VALIDATION OF ENERGY-RELATED VACUOLAR SUBUNIT GENE *Pc_PVA1* FROM WILD HALOPHYTE *Pc* IN SALT SENSITIVE COMMERCIAL RICE, BR75

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is my original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

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of Spring, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on 26/8/2020.

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Ethics Statement

No animal was harmed during this experiment.

Abstract

The study was conducted on transgenic rice, BR75, a high-yielding but salt-sensitive genotype previously transformed with the *Pc_PVA1* gene, at the Plant biotechnology Laboratory of Dhaka University. The aim was to identify presence of the transgene and to assess the response of transgenic seedlings to salt stress. The transgene originated from wild halophytic rice *Porteresia coarctata*, endemic to coastal Bangladesh and was expected to confer salt tolerance to the transformed sensitive rice, BR75. The wild type or untransformed BR75 and the two transgenic genotypes derived from its transformation, P-46-1 and P-9-4 were used for this experiment. DNA was extracted from the wild type and T_1 generation of the transgenic plants. PCR analysis followed by gel electrophoresis was performed with the isolated DNA. Then the performance of transgenic BR75 T_2 population in saline condition was investigated. Seedling screening was done following the standard evaluation system (IRRI), and the chlorophyll content of the seedlings were also measured. The results of the validation study were found to be positive, that is to confirm their tolerance status as opposed to the wild type. The two transgenic varieties in this study performed much better than the wild type BR75 in saline condition because these had higher survival rate and chlorophyll content. The present study was conducted with a small subset of the T_2 population. In future, more tests like the leaf disk senescence assay, determination of Na:K ratio under saline stress, mRNA analysis needs to be done for a more detailed evaluation. Finally, the transgenic plants should be trialed in the natural saline environment using standard Biosafety Protocols.

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Eera Ashrafy Anonna

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Acronyms

BBS	Bangladesh Bureau of Statistics
bHLH	Basic helix-loop-helix
BLAST	Basic Local Alignment Search Tool
BRRI	Bangladesh Rice Research Institute
bZIP	Basic leucine zipper
DAE	Department of Agricultural Extension
ddH20	Distilled deionized water
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic acid
IRRI	International Rice Research Institute
MYB	Myoblastosis
RNase	Ribonuclease
rpm	Revolution Per Minute
TAE	Tris acetate EDTA
USDA	United States Department of Agriculture

Chapter 1:

Introduction

Chapter 1: Introduction

Rice is one of the most important cereal crops around the world agronomically and nutritionally. It is the staple food for more than half the world's population including Bangladesh [1]. The growth and productivity of rice are often threatened by many abiotic and biotic stress factors. In Bangladesh, salinity and drought are the two major abiotic stresses for rice production. In addition to that, population outburst and climate change are making our fight harder. Though, in the year 2018-19, over 37 million tons of rice was grown on 11.7 million hectares in Bangladesh (Source: BBS & DAE), we still needed to import 1.1 million tons of rice in the year 2018 (USDA).

Thus to ensure food security, it is very important to enrich the rice gene pool with the genes that confer stress tolerance in rice. Wild relatives of *Oryza sativa* as well as many other non-relatives are important reservoirs of genes that can be used to improve the existing varieties with desired traits. Different approaches has already been taken to develop salinity tolerant rice varieties including traditional breeding and molecular techniques. Bangladesh Rice Research Institute (BRRI) has released several salt tolerant rice varieties through conventional breeding. However, except BRRI dhan47 none of them got popularized due to lack of adaptive trait. Recently salt tolerant BRRI dhan67 has been released which may fare better. However more salt tolerant varieties are needed for the different agroecological zones of Bangladesh. As salt tolerance is a multigenic trait, it is possible to create elite salt tolerant rice varieties by using marker assisted gene pyramiding and transgenic method. However, it is difficult to find a single plant with multiple genes associated with stress tolerance. Therefore, researchers are always in search of promising germplasm source which can be used to develop new stress-tolerant varieties.

1.1 Soil and water salinity, the major abiotic plant stress in Bangladesh

Bangladesh is the second largest delta of the world. About 20% of the total area of the country is under coastal region, which covers 30% of the arable lands [2]. However, the salinity level is increasing in the non- coastal areas as well. In 1973, 0.83 million hectares of land was affected by salinity which increased to 1.02 million hectares by the year 2000 and in 2009 it was 1.05 million hectares [3].

By 2100, sea level by the coast of Bangladesh may rise about 0.88m where only 1m rise can inundate 20% of the total land area of the country [4]. The soil is considered to be affected by salinity if the electrical conductivity exceeds 4 dSm^{-1} at 25°C. A study showed, 0.83 million hectares out of the coastal land of 2.85 million hectares, had an electrical conductivity varying from 1-20 dSm^{-1} [5].

Moreover, Bangladesh is vulnerable to the seasonal cyclones in every monsoon which originates at the Bay of Bengal. Sidr (2007) and Aila (2009) are two such consecutive cyclones that flooded a total of thirty districts across the South Western coastal area of Bangladesh [6]. As a result of saline water intrusion, salinity in the coastal area has become more severe and cultivable lands are becoming abandoned.

1.2 Soil salinity: A great threat to our food security

Compared to the total land area, Bangladesh has got a huge population of about 17 million. Bangladesh is one of the most densely populated countries in the world with 3310 people per square mile [7]. So, with the limited land and resources, it has so many mouths to feed. Overpopulation has already put an enormous pressure on the arable land and on top of that

salinization and deterioration of water resources are affecting irrigated agriculture by reducing arable land and crop yields.

Bangladesh is one of the ten most affected countries of climate change during the last two decades [8]. Agriculture is the main driving force for the economy of Bangladesh and 48 percent of the rural population of the country depends on agriculture [9]. It is projected that by 2050 in the context of climate change, the food production in Bangladesh will grow by about 53 percent over the level produced in 2010 which is 17 percentage points less than it would be in the case without climate change. Moreover, 8.9 million people in Bangladesh are projected to be at risk of hunger in 2050 as a result of climate change [10].

Around 30% of the arable land of Bangladesh is located at the southwest coastal region [11]. Moreover, the scarcity of water in the Northwest part of the country has led the government of Bangladesh to emphasize on shifting the production of rice to Southern part since surface water is abundant there [12]. However, due to salinity farmers can grow only during monsoon and the crop yield is low in that region.

1.3 *Porteresia coarctata* (Roxb) tateoka, a unique germplasm source for developing salt-tolerant rice varieties

Porteresia coarctata is locally referred to as Uridhan. Unlike the cultivated rice varieties, however, *P. coarctata* has an underground rhizome system. From each node of the rhizome the shoots come out to form a grassy bush. It is also genetically different from cultivated rice having 48 chromosomes (tetraploid) whereas it is 24 in rice (diploid). *P. coarctata* can happily grow and complete its life cycle in 200- 400 mM NaCl. Though it may set a few grains, usually they do not germinate. Rather, it self-propagates through rhizomes [13, 14]

P. coarctata is an established halophyte (salt-loving plant) with identified saline tolerance mechanisms. Some genes identified from *P. coarctata* are directly associated with salinity tolerance [14]. *P. coarctata* plants can accumulate sodium and chloride ions. Salt is stored in the salt glands or salt hairs found on the upper surface of the leaf. Later, the accumulated salt is secreted from the leaves. Study showed that, even after growing in 25% artificial seawater (Na: K ratio 34) for 6 weeks, a Na:K ratio of 0.7 was maintained in the plants which is extremely low compared to the growth medium. [15]

In a comparative study, *P. coarctata* was found to have better growth than cultivated *O. sativa* rice varieties. In a recent study by Baillo, 15158 genes were identified in which are involved in salinity and submergence response. In the study, different transcription factor families, including *MYB, bHLH, AP2-EREBP, WRKY, bZIP* and *NAC*, were identified which are associated with stress-response [16]. Recently, a draft of the whole genome sequence of *P. coarctata* has been published [17].

Therefore, *P. coarctata* is an efficient candidate and a rich source of germplasm for salinity tolerance which gives rise to the possibility of introducing some of its genes that confer salt tolerance into cultivated rice. As it is genetically distant from cultivated rice and also a tetraploid having 48 chromosomes, it is very difficult to cross with cultivated rice. However, the genes associated with salinity tolerance can be identified and cloned and can be introduced in our popular rice varieties to confer salt tolerance.

Figure 1.1 : *Porteresia coarctata growing in the net house of Plant biotechnology lab, DU.*

1.4 Vacuolar Proton ATPase subunit c (*Pc_PVA1*), a promising gene from *P. coarctata*

Hundreds of genes and transcription factors have been identified by transcriptome analysis of *P. coarctata* at different salt concentration [18]. Isolation and characterization of several genes related to the salt-tolerance have already been done from *Porteresia*. From literature mining, we have also identified some of the genes which are directly involved with salt tolerance, for example, Sodium Potassium channel, Metallothionein *Type 3,* Vacuolar H^+ATPase Subunit c gene, Non-selective cation channel etc. Among them, Vacuolar H^+ATPase Subunit c gene (PVA1) is one of the most promising genes.

The vacuolar H^+-ATPase (V-ATPase) is found in every living organism [19, 20]. It can pump protons from the cytoplasm into the vacuole using the energy released by ATP hydrolysis. Thus, the pump moves protons against the electrochemical gradient and regulates the cytoplasmic pH when energy is available [19]. It has two functional domains - the peripheral V_1 domain and membrane integral V_0 domain. The V_1 domain protruding in the cytoplasm takes part in ATP hydrolysis. It has eight different subunits (A–H). On the other hand, the V_0 domain consists of at least five different subunits (a–d). The V_0 domain fixated at the membrane takes part in proton translocation [21]. Since subunits A, B and c are found in all V-ATPases, these subunits are assumed to have significant roles and identified as the major subunits. [22].

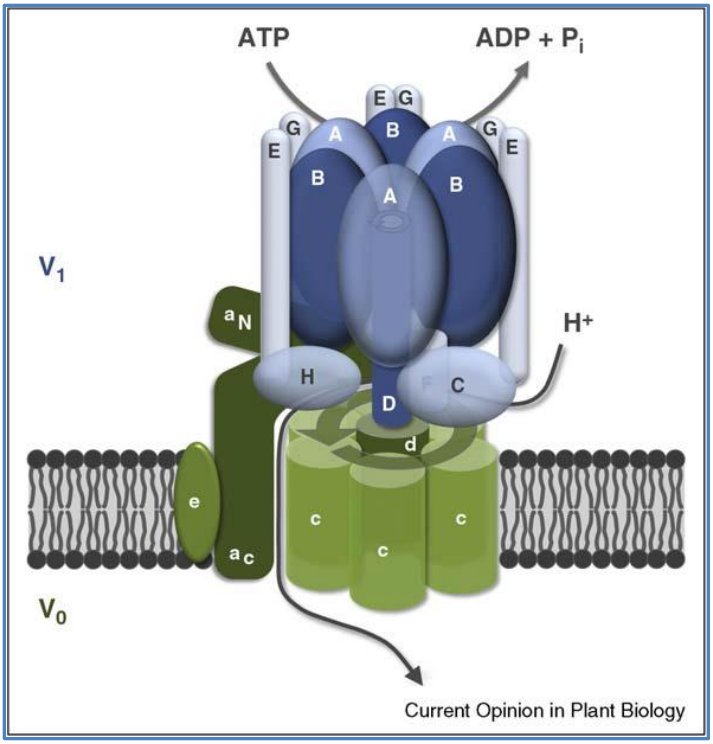

Figure 1.2: *A simplified model of V-ATPase. V-ATPase consists of two domains- the membrane integral V_0 domain and the peripheral V_1 domain. These two domains are kept together by three pairs of subunit E and G. V_1 domain is responsible for the hydrolysis of ATP, V_0 domain transports proton inside the membrane. The V_1 domain consists of six A and B alternating subunits and the V_0 domain consists of a proteolipid-ring made of total six units of subunit c. The ring is adjacent to subunits a and e. Subunits D, F, and d constitute the central stalk. Rotation of the c-ring allows the transport of proton [23]. (Figure taken from Schumacher and Krebs, 2010)*

V-ATPase is highly abundant in tonoplast (6.3-35% of the total tonoplast protein). Both in normal and stressed condition V-ATPase plays great role in plants. In normal condition, it elevates plant growth by energizing secondary transport and maintaining solute homeostasis. Survival of the plant in stressed condition strongly depends on the changes in the expression and activity of different subunits of V-ATPase.

Under salt stress, expression of V-ATPase is increased and excess sodium is removed from the cytoplasm through vacuolar accumulation. A secondary active Na^+/H^+ antiport at the tonoplast regulates this accumulation which is energized by the Proton Motive Force generated by V-ATPase. Though Na^+/H^+ antiporter (NHX) has its own H^+ transport ability it is not enough when huge amount of NaCl appears at once. V-ATPases coordinates with NHX antiporters that mediate the transport of Na^+. V-H^+-ATPases also respond to salt stress through regulation mechanisms at transcriptional and as well as at post-transcriptional level. [24-26]

In two different studies conducted by two groups of Chinese scientists, transgenic tobacco lines were developed overexpressing the LbVHA-c1 gene, or the V-ATPase subunit c gene from *Limonium bicolor*, [27] and V-ATPase subunit c gene from the salt-tolerant plants *Iris lacteal* [28]. In both experiments, it was found that under salt stress, activity of superoxide dismutase (SOD) was significantly increased in the transgenic plants compared to the wild-type tobacco plants. The activity of peroxidase (POD) was also increased. Moreover, the transgenic tobacco plants displayed better growth under salt stress.

From a halophyte grass, *Spartina alterniflora*, *Vacuolar ATPase subunit c1 (SaVHAc1*) was cloned and expressed in rice. It was found that, the *SaVHAc1*-expressing rice plants were more tolerant to salt stress than the wild-type rice plants [29].

In another study, the expression of each subunit of VHA from wheat in wild-type *Arabidopsis thaliana* was observed under salt stress. In the study, the c subunit was found to have an increased expression at the later periods which was much delayed compared to the other subunits. This suggests that, c subunit is less sensitive to salt stress than the others. Moreover, among V-ATPase the sequence of the c subunit was also found to be most conserved, from which it can be assumed that subunit c is an important limiting factor for the functionality of V-ATPase. Thus, the plants with the c subunit transgene were the more salt tolerant [30].

Moreover, the study of Padmanaban (2004) revealed that being the most abundant subunit of V_0, subunit c can alter or modulate the activity of fully V_1V_o ATPase pumps [25]. Other studies also revealed that as the duration of the salinity stress increased, the transcription of c-subunit of *P. coarctata* also increased [13, 31]. Though *Porteresia* is a wild relative of rice, the *PVA1* gene and proteins are not same. BLAST result for *PVA1* gene of *Porteresia coarctata* (AF286464.1) and *Oryza sativa* (indica cultivar) (CT828125.1) showed 88.96% identity and protein BLAST showed 99.4% identity. Compared to *OsPVA1*, *Pc_PVA1* has only a Valine substitution in in place of Isoleucine at 24^{th} amino acid position.

The extensive study of *Pc_PVA1* was done by Senthikumar et al. in 2005. They found that *PVA1* has multiple coding regions in *P. coarctata* and *PVA1* is significantly upregulated by salt stress (500 mM NaCl for 48 hours) at both transcriptional and translational level [32]. Thus, *Pc_PVA1* gene from the wild halophyte *Porteresia coarctata* is a very promising gene to increase the efficiency of Na^+ transport in sensitive rice varieties and confer salt tolerance in them.

1.5 Background of the study

(Research at Plant Biotechnology Laboratory, DU for introducing saline tolerant variety)

Keeping in mind the climate change effects and growing population of Bangladesh the Plant biotechnology lab, DU has been working relentlessly on developing different stress tolerant transgenic rice variety for over 20 years. Some salt tolerant varieties that are available include BRRI dhan47, BRRI dhan61 and BRRI dhan67 BRRI dhan53, BRRI dhan54 and BRRI dhan73 which can withstand 6-8 dS/m. However, they are not enough to cope up with the upcoming challenge.

For the study conducted by plant biotechnology laboratory, wild halophytic rice *P. coarctata*, which is locally called 'Uri dhan', was collected from coastal region of Bangladesh and grown in the net house conditions. High yielding salt sensitive BRRI dhan75 used in this study is green super T. Aman rice which has growth duration of 115 days and average grain yield is 5.5 ton/ha. (Source: BRRI)

RNA was isolated from *P. coarctata* and cDNA was prepared. Then PCR amplification of the targeted gene Vacuolar H^+ATPase subunit c (*PVA1*) was done. The gene was cloned into an Entry Vector (pENTR TOPO D) and the construct was transferred into *E. coli* DH5a strain by heat shock transformation. Confirmation of cloning was done by Gene specific PCR, Restriction Digestion and finally DNA sequencing. After confirmation, cloned gene from the entry vector was transferred to a plant compatible Destination vector (pH7WG2) and Heat shock Transformation was performed on *E. coli* DH5a with targeted gene construct. After confirmation, the vector was transformed into a plant transformation vector and BR75 was transformed using *in planta* transformation.

Thus, the high yielding, but salt-sensitive BRRI Dhan 75 (BR75) was transformed with the construct by Tissue-culture independent *in planta* transformation for developing as salt-tolerant variety.

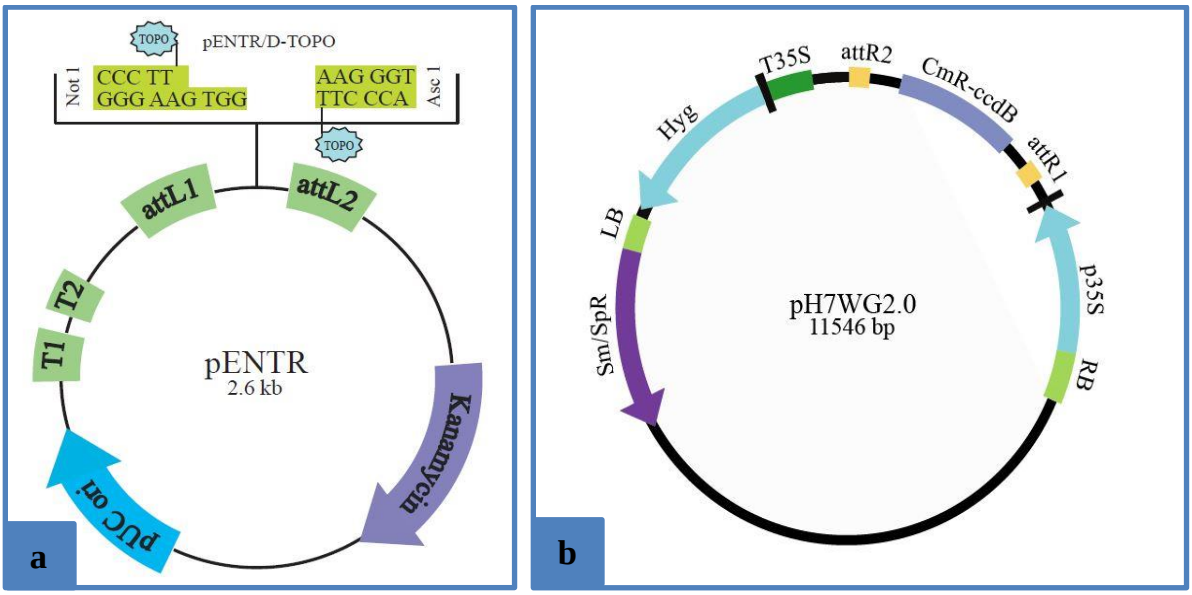

Figure 1.3: *Vectors used for the transformation of Pc_PVA1. a) pENTR TOPO-D vector containing TOPO recognition site, Kanamycin resistance gene , attL site, m13 priming site, pUc origin. b) A plant compatible vector pH7WG2.0, containing attR site, primers for expression in plant system, Hygromycin and streptomycin-spectinomycin resistance gene.*

1.6 Objective

This study is a part of the study conducted in the Plant Biotechnology laboratory of DU which was mentioned in section 1.5. This study aims to validate the presence of the transgene *Pc_PVA1* in transgenic BRRI Dhan75. Under this circumstances the present study has been formulated with the following objectives-

- Confirming the presence of transgene in BRRI Dhan75 T_1 population.
    - ✓ Polymerase chain reaction followed by Gel electrophoresis
- Seedling screening of transgenic BRRI Dhan75 to assess its salt tolerance ability.

Chapter 2:

Materials & Methods

Chapter 2: Materials and Methods

This study has been carried out in Plant Biotechnology laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka. The study aims to confirm the presence of the targeted gene Vacuolar H^+-ATPase subunit-c (PVA1) in the T_2 generation plants, as well as to analyze the performance of transgenic BRRI Dhan75 T_2 population in saline condition at seedling stage. The materials and methods used in this study are described below.

2.1 Materials

2.1.1 Plant material

- T_1 plants from the transgenic population *Pc_PVA1*-BR75.
- T_2 Seeds of transgenic BR75 with *Pc_PVA1* gene. Two previously confirmed transgenic plants P-46-1 and P-9-4 were selected for salinity stress screening at seedling stress.
- BR75 seeds as sensitive wild type control and naturally salt-tolerant variety Pokkali as positive control plants.

2.1.2 PCR components

10 × PCR buffer, 50mM of $MgCl_2$, 10mM of dNTP mixture, *Pc_PVA1* forward and reverse primer, Taq DNA polymerase, DMSO, nuclease free water.

2.1.3 Preparation 25mL 1% agarose gel

0.25 g of agar was added into 25 mL TAE buffer.

2.1.4 Components of stock solutions for Yoshida stock media for hydroponics

Table 2.1: Components of stock solutions for Yoshida stock media for hydroponics

Elements	**Reagent**	**Amount (g/L)**
1. N	NH_4NO_3	91.40
2. P	$NaH_2PO_4.H_2O$	35.60
3. K	K_2SO_4	71.40
4. Ca	$CaCl_2.2H_2O$	117.35
5. Mg	$MgSO_4.7H_2O$	324.0
6. **Micro element**		
a. Mn	$MnCl_34H_2O$	1.50
b. Mo	$(NH_4)_6Mo_7O_{24}.24H_2O$	0.074
c. B	H_3BO_3	0.934
d. Cu	$CuSO_4.5H_2O$	0.031
e. Fe	$FeCl_3.6H_2O$	7.70
f. Citric acid	$C_6H_8O_7.H_2O$	11.9
g. Zn	$ZnSO_4.7H_2O$	0.035

For the preparation of 1L Yoshida solution 1.25 mL of each macro and micro element was taken and distilled water was added to make the volume 1 L. pH was set at 5.

2.1.5 Preparation of 80% acetone for measurement of chlorophyll content

To prepare 80% acetone, 400ml 100% acetone was mixed with 100ml distilled water.

2.2 Methodology

2.2.1 DNA extraction

Reagents for DNA extraction

Liquid N_2, DNA extraction buffer, Phenol-Chloform-Isoamyl alcohol((25:24:1), RNAase A, Na Acetate, ice cold ethanol.

DNA extraction buffer

100 mL DNA extraction buffer was prepared by mixing 10 mL 1M Tris HCl, 2 mL 0.5 M EDTA, 10mL 10% SDS, 2 mL 5M NaCl and finally adding 76 mL of distilled water to make the volume 100 mL. pH was adjusted to 8.

PCI

Phenol: chloroform: isoamyl alcohol = 25:24:1. For preparing the PCI solution absolute phenol, chloroform and isoamyl alcohol was mixed together in 25:24:1 ratio.

DNA extraction method

All the plants were grown in net house of University of Dhaka. Small portion of flag leaves from T_1 plants were collected in the eppendorfs containing distilled water. Then the leaves were taken to the laboratory and DNA extraction was performed following the short method:

i. Plant leaves were pulverized in liquid N_2 and grinded by mortar and pestle.
ii. Leaf powder was taken into a 1.5 mL eppendorf tube.

iii. 650 µL of DNA extraction buffer was added and vortex was done until mixture becomes homogenized.

iv. 650 µL of PCI solution was added and vortex was done.

v. Centrifugation was done at 10000 rpm at 4°C for 15 min.

vi. 400 µL of supernatant was transferred to a fresh 2 mL eppendorf tube.

vii. 400 µL of PCI solution was added and vortex was done.

viii. Centrifugation was done at 10000 rpm at 4°C for 10 min.

ix. Supernatant was transferred to a fresh 2 mL eppendorf tube.

x. 5 µL of RNase A was added and kept at 37 °C for 40 minutes.

xi. 1 mL of isopropanol was added to the eppendorf tubes.

xii. 0.1% volume of Na Acetate was added and the eppendorf tubes were kept overnight at -20°C.

xiii. Next day the tubes were centrifuged at 10000 rpm at 4 °C for 15 minutes.

xiv. Supernatant was discarded and 1 mL of ice cold 70% ethanol was added to the tubes. Pellets are mixed in Ethanol.

xv. Again the tubes were centrifuged at 10000 rpm at 4 °C for 10 minutes.

xvi. Supernatant was discarded and again 1 mL of ice cold 70% ethanol was added to the tubes for better washing.

xvii. Then centrifuged at 10000 rpm at 4 °C for 5 minutes.

xviii. Each drop of ethanol was removed by slowly pouring out the solution and puffed on tissue paper.

xix. Pellets were dried at room temperature.

xx. Lastly, 50 µL of TE buffer was added to the DNA pellet.

2.2.2 Measuring the concentration of DNA using Nanodrop spectrophotometer:

To measure the concentration of DNA samples the Nanodrop spectrophotometer ND1000 was used. The procedure to measure the nucleic acid concentration is described below:

1. The software for the spectrophotometer was opened on the computer. To measure DNA sample nucleic acid option was selected. The wavelength was fixed at 260 and 280 nm.
2. The upper and lower arm of the machine was first cleaned with soft cotton bud and Nuclease free water.
3. After initialization, the blank reading was taken using the buffer used to dissolve the DNA.
4. 1 µL of each sample was loaded onto the lower arm, the lid was then closed and the OD was measured.
5. The software showed the concentration of the sample in ng/µl, its Standard curve with the absorbance ratio of 260 nm to 280. Concentration was recorded.
6. The upper and lower arm of the spectrophotometer was cleaned properly using nuclease fee water when the measurement was over.

2.2.3 Dilution of DNA sample:

After measuring the concentration, DNA samples were diluted to the concentration of 50ng/µL by adding required amount of TE buffer. Required amount of buffer was calculated using the obtained DNA concentration.

2.2.4 PCR analysis of T_1 generation BR75 transgenic plants:

Transgenic rice plants were screened by PCR analysis using 50 ng/µL rice genomic DNA as template from T_1 generation plants. *Pc_PVA1* (Accession No.AF286464.1) - specific primers were used for amplification to replicate 838 bp region. For amplification of the targeted gene, the reaction was performed with an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 61 °C for 50s and extension at 72 °C for 5s, and final extension at 72 °C for 7 min.

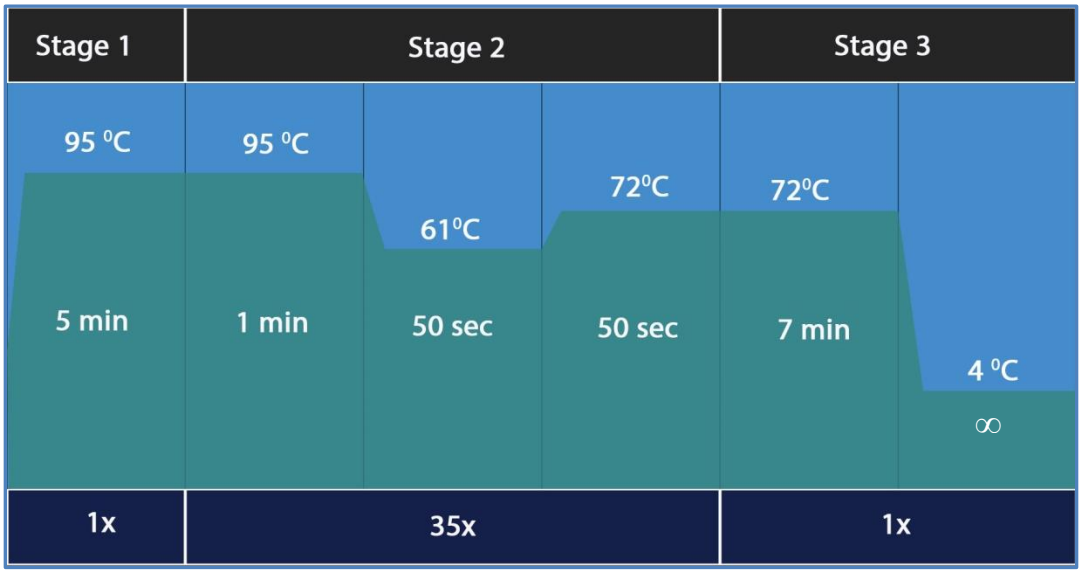

Figure 2.1: *Thermal cycling program for the PCR amplification of PVA1*

PCR analysis was carried out in a 15 µl reaction mixture. Every gene specific PCR needs some optimization of the reagents and annealing temperature. The specific condition for amplifying *Pc_PVA1* gene has been described in table 2.2 and 2.3.

Table 2.2: Composition of Master mixture

Components	**Amount required per unit (µL)**
PCR buffer (10X)	1.5
dNTP 10mM	0.5
MgCl2 50 mM	0.8
Forward primer(50ng/ µL)	0.5
Reverse primer(50ng/ µL)	0.5
Taq DNA polymerase (20X diluted)	0.5
Total	**4.3**

Table 2.3: Preparation of sample, negative and positive control with DNA, DMSO and ddH2O for PCR

Tube usage	**DNA (50ng/ µl)**	**DMSO (20%)**	**ddH20 (Adjustable)**	**Total volume with Mastermix**
Sample	1.5 µl	3.0µl	6.2µl	15 µl
Negative control	0.0 µl	3.0µl	7.7µl	15 µl
Positive control	1.5 µl	3.0µl	6.2µl	15 µl

2.2.5 Reaction mixture preparation for PCR:

To prepare the reaction mixture the steps mentioned below were followed:

1. Sample DNA, 20% DMSO and nuclease free water were pippeted in the labeled PCR tubes with the amounts mentioned in Table: 2.2.2
2. The mixture was then denatured at 95°C for 5 min and immediately transferred into ice.
3. Master mixture was prepared according to the table 2.2.1.

4. 4.3 µl of master mixture was added to the tubes after thoroughly mixing and spinning.
5. The PCR tubes were thoroughly mixed and after spinning any bubble produced was taken care of.
6. Finally, the tubes were transferred to Thermocycler and the specific thermal cycling program was selected for the amplification reaction.

2.2.6 Gel electrophoresis

PCR products were checked for the amplified *Pc_PVA1* gene. 5 µL of loading dye was added to the PCR tubes and 10 µL from each of the sample was loaded on the 1% agarose gel. 1 kb+ DNA ladder was used. Electricity was run for 1 hour at 100 volts until the dye reached near the end of the gel.

2.2.7 Gel staining:

The gel was kept in the ethidium bromide solution (0.5µg/ml) for 15-20 minutes at room temperature. After that, residual stain was removed by placing the gel in distilled water.

2.2.8 Visualization of the bands:

Banding pattern was observed and captured by Alpha imager mini Gel documentation system. AlphaView software is included in this system. Before taking photograph, agarose gel was exposed to UV-Illumination. After capturing the images necessary modifications were made through the software to visualize the bands more clearly and properly.

2.2.9 Seed selection for seedling screening

Based on the result of the gel electrophoresis, P-9-4, P-46-1 seeds were selected. Wild type BR75 was selected as the sensitive control. Pokkali seeds were taken as salt tolerant variety to be used as positive control in the screening.

2.2.10 Seed Dormancy Break

Seeds were kept at 50°C in incubator for 48 hours for breaking seed dormancy. The seeds were placed in a petri dish after covering them with filter paper. Then the seeds were kept at room temperature for 1 day.

2.2.11 Germination of the seeds

Seeds were placed on moist filter paper in petri plates and kept at 37°C for germination. Seeds were soaked in tap water. The water in petri dish was changed every day until germination.

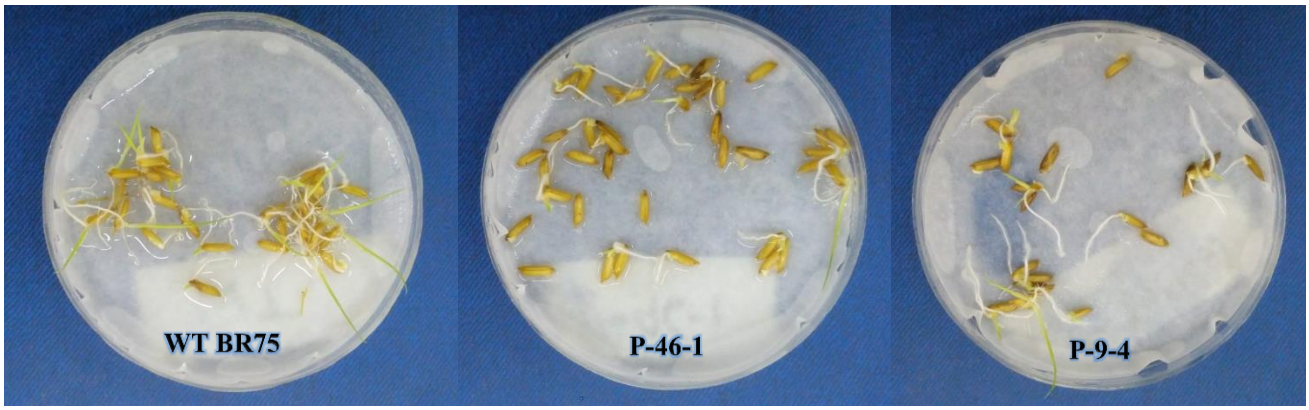

Figure 2.2: *Germinated seeds after 4 days.*

2.2.12 Salinity Stress Screening at Seedling Stage

Phenotypic screening for salinity tolerance was done on *Pc_PVA1*-BR75 T_2 population at seedling stage. WT (BR75) was used in the screening as the sensitive control. Floater was prepared and labeled accordingly prior to the transfer of germinated seeds. Germinated seeds were sown in netted styrofoam floated in PVC trays containing 10 L Yoshida solution. The germinated seeds were allowed to grow for 14 days. Then, NaCl stress was applied gradually starting from 6 to 12 dS/m at 24 h increments of 2 dS/m. After 9 days, when the sensitive WT BR75 was nearly dead, the tolerance- related traits (SES, Root length, shoot length and chlorophyll content) of all stressed plants were measured.

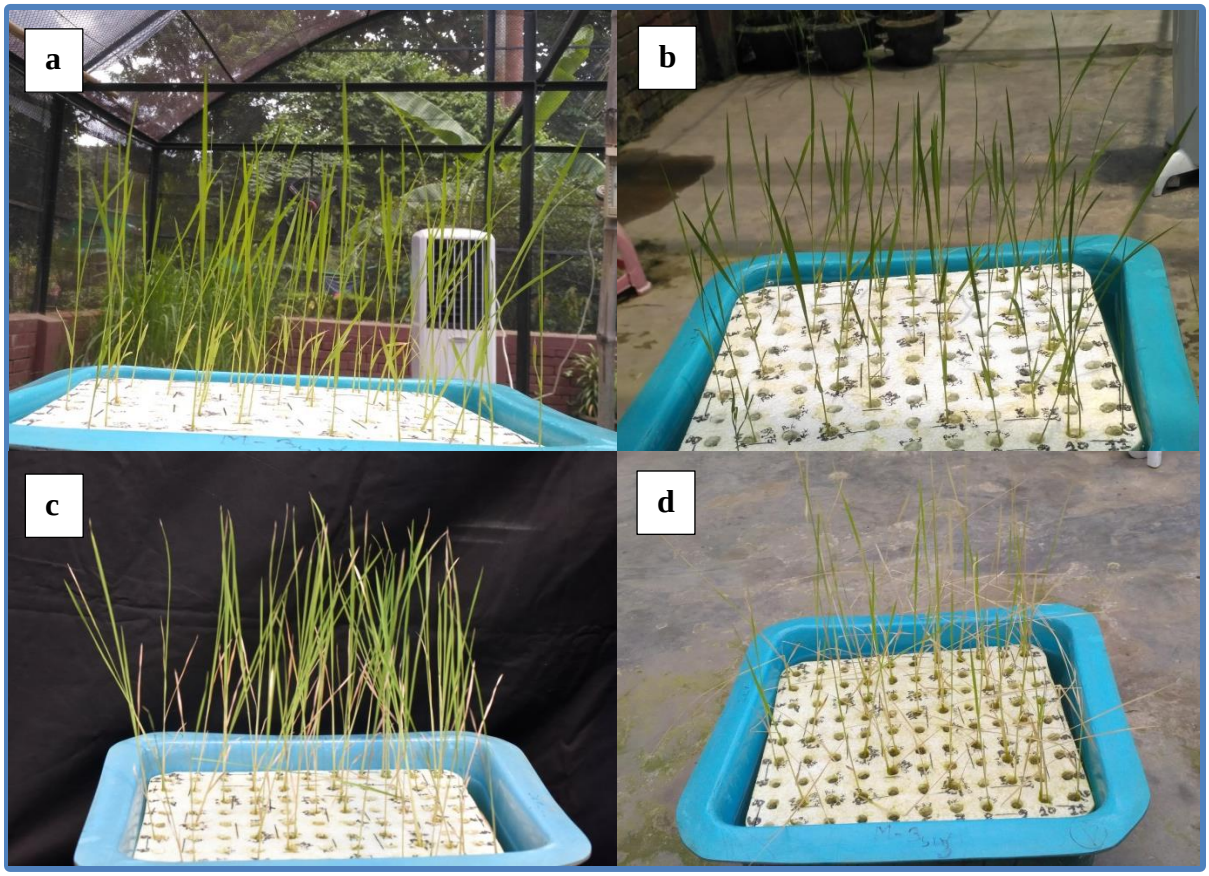

Figure 2.3: *Condition of seedlings before and after putting under salinity stress. a) Day 8 in hydroponics; no salt stress. b) Day 10 in hydroponics; no salt stress. c) Day 17 in hydroponics; 120mM salt stress. d) 4 days after applying 120 mM salt stress.*

2.2.13 Measurement of shoot length root length and weight

After keeping the seedlings in salinity for 9 days, shoot length, root length of the plants were measured using a centimeter scale.

The shoot and root of the seedlings were separated and weighed using an electrical balance. Shoot and root were cut into small pieces for convenience.

2.2.14 Measurement of Chlorophyll Content

Chlorophyll content is a key marker for analyzing plant photosynthesis efficiency at different stress condition. Leaves were cut into pieces and 50 mg put into a bottle containing 12.5 ml of 80% acetone and kept in dark for 2 days. After 48 hours, absorbance of leaf tissue extract was measured at wavelength of 645, 652 and 663 nm by using a spectrophotometer. At first the blank was set using 80% acetone as absorbance 0. The chlorophyll extracts were transferred into the cuvette to take absorbance at 3 different wavelengths. Average absorbance was calculated and recorded. The total amount of chlorophyll content was calculated using the formula:

$$A=ECd$$

A = absorbance,

E = extinction coefficient = 36 mL/mg.cm,

C = Chlorophyll concentration and

d = distance of the light path 1cm.

2.2.15 Data Analysis

Collected data were analyzed using Microsoft excel 2010. Necessary calculations were done and graphs were produced by the software to interpret the result.

Chapter 3:

Result

Chapter 3: Result

This study aims to assess the salt tolerance ability of BRRI Dhan75 previously transformed with *vacuolar proton ATPase subunit c (PVA1)* of *Porteresia coarctata tateoka*. BRRI Dhan75 is a high yielding but salt sensitive variety, on the other hand *P. coarctata* is a distant relative of rice and highly salt tolerant, happily grows upto 400-500mM Salt. *PVA1* is one of the key regulatory genes which have essential roles for maintaining cellular balance during plant salinity stress.

The transgenic population has been developed in Plant Biotechnology Laboratory, University of Dhaka. Here, the transgenic plants have been confirmed for presence of the targeted gene by gene-specific primer at T_1 generation, and then the selected plants have been screened for salt tolerance at 120 mM salt stress at seedling stage.

3.1 PCR confirmation of T_1 generation plants of BR75 transformed with *PVA1* gene

The presence of transgene was confirmed by PCR amplification of *Pc_PVA1* gene (figure 3.1). The size of *Pc_PVA1* gene is 838bp. The details condition of PCR reaction has been described in method and materials section. Two PCR positive lines, P-46-1 and P-9-4 were selected for further screening.

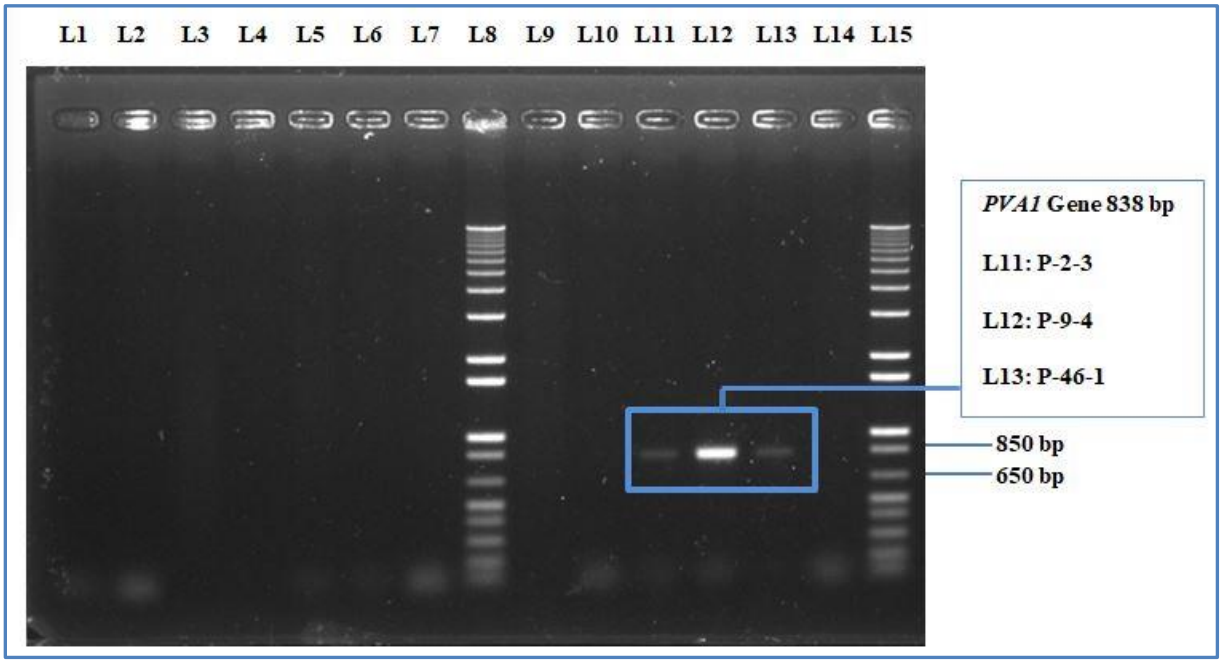

Figure 3.1: *Confirmation of the presence of Pc_PVA1 gene. L1, L2: negative control; L3: wild type; L4-L7: T_1 plants; L8: 1kb+ Ladder, L9-14: T_1 plants, L15: 1kb+ Ladder.*

3.2 Seedling Stress screening

3.2.1 Standard Evaluation System (SES) score of rice:

The rice seedlings were taken out from growth media on the 10th day of 120 mM salt stress when most of the wild type plants were dead. P-9-4 and P-46-1 plants were much healthier and greener than the wild type BR75 plants.

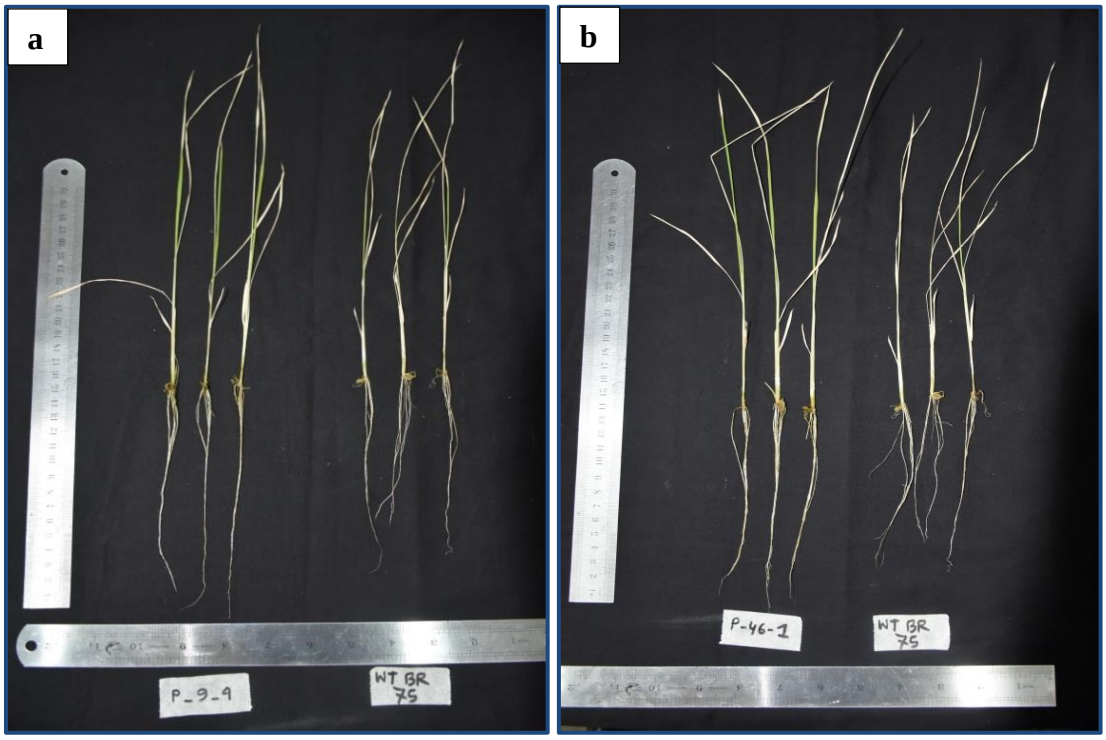

Figure 3.2: *Comparison among the seedlings of transgenic P-9-4, P-46-1 and wild type BR75 after withstanding 120mM salt stress. a) Seedlings of P-9-4 (left) compared to the wild type BR75 (right). b) Seedlings of P-46-1 (left) compared to the wild type BR75 (right).*

The SES score of the selected plants has been presented in figure. The performance for salt tolerance was very clear on the 4th day after salinization. However, the final response was recorded on day 7. After 9 days of stress, most of BR75 seedlings had died and salinity injury in P-46-1 and P-9-4 was severe. The salt-tolerance level of P-9-4 was lower than that of P-46-1 but they could also be considered as tolerant when compared with BR75.

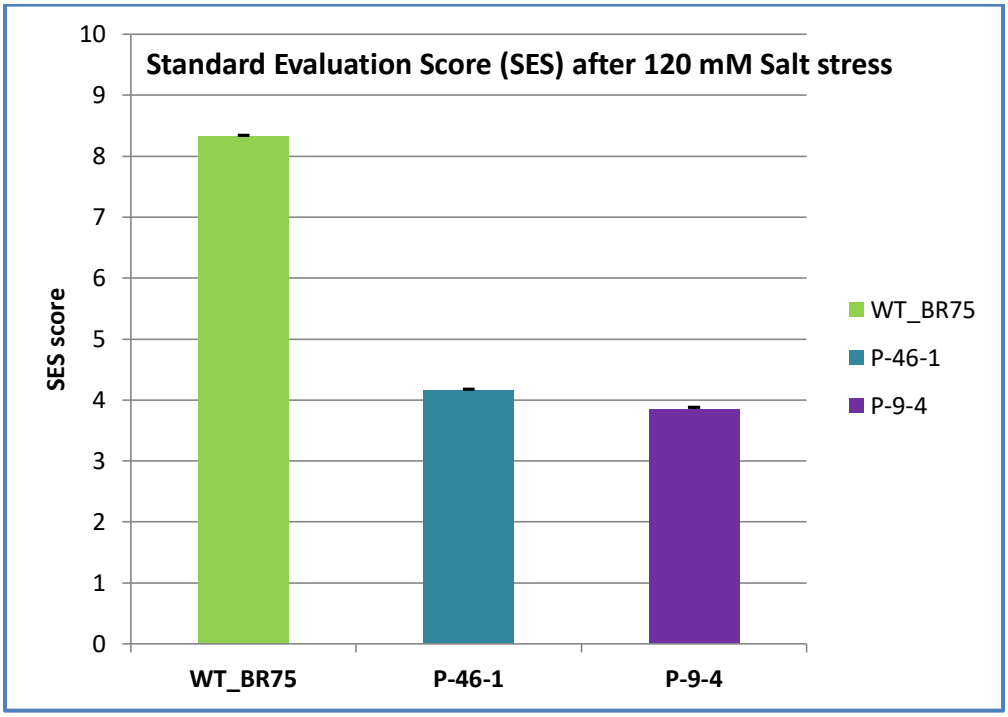

Figure 3.3: *Evaluation score of visual salt injury at seedling stage after 7 days in 120 mM salt stress. Both transgenic lines showed lower damage to plants that means higher salt tolerance than wild type plant.*

*Standard evaluation score of visual salt injury at seedling stage.

1: Normal growth, no leaf symptoms;

3: Nearly normal growth, only the lowest leaf is dry;

5: Growth is severely retarded, and two basal leaves are dry;

7: Complete cessation of growth, only apical leaf survives;

9: Dead seedlings

3.2.2 Comparison of root and shoot parameters:

To assess the salt stress effects, parameters like root length, root weight, shoot length and shoot weight were considered.

In case of root length, transgenic line P-46-1 has shown 4.99% higher, P-9-4 has shown 4.42% higher length than wild type BR75 line. In case of root weight, transgenic line P-46-1 has shown 48.64% higher weight than wild type BR75 line, the output is statistically significant at 5% level (P-value 0.02); line P-9-4 has shown 30.58% higher weight, with a P-value of 0.124.

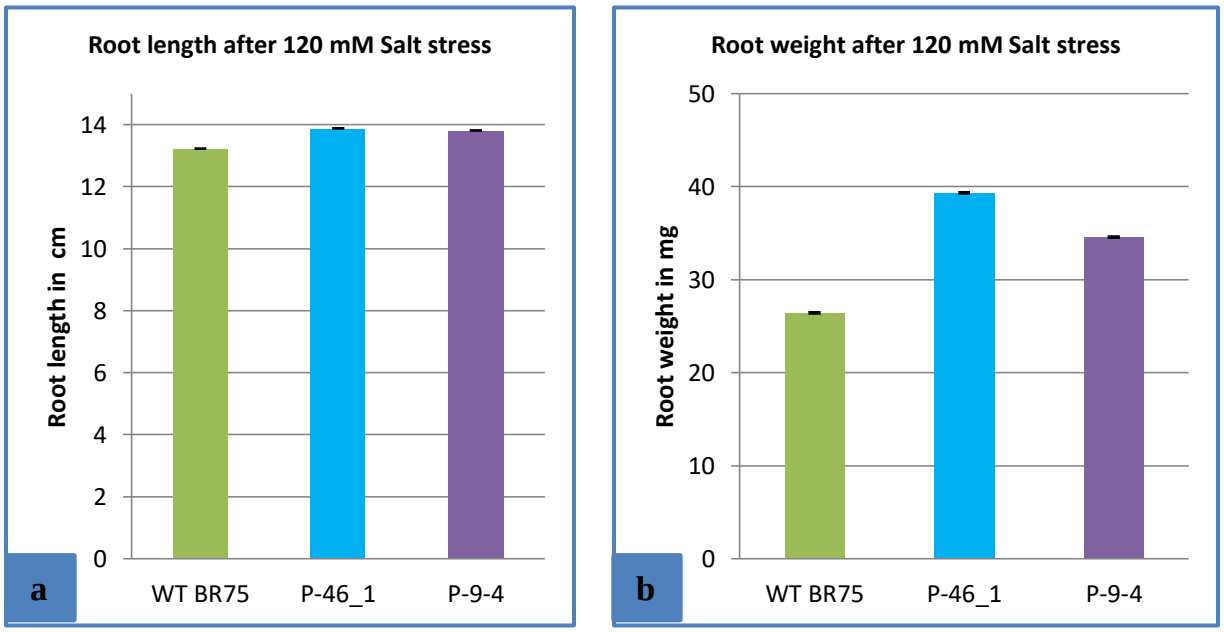

Figure 3.4 : *Root parameters of transgenic lines. (a) Average root length; Line P-46-1 has shown 4.99% higher, P-9-4 has shown 4.42% higher length than wild type BR75 line. (b) Average root weight; Line P-46-1 has shown 48.64% and P-9-4 has shown 30.58% higher weight than wild type BR75.*

Shoot parameters have also shown promising result. Transgenic line P-46-1 has shown 2.41% and P-9-4 has shown 2.11% higher length than wild type plant in 120 mM salt stress. In case of shoot weight, P-46-1 has shown 30% and P-9-4 has shown 18.45% higher shoot weight than wild type BR75 in 120 mM salt stress. The shoot weight output of the transgenic line P-46-1 is significant at 5% level of significance (P-value 0.014).

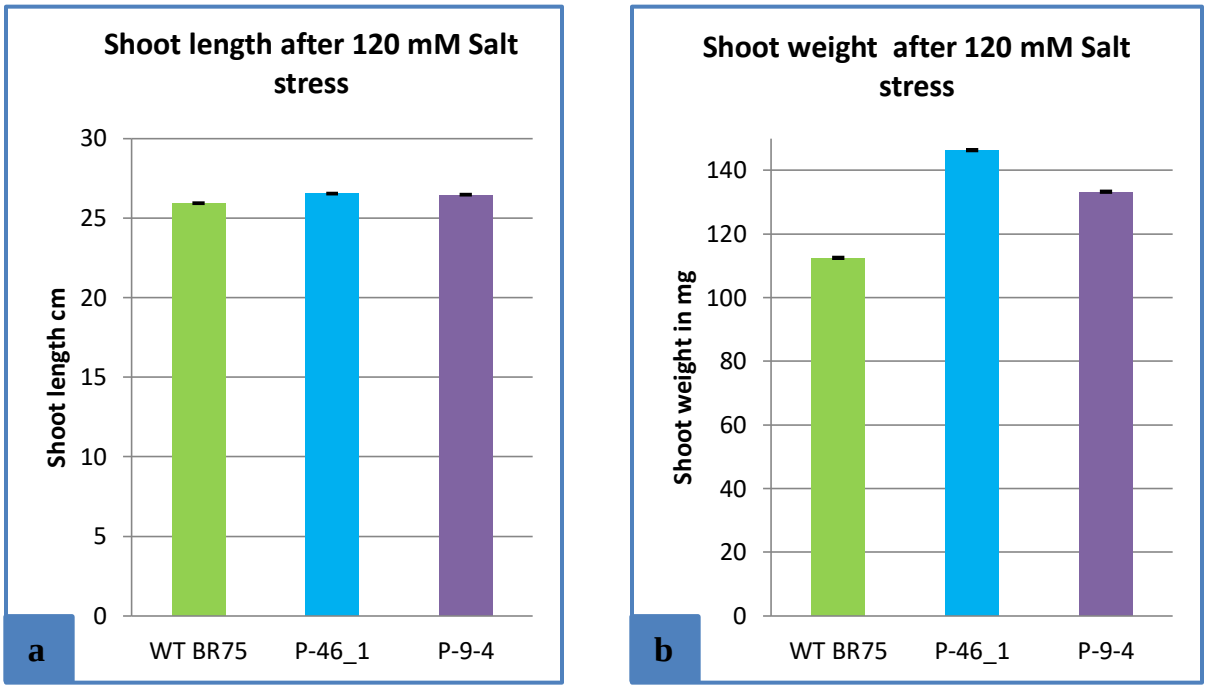

Figure 3.5: *Shoot parameters of transgenic lines. (a) Average shoot length; Line P-46-1 has shown 3.91% higher, P-9-4 has shown 1.97% higher length than wild type BR75 line. (b) Average shoot weight; Line P-46-1 has shown 49% and P-9-4 has shown 31% higher weight than wild type BR75.*

3.3 Determination of the Chlorophyll Concentration of T_2 generation plants of BR75 transformed with *PVA1* gene:

Chlorophyll content was measured from the plants that remained greener than the wild type plant. It was found that the transformed plant showed less amount of chlorophyll loss in the 120mM NaCl solution than the wild type. Transgenic line P-46-1 has shown 34% and P-9-4 has shown 17.86% higher chlorophyll content than wild type plant in 120 mM salt stress.

The figure below (Figure 3.3) shows the total chlorophyll content in both transgenic lines in control and 120 mM salt stress.

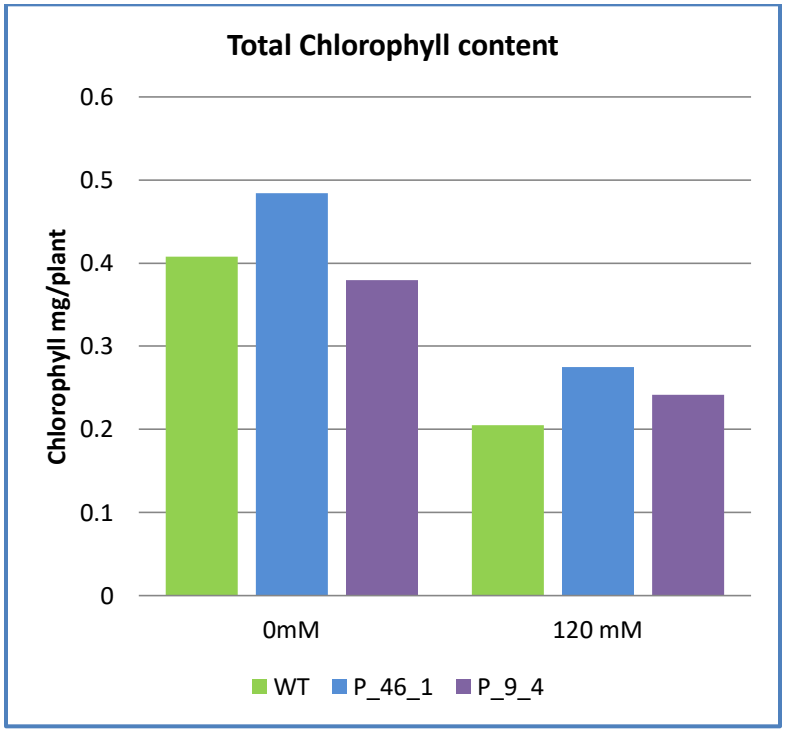

Figure 3.6: *Determination of total Chlorophyll content of T_2 plants. Chlorophyll content reduced significantly in wild type plant than transgenic plant at 120 mM NaCl salt solution. Transgenic line P-46-1 and P-9-4 have shown 34% and 17.86% higher chlorophyll content than non-transformed BR75 respectively.*

Chapter 4:

Discussion & Conclusion

Chapter 4: Discussion and Conclusion

In the present context of climate change and population outburst, the challenge to ensure food security for the people is continuously intensifying for Bangladesh as the resources are limited. To overcome this challenge, transgenic rice varieties with improved stress tolerance and yield are very promising. The scientists of Bangladesh are working relentlessly to enrich the rice genome with desired traits and release new varieties. Already BRRI has released a number of salt and drought tolerant varieties. Among the salt tolerant varieties BRRI47, BRRI61, BRRI54, BRRI73 have shown to be very successful which are also high yielding. (Bangladesh Rice Knowledge Bank)

With the same motto, the Plant biotechnology laboratory situated in the Department of Biochemistry and Molecular Biology of Dhaka University has been searching for promising genes to confer different biotic and abiotic stress tolerance in rice. Incorporating the *Pc_PVA1* gene into BR75 from the halophytic plant *Porteresia coarctata* to confer salt tolerance, was a part of the same initiative.

In this study, T_1 and T_2 generation seedlings of BR75, previously transformed with *Pc_PVA1* gene, has been investigated for salt tolerance. The germinated rice plants were grown in Yoshida's Nutrient solution for three weeks by using Completely Randomized Design to ensure each type of plant receives a homogeneous nutritional treatment. NaCl was gradually added at the concentration of 60-120mM to the nutrient solution as salinity treatment. Standard Evaluation Score (SES), shoot and root parameters, chlorophyll content were determined after exposure to 9 days of salt treatment.

Different visual symptoms were visible as a result of salinity stress in all three types of rice seedlings in the experiment. The SES score indicated that the most salinity tolerant types were P-46-1 with no dead plant and the second one was P-9-4 which was moderately tolerant. The transgenic lines didn't show significant changes in root and shoot length but there was a change in root and shoot weight.

In case of shoot and root weight, the transgenic line P-46-1 has shown the lowest P-values, which is statistically significant at 5% level. In case of shoot weight, P-46-1 has shown 30% and P-9-4 has shown 18.45% higher shoot weight than wild type BR75 in 120 mM salt stress. The shoot weight output for line P-46-1 is significant at 5% level of significance (P-value 0.014). In case of root weight, transgenic line P-46-1 has shown 48.64% higher weight than wild type BR75 line, the output is statistically significant at 5% level (P-value 0.02). Line P-9-4 has shown 30.58% higher weight with a P-value of 0.124. Since, the experiment was conducted on a small population of plants, greater P-values in case of the other parameters cannot be taken into consideration without performing the experiment for a larger population.

Wild type BR75 was used as control plants in salt screening to assess the salt injury of young seedling. A drawback in this experiment was the absence of salt tolerant positive control in the seedling screening stage. This happened due to the seeds not being able to germinate. However, T_2 generation was not screened to check the presence of the transgene. It is important to check the transgenic condition in each generation since the trans-gene may be segregated following the Mendel's law of segregation. Though three different transgenic plants produced band in the gel electrophoresis, the P-2-3 could not be included in this experiment as the T_2 seeds did not germinate. Several factors that might have effects include the storage maintenance, quality of seeds, and optimum conditions for germination.

The two transgenic varieties P-46-1 and P-9-4 in this study performed better than the wild type BR75 in saline condition having higher survival rate and chlorophyll content. The present study was conducted in a small periphery. In future, the transgenic lines will be screened at reproductive stage for yield parameters, Na:K ratio. Southern analysis needs to be done for a more detailed evaluation. Finally, the transgenic plants should be trialed in the natural saline environment following the biosafety protocols.

Therefore, the study was able to confirm the presence of *Pc_PVA1* to establish two of the transgenic lines, which can be studied further for developing a salt-tolerant as well as high yielding rice variety.

Chapter 5:

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Chapter 5: References

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Appendix

CLUSTAL Omega (1.2.4) multiple sequence alignment of *Pc_PVA1* (AF286464.1) and *Oryza sativa* (indica cultivar, CT828125.1) cDNA full insert :

```
AF286464.1             CGACCCGGTGCGAGAGAGGAGGAAGAGATCGAGCTCGCCTCAGGAGGAGGAAGAAGAAGA  60
CT828125.1:129-903     ----------------------------------------------------AGAAGGAA  8
                                                                             *    *

AF286464.1             GGAAGAGCAAGATGTCGTCGGTTTTCAGCGGCGATGAGACAGCGCCCTTCTTCGGCTTCC  120
CT828125.1:129-903     GAGGAGCAAAGATGTCGTCGGTGTTCAGCGGCGACGAGACGGCGCCCTTCTTCGGCTTCC  68
                       *       ************** *********** ***** ******************

AF286464.1             TCGGCGCCGCCTCGGCCCTCGTCTTCTCATGCATGGGGGCAGCGTACGGGACGGCGAAGA  180
CT828125.1:129-903     TCGGCGCCGCCGCCGCCCTCATCTTCTCATGCATGGGCGCCGCCTACGGCACGGCCAAGA  128
                       *********** * ****** **************** ** ** ***** ***** ****

AF286464.1             GTGGCGTCGGCGTGGCGTCCATGGGTGTGATGCGCCCCGAGCTCGTCATGAAGTCCATCG  240
CT828125.1:129-903     GCGGCGTCGGCGTGGCGTCCATGGGCGTCATGCGCCCGGAGCTCGTCATGAAGTCCATCG  188
                       * ********************** ** ******** **********************

AF286464.1             TGCCAGTCGTCATGGCTGGTGTGCTCGGTATCTACGGGCTTATCATTGCCGTCATCATCA  300
CT828125.1:129-903     TGCCCGTCGTCATGGCCGGGGTGCTCGGGATCTACGGCCTCATCATCGCCGTCATCATCA  248
                       **** *********** ** ******** ******** ** ***** *************

AF286464.1             GTACCGGGATTAACCCCAAGGCGAAGCCGTACTACCTCTTCGATGGATACGCGCATCTCT  360
CT828125.1:129-903     GCACAGGGATCAACCCCAAGGCCAAGCCCTACTACCTCTTCGACGGCTACGCGCACCTCT  308
                       * ** ***** *********** ***** ************** ** ******** ****

AF286464.1             CCTCAGGGCTTGCCTGTGGCCTTGCTGGTCTCGCCGCAGGCATGGCCATCGGCATCGTCG  420
CT828125.1:129-903     CCTCCGGCCTCGCCTGCGGCCTCGCCGGCCTCGCCGCCGGAATGGCCATCGGCATCGTCG  368
                       **** ** ** ***** ***** ** ** ******** ** ******************

AF286464.1             GTGATGCTGGTGTTAGGGCAAATGCACAACAACCAAAGCTTTTCGTGGGCATGATCCTCA  480
CT828125.1:129-903     GCGACGCCGGCGTCAGGGCAAATGCACAACAACCAAAACTGTTCGTGGGCATGATCCTCA  428
                       * ** ** ** ** ********************** ** ******************

AF286464.1             TCCTCATTTTCGCTGAAGCTCTTGCTCTGTATGGTCTCATTGTGGGCATCATCCTCTCAT  540
CT828125.1:129-903     TCCTCATCTTCGCAGAAGCGCTTGCACTCTATGGTCTCATTGTGGGCATCATCCTCTCAT  488
                       ******* ***** ***** ***** ** ********************************

AF286464.1             CCCGTGCTGGTCAATCCCGTGCAGATTAAGCACCTTGCAGTACCAATCCGCAGTTATTCC  600
CT828125.1:129-903     CCCGTGCCGGCCAATCTCGTGCAGATTAGGCACTTTGCGGTACCATACCGCTGTTATTCC  548
                       ******* ** ***** *********** **** **** ******  **** ********

AF286464.1             ACTTGTTATATTCTTGAGAAAACCTAAAACTT----GGGAGCTCTAGTTTTAATGTATTA  656
CT828125.1:129-903     ACTGGCTATATTCTTGAGAAAACCTGAAACTTACTTGGGAGCTCTAGTTTAATGTATTAA  608
                       *** * ****************** ******    ************** *     * *

AF286464.1             AAGATCGATTTATAGCTTAAGGAAGGTGGCACTTCCAGTCCTTTTTCGTTTCTTTGGTGG  716
CT828125.1:129-903     AAGATCGATTTGTAGCTTAAGGAAGGTGGCACTTCCAGTCCTTTTTGTTTCTTTT-GTGG  667
                       *********** ********************************  **  *** ****

AF286464.1             TGATTCATGCAGAGTTTTTTTTGGGTTAGGCTGGATTTGCTGCTCCTGAGCAAATGGATT  776
CT828125.1:129-903     TGGTTCATGCA--AAGTTTTTTGGGTTAGGCTGGATTTGCTGCTCCTGAGCAAACGGATT  725
                       ** ********     ************************************* *****

AF286464.1             TAATCCTATTCGTGGTGAATAAAGAACACGGCACTGTAGCAAAATAAAATTACAATAAAA  836
CT828125.1:129-903     TAATCTCATTCGTGGGGAATAAAAAACACGGCATTGTAGCAAAAAAAAAT----------  775
                       *****  ******** ******* ********* ********** *****

AF286464.1             AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA   876
CT828125.1:129-903     ----------------------------------------   775
```

CLUSTAL Omega (1.2.4) multiple sequence alignment of *Porteresia coarctata* and *Oryza sativa* indica for Vacuolar H^+ ATPase subunit c protein:

```
tr|Q94G11|Q94G11_ORYCO    MSSVFSGDETAPFFGFLGAASALVFSCMGAAYGTAKSGVGVASMGVMRPELVMKSIVPVV   60
sp|A2ZBW5|VATL ORYSI      MSSVFSGDETAPFFGFLGAASALIFSCMGAAYGTAKSGVGVASMGVMRPELVMKSIVPVV   60
                          **********************:*********************************

tr|Q94G11|Q94G11_ORYCO    MAGVLGIYGLIIAVIISTGINPKAKPYYLFDGYAHLSSGLACGLAGLAAGMAIGIVGDAG  120
sp|A2ZBW5|VATL_ORYSI      MAGVLGIYGLIIAVIISTGINPKAKPYYLFDGYAHLSSGLACGLAGLAAGMAIGIVGDAG  120
                          ************************************************************

tr|Q94G11|Q94G11_ORYCO    VRANAQQPKLFVGMILILIFAEALALYGLIVGIILSSRAGQSRAD     165
sp|A2ZBW5|VATL ORYSI      VRANAQQPKLFVGMILILIFAEALALYGLIVGIILSSRAGQSRAD     165
                          *****************************************
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