved physiological and agronomic performance under salinity stress, without compromising yield potential under normal conditions.

At the seedling stage, *OcPVA1* transgenic lines, particularly MUH_117, MUH_113, and MUH_99, exhibited higher biomass accumulation, enhanced root and shoot growth, and superior chlorophyll retention under salt stress compared to the wild type. These traits are indicative of better osmotic balance and photosynthetic stability, likely mediated by the protective role of *OcPVA1* in stress response pathways. The electrolyte leakage assay further confirmed enhanced membrane integrity in transgenic plants under salt stress. Lines such as MUH_117 maintained very low electrolyte leakage even after prolonged exposure to 100 mM NaCl, suggesting that *OcPVA1* expression helps mitigate oxidative and osmotic damage at the cellular level.

A critical aspect of salt tolerance involves ionic homeostasis, particularly maintaining low Na^+/K^+ ratios in both roots and shoots. The *OcPVA1*-overexpressing lines demonstrated a significantly better capacity to restrict Na^+ accumulation and retain K^+ under salinity stress. MUH_113 and MUH_99 consistently maintained low Na^+/K^+ ratios, highlighting a possible role of *OcPVA1* in regulating ion transport or compartmentalization, although the specific molecular mechanisms warrant further investigation.

At the reproductive stage, transgenic lines showed remarkable resilience under salinity. While the wild type suffered severe yield losses at 100 mM NaCl, lines like MUH_117 and MUH_113 maintained high grain yields and spikelet fertility. This finding is crucial since salinity at the reproductive phase often leads to sterility and crop failure in rice. The yield stability observed in *OcPVA1* lines under salt stress positions this gene as a promising candidate for rice improvement programs targeting saline-prone regions. The qRT-PCR analysis confirmed that the top-performing lines had significantly higher levels of *OcPVA1* transcript accumulation. The expression pattern correlates well with physiological performance, especially in MUH_113, which showed the highest expression along with improved ionic balance and yield.

Overall, the findings suggest that *OcPVA1* plays a multifaceted role in enhancing salinity tolerance, likely through mechanisms involving ion homeostasis, membrane protection, and stress-responsive metabolic regulation. Importantly, the gene does not impose any yield penalty under non-stress conditions, which is a common drawback in many stress-tolerance transgenic approaches.

Enhanced salt tolerance in *OcMT3*-expressing transgenic rice lines

Metallothioneins (MTs) are low molecular weight, cysteine-rich proteins that chelate metal ions through mercaptide bonds formed with their abundant cysteine residues. This binding helps detoxify metals by buffering cytosolic metal concentrations (Cobbett and Goldsbrough, 2002). Analysis of various expressed sequence tag (EST) databases shows that MTs are amongst the highly abundant transcripts in plants (Matsumura et al. 1999).

Physiological assessments revealed that *OcMT3*-expressing lines maintained significantly higher chlorophyll content and lower electrolyte leakage compared to the wild-type under 100 mM NaCl

stress. This indicates that *OcMT3* plays a crucial role in protecting the photosynthetic apparatus and preserving membrane integrity during salt-induced oxidative stress. These findings align with previous reports that metallothioneins contribute to scavenging reactive oxygen species and stabilizing cellular structures under abiotic stresses (Cobbett and Goldsbrough, 2002; Zimeri et al., 2005).

Improved ionic homeostasis was a key determinant of enhanced salt tolerance in *OcMT3* transgenics. Both root and shoot tissues of transgenic lines exhibited significantly reduced Na^+/K^+ ratios relative to wild-type plants, reflecting a better ability to exclude toxic sodium ions or retain potassium essential for cellular function. Notably, line P-14-1 consistently showed the most favorable ionic balance, correlating with its superior physiological performance and yield stability under salinity. This suggests that *OcMT3* may facilitate ion transport regulation or sequestration mechanisms, limiting sodium toxicity at the whole-plant level (Kumar et al., 2013; Gupta and Huang, 2014).

Agronomic trait evaluation under control conditions confirmed that *OcMT3* overexpression does not negatively impact growth, development, or yield, indicating that the transgene does not impose fitness costs in the absence of stress (Zhao et al., 2011). Under salt stress, the superior maintenance of grain yield in transgenic lines, especially P-13-2 and P-14-1, underscores the practical potential of *OcMT3* for improving crop productivity in saline environments (Munns & Tester, 2008). P-14-1, P_28_1 and P_34_1 have shown better performance at histochemical detection of superoxide radicals under salt stress.

Furthermore, semi-quantitative RT-PCR analysis demonstrated that *OcMT3* is a stress-inducible gene, with its expression strongly upregulated in transgenic lines under salt treatment. This inducible expression pattern mirrors that of the native gene in *O. coarctata*, suggesting that the transgenic plants have successfully adopted the natural adaptive mechanism of this halophytic species. The differential expression levels among lines may account for variation in salt tolerance, highlighting the importance of expression regulation in transgene efficacy (Sharma & Dietz, 2009).

In summary, the enhanced salt tolerance observed in *OcMT3* transgenic rice likely results from a combination of membrane protection, improved ion homeostasis, and stress-responsive gene expression. These results position *OcMT3* as a promising candidate gene for engineering salt tolerance in rice, contributing to sustainable crop production on salt-affected soils. Future work could focus on detailed molecular mechanisms of *OcMT3* action and field-level validation of the top-performing lines.

Comparative Evaluation of Salt Tolerance in *OcAsr1, OcPVA1,* and *OcMT3* Transgenic Rice Lines

The comparison of *OcAsr1*, *OcPVA1*, and *OcMT3* transgenic rice lines under salt stress highlights their distinct advantages. *OcAsr1* lines show the highest yield stability, superior biomass, strong gene expression, and effective ion homeostasis with reduced Na^+/K^+ ratios. *OcPVA1* lines exhibit rapid gene induction, excellent ionic balance, and improved chlorophyll retention, resulting in stable yield and fertility under salinity. *OcMT3* lines mainly enhance oxidative stress tolerance and membrane stability, with moderate improvements in yield and ion balance. Overall, *OcAsr1* performs best across key traits, closely followed by *OcPVA1*, while *OcMT3* provides protection against oxidative damage. These findings suggest that combining these genes could develop rice varieties with robust salt tolerance.

Table 5.14: Summary of physiological and agronomic traits in *OcAsr1*, *OcPVA1*, and *OcMT3* transgenic lines

Parameter	*OcAsr1* Transgenics	*OcPVA1* Transgenics	*OcMT3* Transgenics
Yield under salt stress	Highest yield stability; lines P_73_2, P_76_2, P_70_1 maintained 40–50% higher yield than WT at 100 mM NaCl	Lines MUH_117 and MUH_113 maintained high grain yield and spikelet fertility under 100 mM NaCl	Lines P-13-2 and P-14-1 showed superior grain yield maintenance under 100 mM NaCl
Ion homeostasis (Na^+/K^+ ratio)	30–50% lower root Na^+/K^+ and 20–40% lower shoot Na^+/K^+ ratios than WT	Significantly lower Na^+/K^+ ratios in roots and shoots; MUH_113 and MUH_99 had consistently low ratios	Significant reduction in Na^+/K^+ ratios in root and shoot; P-14-1 best ionic balance
Gene expression	Strong expression with confirmed stable integration; expression peaks at 24h post salt stress	High transcript levels correlated with superior physiological performance; early and broad induction (6-24hours)	Strong salt-inducible expression matching native gene regulation (peak at 48 hours)
Biomass and growth	Enhanced root/shoot biomass and chlorophyll retention; lines P_70_1 and P_76_2 notable	Higher biomass accumulation and chlorophyll retention, especially MUH_117 and MUH_113	Improved chlorophyll retention; reduced electrolyte leakage; biomass improved
Membrane integrity	22–35% reduction in electrolyte leakage indicating better stability	Very low (20-27%) electrolyte leakage even after prolonged salt exposure	20-27%) lower electrolyte leakage than wild type supporting membrane protection
Overall advantages	Best yield performance and ion regulation; strong osmoprotection and photosynthetic support	Excellent early stress response with ion compartmentalization; yield stability without penalty	Effective in metal chelation and oxidative stress protection; good yield and ionic balance

5.7 Future Direction

Future research should focus on evaluating the top-performing *OcAsr1*, *OcPVA1,* and *OcMT3* transgenic rice lines under field conditions across diverse saline environments to validate their salt tolerance and yield stability. Combining these genes through pyramiding approaches may enhance tolerance by leveraging their complementary mechanisms, including osmoprotection, ion homeostasis, and oxidative stress mitigation. Further molecular studies are needed to elucidate the underlying pathways and regulatory networks associated with each gene, which could guide precision breeding or genome editing strategies such as CRISPR/Cas9 to optimize endogenous gene function in elite cultivars. Additionally, integrated metabolomic and proteomic analyses would provide deeper insights into the physiological adaptations mediated by these transgenes. Finally, assessing the performance of these lines under combined abiotic stresses and conducting thorough biosafety evaluations will be essential steps toward their practical deployment in saline agriculture.

5.8 Conclusion

This research successfully established that the introduction of halophyte-derived genes *OcAsr1*, *OcPVA1* and *OcMT3* into rice can substantially improve its ability to tolerate saline conditions. Each gene imparts unique benefits, ranging from enhanced ion regulation and osmotic adjustment to protection against oxidative damage, resulting in transgenic lines that outperform wild-type plants in growth, physiological resilience, and grain yield under salt stress. Importantly, these improvements do not come at the cost of reduced performance under normal conditions, underscoring their agronomic viability. These promising results open new avenues for breeding salt-tolerant rice, emphasizing the value of exploring wild species for stress adaptation genes. Moving forward, combining these genes and testing them under field environments will be vital steps to fully realize their potential in crop improvement programs aimed at securing food production in salt-affected areas.

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Appendix 1

Gene sequences

Oryza coarctata abscisic stress ripening protein mRNA, complete cds

GenBank: KM349310.1

>KM349310.1 Oryza coarctata abscisic stress ripening protein mRNA, complete cds

ATGGCGGAGGAGAAGCACCACCACCACCTTTTCCACCACAAGAAGGACAACGAGGAGAGGCCCGTCGGAG
AGTACGGCGAGGGAGGGTACACGGCGGAGACGGTGACCACGGAGGTGATCACCACCGGCCAGGACGAGTA
CGAGAGCTACAACAAGCAGGAGAAGGAGCACAAGCACAAGCAGCACCTCGGCGAGGCCGGCGCCCTCGCC
GCCGGCGCCTTCGCCCTGTACGAGAAGCACGAGGCGAAGAAGGACCCGGAGAACGCGCACAGGCACAAGA
TTACGGAGGAGATCGCGGCCACGGCGGCGGTCGGCGCCGGCGGCTACGCCTTCCACGAGCACCACGAGAA
GAAGAAGGACCACAAGAGCGCCGAGGAGGCCAGCGGCGAGAAGAAGCACCACCTCTTCGGCTGA

Porteresia coarctata V-ATPase subunit c (PVA1) mRNA, complete cds

GenBank: AF286464.1

>AF286464.1 Porteresia coarctata V-ATPase subunit c (PVA1) mRNA, complete cds

CGACCCGGTGCGAGAGAGGAGGAAGAGATCGAGCTCGCCTCAGGAGGAGGAAGAAGAAGAGGAAGAGCAA
GATGTCGTCGGTTTTCAGCGGCGATGAGACAGCGCCCTTCTTCGGCTTCCTCGGCGCCGCCTCGGCCCTC
GTCTTCTCATGCATGGGGGCAGCGTACGGGACGGCGAAGAGTGGCGTCGGCGTGGCGTCCATGGGTGTGA
TGCGCCCCGAGCTCGTCATGAAGTCCATCGTGCCAGTCGTCATGGCTGGTGTGCTCGGTATCTACGGGCT
TATCATTGCCGTCATCATCAGTACCGGGATTAACCCCAAGGCGAAGCCGTACTACCTCTTCGATGGATAC
GCGCATCTCTCCTCAGGGCTTGCCTGTGGCCTTGCTGGTCTCGCCGCAGGCATGGCCATCGGCATCGTCG
GTGATGCTGGTGTTAGGGCAAATGCACAACAACCAAAGCTTTTCGTGGGCATGATCCTCATCCTCATTTT
CGCTGAAGCTCTTGCTCTGTATGGTCTCATTGTGGGCATCATCCTCTCATCCCGTGCTGGTCAATCCCGT
GCAGATTAAGCACCTTGCAGTACCAATCCGCAGTTATTCCACTTGTTATATTCTTGAGAAAACCTAAAAC
TTGGGAGCTCTAGTTTTAATGTATTAAAGATCGATTTATAGCTTAAGGAAGGTGGCACTTCCAGTCCTTT
TTCGTTTCTTTGGTGGTGATTCATGCAGAGTTTTTTTTGGGTTAGGCTGGATTTGCTGCTCCTGAGCAAA
TGGATTTAATCCTATTCGTGGTGAATAAAGAACACGGCACTGTAGCAAAATAAAATTACAATAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Porteresia coarctata metallothionein mRNA, complete cds

GenBank: AF257465.1

>AF257465.1 Porteresia coarctata metallothionein mRNA, complete cds

CTTAAGCGAAAGCAGCAGCTAGCAGCACAAGGAATTCATCGCTCGCTTCAGCTAATCTCTTCTTCGATCA
TGTCGGACAAGTGCGGCAACTGCGACTGCGCTGACAAGTCTCAGTGCGTGAAGAAGGGAAACAGCTATGG
CGTCGTGCTAGTCGACACGGAGAAGAGCCACTTGGAGGAGATCGCCGCCGCCGGCGCTGAGAACGACGGG
TGCAAGTGCGGCTCCAGCTGCTCCTGCGGCACCGACTGCAAGTGCGGCAAGTGAAGCGCTATGATCACTC
TCGATCGCCGGCGCCGTACGTCATGCACCTACCTTAGTCATCTCACAATTAAAATCGCCCTACGTATGTG
TGGTTTGTGTGTTGGATTGTTGAACCCCTGTGCTGTGTGATTTCGCGATTAAGCTGGTCGCTTTTGGGAG
TATATATGTATCAAATTATGCCTTGTGTTATGCCATCATGTGATATGTCATGTACCCGTGCTGCTATACG
CAGTGAAATTAATTAATTAATGCAGCGTGTATTTGTGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAA

Oryza coarctata clone 20a metallothionein (MT) mRNA, complete cds

GenBank: OP382350.1

>OP382350.1 Oryza coarctata clone 20a metallothionein (MT) mRNA, complete cds
CTTAAGCGAAAGCAGCAGCTAGCAGCACAAGGAATTCATCGCTCGCTTCAGCTAATCTCTTCTTCGATCA
TGTCGGACAAGTGCGGCAACTGCGACTGCGCTGACAAGTCTCAGTGCGTGAAGAAGGGAAACAGCTATGG
CGTCGTGCTAGTCGACACGGAGAAGAGCCACTTGGAGGAGATCGCCGCCGCCGGCGCTGAGAACGACGGG
TGCAAGTGCGGCTCCAGCTGCTCCTGCGGCACCGACTGCAAGTGCGGCAAGTGAAGCGCTATGATCACTC
TCGATCGCCGGCGCCGTACGTCATGCACCTACCTTAATCATCTCACAATAAAAATCGCCCTACGTATGTG
TGGTTTGTGTGTTGGATTGTTGAACTAGCTAAACCCCTGTGCTGTGTGATATCGCGATTAAGCTGGTCGC
TTCTGGGAGTATATATGTATCAACTTATGCCTTGTGTTATGCCATCATGTGATATGTCATGTACCCGTGC
TGCTATACGCAGTGAAATTAATTAATTAATGCAGCGTGTATTTGTGTGA

Appendix 2: Abstract of the published journals from this PhD research

Plant Tissue Cult. & Biotech. 27(1): 63-76, 2017 (June)

PTC&B

Characterization of Progenies from Intergeneric Hybridization Between *Oryza sativa* L. and *Porteresia coarctata* (Roxb.) Tateoka

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Key words: Porteresia coarctata, Induced-tetraploid, Intergeneric hybridization, SSR marker

Abstract

Porteresia coarctata (Roxb.) Tateoka is an endemic halophyte growing all over the coastal belt of Bangladesh, propagating through rhizomes and setting a few rice-like grains. So exploiting the genetic potential of this wild rice as salt tolerant donor in possible wide crosses with rice (2n = 24) could be useful. We attempted intergeneric hybridization between *Oryza sativa* L. and *P. coarctata*. The survival rate of hybrid progenies in embryo culture was low but among them 2 hybrid plants were successfully matured from the intergeneric cross between the cultivated induced tetraploid of rice, Latisail (2n = 4x = 48) and *P. coarctata* (2n = 48). The hybrid plants could be successfully established in soil and were not like either of the parents in morphology although some of their features were similar to their maternal parent, Latisail (4x). Both of the hybrids were investigated through physiological analysis under salinity stress and molecular analyses with rice specific SSR markers. Molecular analysis of the F_1 DNA with only 3 SSR markers, RM581, RM20224 and RM25271, out of 36 others tested, showed bands specific to both of the parents, while all had common bands with the maternal parent. Dendrogram analysis of the hybrids with the 36 SSR markers, show that *P. coarctata* forms a different clade and is clearly separated from Latisail and the hybrids. The putative hybrids however made a subgroup with Latisail. These observations could be possibly explained if chromosome loss of the paternal

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RESEARCH ARTICLE

Anatomical and karyotypic comparison of induced tetraploid of *Oryza sativa* var Latisail with the allotetraploid halophytic wild rice *Oryza coarctata*

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Abstract The allotetraploid wild rice, *Oryza coarctata* grows in sea water and is the only halophyte which sets rice-like grains. It is a unique genetic resource for introducing salt tolerance into elite rice cultivars of *Oryza sativa*. Hybridization between these two remains unsuccessful. Our aim was to induce tetraploidization in the traditional Latisail cultivar of rice and compare its anatomical features to *O. coarctata*. Colchicine was used on the apical shoot tips of germinating seeds to produce the induced autotetraploid of *Oryza sativa*. Detailed anatomical comparisons of the leaves, stems, roots and chromosomes of the diploid and induced tetraploid of the Latisail variant of *O. sativa* and wild *O. coarctata* were carried out. Chromosomes of the induced tetraploid of *O. sativa* were shorter than diploid *O. sativa*, but longer than the 48 chromosomes in *O. coarctata*. The anatomical features of the induced tetraploid, like the number and size of bundle sheath cells, vascular bundle sizes and cuticle sizes were closer to that of the perennial allotetraploid, *O. coarctata*. It had more than twice the number of bulliform cells compared to the diploid and the number was similar to those found in *O. coarctata*. Crosses between the induced tetraploid and *O. coarctata* produced fertile offspring resembling *O. sativa* with evidence of small introgressions from *O. coarctata* pollens. The induced tetraploid of the Latisail variant of *O. sativa* and its progeny with *O. coarctata* are valuable plant genetic resources and may help introduce the vigorous halophytic traits of *O. coarctata* into commercial rice.

Keywords *Oryza sativa* L. var Latisail · *Oryza coarctata* (Roxb.) Tateoka · Induced tetraploid · Karyotype · Stomata · Bulliform cells

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Appendix 3: Media Compositions

Table 1.1: Composition of YM Media

Components	Volume (for 100 mL)
Yeast extract	0.04 g
Mannitol	1.0 g
NaCl	0.01 g
$MgSO_4$	0.02 g
Dry K_2HPO_4	0.036 g
Agar (for solid)	1.5 g

*pH is adjusted to 7.0 before adding agar.

Table 1.2: Composition of Bacterial Re-suspension Media

Components	Volume for 100 mL
Sucrose	6.84 g
Glucose	3.6 g
AB buffer	5 mL
AB salt	5 mL

*100 mL volume is made with ddH_2O and pH is adjusted to 5.6 and autoclaved.
Finally, Acetosyringone is added to the final concentration of 200 µg/mL.

Table 1.3: Composition of Hydroponic Solution

Macronutrients:

Element	Reagent (AR grade)	Preparation solution (g/4L)
N	Ammonium nitrate (NH_4NO_3)	365.6
P	Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$)	142.4
K	Potassium sulfate (K_2SO_4)	285.6
Ca	Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$)	469.4
Mg	Magnesium sulfate. Seven-hydrate ($MgSO_4 \cdot 7H_2O$)	1296.0

Micronutrients:
Dissolve each reagent separately and mix in 2 L distilled water then add 200 mL H_2SO_4 and make up volume to 4 L

Element	Reagent (AR grade)	Preparation solution (g/4L)
Mn	Manganous chloride, 4-hydrate ($MnCl_2 \cdot 4H_2O$)	6.000

Mo	Ammonium molybdate, 4-hydrate $[(NH_4)_6Mo_7O_{24}\cdot 4H_2O]$	0.296
Zn	Zn-Sulfate, 7-hydrate $(ZnSO_4\cdot 7H_2O)$	0.140
B	Boric acid (H_3BO_3)	3.736
Cu	Cupric sulfate, 5-hydrate $(CuSO_4\cdot 5H_2O)$	0.124
Fe	Ferric chloride, 6-hydrate $(FeCl_3\cdot 6H_2O)$	30.800
	Citric acid monohydrate $(C_6H_8O_7)$	47.600