CLONING OF AN ANTIPORTER GENE (NHX1) FROM PCR AMPLICONS INTO A RECOMBINATION COMPETENT VECTOR CONTAINING A CONSTITUTIVE PROMOTER (CaMV35S)

A DISSERTATION SUBMITTED TO THE BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY.

MS Thesis

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Certificate

This is to certify that the research work embodying the results reported in this thesis entitled "***Cloning of an Antiporter Gene (NHX1) from PCR amplicons into a recombination competent vector containing a constitutive promoter (CaMV35S)***" submitted by Samsad Razzaque, has been carried out under my supervision in Plant Biotechnology Lab, Department of Biochemistry and Molecular Biology, University of Dhaka. It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka.

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ABSTRACT

The costal belt of Bangladesh is under the threat of increasing salinity and thus reducing plant productivity. Consequently, salinity has become a major concern for ensuring food security. Salt tolerance is known to be a complex trait both genetically as well as physiologically and conferring this tolerance by introducing a single gene is therefore difficult. However, over expression of the vacuolar antiporter gene, NHX1 has been reported to provide salinity tolerance to a good extent. This work was carried out to clone the coding sequence of the rice antiporter gene (applying Gateway Technology) in a binary vector (pH7WG2.0) from where this can be easily introduced into a plant genome with a highly efficient constitutive promoter (CaMV35S). In this study, NHX1 gene was first cloned into pENTR and confirmed the insertion through PCR, Restriction Digestion and Sequencing technique. Then the gene of interest was recombined from pENTR to the Destination vector (pH7WG2.0) and recombination was confirmed by PCR and Restriction Digestion respectively. Finally, recombinant vector was then transformed into *Agrobacterium* (the transformation was also confirmed by PCR and Restriction Digestion) to perform *Agrobacterium* mediated transformation into tomato plant.

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List of Abbreviations

bp	Base pair
BRRI	Bangladesh Rice Research Institute
C	Celsius
cDNA	Complementary DNA
ddH_2O	Distilled deionized water
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide tripohosphates
e.g.	For example
EC	Electrical conductivity
et al.	and others
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
etc	etcetras
Fig.	figure
i.e.	that is
IRRI	International Rice Research Institute
kb	kilobase
LB	Luria Broth
M	molar
mM	millimolar
mg	milligram
µg	microgram

ml	milliliter
µl	microliter
min	minute
NaCl	Sodium chloride
NaOH	Sodium hydroxide
No./no.	Number
O.D.	Optical density
PCR	Polymerase Chain Reaction
pH	Negative logarithm of hydrogen ion concentration
rpm	Revolution per minute
RNase	Ribonuclease
RT	Room temperatute
SDS	Sodium dodecyl sulphate
sec	second
TAE	Tris acetate EDTA
ng	nanogram
UV	Ultra violet
vol	volume

CHAPTER 1: *INTRODUCTION*

Introduction

1.1 General overview of tomato:

Tomato may refer to both the plant (*Solanum lycopersicum*) and the edible, typically red, fruit which it bears. Originating in South America, the tomato was spread around the world following the Spanish colonization of the Americas, and its many varieties are now widely grown, often in greenhouses in cooler climates. The tomato fruit is consumed in diverse ways, including raw, as an ingredient in many dishes and sauces, and in drinks. While it is botanically a fruit, it is considered a vegetable for culinary purposes, which has caused some confusion. The fruit is rich in lycopene, which is defined as a strong antioxidant. The tomato belongs to the nightshade family. The plants typically grow to 1–3 meters (3–10 ft) in height and have a weak stem that often sprawls over the ground and vines over other plants. It is a perennial in its native habitat, although often grown outdoors in temperate climates as an annual.

1.2 Taxonomical characteristics:

Tomatoes are one of the most widely produced and consumed vegetables in the world, both for the fresh fruit market and the processed food industries .It has vigorous tap root, extensive fibrous roots, solid, hairy stems and spirally arranged, mainly oval leaves. The fruit is a fleshy, round or lobed, smooth or furrowed, red, pink or yellow berry with numerous flat, slightly curved seeds.

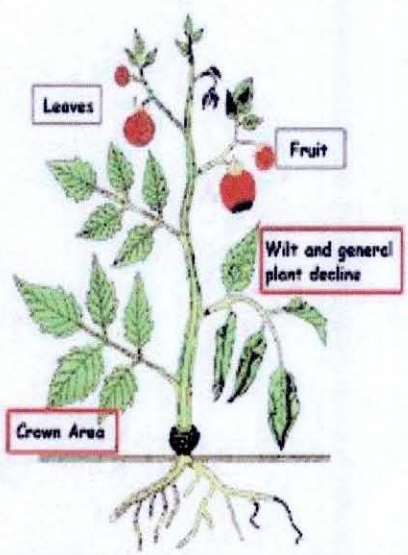


Figure 1.1: Tomato Plant

1.3 Scientific Classification:

Kingdom Plantae – Plants

Subkingdom Tracheobionta – Vascular plants

Super division Spermatophyta – Seed plants

Division Magnoliophyta – Flowering plants

Class Magnoliopsida – Dicotyledons

Subclass Asteridae

Order Solanales

Family Solanaceae – Potato family

Genus Solanum L. – nightshade

Species *Solanum lycopersicum L.*

Source: NCBI Taxonomy Browser
(http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/)

1.4 Natural Benefits and Curative Properties of Tomato

The tomato is one of the most powerful deobstruents of the Materia Medica. It removes disease particles and opens natural channels of the body. It is gentle natural stimulant for kidneys and helps to wash away the toxins which cause diseases and contaminate our system. They are at their best when they are fully ripe. Their vitamin C content increase as they ripen. Tomato juice is probably one of the most widely used juices. Fresh, raw tomato juice is most beneficial and has an alkaline reaction, when digested, in concentrated form. Tomato as an external application can be used as a cosmetic. Its pulp should be applied liberally on the face and left there for an hour and then' washed with warm water. Repeated daily, it will give good complexion and remove ugly-looking pimples in a short time.

1.4.1 Acidosis:

The tomato is essentially an alkaline vegetable. Its acid taste is due to malic acid which is about 0.5'per cent. It also contains 0.52 to 1.81 per cent citric acid and only a trace of oxalic acid. These acids in tomatoes, in combination with sodium and potassium either form sodium or potassium acid malate, citrate or oxalate. Their end products, when oxidized in the body, are carbon dioxide, water and the carbonates of potassium and sodium. The latter has alkaline reaction. Tomatoes thus leave an alkaline ash in the process of being oxidized by the body. This increases the alkalinity of the blood and decreases the urine and neutralizes the acid compounds of the body such as phosphates, urea and ammonia. It is, therefore, highly beneficial in the treatment of acidosis and other disease associated with too much acid in the system.

1.4.2 Diabetes:

Because of its low carbohydrate contents, it is very good food for diabetic patients and for those who want to reduce their body weight. It is said to be very effective in controlling the percentage of sugar in the urine of diabetic patients.

1.4.3 Eye Disorders:

Being a rich source of vitamin A, tomatoes are a dependable preventive against night blindness short sightedness and other diseases of the eye caused by the deficiency of the vitamins. Tomato leaves are useful in optic nerve and eye weakness. A small handful of the freshly plucked leaves should be covered with soft hot water for 15 minutes. The water should then strained. It forms a good tonic for the eyes and optic nerve when a teaspoonful of this water is taken before meals three times daily.

1.4.4 Urinary Disorder:

Eating a tomato early in the morning is found to be very effective medicine to prevent the formation of urinary calculi or stone by supplying sufficient quantity of acids and Vitamins A and C. It is proved that deficiency of vitamins A and C and the recurrent urinary tract infections are among the most important factors in the formation of calculi i.e. stone. Tomato restricts the acid value of urine to 5.5 or less, thereby reducing the chances of infections by increasing the acidity of the urine.

1.4.5 Obesity:

Tomatoes are highly beneficial in the treatment of obesity. One or two ripe tomatoes taken early morning, without breakfast, for a couple of months is considered a safe method of reduction in weight and at the same time, it also supplies the essential food elements to preserve the health.

1.4.6 Intestinal and Liver Disorders:

A glassful of fresh tomato juice, mixed with a pinch of salt and pepper, taken early in the morning is considered an effective remedy for morning sickness, biliousness, sluggishness and diminished responsiveness of the liver, jaundice, indigestion, excessive formation of gas in the intestines, constipation, diarrhea due to indigestion burning in the gastrointestinal tract and constant burning sensation in the chest due to hiatus hernia, a condition in which stomach passes partly or completely into chest.

1.4.7 Respiratory Disorders:

A glassful of fresh tomato juice mixed with honey, a pinch of powdered cardamom seeds, taken after swallowing three peeled cloves of garlic every night before going to bed, is considered highly beneficial in the treatment of tuberculosis and other lung infections. It increases the body's resistance and prevents drug resistance and the relapse which are so common in tubercular patients. In asthmatics, it reduces the congestion in the bronchioles and checks the hyper secretion of mucous and reduces the spasms.

1.4.8 Painful Joints:

The juice of the whole plant including leaves, mixed with equal quantity of till-oil, is heated until all the watery part is evaporated and the oil is preserved in a bottle This oil, massaged over painful joints and sprains and fomented with dry heal gives a great relief.

1.5 Research on the molecular biology of tomato:

Tomatoes have got the worldwide acceptance as vegetables and usually served in every type of food festival and also used with all type of food as salad. However, whole genome sequencing of tomato is in the pipeline and a few organizations have already taken the responsibility to

sequence the genome of tomato. Moreover, the genetic transformation of tomato has been a successful approach to introduce foreign gene to have a functional characteristics. Abiotic stresses have been a vital concern to maximize the productivity of the tomato. Salinity is a major concern to ensure better productivity of the tomato production. Coastal region of Bangladesh is under a great threat of salinization and reducing the crop productivity. So that genetic transformation with the salt tolerant gene in tomato can be solution to reduce the threat against the salinity. Some approaches have already been reported with a few genes like antiporters to confer salinity tolerant in plant. Zhang et al, reported such transformation technique of tomato with an antiporters with NHX1 gene from tomato. A total diversification with the genomes of tomato is still infancy. Due its production range according to its requirement is rapidly increasing, so that further modification with transgenic approach is really appreciable and to be conducted as to secure food security with the better nutrition as the food contains better nutrients value as well.

1.6 Objective of the study:

Due to current concern of the salinity problem at the coastal belt of Bangladesh, it has been a major need to face the environmental changes with the best possible best outcomes. Here it is to be mentioned that, popularity of tomato in food habit is increasing very vastly and the requirement for that vegetable is also huge. Bangladesh is imports processed tomato and here is a chance to produce the maximum production and it is only possible by doing genetic transformation of tomato to face all the stresses that inhibit the maximum production. However the aim target of my research is to construct a recombinant vector with NHX1 gene form rice, so that it can be easily incorporated with the genome of farmer popular varieties of tomato to confer salt tolerance activity.

CHAPTER 2 : *REVIEW OF LITERATURE*

Literature Review

2.1 Introduction:

Plant growth and development are adversely affected by salinity – a major environmental stress that limits agricultural production. The physiological mechanisms by which growth and development of crop plants are affected by salinity are stated below. The initial phase of growth reduction is due to an osmotic effect, is similar to the initial response to water stress and shows little genotypic differences. The second, slower effect is the result of salt toxicity in leaves. In the second phase a salt sensitive species or genotype differs from a more salt tolerant one by its inability to prevent salt accumulation in leaves to toxic levels. Most crop plants are salt tolerant at germination but salt sensitive during emergence and vegetative development. Root and shoot growth is inhibited by salinity; however, supplemental Ca partly alleviates the growth inhibition. The Ca effect appears related to the maintenance of plasma membrane selectivity for K over Na. Reproductive development is considered less sensitive to salt stress than vegetative growth, although in wheat salt stress can hasten reproductive growth, inhibit spike development and decrease the yield potential, whereas in the more salt sensitive rice, low yield is primarily associated with reduction in tillers, and by sterile spikelets in some cultivars. Plants with improved salt tolerance must thrive under saline field conditions with numerous additional stresses. Salinity shows interactions with several stresses, among others with boron toxicity, but the mechanisms of salinity-boron interactions are still poorly known. To better understand crop tolerance under saline field conditions, future research should focus on tolerance of crops to a combination of stresses

2.2 Definition of salinity:

Salinity is one of the most important abiotic stresses for agricultural crops. High concentrations of salt in the soil cause hyper osmotic and ionic stresses, which, in turn, may generate secondary stresses such as oxidative stress, etc. The complexity and polygenic nature of the salt tolerance trait has seriously limited the efforts to develop salt-tolerant crop varieties. The molecular dissection of plant responses to salt stress, discovery of novel structural and regulatory genes involved in stress adaptation, and transgenic and molecular marker strategies used for

engineering salt tolerance in plants. Applying different novel techniques, a number of stress-related genes have been characterized including the ones that encode for important enzymes or a biochemical pathway, participate in signalling pathways or act as transcriptional regulators for coordinated regulation of stress related genes. Some of these genes have been successfully transferred in model plant species including Arabidopsis, rice and tobacco, and a marginal to significant improvement in salt-tolerance has been reported. In addition to transgenic approaches, molecular markers can be used for linkage mapping of genes/QTLs for salinity tolerance trait, marker-assisted transfer and pyramiding of such QTLs into agronomically desirable genotypes and or for map-based cloning of genes. Application of transgenic and molecular marker research coupled with rapid gene discovery via functional genomic research in plants shall provide effective means for designing salt-tolerant crops.

2.3 Factors affecting soil salinity:

Several factors affect the amount and composition of salts in soils:

2.3.1 Irrigation water quality:

The total amount of dissolved salts in the irrigation water, and their composition, influence the soil salinity. Therefore, various parameters, such as source water EC and its minerals content should be tested.

2.3.2 Fertilizers applied:

The type and amount of fertilizers applied to soil, affect its salinity. Some fertilizers contain high levels of potentially harmful salts, such as potassium chloride or ammonium sulphate. Overuse and misuse of fertilizers leads to salinity buildup, and should be avoided.

2.3.3 Irrigation regimen and type of irrigation system:

The higher the water quantity applied, the closer soil salinity is to irrigation water salts concentration. When the soil dries, the concentration of salts in the soil solution is increased.

Since salts move with the wetting front, the salts accumulate in specific profiles according to the irrigation regimen and the type of irrigation used. For instance, when irrigating using sprinklers,

water and salts move deeper, according to the soil's infiltration capacity and the water quantity, until they stop at a certain depth. When using drip irrigation - there is also a lateral movement of water and salts.

2.3.4 Field's characteristics and agricultural history:

A poorly drained soil might reach salinity level that is harmful to the plants and to the whole crop. A soil that was not flushed after a previous growing cycle might contain high level of accumulated salts.

2.4 Effects of salinity:

2.4.1 Direct soil salinity damages

2.4.1.1 Decreased water uptake:

High salts concentration results in high osmotic potential of the soil solution, so the plant has to use more energy to absorb water. Under extreme salinity conditions, plants may be unable to absorb water and will wilt, even when the surrounding soil is saturated.

2.4.1.2 Ion-specific toxicity:

When a plant absorbs water containing ions of harmful salts (e.g. Sodium, Chloride, excess of Boron etc.), visual symptoms might appear, such as stunted plant growth, small leaves, marginal necrosis of leaves or fruit distortions.

2.4.1.3 Indirect soil salinity damages

2.4.1.4 Interference with uptake of essential nutrients:

An imbalance in the salts content may result in a harmful competition between elements. This condition is called "antagonism", i.e. an excess of one ion limits the uptake of another ion. For example, excess of chloride reduces the uptake of nitrate, excess of phosphor reduces the uptake of manganese, and excess of potassium limits the uptake of calcium.

2.4.1.5 Sodium effect on soil structure:

In saline soils, sodium replaces calcium and magnesium, which are adsorbed to the surface of clay particles in the soil. Thus, aggregation of soil particles is reduced, and the soil will tend to disperse. When wet, a sodic soil tends to seal, its permeability is dramatically reduced, and thus water infiltration capacity is reduced as well. When dry, a sodic soil becomes hard has the tendency to crack. This may result in damages to roots.

It should be noted that salinity by itself actually improves soil structure and eliminates to some degree the negative effect of sodium ions, but of course, salinity cannot be increased without affecting plants growth.

2.5 Salinity Stress and Plant Development:

Salinity affects plants in different ways such as osmotic effects, specific-ion toxicity and/or nutritional disorders (Läuchli and Epstein, 1990). The extent by which one mechanism affects the plant over the others depends upon many factors including the species, genotype, plant age, ionic strength and composition of the salinizing solution, and the organ in question. Plants undergo characteristic changes from the time salinity stress is imposed until they reach maturity (Munns, 2002a). This author describes these changes over different time scales in the plant's development. Moments after salinization, cells dehydrate and shrink, but regain their original volume hours later. Despite this recovery, cell elongation and to a lesser extent cell division, are reduced leading to lower rates of leaf and root growth. Over the next days, reductions in cell division and elongation translate into slower leaf appearance and size. Plants that are severely salt-stressed often develop visual injury due to excessive salt uptake. After weeks, lateral shoot development is affected and after months, clear differences in overall growth and injury are observed between salt-stressed plants and their non-stressed controls. Understanding these temporal differences in response to salinity, Munns (2002a, 2005) developed the concept of the 'two-phase growth response to salinity'. The first phase of growth reduction happens quickly (within minutes) after exposure to salinity. This response is due to the osmotic changes outside the root causing changes in cell-water relations (osmotic effect). The osmotic effect initially reduces the ability of the plant to absorb water. This effect is similar to water stress and shows

little genotypic differences. Several minutes after the initial decrease in leaf growth, there is a gradual recovery of the growth rate until a new steady state is reached, dependent upon the salt concentration outside the root (Munns, 2002a). The second much slower effect, taking days, weeks or months is the result of salt accumulation in leaves, leading to salt toxicity in the plant, primarily in the older leaves (i.e. salt-specific effect). This salt toxicity can result in the death of leaves and reduce the total photosynthetic leaf area. As a result, there is a reduction in the supply of photosynthate to the plant, affecting the overall carbon balance necessary to sustain growth (Munns, 2002a). Salt toxicity primarily occurs in the older leaves where Na and Cl build up in the transpiring leaves over a long period of time, resulting in high salt concentration and leaf death. Leaf injury and death is probably due to the high salt load in the leaf that exceeds the capacity of salt compartmentation in the vacuoles, causing salt to build up in the cytoplasm to toxic levels (Munns and Termaat, 1986; Munns 2002a; 2005; Munns et al, 2006). The rate at which leaves die and thus reduce the total photosynthetic leaf area determines the survival of the plant. If new leaves are produced at a rate greater than the rate at which old leaves die, there are enough photosynthesizing leaves for the plant to flower and produce seeds, although at reduced numbers. If, however, old leaves die faster than new leaves develop, the plant may not survive long enough to supply sufficient photosynthate to the reproductive organs and produce viable seeds. Based on this two-phase concept, the initial growth reduction for both salt sensitive and salt tolerant plants is caused by an osmotic effect of the salts in the medium outside the roots. In contrast, in the second phase, a salt-sensitive species or genotype differs from a more salt tolerant one by its inability to prevent salt from accumulating in transpiring leaves to toxic levels (Munns et al, 2006). In light of the different mechanisms of plant response to salinity (Läuchli and Epstein, 1990) and characteristic sequential changes which the plants endure after being exposed to salinity (Munns, 2002a), are their specific developmental stages where the plants are more or less sensitive to salinity?

2.6 Salt Sensitivity in Relation to Developmental Growth Stage:

It has long been recognized that a crop's sensitivity to salinity varies from one developmental growth stage to the next (Bernstein and Hayward, 1958). Although there are exceptions, the majority of the research indicates that most annual crops are tolerant at germination but are sensitive during emergence and early vegetative development (Läuchli and Epstein, 1990; Maas

and Grattan, 1999). As plants mature, they become progressively more tolerant to salinity, particularly at later stages of development. While these statements are generally true (with the exception of perhaps a few crops), it is important to emphasize that the definition of salt tolerance is not the same for each growth stage. During germination and emergence, tolerance is based on percent survival, while during the later developmental stages, tolerance is usually based on relative growth reductions. Salinity affects both vegetative and reproductive development which has profound implications depending on whether the harvested organ is a stem, leaf, root, shoot, fruit, fiber or grain. Salinity often reduces shoot growth more than root growth (Läuchli and Epstein, 1990) and can reduce the number of florets per ear, increase sterility and affect the time of flowering and maturity in both wheat (Maas and Poss, 1989a) and rice (Khatun et al. 1995). Since salt-tolerance from an agronomic or horticulturist perspective is based on the yield of the harvestable organ, relative to that in non-stressed environments, understanding how salinity affects vegetative and reproductive development is important for developing management strategies that can minimize stress at critical times.

2.7 Monocots differ from dicots in salt tolerance:

Dicotyledonous species vary more than monocotyledonous species in the extent to which tolerance is associated with low shoot Na^+. For example a highly salt-tolerant wild relative of tomato, *Lycopersicon peruvianum*, accumulates higher concentrations of Na^+ than the salt-sensitive domesticated tomato, *L. esculentum* (Tal, 1971; Santa-Cruz *et al.*, 1999). Importantly, the sensitivity of salt-sensitive (Zhu *et al.*, 1998) and salt-accumulating (Nublat *et al.*, 2001) mutants does not appear to be closely related to shoot levels of Na^+. For example, the salt sensitive mutants of Arabidopsis, *sos1*, has a lower shoot Na^+ content and lower Na^+ influx than wild-type plants (at least in the presence of low K^+) (Ding and Zhu, 1997).

2.8 Mechanism of salt tolerance in plant:

Two basic genetic approaches that are currently being used to improve stress tolerance include: (i) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of quantitative trait loci (QTLs – regions of a genome thatare associated with the variation of a quantitative trait of interest) (Foolad, M.R. 2004.,

Lindsay, M.P. et al., 2004) and subsequent marker-assisted selection, and (ii) generation of transgenic plants to introduce novelgenes or to alter expression levels of the existing genes to affect the degree of salt stress tolerance. The role of antioxidants, osmoregulation, signalling and transcriptional control have been recently reviewed (Seki, M. et al., 2003, Zhang, J.Z. et al. 2004, Chinnusamy, V. et al. 2005)

Plant adaptations to salinity are of three distinct types: a) osmotic stress tolerance; b)Na+ exclusion; and c) tissue tolerance, i.e., tolerance of tissue to accumulated Na+, and possibly Cl−. (Flowers, T.J. 2004)

2.9 Marker-assisted breeding:

The direct selection of superior salt-tolerant genotypes under field conditions is hindered by the significant influence that environmental factors have on the response of plants to salinity (Richards, R.A. 1996) There is also evidence supporting the notion that salt tolerance is a complex trait involving the function of many genes (Foolad, M.R. 2004) Salt tolerance in plants appears to be a developmentally regulated process and the tolerance of the plants at one stage of development is not always correlated with tolerance at other stages (Foolad, M.R. 2004, Greenway, H et al.,1980, Tal, M et al., 1983)

The development of molecular biology techniques has enabled the development of DNA markers that can be used to identify QTLs. The use of QTLs has improved the efficiency of selection, in particular for those traits that are controlled by several genes and are highly influenced by environmental factors(Flowers, T.J. 2004)QTLs and marker-assisted selection provide several advantages over direct phenotypic screening, particularly because the PCR-based methodologies used to detect the markers reduce the time needed to screen individuals and reduce the impact of environmental effects on the trait under study. There is considerable evidence to support the view that salt tolerance and its sub-traits are determined by multiple QTLs and that both additive and dominance effects are important in the inheritance of many of the traits associated with salt tolerance (Flowers, T.J. 2004, Richards, R.A. 1996, Gregorio, G.B. et al., 2002) The development of high-density DNA maps that incorporate micro satellite markers, RFLP (restriction fragment-length polymorphisms) and AFLP (amplified fragment-length

polymorphisms), and advances in marker-assisted selection techniques will facilitate pyramiding traits of interest to attain substantial improvement in crop salt tolerance

2.10 Salt Tolerances by Transgenic approach:

Physiologically, salinity (i) imposes an initial water-deficit that results from the relatively high solute concentrations in the soil, (ii) causes ion-specific stresses resulting from altered K^+/Na^+ ratios and (iii) leads to build up in $Na^+$and Cl^- concentrations that are detrimental to plants. Plants respond to salinity using two different types of responses. Salt-sensitive plants restrict the uptake of salt and adjust their osmotic pressure by the synthesis of compatible solutes (e.g. proline, glycinebetaine and sugars) (Tal, M et al., 1983). Salt-tolerant plants sequester and accumulate salt into the cell vacuoles, controlling the salt concentrations in the cytosol and maintaining a high cytosolic K^+/Na^+ ratio in their cells (Glenn, E.P. et al. 1999) Ion exclusion mechanisms could provide a degree of tolerance to relatively low concentrations of NaCl but would not work at high concentrations of salt, resulting in the inhibition of key metabolic processes and concomitant growth inhibition.

2.11 The vacuolar Na^+/H^+ antiporter:

The Na^+/H^+ antiporters (exchanger) catalyze the exchange of Na^+ for H^+ across membranes. Although the antiporters are found in animals, yeasts, bacteria, and plants, antiporter activity in the vacuolar membranes has only been reported in yeast, algae, and plants (Blumwald *et al.*, 2000). In plants, the Na^+/H^+ antiporter in vacuolar membranes transports Na^+ from the cytoplasm to vacuoles using the electrochemical H^+ gradient generated by two H^+-pumps, vacuolar H^+-inorganic pyrophosphatase and vacuolar H^+-ATPase. The plant cells treated with high salinity must maintain a higher K^+/Na^+ ratio in the cytoplasm and control the osmotic balance of the cell with the environment by accumulating Na^+ in the vacuoles. In this process, the vacuolar Na^+/H^+ antiporter is thought to play an important role(s).

2.12 Ion homeostasis maintained through vacuolar antiporter:

Although Na^+ is required in some plants, particularly halophytes (Glenn *et al.,* 1999), a high NaCl concentration is a toxic factor for plant growth. The alteration of ion ratios in plants is due to the influx of Na^+ through pathways that function in the acquisition of K^+ (Blumwald *et al.,*

2000). The sensitivity to salt of cytosolic enzymes is similar in both glycophytes and halophytes, indicating that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in high salt (Glenn *et al.,* 1999). Strategies that plants could use in order to maintain a high K^+/Na^+ ratio in the cytosol include:

1. Extrusion of Na^+ ions out of the cell
2. Vacuolar compartmentation of Na^+ ions

Under typical physiological conditions, plants maintain a high cytosolic K^+/Na^+ ratio. Given the negative membrane potential difference at the plasma membrane (-140 Although Na^+ is required in some plants, particularly halophytes (Glenn *et al.,* 1999), a high NaCl concentration is a toxic factor for plant growth. The alteration of ion ratios in plants is due to the influx of Na^+ through pathways that function in the acquisition of K^+ (Blumwald *et al.,* 2000). The sensitivity to salt of cytosolic enzymes is similar in both glycophytes and halophytes, indicating that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in high salt (Glenn *et al.,* 1999). Strategies that plants could use in order to maintain a high K^+/Na^+ ratio in the cytosol include: (i) extrusion of Na^+ ions out of the cell and (ii) vacuolar compartmentation of mV) (Higinbotham, 1973) , a rise in extracellular Na^+ concentration will establish a large electrochemical gradient favoring the passive transport of Na^+ into the cells. Na^+ extrusion from plant cells is powered by the operation of the plasma membrane H^+-ATPase generating an electrochemical H^+ gradient that allows plasma membrane Na^+/H^+ antiporters to couple the passive movement of H^+ inside the cells, along its electrochemical potential, to the active extrusion of Na^+ (Blumwald *et al.,* 2000). Recently, *AtSOS1* from *Arabidopsis thaliana* has been shown to encode a plasma membrane Na^+/H^+ antiport with significant sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria and fungi (Shi *et al.,* 2000). The overexpression of *SOS1* improved the salt tolerance of *Arabidopsis*, demonstrating that improved salt tolerance can be attained by limiting Na^+ accumulation in plant cells (Shi *et al.,* 2003). The compartmentation of Na^+ ions into vacuoles also provides an efficient mechanism to avert the toxic effects of Na^+ in the cytosol.

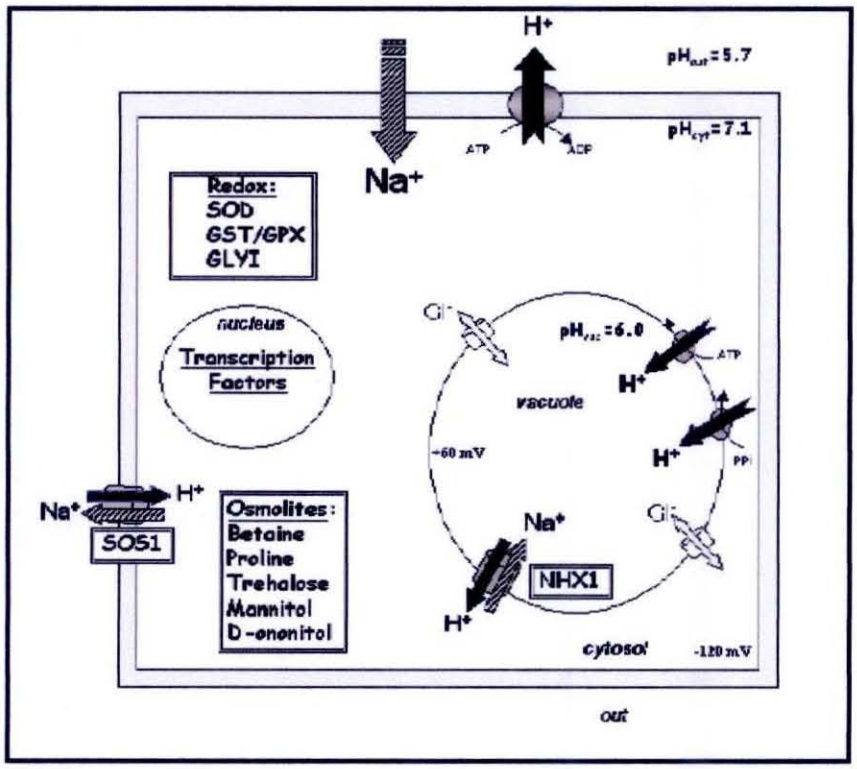


Figure 2.1. Schematic representation of primary and secondary transport in the plant cells. Electrogenic H^+ transport (H^+-ATPase in the plasma membrane and vacuolar membrane, H^+-PPiase in the vacuolar membrane) generates gradients of pH and electrical potential difference across the cell and vacuolar membranes. Na^+ ions enter the cell and can be translocated out of the cell or into the vacuole by the action of a plasma membrane Na^+/H^+ antiporter (SOS1) or a vacuolar Na^+/H^+ antiporter (NHX1), respectively.

The transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter that is driven by the electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-PPiase (Blumwald, 1987). The overexpression of a *AtNHX1,* a vacuolar Na^+/H^+ antiporter from *Arabidopsis,* in *Arabidopsis* resulted in transgenic plants that were able to grow in high salt concentrations (Apse *et al.,* 1999). The paramount role of Na^+ compartmentation in plant salt tolerance has been further demonstrated in transgenic tomato and Canola plants overexpressing AtNHX1 (Zhang *et al.,* 2001). Additional evidence supporting the

role of vacuolar transport in salt tolerance has been provided by *A. thaliana* plants overexpressing a vacuolar H^+-PPiase (Gaxiola *et al.*, 2001). Transgenic plants overexpressing *AVP1,* coding for the vacuolar H^+-pyrophosphatase, displayed enhanced salt tolerance that was correlated with the increased ion content of the plants. These results suggest that the enhanced vacuolar H^+-pumping in the transgenic plants provided additional driving force for vacuolar sodium accumulation via the vacuolar Na^+/H^+ antiporter.

2.13 Over expression of vacuolar Na^+/H^+ antiporters in monocots and dicots:

Na^+/H^+ antiporters are membrane proteins that play a major role in cellular pH and ion homeostasis. Vacuolar Na^+/H^+ antiporters are responsible for the compartmentalization of Na^+ into the vacuoles and are therefore thought to play an important role in salt tolerance. One of the principal mechanisms that plants adopt to survive under salt stress is to accumulate Na^+ and Cl^- ions in the vacuoles of root cells, while K^+ ions accumulate in the cytosol. Halophytes have a greater capacity to compartmentalize K^+ in the cytosol and Na^+ ions in the vacuoles. In the long run plants tend to store excess sodium in the vacuoles of older leaves and get rid of the excess Na^+ by shedding the older leaves. NaCl stored in vacuolar space also acts as an osmoticum, maintaining an osmotic potential that drives water into the cells. Vacuolar Na^+/H^+ antiporters have been investigated as the key to salt tolerance in yeast and plants. In plants, a vacuolar Na^+/H^+ antiporter gene from Arabidopsis, *AtNHX1,* was cloned as the first plant homolog to the yeast prevacuolar Na^+/H^+ antiporter, *ScNHX1* (Gaxiola *et al.* 1999). In 1999, Apse *et al.* found that over-expression of *AtNHX1* in *Arabidopsis* plants promotes sustained growth and development in soil watered with up to 200mM NaCl. Later Zhang *et al.* reported in 2001 that transgenic tomato plants over-expressing *AtNHX1* were able to grow, flower and produce fruit in presence of 200 mM NaCl. The tomato fruit had very low sodium content although the leaves accumulated high concentrations. The same year, Zhang *et al.* reported transgenic *Brassica napus* plants over-expressing *AtNHX1*, which are able to grow, flower and produce seeds in 200 mM NaCl. Therefore, in contrast to the notion that multiple traits introduced by breeding into crop plants are necessary to obtain salt-tolerant plants, the modification of a single trait significantly improved the salinity tolerance of these crop plants. However, the studies described

above have all been done in dicots. There are important differences between the transport system and physiology of monocots and dicots.

OsNHX1 has been overexpressed in rice. In 1999, Fukuda *et al.* isolated a rice cDNA clone of *OsNHX1*. The deduced amino acid sequence of this cDNA had homology with the putative Na^+/H^+ exchanger in *Saccharomyces cerevisiae ScNHX1*. The sequence was also found to be similar to that of *NHX1* and *NHE* isoforms in mammals. The expression of the gene was found to increase by salt stress. In 2004, the same group has made another publication on *OsNHX1*. They have found that OsNHX1 had the ability to suppress the Na^+, Li^+, K^+ and hygromycin sensitivity of yeast *nhx1* mutants. These results indicated that OsNHX1 protein has the same activity as the yeast protein ScNHX1 and it functions as a vacuolar $(Na^+, K^+)/H^+$ antiporter. They confirmed the localization of OsNHX1 on the tonoplasts. They found that the expression of *OsNHX1* is regulated by salt stress, and this regulation is mainly due to ionic stress. They also found that overexpression of *OsNHX1* improved the salt tolerance of transgenic rice cells and plants. The transgenic plants were able to maintain growth in 0.1 M NaCl, but the wild-type plants eventually died. Another group, Ohta *et al.* engineered a salt-sensitive rice cultivar (*Oryza sativa* cv. Kinuhikari) to express a vacuolar-type Na^+/H^+ antiporter gene from a halophytic plant, *Atriplex gmelini* (AgNHX1) in 2002, *AgNHX1* was expressed under CaMV35S promoter. The activity of the vacuolar-type Na^+/H^+ antiporter in the transgenic rice plants was eight-fold higher than that in wild-type rice plants. Transgenic plants over-expressing *AgNHX1* could survive under conditions of 300 mM NaCl for 3 days while the wild-type rice plants could not.

In tomato, molecular cloning approach with an overexpressing a vacuolar Na+/H+ antiport was performed and the transgenic tomato were able to grow, flower, and produce fruit in the presence of 200 mM sodium chloride. Although the leaves accumulated high sodium concentrations, the tomato fruit displayed very low sodium content. Contrary to the notion that multiple traits introduced by breeding into crop plants are needed to obtain salt-tolerant plants, the modification of a single trait significantly improved the salinity tolerance of this crop plant. These results demonstrate that with a combination of breeding and transgenic plants it could be possible to produce salt-tolerant crops with far fewer target traits than had been anticipated. The accumulation of sodium in the leaves and not in the fruit demonstrates the utility of such a modification in preserving the quality of the fruit (Zhang and Blumwal, 2011).

CHAPTER 3 : *METHODS AND MATERIALS*

Materials and Methods:

3.1 Bioinformatics Analysis:

It was necessary to do the analysis with the bioinformatics tools to have a clear picture of the targeted gene to be cloned. Discussions of methods of different bioinformatics analysis carried out are given below. Unless otherwise mentioned, default parameters of the tools/ software were used.

3.1.1 Databases:

3.1.1.1 NCBI:

Established in 1988 as a national resource for molecular biology information, the National Center for Biotechnology Information (NCBI) creates public databases, conducts research in computational biology, develops software tools for analyzing genome data, and disseminates biomedical information - all for the better understanding of molecular processes affecting human health and disease.NCBI data bases was used pointed into the following web address: (http://www.ncbi.nlm.nih.gov) .

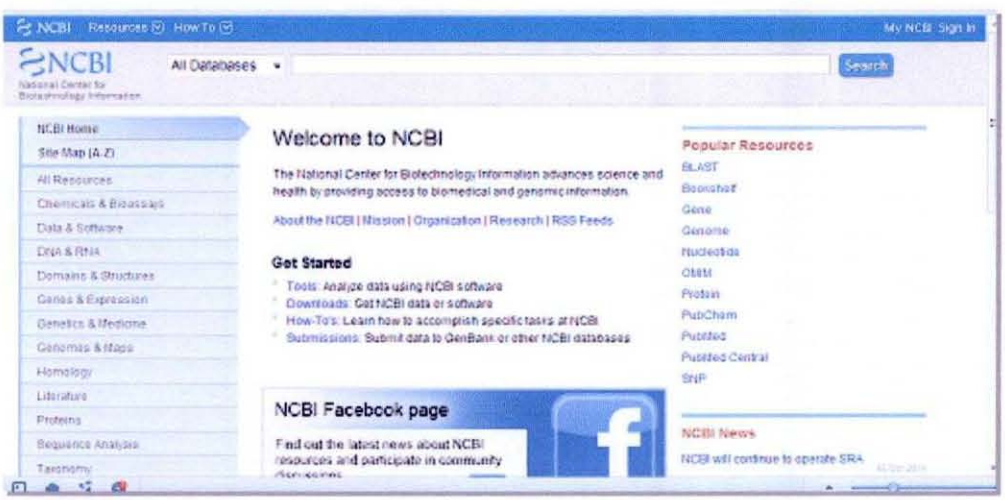


Figure 3.1: NCBI Home Page

3.1.1.2Gramene:

As an information resource, Gramene's purpose is to provide added value to data sets available within the public sector, which will facilitate researchers' ability to understand the grass genomes and take advantage of genomic sequence known in one species for identifying and understanding corresponding genes, pathways and phenotypes in other grass species. This is achieved by building automated and curated relationships between cereals for both sequence and biology. The automated and curated relationships are queried and displayed using controlled vocabularies and web-based displays. The controlled vocabularies (Ontologies), currently being used includes Gene ontology, Plant ontology, Trait ontology, Environment ontology and Gramene Taxonomy ontology. The web-based displays for phenotypes include the Genes and Quantitative Trait Loci (QTL) modules. Sequence based relationships are displayed in the Genomes module using the genome browser adapted from Ensembl, in the Maps module using the comparative map viewer (CMap) from GMOD, and in the Proteins module displays. BLAST is used to search for similar sequences. Literature supporting all the above data is organized in the Literature database. Gramene database was accessed by clicking into the following wed address: (http://www.gramene.org).

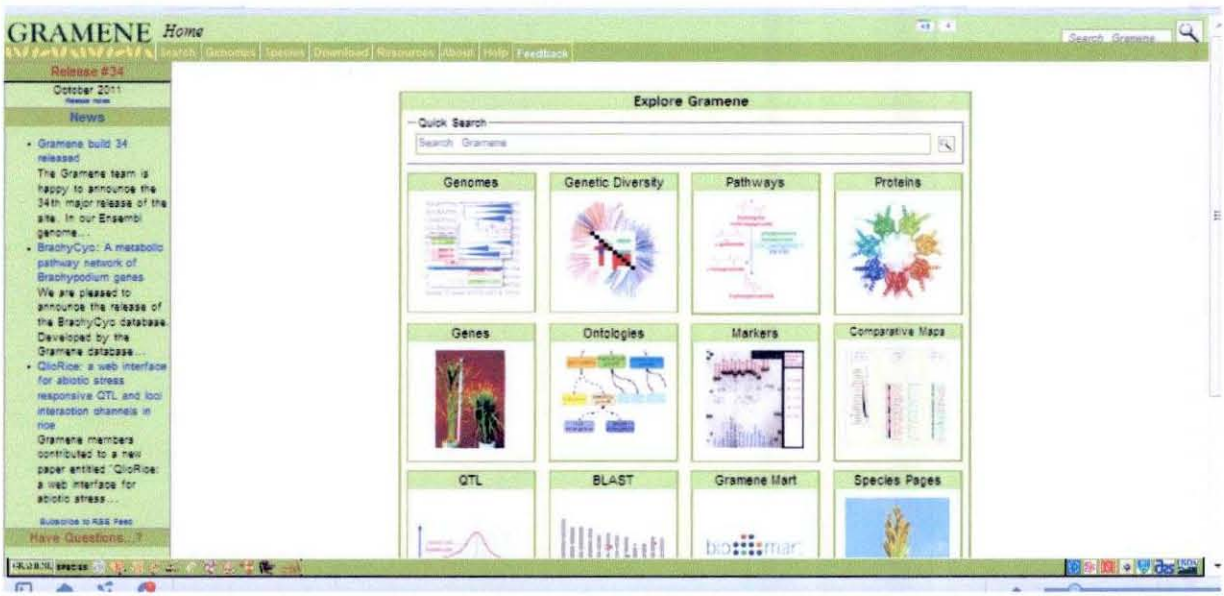


Figure 3.2: Gramene Home Page

3.1.1.3 EMBL-EBI:

The European Bioinformatics Institute (EBI) is an academic research institute located on the Wellcome Trust Genome Campus in Hinxton near Cambridge (UK), part of the European Molecular Biology Laboratory (EMBL). EMBL-EBI is also an open accessed database and can get into it through clicking into http://www.ebi.ac.uk .

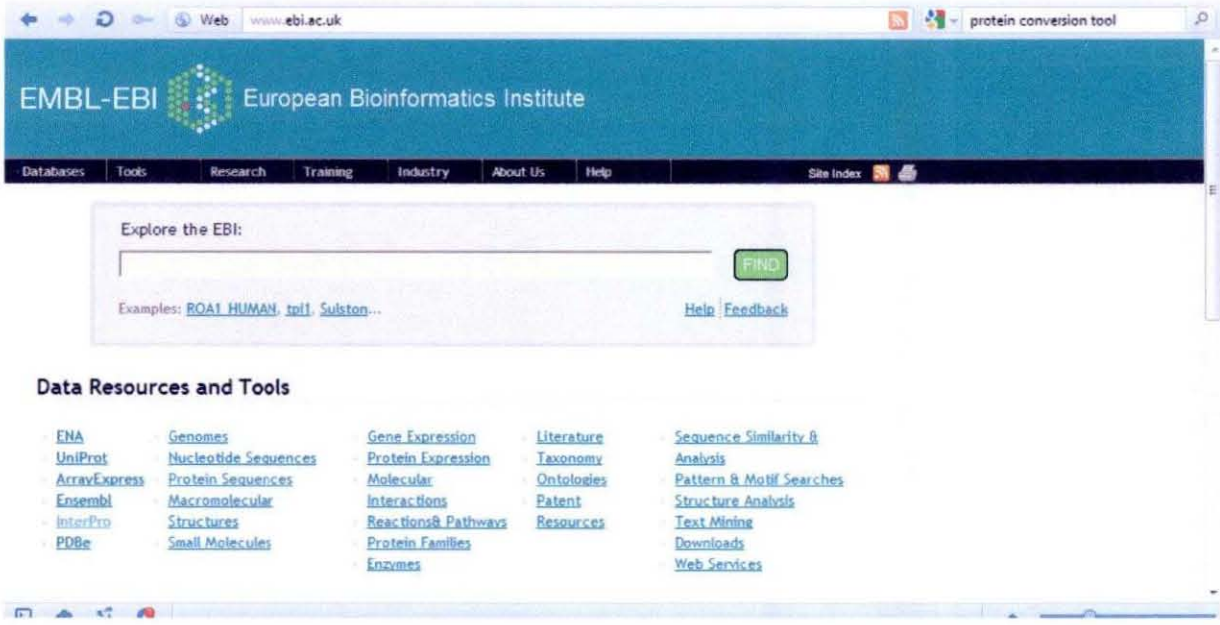


Figure 3.3: EMBL-EBI Home Page

3.1.2 Tools

Several tools were used for bioinformatics analysis of target sequences in this study. A brief description of these tools is given below:

3.1.2.1 BLAST

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. This tool can be accessed by clicking into http://blast.ncbi.nlm.nih.gov/Blast .

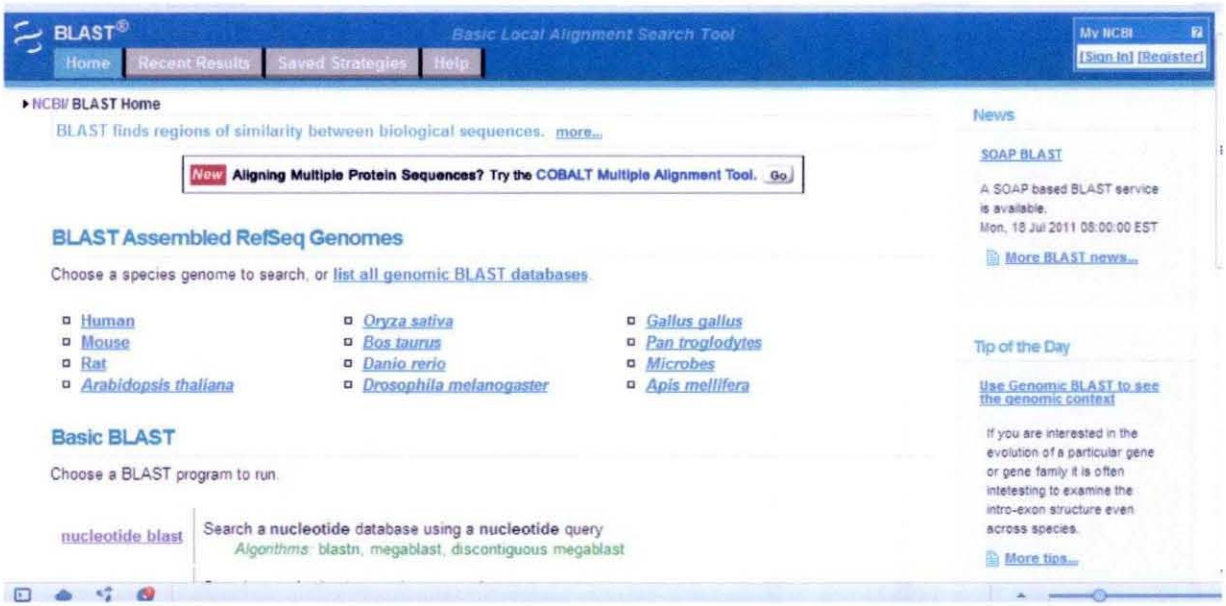


Figure 3.4: NCBI BLAST Home Page

3.1.2.2 ClustalW:

ClustalW2 is a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. Evolutionary relationships can be seen via viewing Cladograms or Phylograms (Thompson *et al.* 1994).
(http://www.ebi.ac.uk/Tools/msa/clustalw2/).

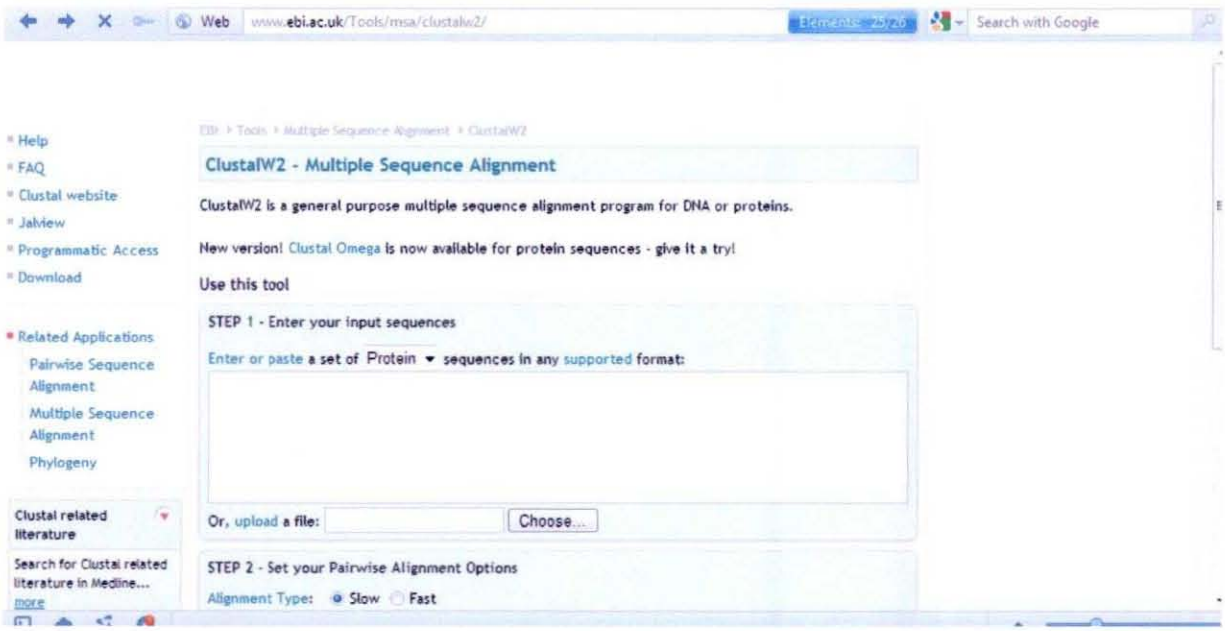


Figure 3.5: ClustalW Home Page

3.1.2.3 InterProScan:

InterProScan is a tool that combines different protein signature recognition methods native to the InterPro member databases into one resource with look up of corresponding InterPro and GO annotation. This service allows you to query your protein sequence against InterPro. This toll can be easily accessed by clicking http://www.ebi.ac.uk/Tools/pfa/iprscan .

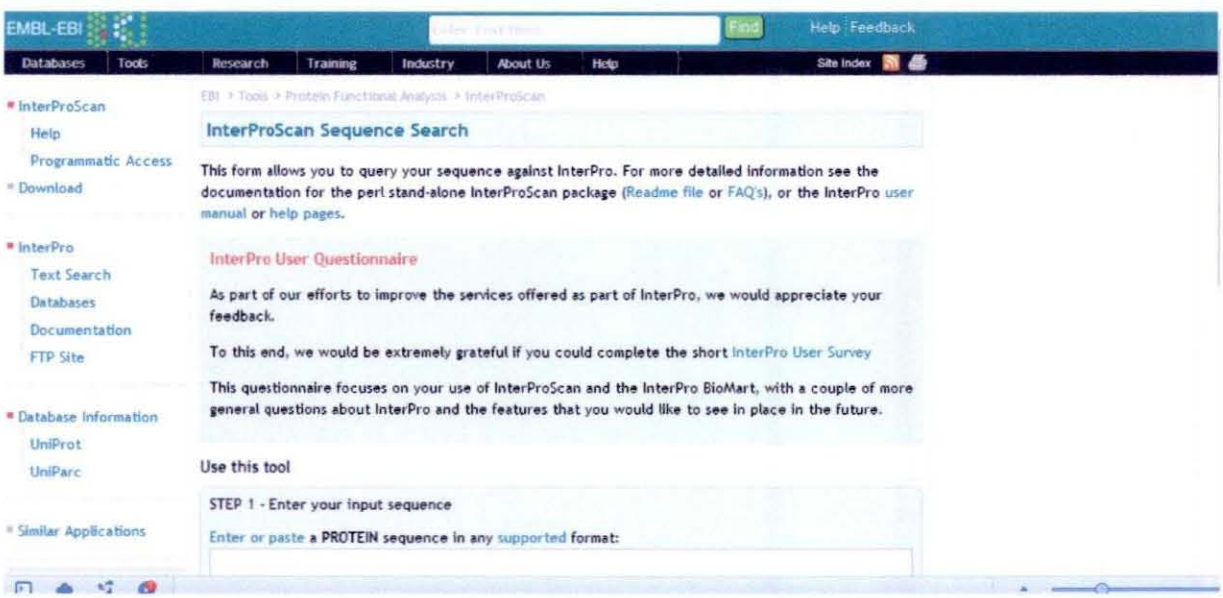


Figure 3.6: InterProScan Home Page

3.1.2.4 Transeq:

Transeq translates nucleic acid sequences to the corresponding peptide sequence. It can translate in any of the 3 forward or three reverse sense frames, or in all three forward or reverse frames, or in all six frames. Web address: http://www.ebi.ac.uk/Tools/emboss/transeq .

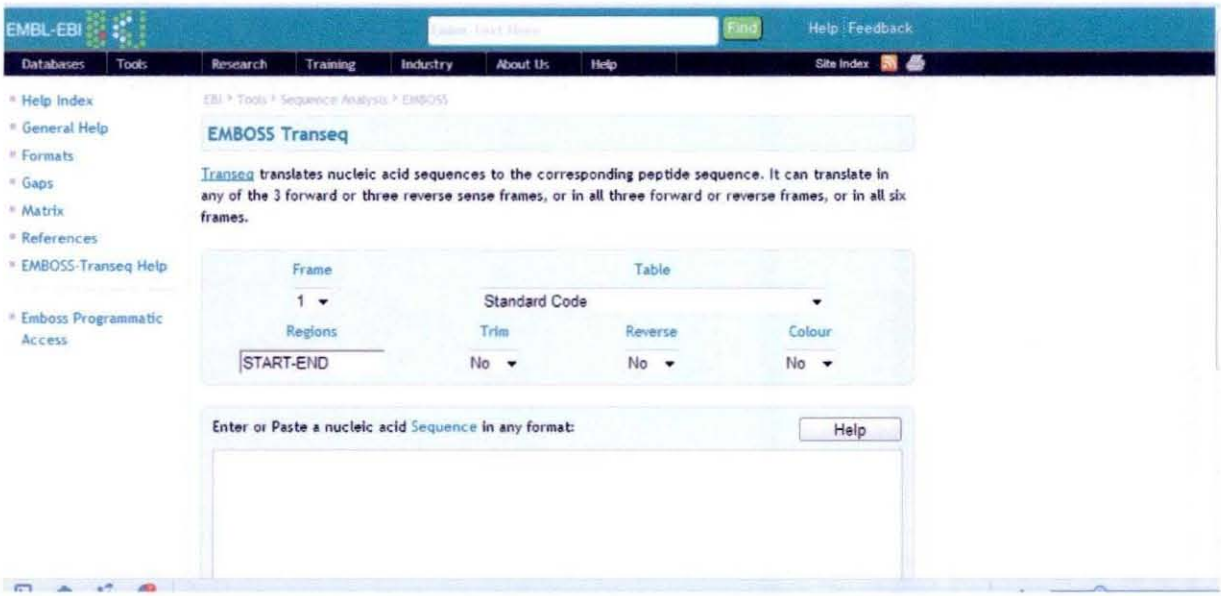


Figure 3.7: Transeq Home Page

3.1.3 Designing of Primer:

Following steps were followed to design primers:
1. Web browser was pointed to Primer3Plus URL (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

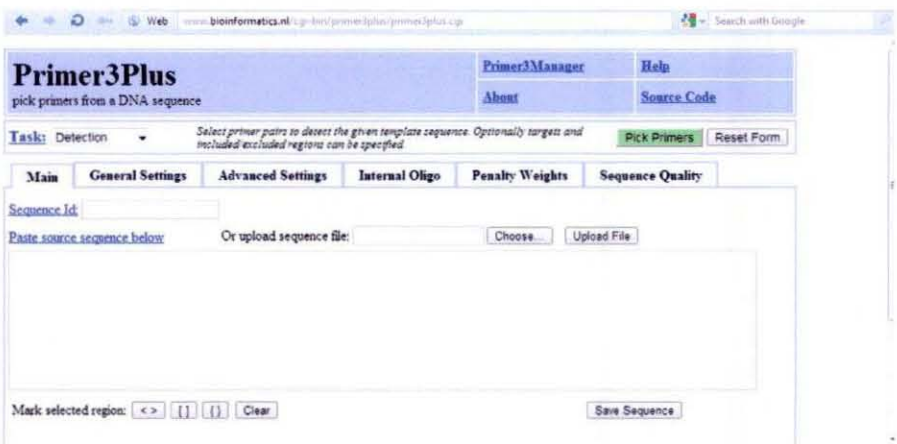


Figure 3.8: Primer3Plus Home Page

3.1.3.1 Designed primer verification:

IDT (Integrated DNA Technology) was used to verify the quality of the designed primer for melting temperature, self-dimer , hetero-dimer , hairpin etc. This software can easily can be accessed by clicking http://www.idtdna.com/analyzer/Applications/OligoAnalyzer .

Figure 3.9: IDT Home Page

3.2 Wet Lab Experiment:

3.2.1 Strategy followed to construct a recombinant vector:

3.2.1.1 Gateway Technology:

Gateway is a universal cloning technology based on the site-specific recombination properties of bacteriophage lambda. The Gateway Technology provides a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression, and can be schematically represented as follows:

attL1-gene-attL2 × attR1-ccdB-attR2 ⇔ attB1-gene-attB2 × attP1-ccdB-attP2

(entry clone) **(destination vector)** **(expression clone)** **(pDONR.)**

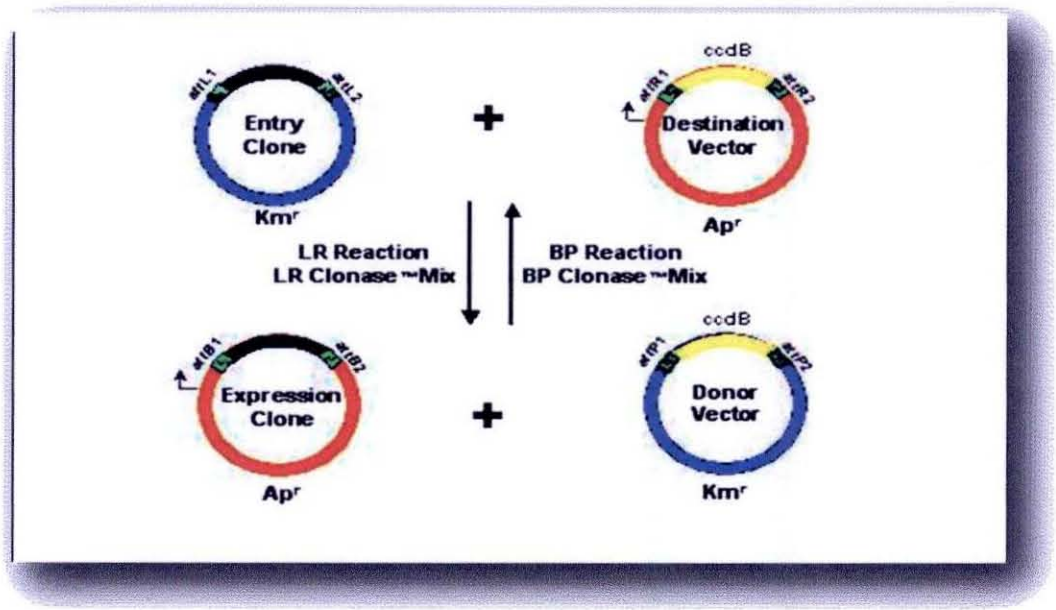


Figure 3.10: Gateway Cloning Technology

The attB × attP reaction is mediated by Gateway BP Clonase ll enzyme mix; the attL × attR reaction is mediated by Gateway LR Clonase ll enzyme mix. ccdB is the F plasmid-encoded gene that inhibits growth of E. coli and "gene" represents any DNA segment of interest (e.g. PCR product, cDNA, genomic DNA).

Gateway LR Clonase ll enzyme mix is a proprietary enzyme formulation containing the bacteriophage lambda recombination proteins Integrase (Int) and Excisionase (Xis), and the E. coli-encoded protein Integration Host Factor (IHF). Gateway LR Clonase ll enzyme mix promotes in vitro recombination between an entry clone (attL-flanked "gene") and any number of attR-containing destination vectors to generate attB-containing expression clones. "Genes" present in expression clones can be transferred back into an entry vector by mixing with an attP Vector (**pH7WG2.0**) and adding Gateway LR Clonase ll enzyme mix.

3.2.1.2 Required Materials for Gateway Technology:

- Entry Vector (pENTR)
- Destination Vector (pH7WG2.0)

3.2.1.3 Description of vectors used for cloning:

3.2.1.4 Features of the Cloning Vector:

The pENTR/D-TOPO vectors are designed to facilitate rapid, directional TOPO Cloning of blunt-end PCR products for entry into the Gateway System

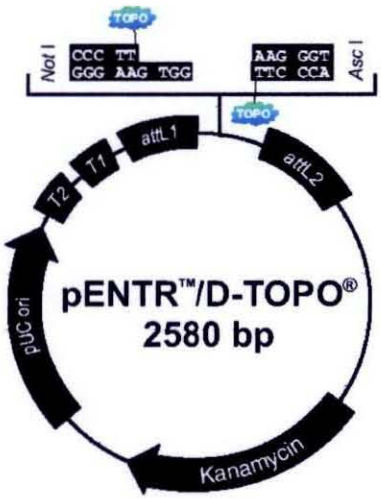


Figure 3.11: Genetic Map of pENTR /D-TOPO Vector

1. attL1 and attL2 sites for site-specific Recombination of the entry clone with a Gateway Destination vector.
2. Directional TOPO Cloning site for rapid and efficient directional cloning of blunt-end PCR products.
3. Kanamycin resistance gene for selection in *E. coli*
4. pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

3.2.1.5 Mechanism of Directional TOPO cloning by pENTR /D-TOPO Vector:

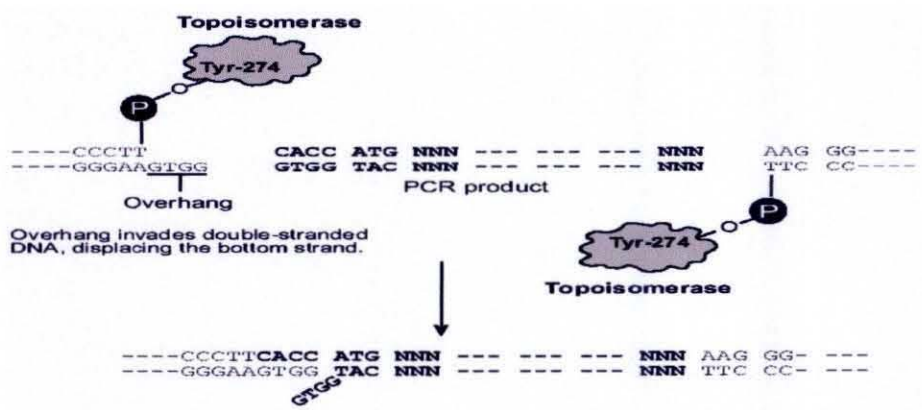


Figure 3.12: Mechanism of Directional TOPO Cloning

Topoisomerase I from Vaccinia virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone in one strand. The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase. TOPO Cloning exploits this reaction to efficiently clone PCR products.

Directional joining of double-strand DNA using TOPO-charged oligonucleotides occurs by adding a 3' single-stranded end (overhang) to the incoming DNA. This single-stranded overhang is identical to the 5' end of the TOPO-charged DNA fragment. This idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO-charged DNA and adapting it to a 'whole vector' format. In this system, PCR products are directionally cloned by adding four bases to the forward primer (**CACC**). The overhang in the cloning vector (**GTGG**) invades the 5' end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.

3.2.1.6 Vector of Destination vector and its characteristics:

3.2.1.7 Destination Vector pH7WG2.0

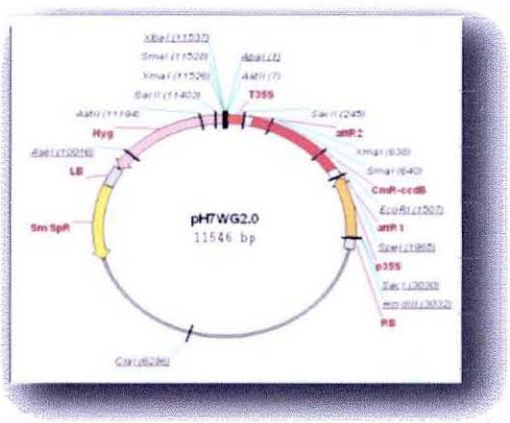


Figure 3.13: Destination vector map

3.2.1.8 Features of pH7WG2.0:

3.2.1.9 pH7WG2.0 is a binary vector and contains the following location:

- Recombination site attr1 and attr2
- CaMV35S constitutive promoter
- Hygromycin resistance for selection in plants
- Spectinomycin and Streptomycin resistance for selection in bacteria
- A marker in the empty vector (ccdB) which will be replaced by the insert after the LR reaction
- A terminator

3.2.2.1 Target sequence to be cloned (only CDS of OsNHX1):

3.2.2.2 PCR amplification of the coding sequence of vacuolar antiporter gene:

3.2.2.3 Source of template:

Coding sequence of vacuolar antiporter gene, NHX1 from salt tolerant landrace Nipponbare, was amplified by using pENTR.OsNHX1_1.9 construct as template. pENTR.OsNHX1_1.9 construct was prepared in our lab from the cDNA of vacuolar NHX1 gene (Nipponbare) amplified by RT-PCR. The cloned vector with NHX1 contains 5' UTR and the current research objective was to eliminate the UTR portion and to clone only the Coding sequence of the Vaculour antiporter NHX1 gene.

3.2.2.4 Target Nucleotide Sequences:

3.2.2.5 Target Sequence of OsNHX1 (1608 bp)

ATGGGGATGGAGGTGGCGGCGGCGCGGCTGGGGGCTCTGTACACGACCTCCGACTACGCGTCGGTGGTGTCCATCAACCTGTTCGTC
GCGCTGCTCTGCGCCTGCATCGTCCTCGGCCACCTCCTCGAGGAGAATCGCTGGGTCAATGAGTCCATCACCGCGCTCATCATCGGGC
TCTGCACCGGCGTGGTGATCTTGCTGATGACCAAAGGGAAGAGCTCGCACTTATTCGTCTTCAGTGAGGATCTCTTCTTCATCTACC
TCCTCCCTCCGATCATCTTCAATGCAGGTTTTCAGGTAAAGAAAAAGCAATTCTTCCGGAATTTCATGACGATCACATTATTTGGAG
CCGTCGGGACAATGATATCCTTTTTCACAATATCTATTGCTGCCATTGCAATATTCAGCAGAATGAACATTGGAACGCTGGATGTA
GGAGATTTTCTTGCAATTGGAGCCATCTTTTCTGCGACAGATTCTGTCTGCACATTGCAGGTCCTCAATCAGGATGAGACACCCTTT
TTGTACAGTCTGGTATTCGGTGAAGGTGTTGTGAACGATGCTACATCAATTGTGCTTTTCAACGCACTACAGAACTTTGATCTTGT
CCACATAGATGCGGCTGTCGTTCTGAAATTCTTGGGGAACTTCTTTTATTTATTTTTGTCGAGCACCTTCCTTGGAGTATTTGCTGG
ATTGCTCAGTGCATACATAATCAAGAAGCTATACATTGGAAGGCATTCTACTGACCGTGAGGTTGCCCTTATGATGCTCATGGCTT
ACCTTTCATATATGCTGGCTGAGTTGCTAGATTTGAGCGGCATTCTCACCGTATTCTTCTGTGGTATTGTAATGTCACATTACACTT
GGCATAACGTCACAGAGAGTTCAAGAGTTACAACAAAGCACGCATTTGCAACTCTGTCCTTCATTGCTGAGACTTTTCTCTTCCTGT
ATGTTGGGATGGATGCATTGGATATTGAAAAATGGGAGTTTGCCAGTGACAGACCTGGCAAATCCATTGGGATAAGCTCAATTTTG
CTAGGATTGGTTCTGATTGGAAGAGCTGCTTTTGTATTCCCGCTGTCGTTCTTGTCGAACCTAACAAAGAAGGCACCGAATGAAAA
AATAACCTGGAGACAGCAAGTTGTAATATGGTGGGCTGGGCTGATGAGAGGAGCTGTGTCGATTGCTCTTGCTTACAATAAGTTTA
CAAGATCTGGCCATACTCAGCTGCACGGCAATGCAATAATGATCACCAGCACCATCACTGTCGTTCTTTTTAGCACTATGGTATTTG
GGATGATGACAAAGCCATTGATCAGGCTGCTGCTACCGGCCTCAGGCCATCCTGTCACCTCTGAGCCTTCATCACCAAAGTCCCTGC
ATTCTCCTCTCCTGACAAGCATGCAAGGTTCTGACCTCGAGAGTACAACCAACATTGTGAGGCCTTCCAGCCTCCGGATGCTCCTCA
CCAAGCCGACCCACACTGTCCACTACTACTGGCGCAAGTTCGACGACGCGCTGATGCGACCGATGTTTGGCGGGCGCGGGTTCGTGC
CCTTCTCCCCTGGATCACCAACCGAGCAGAGCCATGGAGGAAGATGA

3.2.2.6 Sequence amplified by Polymerase Chain Reaction (PCR):

3.2.2.6.1 Materials:

The following components are required for PCR and all components were purchased from Invitrogen:

- 10X PCR reaction buffer
- 50 mM $MgCl_2$
- 10mM dNTPs
- Primers (100ng/µl):

- **Forward Primer**

Primer: **OsNHX1_F_1.6**

Sequence	Length	Tm	%GC
CACCATGGGGATGGAGGTGGCG	22	61.5	%68.2

- **Reverse Primer**

Primer: **OsNHX1_R_1.6**

Sequence	Length	Tm	%GC
TCATCTTCCTCCATGGCTCTGC	22	59.0	%54.5

- Taq polymerase with pfx (10:1)
- 20% DMSO (Dimethyl sulfoxide)

(20% DMSO was prepared by mixing 200µl of DMSO in 800µl of deionized sterile water and was stored at-20^{0}C)

- DNA template
- Plasmid (1ng/µl)
- TE solution for dilution of the template DNA
  - i. 10mM Tris HCl (pH 8.0)
  - ii. 0.1mM EDTA (pH 8.0)
- Autoclaved ultra-pure water (PCR H_2O)

N. B. In each reaction, the volume of the PCR buffer used was one-tenth of the total reaction volume. Taq DNA polymerase was added just before the start of the reaction.

Plasmid DNA and 20% DMSO was dispensed in the labeled tubes prior to adding the master mixture for well DNA de-naturation as follows:

	DNA(1ng/µl)	20%DMSO
Sample tube	1.0µl	3.0µl
Control tube	0.0µl	3.0µl

N.B. Control tube is required to detect any kind of contamination, which causes false positive amplification. Finally, the tubes were subjected to momentary spin and transferred to Thermo-cycler for the amplification reaction.

3.2.2.6.2 PCR amplification:

Using the mentioned primers, insert was amplified as following reaction condition and PCR

PCR Reaction :

10X PCR buffer	2.5µl
10mM dNTP mix	1.0µl
50mM $MgCl_2$	1.8µl
20% DMSO	3.0µl
OsNHX1_F_1.6	2.0µl
OsNHX1_R_.6	2.0µl
Taq polymerase: pfx(10:1)	0.3µl
Template	1.0µl
PCR H_2O	13.0µl
Total	25.0µl

PCR program:

Step 1:	95°C for 5 min
Step 2:	95°C for 1 min
Step 3:	61°C for 1 sec
Step 4:	72°C for 2 min
Step 5:	repeat 2 to 4 for 35 cycles
Step 6:	72°C for 15 min

3.2.2.6.3 Visualization of the amplified PCR product:

The amplified PCR product was detected by the help of 0.8% agarose gel electrophoresis and 1 kb plus ladder marker DNA. Type of gel selection generally depends on the length and nature of the amplified product.

3.2.2.6.4 Concentration check of the PCR product:

The purity and concentration of amplified product can be checked from the band in agarose gels. For this 0.8% agarose is ideal. Concentration was estimated by using known concentrations of bacteriophage lambda DNA (50ng/μL).

3.2.2.6.5 Procedure:

1. According to percentage and the volume of gel desired, 50x TAE buffer was measured and the appropriate weight of agarose was taken in a flask, and made the final volume by adding distilled water. The flask was placed in a microwave on high temperature for 2-3 minutes until the gel solution was completely clear.
2. The solution was air-cooled until the temperature is below 60^0 C.
3. The cooled solution was poured into the gel tray. The comb(s) were placed into the slot(s) and it was made sure that there were no bubbles around the combs. The gel was kept approximately 30-45 min to be condensed.
4. The gel was placed in the tank filled with 1x TAE buffer. Enough TAE buffer was added to cover the gel and the comb(s) was removed gently.
5. The DNA samples (with loading buffer) were loaded in the slots leaving room at both the ends for size standards (lambda DNA).
6. The gel tank was covered. Then the leads were connected and the power supply was turned on.
7. Then 100V was applied and the electrophoresis was done until the blue dye migrated three fourths of the length of the gel, then the power supply was turned off and the leads were disconnected.

8. The gel was stained for 15 min, immersing it in water containing ethidium bromide (0.5 gm/ml).
9. The gel was photographed under UV illumination using AlphaEase FC Imaging System

3.2.2.6.6 QIAquick Gel Extraction Kit Protocol (using a microfuge)

It is strongly recommended to use gel extracted DNA while performing cloning reaction, so that the amplified product by the PCR was extracted from the gel using the following protocol. This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. More than one QIAquick column should be used for gel slices >400 mg.

Notes:

1. The yellow color of Buffer QG indicates a pH ≤ 7.5.
2. 96-100% ethanol was added to Buffer PE before use.
3. 100% isopropanol and a heating block or water bath at 50°C were required.
4. All centrifugation steps were carried out at ≥ 10,000 g (~ 13,000 rpm) in a conventional table-top microcentrifuge.
5. 3 M sodium acetate, pH 5.0 may be necessary to decrease the pH of Buffer QG.

3.2.2.6.7 Procedure:

1. The DNA fragment was excised from agarose gel using a clean scalpel and in a pre-weighed eppendorf tube

2. The size of the gel was minimized by removing extra agarose.

3. The gel slice was weighed in a colorless tube. Three volumes of buffer QG was added to one volume of gel (100 mg ~100 µl). For example, 300 µl of Buffer QG was added to each 100 mg of gel. 6 volumes of Buffer QG were added for > 2% agarose gel.

4. The tube was incubated at 50°C for 10 min or until the gel slice has completely dissolved. The tube was vortexed every 2-3 minutes during incubation to help dissolve the gel.

IMPORTANT: The agarose should be solubilized completely. Incubation time should be increased for gels >2%.

5. After the gel slice had dissolved completely, the color of the mixture was checked.If the color was not yellow (similar to Buffer QG without dissolved agarose) but had turned orange or violet, 10 µl of 3M sodium acetate, pH 5.0 was added and mixed. The adsorption of DNA to the QIAquick membrane is efficient only at pH $\leq$ 7.5. Buffer QG contains a pH indicator which is yellow at pH $\leq$ 7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

6. One gel volume of isopropanol was added to the sample and mixed. For example if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments < 500 bp and > 4 k. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. The sample should not be centrifuged at this stage.

7. A QIAquick spin column was placed in a provided 2-ml collection tube.

8. The sample was applied to the QIAquick colum, to bind DNA, and centrifuged for 1 min. The maximum volume of the column reservoir is 800 µl. The sample should be loaded and spinned again if the sample volume was more than 800

9. The flow-through was discarded and the QIAquick column was placed back in the same collection tube.

10. 0.5 ml of Buffer QG was added to QIAquick column and centrifuged for 1 min. This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription or microinjection. 0.75 ml of Buffer PE was added to QIAquick column and centrifuged for 1 min.

Note: If the DNA is intended to be used for salt sensitive applications, such as blunt-end ligation, and direct sequencing, the column should be allowed to stand 2-5 min after addition of Buffer PE, before centrifuging.

11. The flow-through should be discarded and the QIAquick column should be centrifuged for an additional 1 min at ≥ 10,000 g (~13,000 rpm).

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

12. The QIAquick column was placed into a clean 1.5-ml microfuge tube.

13. To elute DNA, 50 µl of Buffer EB (10 mm Tris-Cl, pH 8.5) or water was added to the center of the QIAquick membrane and the column was centrifuged for 1 min at maximum speed. Alternatively, for increased DNA concentration, 30 µl elution buffer was added to the center of the QIAquick membrane, the column was allowed to stand for 1 min, and then centrifuged for 1 min.

IMPORTANT: The elution buffer should be dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average elute volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, it should be made sure that the pH value is within this range, and the DNA should be stored at -20°C as DNA may degrade in

the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

14. Concentration of the gel extract was then measured by using 0.8% agarose gel.

3.2.3 TOPO cloning reaction (Invitrogen):

3.2.3.1 Amount of PCR product required:

Amount of insert / Size of insert = Amount of Vector / Size of Vector

Here, Size of insert = 1608 bp

Size of Vector = 2580 bp

Amount of Vector = 10ng (as vector concentration is 20ng/ul and 0.5ul was taken for the reaction)

Therefore, Amount of PCR product (insert) required = 8.96ng = ~ 9.0ng

3.2.3.2 Reaction Protocol:

The table below describes the set up of TOPO cloning reaction (3 µl) for transformation into chemically competent *E. coli* cell.

Reagent	Amount Taken
Fresh PCR product	0.2 µl (9 ng)
Salt Solution	0.5 µl
Sterile Water	1.8 µl (For a total volume of 3 µl)
TOPO® vector	0.5 µl (10 ng)
Final Volume	**3 µl**

1. The reaction components was dropped upon drop gently and incubated for 30 minutes at room temperature (22-23°C). (N.B For most applications, 5 minutes yields plenty of colonies for analysis. Depending on the needs, the length of the TOPO cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or a pool of PCR product is used for TOPO cloning, increasing the reaction time will yield more colonies.)

2. The reaction was placed on ice and preceded for Transformation.

3.2.3.3 Bacterial transformation by heat-shock method:

3.2.3.3.1 Preparation of competent cells:

Materials

- $CaCl_2$ solution (60mM $CaCl_2$, 15% glycerol, 10mM PIPES pH 7.0)
- LB (Luria-Bertani) medium liquid (5g Yeast extract, 10 g Peptone, 10 g NaCl per liter pH 7.0)
- E.coli cell DH5α strain.

Procedure

1. A single colony of *E.coli* cell was incubated into 5 ml LB medium overnight at 37°C with shaking (250 rpm).

2. 1 ml of this culture was taken into a 100 ml LB medium in a 250 ml conical flask. The medium was incubated at 37°C with shaking (250 rpm) until optical density of this medium at 590 nm reaches 0.375.

3. The culture was then aliquot into 2 pre-chilled sterile sorval tubes and placed on ice for 5 to 10 minutes. It was then centrifuged for 7 minutes at 1600 g and 4°C.

4. Each palette was resuspended in 10 ml of ice-cold $CaCl_2$ solution. It was centrifuged at 1100 g for 5 minutes at 4°C.
5. The palettes were resuspended again in 10 ml of ice-cold $CaCl_2$ solution and kept on ice for 30 minutes. It was then centrifuged at 1100 g for 5 minutes at 4°C.

6. Each palette was then resuspended in 2 ml ice-cold $CaCl_2$ solution completely. 250µl of this solution was aliquot in each sterile pre-chilled microcentrifuge tube and stored at -80°C for later use in transformation.

3.2.3.4 Transformation by heat-shock:

Materials

- Ice
- Water bath at 42°C
- SOC media (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose)
- LB plates containing 50 µg/ml ampicillin or 50 µg/ml kanamycin
- 42°C water bath
- 37°C shaking and non-shaking incubator

3.2.3.4.1 Procedure for bacterial transformation by heat shock

1. Aliquots of frozen competent cell were thawed on ice for five minutes.

2. 3 µl of TOPO reaction mixture was taken in a cold 1.5 ml polypropylene microfuge tube and placed on ice.

3. 37.5 µl of freshly thawed competent cells were added to the tube. The contents of the tube was mixed by tapping very gently and incubated on ice ~ 30 minute.

4. The cells were heat shocked at 42°C in a water bath for 90 seconds.

5. The tube was transferred immediately on ice and incubated for 10-15 minutes.

6. 375 µl of SOC media was added to the tube and the tube was incubated at 37°C with mild shaking for 30 minutes.

7. The tube was then centrifuged at 4000 rpm for 3 minutes.

8. 150 ul supernatant was discarded and cells were then resuspended in rest supernatant by pipetting ups and downs very slowly.

9. The cells were then plated on LB^{kana+} plate.

10. The plate was then incubated at 37°C overnight.

3.2.3.4.2 Patch Culture of the Eletroporated cells:

1. LB agar plates were prepared containing the appropriate antibiotic.

2. Patch culture papers were numbered and bound to plates with tape.

3. Single colonies were then subcultured and incubated overnight.

3.2.3.4.3 Identification of Positive colonies by Lysate PCR:

Materials:

- PCR Mix (contains all the components for PCR except template)
- Appropriate forward and reverse PCR primers.

- **Forward Primer**

Primer: **OsNHX1_679_F**

Sequence	Length	Tm	%GC
GCTGGATTGCTCAGTGCATA	20	60.4	50

- **Reverse Primer**

Primer: **OsNHX1_679_R**

Sequence	Length	Tm	%GC
AAGGCTCAGAGGTGACAGGA	20	62.4	55

Reaction mix:

10X PCR buffer	1.5µl
1mM dNTP mix	1.5µl
50mM $MgCl_2$	0.4µl
20% DMSO	2.0µl
Primer-F	0.5µl
Primer-R	0.5µl
Taq polymerase	0.5µl
PCR H_2O	8.1µl
Total	15.0µ

PCR program:

Step 1:	95°C for 10 min
Step 2:	95°C for 1 min
Step 3:	61.4°C for1 min
Step 4:	72°C for 1 min
Step 5:	repeat 2 to 4 for 35 cycles
Step 6:	72°C for 10 min

3.2.3.4.4 Procedure:

1. For each sample, PCR Mix is taken into a 0.5 ml microcentrifuge tube.
2. Colonies are picked and resuspended individually in 15 µl of the PCR mixture.
3. The reaction is incubated for 10 minutes at 95°C to lyse the cells and inactivate nucleases.
4. Amplified for 35 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualization by agarose gel electrophoresis.

3.2.3.5 Plasmid isolation from positive colonies:

3.2.3.5.1 Mini-preparation of plasmid DNA by alkaline lysis method (modified from Sambrook et. al. 1989)

Reagent

Solution I (200 ml)		Solution II (5 ml)		Solution III (5 ml)	
Glucose	1.8 g	10% SDS	0.5 ml	5M KOAc	3.00 ml
1M Tris(pH 8.0)	5.0 ml	5N NaOH	0.2 ml	Glacial acetic acid	1.25 ___

Procedure

1. A single colony of DH5α/BjGlyI_TOPO-D (for example) was picked up and used to inoculate 5.0 ml LB medium, containing 100μg kanamycin per ml. the bacterial culture was incubated at 37°C overnight with shaking (200 rpm).

2. The overnight culture was transferred to a 15 ml Falcon tube and the cells were centrifuged at 4,000 rpm for 10 minutes at 4°C.

3. The supernatant was poured off and the cells in pellet was resuspended in 200 μl solution I and transferred to a 1.5 ml Eppendorf microcentrifuse tube. The tube was incubated in room temperature for 5 minutes.

4. 400μl of freshly prepared solution II was added to the tube. The tube was inverted several times to mix the content and placed on ice strictly for 5 minutes.

5. 300 μl of solution III was added to the tube and mixed well by inversion. The mixture was kept on ice for 3-5 minutes.

6. The tube was centrifuged at 4°C 12,000 rpm for 10 minutes.

7. The supernatant was transferred to a fresh tube. 600 μl of chloroform was added to the tube. The tube was inverted several times to mix the content and then centrifuged at 12,000 rpm for 5 minutes at 4°C.

8. The supernatant was transferred to a fresh Eppendorf tube. 0.6 to 1 volume of ice cold Isopropanol was added to the supernatant. The tube was inverted several times to mix the content. The tube was then kept still for 15-30 minutes at room temperature.

9. The tube was then centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was then poured off and the pellet containing DNA was washed with 70% ethanol. The tube was centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was removed carefully with a pipette and the palette was air-dried.

10. The palette was dissolved in 39µl TE (10 mM TrisHCl, 1mM EDTA) buffer. 1µl of RNaseA (10 mg/ml) was added to the tube and incubated at 37°C for 20 minutes.

11. 1-2 µl of the plasmid preparation was run on 0.8% agarose gel to determine the concentration of the plasmid DNA.

12. The plasmid preparation was stored at -20°C.

3.2.3.5.2 Plasmid Isolation by Kit:

The vector plasmid pENTR\D-TOPO was isolated from the strain DH5α using FastPlasmid™ Mini kit (*promega)*. The kit components and procedures are described as follows:

3.2.3.5.3 FastPlasmid™ Mini Kit Components and Procedure:

Kit components

Components	**Storage Temperature**
Lysis Solution	4°C
RNase Solution	4°C

Lysozyme	4°C
Wash Buffer Concentrate	Room Temp.
Elution Buffer	Room Temp.
FastPlasmid Spin Column Assembly (Spin Column and Waste Tube)	Room Temp.
Collection Tube	Room Temp.

Procedure:

1. Bacterial growth (5ml) in screw cap tube after overnight shaking centrifuged at 4,000 rpm for 12 mins
2. Removing the supernatant and kept the pallet as dry as possible
3. Then added 250 micro liter of Cell Resuspension solution in the screw cap tube and after dissolving the pellet by vortexing , mixed solution then placed into a 1.5 ml eppendorf tube
4. Add 250 micro liter of cell lysis buffer into the tube inverting 4 times and kept at room temperature for 5 min (No vortexing is recommended)
5. After that add 10 micro liter of Alkaline protease into the tube inverting 4 times and kept at room temperature for 5 min(No vortexing is recommended)
6. Add 350 micro liter of Neutrilization Buffer and centrifugate at room temperature at maximum speed (13,000 rpm) for 20 mins
7. After that the supernatant is transferred into fresh tube column from the kit box and centrifuge again at maximum speed for 1 min
8. Then 750 micro liter of prepared wash solution (Column wash solution with 99% Ethanol) added into the column tube and centrifuge for 1 min at maximum speed
9. Add 250 micro liter of more prepared wash solution and centrifuge at maximum speed for 1 min at room temperature
10. Then the upper cap tube replaced into gain another fresh eppendorf tube and added 40 micro liter of nuclease free water and keep that for 1 hour at room temperature
11. After 1 hour, the tube is centrifuged at room temperature at a maximum speed of 13000 rpm for 1 min , thus the plasmid is ready to check through running the agarose gel.

3.2.3.5.4 DNA quality check and quantification

3.2.3.5.4.1 Introduction

Once DNA was prepared, it is important that the quality of DNA be checked for obtaining good results and for long-term storage. It is also important to know that one should know exactly how much DNA is available for the experiment and the concentration of DNA in known volume of TE buffer.

3.2.3.5.4.2 Procedure for DNA quantification using Spectrophotometer (Beckman)

1. The spectrophotometer settings were set at 260nm.
2. The cuvette was washed with first distilled water and then TE buffer and dried completely.
3. TE buffer was used for calibration.
4. Measurements were taken of samples with TE and the TE was discarded.
5. 5.0 µl of DNA samples was put in the cuvette and 95 µl of TE was added. The mixture was mixed and the cuvette was put in the compartment. Leakage of light was avoided.
6. The OD values were read directly from the display. This gave DNA concentration. These values were multiplied by 1000. (e.g., If OD was 0.2 then the DNA conc. is 200 µg/ml).

3.2.3.5.4.3 Assessment of purity of the samples

1. OD of the samples was read at 260 nm.
2. OD of the same samples was also read at 280 nm.
3. A ratio value of OD_{260}/OD_{280} =1.8 indicates a highly pure DNA preparation.
4. Lesser ratio values implied significant presence of contaminants-generally proteins.
5. Ratio of 2.00 indicated high concentration of RNA contamination.

3.2.3.5.4.4 Checking the quality and quantity of DNA by agarose gel electrophoresis:

The purity and concentration of genomic DNA can be checked from the compactness of the band in agarose gels or whether there is smearing. For genomic DNA 0.8% agarose is ideal. Concentration of the DNA was estimated by using known concentrations of bacteriophage lambda DNA.

Procedure

1. A large gel tray and combs were obtained. The ends of the tray were taped; and placed on a level surface.
2. 250 ml of 1x TAE buffer was measured and the appropriate weight of agarose in a flask, according to percentage of gel desired (e.g., 2 g agarose for 250ml of 0.8%gel).
3. The flask was placed in a microwave on high temperature for 2-3 minutes until the gel solution was completely clear.
4. The solution was air-cooled until the temperature is below 60^0 C.
5. The cooled solution was poured into the gel tray. The comb(s) were placed into the slot(s) and it was made sure that there were no bubbles around the combs. The gel was kept to harden for approximately 30-45 min.
6. The tape was removed and the gel was placed in the tank filled with 1x TAE buffer. Enough TAE buffer was added to cover the gel and the comb(s) was removed gently.
7. The DNA samples (with loading buffer) were loaded in the slots leaving room at both the ends for size standards.
8. The gel tank was covered. Then the leads were connected and the power supply was turned on. The gel was run for at 60V until the blue dye entered the gel (about 10 min).

9. The power was turned up to 80V and the gel ran until the blue dye migrated three fourths of the length of the gel, then the power supply was turned off and the leads were disconnected.
10. The gel was stained for 30-40 min, immersing it in water containing ethidium bromide (0.5 gm/ml).
11. The gel was photographed under UV illumination.

3.2.3.6 PCR Amplification applying OsNHX1_1.6 Primer set from isolated Plasmid :

Using the mentioned primers, insert was amplified as following reaction condition and PCR program.

PCR program:

Step 1:	95°C for 5 min
Step 2:	95°C for 1 min
Step 3:	61°C for 1 sec
Step 4:	72°C for 2 min
Step 5:	repeat 2 to 4 for 35 cycles
Step 6:	72°C for 15 min

Visualization of the amplified PCR product:

The amplified PCR product was detected by the help of 0.8% agarose gel electrophoresis and 1 kb plus ladder marker DNA. Type of gel selection generally depends on the length and nature of the amplified product.

3.2.3.7 Restriction Digestion:

Restriction endonuclease is called the molecular scissor. It can cut DNA by recognizing a specific sequence. The following reaction was to cut the DNA

10X reaction buffer	1.0µl
BSA	0.5µl
DNA	1.0µg
Enzyme (10U/µl)	0.2µl
Sterile water	Up to 10.0µl

This reaction is for digestion with single enzyme. When digestion with more then one enzyme was required, the enzymes were added accordingly and volume was adjusted with water. After digestion the fragments were visualized under UV illumination after separation by agarose gel electrophoresis.

3.2.3.8 Sequencing of cloned OsNHX1 from pENTR_OsNHX1_1.6

After successful completion of PCR reaction and Restriction digestion procedure with different enzymes, the target sequence was then sequenced by the 1st Base company (Singapore). At the time of sequencing four reactions were initiated with the external and primers of the insert.

3.2.4 LR Reaction:

The following procedure was performed for an LR recombination reaction. For a positive control, 50 ng (1 µl) of pENTR.-gus was used.

1. In a 1.5 ml microcentrifuge tube at room temperature the following components were mixed:

Reagent	Amount Taken
Entry clone	1.0 µl (~ 50ng)
Destination vector	1.0 µl (~ 50ng)
LR Clonase Reaction Buffer	0.5 µl
LR Clonase ll Enzyme mix	0.5 µl (10 ng)
Final Volume	3.0 µl

2. The mixture was mixed thoroughly and spinned and incubated at room temperature for 16 hours.

3. 0.25 ul Proteinase K was added and incubated at 37°C water bath for 10 minutes to terminate the reaction.
4. For total 3.25 ul reaction mix, 41 ul freshly thawed competent cells were added to the tube. Then the "Heat Shock" transformation procedure was followed. But here 50mg/L spectinomycin was used for positive colony selection.
5. Recombination was checked by PCR.

3.2.4.1 Confirmation of LR recombination by PCR:

Materials:

- PCR Mix (contains all the components for PCR)
- Appropriate forward and reverse PCR primers.

- **Forward Primer**

Primer: **OsNHX1_F_1.6**

Sequence	Length	Tm	%GC
CACCATGGGGATGGAGGTGGCG	22	61.5	%68.2

- **Reverse Primer**

Primer: **OsNHX1_R_1.6**

Sequence	Length	Tm	%GC
TCATCTTCCTCCATGGCTCTGC	22	59.0	%54.5

PCR amplification:

Using the mentioned primers, insert was amplified as following reaction condition and PCR Program

10X PCR buffer	2.5µl
10mM dNTP mix	1.0µl
50mM $MgCl_2$	1.8µl
20% DMSO	3.0µl
OsNHX1-Dest-2.3F	2.0µl
OsNHX1-2.3-R	2.0µl
Taq polymerase: pfx(10:1)	0.3µl
Template	1.0µl

PCR program:

Step 1:	95°C for 5 min
Step 2:	95°C for 1 min
Step 3:	61°C for 1 sec
Step 4:	72°C for 2 min
Step 5:	repeat 2 to 4 for 35 cycles
Step 6:	72°C for 15 min

3.2.4.2 Restriction Digestion:

The isolated plasmid pH7WG2.0_OsNHX1_1.6 was further confirmed by restriction digestion with the restriction enzymes HindIII, BamHI and SacI.

Restriction Enzyme	Company	Recognitaion site	Cut site
HindIII	Invitrogen	A^AGCT_T	1
BamHI	Invitrogen	GT^AGCG_CC	1
SacI	Invitrogen	GC^GGCC_GC	2

The appropriate components were taken in PCR tubes and mixed well and then digested at 37^0C for 1 hour. The digested products were resolved in 0.8% agarose gel.

Plasmid DNA	1µl

Reaction buffer 2/Reaction buffer 3	1µl
Restriction Enzyme	0.5µl
PCR H_2O	7.5µl
Total	10.0µl

3.2.5 Transformation into *Agrobacterium:*

Transformation of *Agrobacterium tumifaciens* (LBA4404) with pH7WG2.0_OsNHX1_1.6 by electroporation was done for further experiment with the recombinant vector.

3.2.5.1 Preparation of Electrocompetent Cells

1. A freshly prepared Ym agar plate was streaked with the appropriate strain of *Agrobacterium tumefaciens (LBA4404)* and incubated at 28°C for 48-72 hours.
2. 5.0 ml of Ym liquid media containing kanamycin was inoculated with a single colony of *Agrobacterium tumefaciens (LBA4404)*. Cells were grown overnight at 28°C with shaking at 200-300 rpm.
3. 50.0 ml of Ym broth (Spectinomycin and Streptomycin) in a 250 ml Erlenmeyer flask was inoculated with 5 ml of a log phase (fresh overnight culture) *Agrobacterium tumefaciens* culture.
4. The 50 ml culture was incubated at 28°C overnight (~approx. 16 hrs), with shaking at 200-300 rpm.
5. The cells were decanted into sterile 50.0 ml solvall tubes and pelleted by centrifugation at 4000 x g for 10 min at 4°C.
6. The supernatant was carefully poured off and discarded; the centrifuge tubes with the cell pellets were placed on ice.
7. ~ 5.0 ml of sterile, ice-cold 10% glycerol was added to each tube and the cell pellets were resuspended by pipetting up and down or tapping the tube gently; the volume in each of the centrifuge tubes was brought to 50 ml with sterile cold 10% glycerol. The cells were

pelletted by centrifugation at 4000 x g for 10 min at 4°C; the supernatant was poured off and discarded.

8. The cells were washed again as in the previous step.
9. Each of the cell pellets were resuspended in 500 µl of sterile, ice-cold 10% glycerol and transferred to a chilled 1.5 ml eppendorf tube. The cells were pelletted by centrifugation at 4000 x g for 10 min at 4°C; the supernatant was poured off and discarded.
10. The cell pellet was resuspended in 50 µl of sterile, ice-cold 1 M sorbitol; the final cell volume should be ~ 150 µl and cell concentration should be ~ 5 x 10^{10} cells/ml. 45 µl aliquots of the electrocompetent cells were dispensed in sterile 0.2 ml or 0.5 ml microfuge tubes; the cells were frozen in an isopropanol-dry ice bath, then stored at -70°C. The cells are stable for about 6 months under these conditions.

3.2.5.2 Transformation of electrocompetent *Agrobacterium tumefaciens (LBA*4404) cells with pH7WG2.0_OsNHX1_1.6

1. ~40 µl competent cells to be electroporated were pipetted in two eppendorf tubes and the tubes were kept on ice for two minutes. ~5µl plasmid DNA samples (containing ~1µg DNA) was transferred to one of the above tubes.the tubes were tapped gently.
2. The contents of tubes were transferred to two cuvettes (pre cold on ice). One of the above cuvettes (containing only the competent cells) was labeled as negative control.
3. The Micropulser was set to "Agr" mode.
4. The DNA-cell samples transferred to the electroporation cuvettes was tapped to the bottom of the cuvette. The cuvette was placed in the chamber slide. The slide was pushed into the chamber until the cuvette is seated between the contacts in the base of the chamber. The Pulse button was pressed once.
5. The cuvette was removed from the chamber and ~600µl YM broth media (pre warm at 28^0C in tissue culture Lab.) was added to the cuvette and pipetted ups and down ~4-5 times and immediately transferred to the 1.5 ml microfuge tube.
6. The pulse parameters were checked and recorded. The time constant should be about 5 miliseconds. The field strength can be calculated as actual volts (kV) / cuvette gap (cm).

7. The cells were incubated for 3 hrs at 28°C, shaking at 250 rpm. Plate Aliquots of the electroporated cells were plated on YM agar plates containing the appropriate selective media. The plates were incubated for 48 hrs at 28°C (see section 4.2).

3.2.5.3 Patch Culture of the Eletroporated cells

1. YM agar plates were prepared containing the appropriate antibiotic.

2. Patch culture papers were numbered and bound to plates with tape.

3. Single colonies were then sub-cultured and incubated overnight.

3.2.5.4 Confirmation of *Agrobacterium* transformants by lysate PCR

Procedure

1. 15 µl PCR reaction mixtures were prepared.
2. A single colony was picked up using a pipette tip and mixed with the PCR reaction mixture by pipetting up and down.
3. The tubes were short spinned well.
4. The tubes were set in the PCR machine and the PCR program was run. The initial denaturation step was increased to 10 minutes from 5 minutes.

3.2.5.5 PCR amplification

The insert was amplified using two primer sets as stated above inwhich one is an internal primer set and another one is for whole insert of 1.6 Kbp long.

Primer sets are discussed at section 3.2.4.1 1 and Section 3.2.2.6.1

3.2.5.6 Confirmation of Transformants by restriction digestion

The isolated plasmid pH7WG2.0_OsNHX1_1.6 was further confirmed by restriction digestion with the restriction enzymes SacI and BamH1.The appropriate components were taken in PCR tubes and short spinned and then digested at 37^0C for 1 hour. The digested products were resolved in 0.8% agarose gel . The procedure is given below:

PCR H_2O	6.5 µl	PCR H_2O	6.5 µl
React 2 buffer	1.0 µl	React 3 buffer	1.0 µl
Plasmid DNA	2.0 µl	Plasmid DNA	2.0 µl
Restriction enzyme BamH1	0.5 µl	Restriction enzyme SacI	0.5 µl

CHAPTER 4 : *RESULTS*

Result

4.1 Bioinformatics Analysis:

4.1.1 Target Sequences revealed from data bases:

The available antiporter gene was analyzed with the initial target source of Arabidopsis. The coding sequences of the both antiporter genes (OsNHX1 and AtNHX1) were revealed applying both NCBI and Gramene databases (Section 3.1.1.1 and section 3.1.1.2). Revealed sequences are as follows in Fasta format:

4.1.1.1 Oryza sativa Japonica Group OsNHX1 mRNA, complete CDS:

ATGGGGATGGAGGTGGCGGCGGCGCGGCTGGGGGCTCTGTACACGACCTCCGACTACGCGTCGGTGGTGTCCATCAACCTGTTCGTC
GCGCTGCTCTGCGCCTGCATCGTCCTCGGCCACCTCCTCGAGGAGAATCGCTGGGTCAATGAGTCCATCACCGCGCTCATCATCGGGC
TCTGCACCGGCGTGGTGATCTTGCTGATGACCAAAGGGAAGAGCTCGCACTTATTCGTCTTCAGTGAGGATCTCTTCTTCATCTACC
TCCTCCCTCCGATCATCTTCAATGCAGGTTTTCAGGTAAAGAAAAAGCAATTCTTCCGGAATTTCATGACGATCACATTATTTGGAG
CCGTCGGGACAATGATATCCTTTTTCACAATATCTATTGCTGCCATTGCAATATTCAGCAGAATGAACATTGGAACGCTGGATGTA
GGAGATTTTCTTGCAATTGGAGCCATCTTTTCTGCGACAGATTCTGTCTGCACATTGCAGGTCCTCAATCAGGATGAGACACCCTTT
TTGTACAGTCTGGTATTCGGTGAAGGTGTTGTGAACGATGCTACATCAATTGTGCTTTTCAACGCACTACAGAACTTTGATCTTGT
CCACATAGATGCGGCTGTCGTTCTGAAATTCTTGGGGAACTTCTTTTATTTATTTTTGTCGAGCACCTTCCTTGGAGTATTTGCTGG
ATTGCTCAGTGCATACATAATCAAGAAGCTATACATTGGAAGGCATTCTACTGACCGTGAGGTTGCCCTTATGATGCTCATGGCTT
ACCTTTCATATATGCTGGCTGAGTTGCTAGATTTGAGCGGCATTCTCACCGTATTCTTCTGTGGTATTGTAATGTCACATTACACTT
GGCATAACGTCACAGAGAGTTCAAGAGTTACAACAAAGCACGCATTTGCAACTCTGTCCTTCATTGCTGAGACTTTTCTCTTCCTGT
ATGTTGGGATGGATGCATTGGATATTGAAAAATGGGAGTTTGCCAGTGACAGACCTGGCAAATCCATTGGGATAAGCTCAATTTTG
CTAGGATTGGTTCTGATTGGAAGAGCTGCTTTTGTATTCCCGCTGTCGTTCTTGTCGAACCTAACAAAGAAGGCACCGAATGAAAA
AATAACCTGGAGACAGCAAGTTGTAATATGGTGGGCTGGGCTGATGAGAGGAGCTGTGTCGATTGCTCTTGCTTACAATAAGTTTA
CAAGATCTGGCCATACTCAGCTGCACGGCAATGCAATAATGATCACCAGCACCATCACTGTCGTTCTTTTTAGCACTATGGTATTTG
GGATGATGACAAAGCCATTGATCAGGCTGCTGCTACCGGCCTCAGGCCATCCTGTCACCTCTGAGCCTTCATCACCAAAGTCCCTGC
ATTCTCCTCTCCTGACAAGCATGCAAGGTTCTGACCTCGAGAGTACAACCAACATTGTGAGGCCTTCCAGCCTCCGGATGCTCCTCA
CCAAGCCGACCCACACTGTCCACTACTACTGGCGCAAGTTCGACGACGCGCTGATGCGACCGATGTTTGGCGGGCGCGGGTTCGTGC
CCTTCTCCCCTGGATCACCAACCGAGCAGAGCCATGGAGGAAGATGA

4.1.1.2 Arabidopsis thaliana Na+/H+ antiporter mRNA, complete CDS:

ATGTTGGATTCTCTAGTGTCGAAACTGCCTTCGTTATCGACATCTGATCACGCTTCTGTGGTTGCGTTGAATCTCTTTGTTGCACTT
CTTTGTGCTTGTATTGTTCTTGGTCATCTTTTGGAAGAGAATAGATGGATGAACGAATCCATCACCGCCTTGTTGATTGGGCTAGG
CACTGGTGTTACCATTTTGTTGATTAGTAAAGGAAAAAGCTCGCATCTTCTCGTCTTTAGTGAAGATCTTTTCTTCATATATCTTT
TGCCACCCATTATATTCAATGCAGGGTTTCAAGTAAAAAAGAAGCAGTTTTTCCGCAATTTCGTGACTATTATGCTTTTTGGTGCT
GTTGGACTATTATTTCTTGCACAATCATATCTCTAGGTGTAACACAGTTCTTTAAGAAGTTGGACATTGAAACCTTTGACTTGGGT
GATTATCTTGCTATTGGTGCCATATTTGCTGCAACAGATTCAGTATGTACACTGCAGGTTCTGAATCAAGACGAGACACCTTTGCT
TTACAGTCTTGTATTCGGAGAGGGTGTTGTGAATGATGCAACGTCAGTTGTGGTCTTCAACGCGATTCAGAGCTTTGATCTCACTC
ACCTAAACCACGAAGCTGCTTTTCATCTTCTTGGAAACTTCTTGTATTTGTTTCTCCTAAGTACCTTGCTTGGTGCTGCAACCGGTC
TGATAAGTGCGTATGTTATCAAGAAGCTATACTTTGGAAGGCACTCAACTGACCGAGAGGTTGCCCTTATGATGCTTATGGCGTAT
CTTTCTTATATGCTTGCTGAGCTTTTCGACTTGAGCGGTATCCTCACTGTGTTTTTCTGTGGTATTGTGATGTCCCATTACACATGG
CACAATGTAACGGAGAGCTCAAGAATAACAACAAAGCATACCTTTGCAACTTTGTCATTTCTTGCGGAGACATTTATTTTCCTGTA
TGTTGGAATGGATGCCTTGGACATTGACAAGTGGAGATCCGTGAGTGACACACCGGGAACATCGATCGCAGTGAGCTCAATCCTAA
TGGGTCTGGTCATGGTTGGAAGAGCAGCGTTCGTCTTTCCGTTATCGTTTCTATCTAACTTAGCCAAGAAGAATCAAAGCGAGAAA
ATCAACTTTAACATGCAGGTTGTGATTTGGTGGTCTGGTCTCATGAGAGGTGCTGTATCTATGGCTCTTGCATACAACAAGTTTAC
AAGGGCCGGGCACACAGATGTACGCGGGAATGCAATCATGATCACGAGTACGATAACTGTCTGTCTTTTTAGCACAGTGGTGTTTG
GTATGCTGACCAAACCACTCATAAGCTACCTATTACCGCACCAGAACGCCACCACGAGCATGTTATCTGATGACAACACCCCAAAAT
CCATACATATCCCTTTGTTGGACCAAGACTCGTTCATTGAGCCTTCAGGGAACCACAATGTGCCTCGGCCTGACAGTATACGTGGCT
TCTTGACACGGCCCACTCGAACCGTGCATTACTACTGGAGACAATTTGATGACTCCTTCATGCGACCCGTCTTTGGAGGTCGTGGCT
TTGTACCCTTTGTTCCAGGTTCTCCAACTGAGAGAAACCCTCCTGATCTTAGTAAGGCTTGA

4.1.2 BLAST result of the sequences:

Revealed sequences were blasted by NCBI- BLASTn (Section 3.1.2.1) and observed about 73% of maximum identity between the sequences. Sequence alignments of the two genes are at the Appendix.

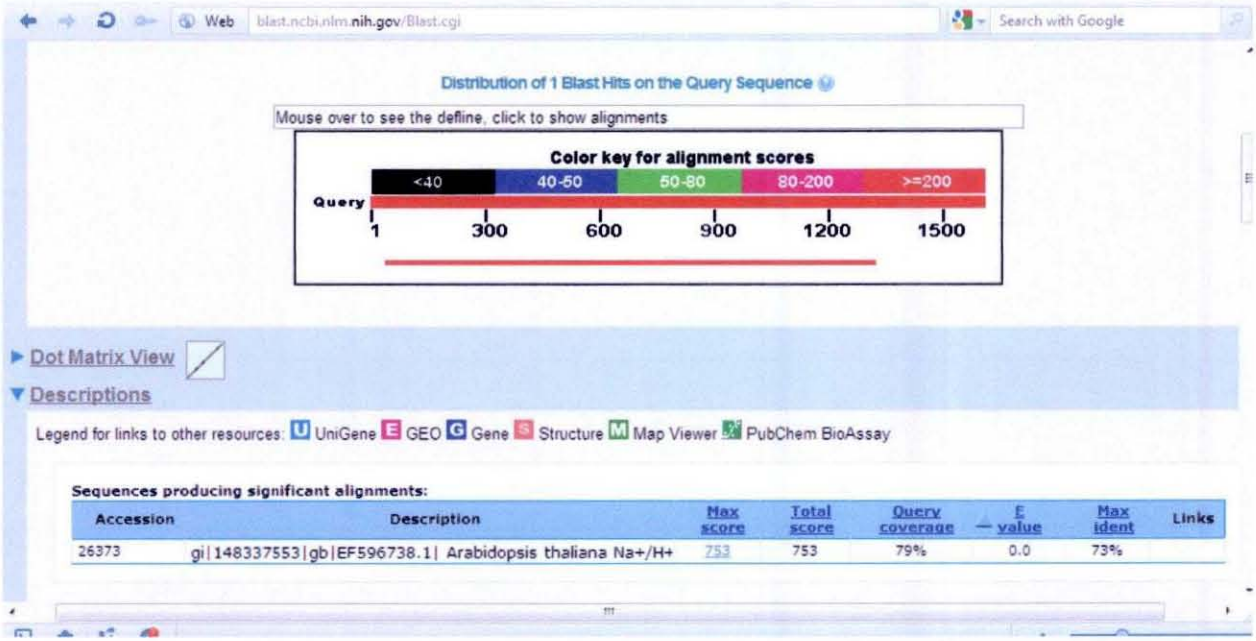


Figure 4.1: BLASTn Result

4.1.3 Conversion of the Nucleotide bases to Protein:

Applying Transeq tool (Section 3.1.2.4) the revealed sequences were then translated into proteins, so that the domain structures at protein level of the two genes would be classified.

Converted amino acid sequences of the genes are as follows:

4.1.3.1 Oryza sativa Japonica Group OsNHX1 mRNA, complete CDS:

MGMEVAAARLGALYTTSDYASVVSINLFVALLCACIVLGHLLEENRWVNESITALIIGLCTGVVILLMTKGKSSHLFVFSEDLFFIYLLP
PIIFNAGFQVKKKQFFRNFMTITLFGAVGTMISFFTISIAAIAIFSRMNIGTLDVGDFLAIGAIFSATDSVCTLQVLNQDETPFLYSLVFGE
GVVNDATSIVLFNALQNFDLVHIDAAVVLKFLGNFFYLFLSSTFLGVFAGLLSAYIIKKLYIGRHSTDREVALMMLMAYLSYMLAELLD
LSGILTVFFCGIVMSHYTWHNVTESSRVTTKHAFATLSFIAETFLFLYVGMDALDIEKWEFASDRPGKSIGISSILLGLVLIGRAAFVFPL
SFLSNLTKKAPNEKITWRQQVVIWWAGLMRGAVSIALAYNKFTRSGHTQLHGNAIMITSTITVVLFSTMVFGMMTKPLIRLLLPASG
HPVTSEPSSPKSLHSPLLTSMQGSDLESTTNIVRPSSLRMLLTKPTHTVHYYWRKFDDALMRPMFGGRGFVPFSPGSPTEQSHGGR*

4.1.3.2 Arabidopsis thaliana Na+/H+ antiporter mRNA, complete CDS:

MLDSLVSKLPSLSTSDHASVVALNLFVALLCACIVLGHLLEENRWMNESITALLIGLGTGVTILLISKGKSSHLLVFSEDLFFIYLLPPIIF
NAGFQVKKKQFFRNFVTIMLFGAVGTIISCTIISLGVTQFFKKLDIETFDLGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSLVFGEGVV

NDATSVVVFNAIQSFDLTHLNHEAAFHLLGNFLYLFLLSTLLGAATGLISAYVIKKLYFGRHSTDREVALMMLMAYLSYMLAELFDLS
GILTVFFCGIVMSHYTWHNVTESSRITTKHTFATLSFLAETFIFLYVGMDALDIDKWRSVSDTPGTSIAVSSILMGLVMVGRAAFVFPL
SFLSNLAKKNQSEKINFNMQVVIWWSGLMRGAVSMALAYNKFTRAGHTDVRGNAIMITSTITVCLFSTVVFGMLTKPLISYLLPHQN
ATTSMLSDDNTPKSIHIPLLDQDSFIEPSGNHNVPRPDSIRGFLTRPTRTVHYYWRQFDDSFMRPVFGGRGFVPFVPGSPTERNPPDL
SKA*

4.1.4 BLAST result of the protein sequences:

Converted amino acid sequences from the both sources were then blasted into BLASTp and observed 96% of maximum identity. The alignments of the two amino acid sequences are at the Appendix.

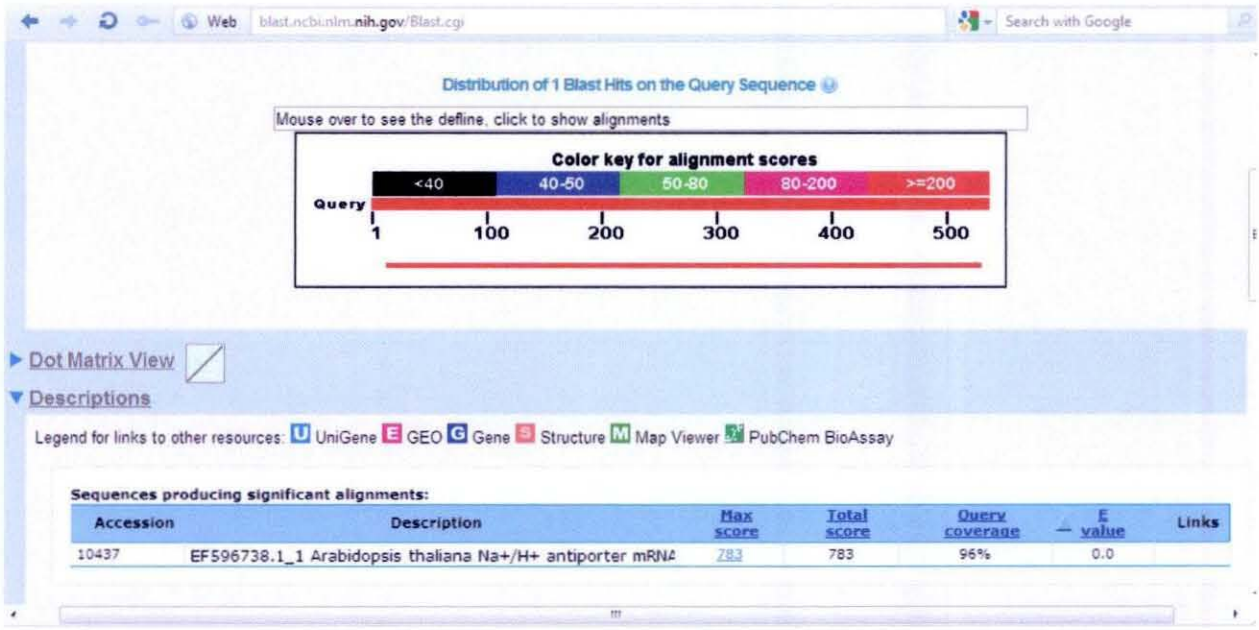


Figure 4.2: BLASTp Result

4.1.5 Domains' clarification:

Converted amino acid bases of the two target genes were then checked out through InterProScan software (Section 3.1.2.3). Protein domains of the nucleotide bases were same and no differences were found in the active site of the both proteins.

4.1.5.1 InterProScan results for the NHX1 gene from Rice:

There were four domains observed. The domains' accession codes are,

1. IPR004709
2. IPR006153
3. IPR01842
4. noIPR

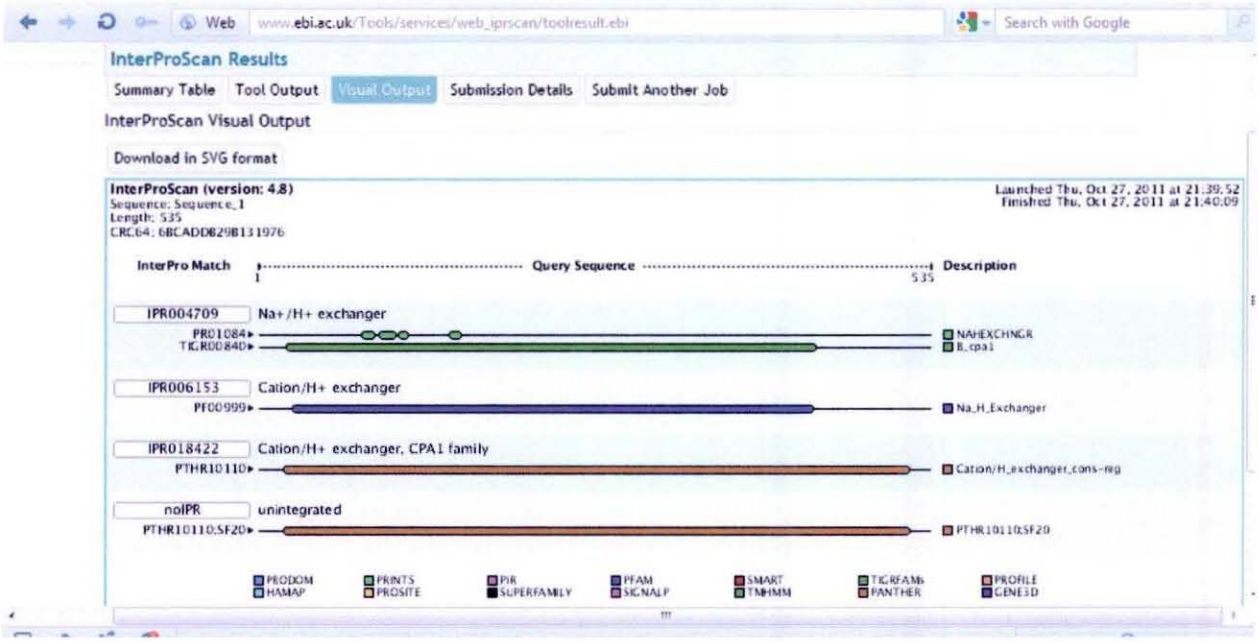


Figure 4.3: Protein domains of the NHX1 genes from Rice

4.1.5.2 InterProScan results for the NHX1 gene from *Arabidopsis*:

Four domains were observed from the amino acid bases of the Antiporter gene from Arabidopsis. These are:

1. IPR004709
2. IPR006153
3. IPR01842
4. noIPR

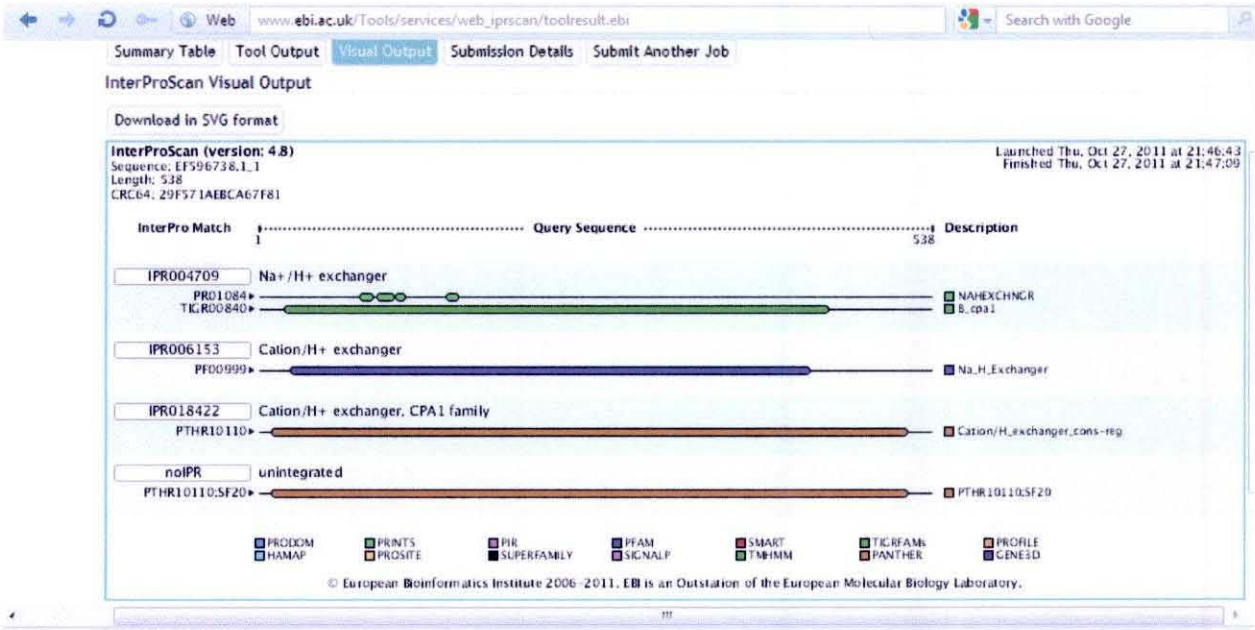


Figure 4.4: Protein domains of the NHX1 genes from *Arabidopsis*

4.1.6 Final Interpretation from the Bioinformatics analysis:

The query sequence was from rice and the ultimate target was to find out the similarities with an antiporter gene (NHX1) from *Arabidopsis*. The query started from blasting both the nucleotide sequences and obtained maximum identity of 73%. Then nucleotide sequences were converted into the amino acid sequences and blasted once again. This time the similarities in protein level were achieved up to the maximum identity of 96%. Then further studies were initiated to check out the domains of the proteins from both the sources and found domains were identical. So, it can be interpreted form all the queries is that the coding sequence of an antiporter gene from rice is almost similar in both the structural and functional activities with the antiporter genes from *Arabidopsis* ,thus the gene from rice can be easily cloned and can be predicted as efficient as the genes from *Arabidopsis*.

4.2 Wet Lab Experiment:

4.2.1 Cloning of coding sequence of the Rice Vacuolar Antiporter Gene:

4.2.1.1 General Overview:

The full length of the vacuolar Na^+/H^+ antiporter gene, *OsNHX1* (NCBI Accession: AB021878) found to play an important role for salinity tolerance in rice (Fukuda et al. 1999).Full length cDNA of this gene has been cloned from rice(Nipponbare) which contains both 5' and 3' UTR with a total length of 2313 bp in size. This study was carried out to clone the coding sequence (1608 bp in size) of the antiporter gene and the coding sequence was amplified from a construct , pENTR_OsNHX1_1.9 (Cloned in Plant Biotechnology Lab, University of Dhaka) , in which the insert was the coding region along with only the 5' UTR(Section 3.2.2.3) .

Desired coding sequence was amplified by PCR with one set of designed primer named as OsNHX1_F_1.6 and OsNHX1_R_1.6 (Section 3.2.2.6.1) . Forward primer contains CACC overhang at 5' end to make sure of the amplified vacuolar antiporter gene to enable directional cloning into pENTR /D-TOPO vector as suggested by invitrogen (Section 3.2.1.5).

4.2.2 Transformation of the source plasmid and confirmation through PCR and Restriction Digestion:

In this study, the plasmid pENTR_OsNHX1_1.9, was transformed into E.coli (DH5α) .After plasmid isolation , the cloned pENTR_OsNHX1_1.9 was then confirmed by PCR and Restriction digestion (Section 3.2.2.3 and Section 3.2.2.1).

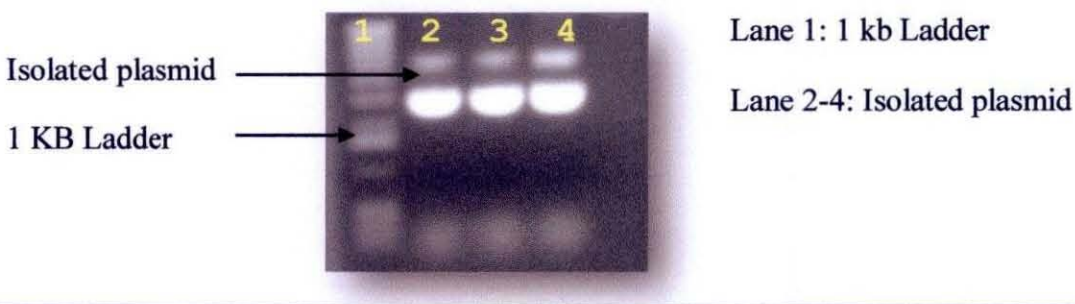


Figure 4.5: Isolated Plasmid of pENTR_OsNHX1_1.9; Lane 1, 2 and indicates plasmid isolated from three different colonies, isolated plasmids were checked running with the DNA marker (1 kb Ladder)

4.2.1 PCR amplification (Desired band size is 1900 bp):

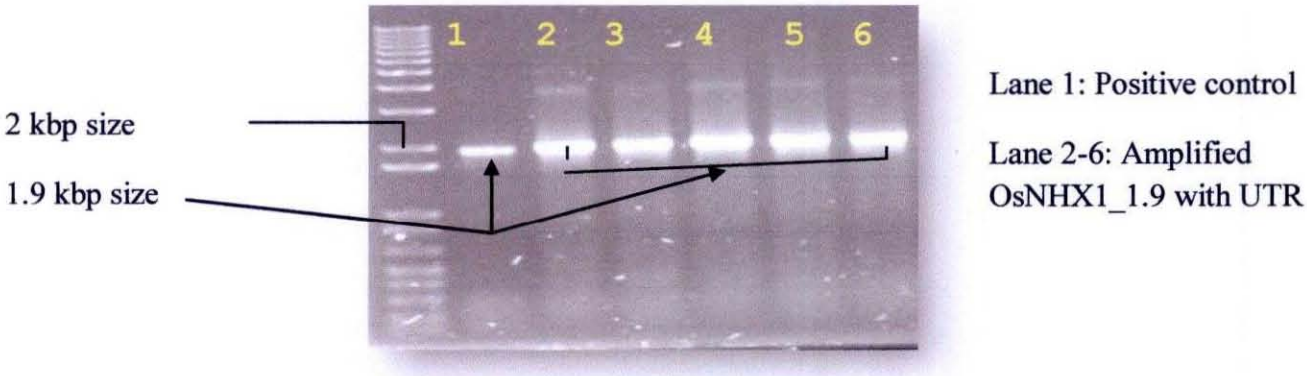
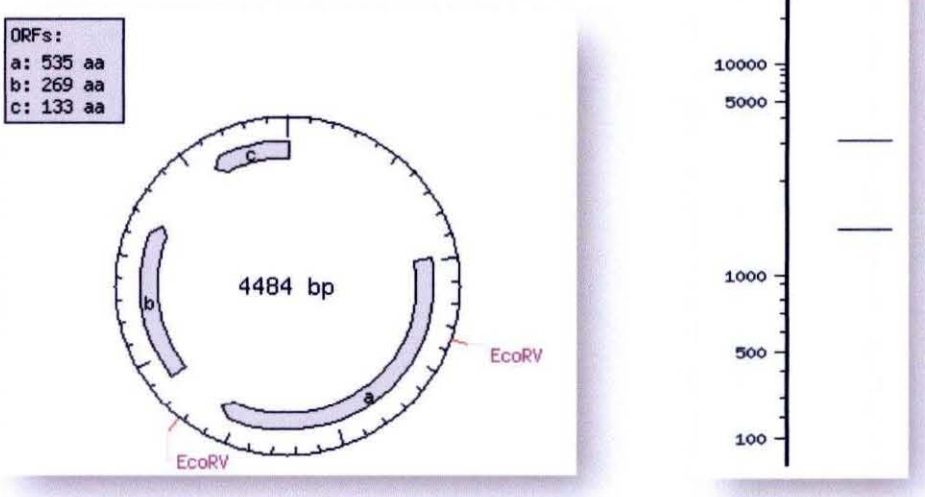


Figure 4.6: PCR amplification for whole insert with 5' UTR (OsNHX1_1.9). Lane 1 is for the positive control and L2- L6 was from isolated plasmid. Amplification came upto the desired band size.

4.2.2.2 Restriction Digestion with pENTR_OsNHX1_1.9:

4.2.2.3 Predicted result for pENTR_OsNHX1_1.9 with the restriction enzyme (EcoRV):



3.1 kbp and 1.3 kbp

4.2.3. 2. Digested photo with EcoRV:

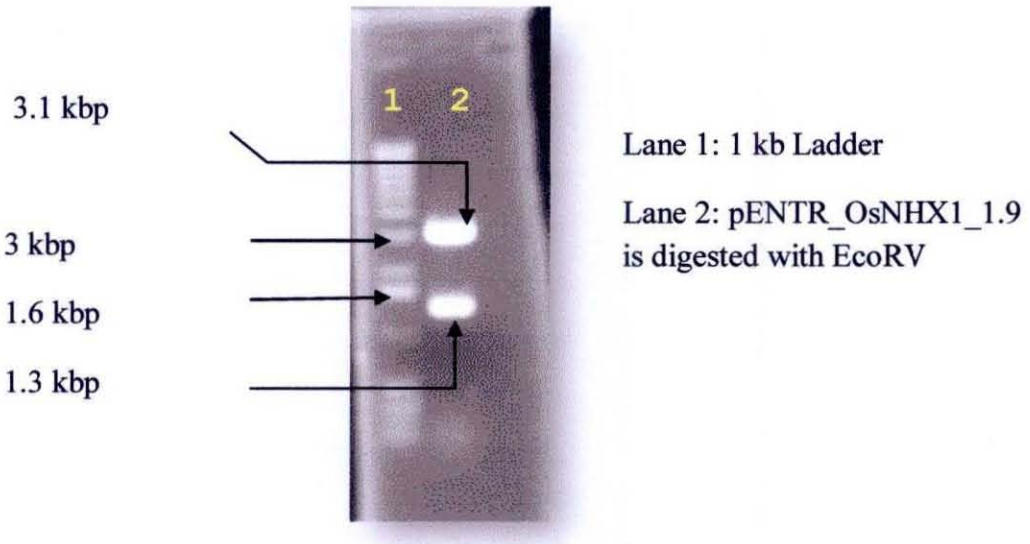


Figure 4.7: pENTR_OsNHX1_1.9 is digested with EcoRV and found bands exactly matched with the predicted band size. L1 is the ladder and the L2 is the sample cut with EcoRV

4.2.4 Amplification of the target sequence:

After confirmation by both PCR and Restriction Digestion, the target sequence was to be amplified with newly designed primer set.Different PCR programming with various parameters was performed to do the amplification and the optimized temperature and PCR cycle was taken as a standard proceudure to amplify the target sequence in every step for the later PCR programming (Section 3.2.2.3).

After the amplification, gel extraction of the PCR product was done to make sure the purity of the target sequence and this step was also strongly recomended by Invitrogen while setting up for a Directional D/TOPO cloning reaction. Amplified DNA was then extracted and visualisation of DNA was performed by electrophoresing it through 0.8% Agarose gel, by which the exact size of my desired product was confirmed using 1kb Ladder DNA as a marker (Section 3.2.3).

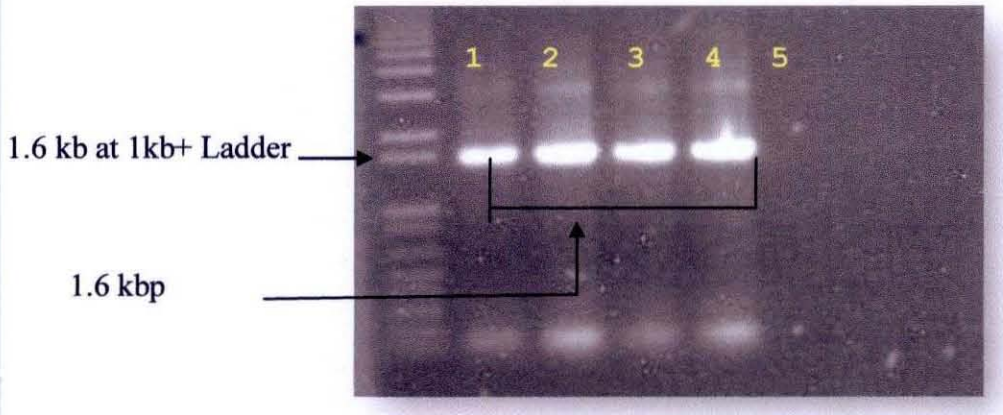


Figure 4.8: PCR amplification with the newly designed primer for only the coding sequence of OsNHX1 (1608 bp in size) . L1-4 is the sample with the insert and L5 indicates negative control without sample.

4.2.4.1 Gel purification Photo of the amplified sequence:

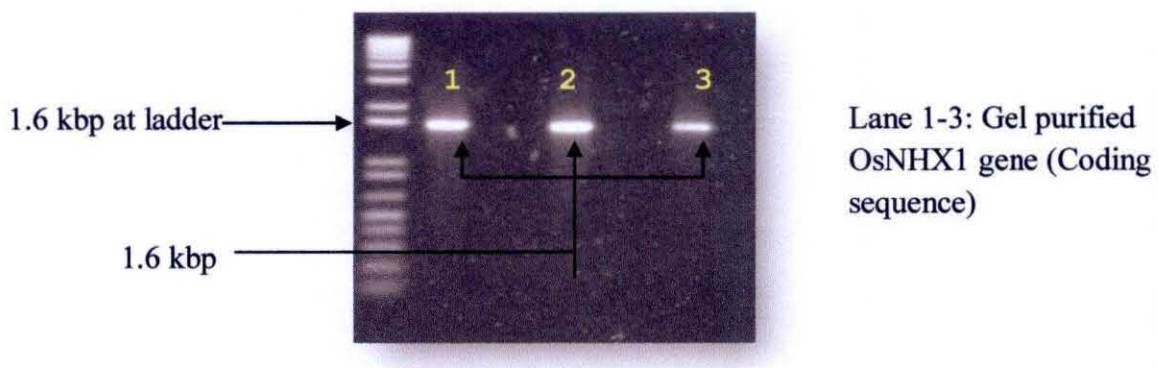


Figure 4.9: Target sequence after gel Purification. Lane 1-3 indicates gel purified photo at the desired size compared with a 1 kb+ ladder's 1.6 kbp position

4.2.5 Cloning into the pENTR/D/TOPO cloning vector and confirmation by PCR and Restriction Digestion:

A cloning reaction was then set up with the empty pENTR vector; the reaction was kept for 30 mins in room temperature and then transformed that into better efficient strain of *E.coli* (DH5α) following heat shock method. After overnight of incubation at 37 degree celsius, a few colonies were obtained in the transformed plate (Section 3.2.3.3).

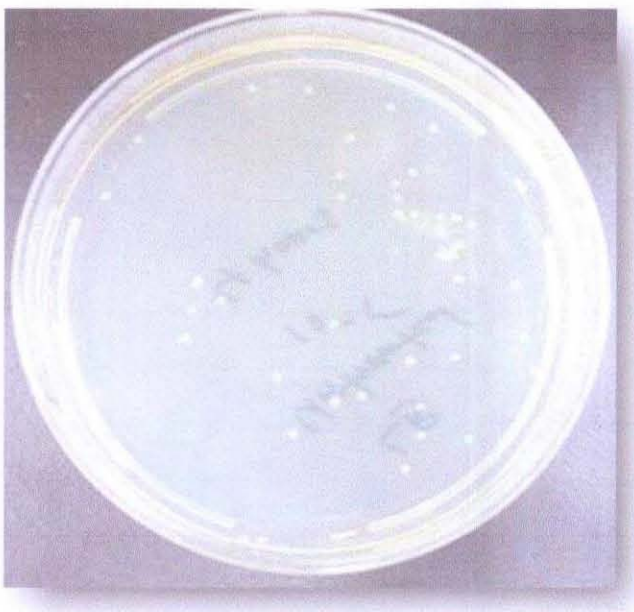


Figure 4.10: pENTR_OsNHX1_1.6 transformed bacterial colony on LB/Kanamycin plate

4.2.6 PCR with two sets of primers after plasmid isolation:

The following day, after transformation, patch culture was done with the obtained colonies. Then plasmid was isolated for further molecular screening. PCR reaction was performed with two set of Primers, one set was for the exact insert and another was vector specific primer which can be stated as external primer (vector specific) (Section 3.2.3.4) in terms of insert as the forward

primer and the reverse primer will be just outside of the insertion site of the foreign sequence of the Entry vector (pENTR).

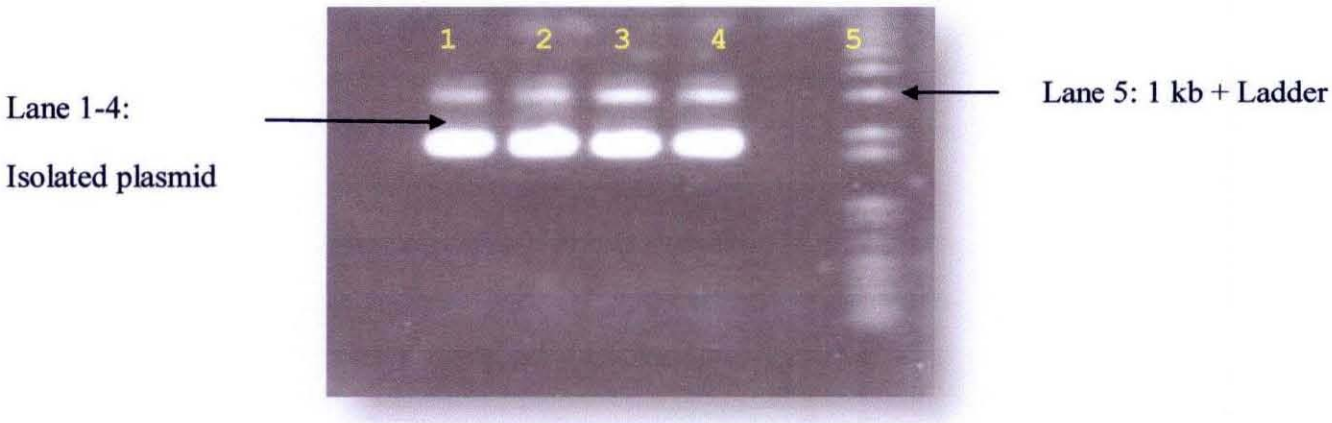


Figure 4.11: Isolated plasmid from the cloned pENTR with the coding sequence of OsNHX1 (pENTR_OsNHX1_1.6)

4.2.6.1 PCR for the whole insert:

Primers only for the coding region of the antiporter gene (OsNHX1) used to detect the insertion of the gene properly by amplifying the target sequence. Optimized PCR programming was used to see the amplification. After PCR programming, PCR products were visualized in 0.8% of agarose gel and sucessfully confirmed the insertion of the targeted sequence into the pENTR vector (Section 3.2.3.6).

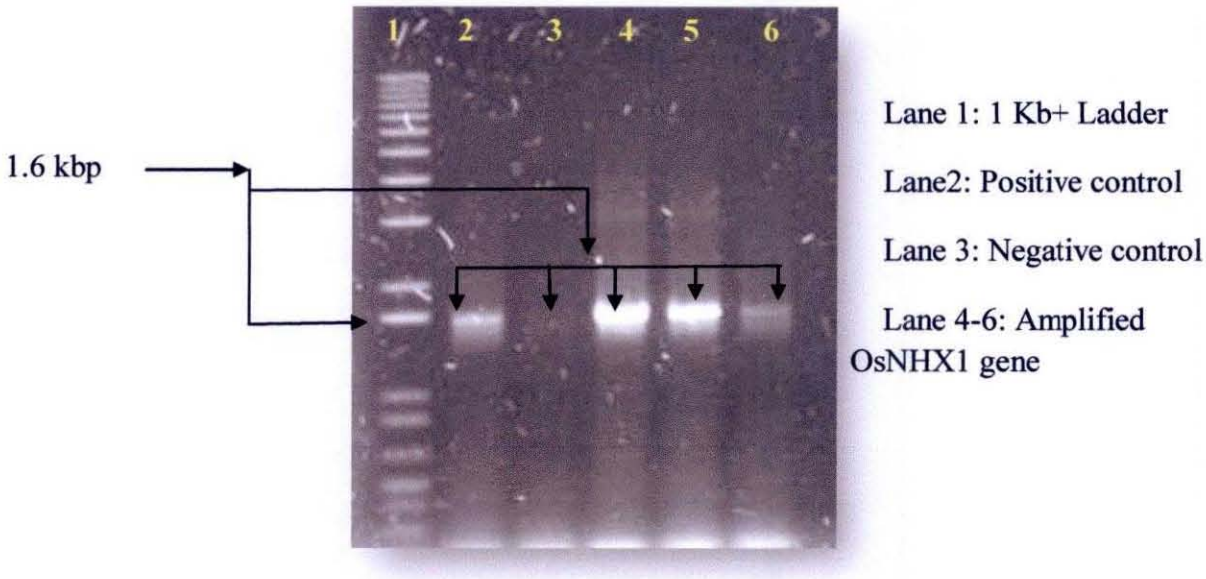


Figure 4.12: PCR product with the whole insert primer

4.2.6.2 PCR with the external primer in terms of insert:

A vector specific primer set was then used, which could amplify only approximately 300 bp if no inserts get incorporated into the pENTR vector. But if the sequence gets incorporated into the pENTR vector then the amplified product will be visualized according to the sum of the size of insert and the usual amplification of 300 bp DNA. So, the sequence is expected at 1900bp sized band.

Result

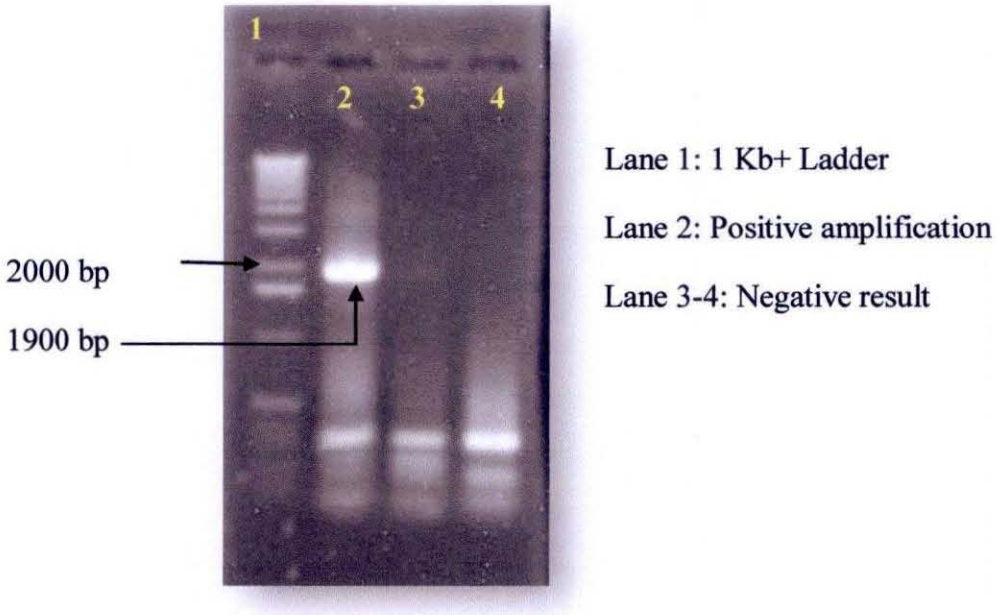


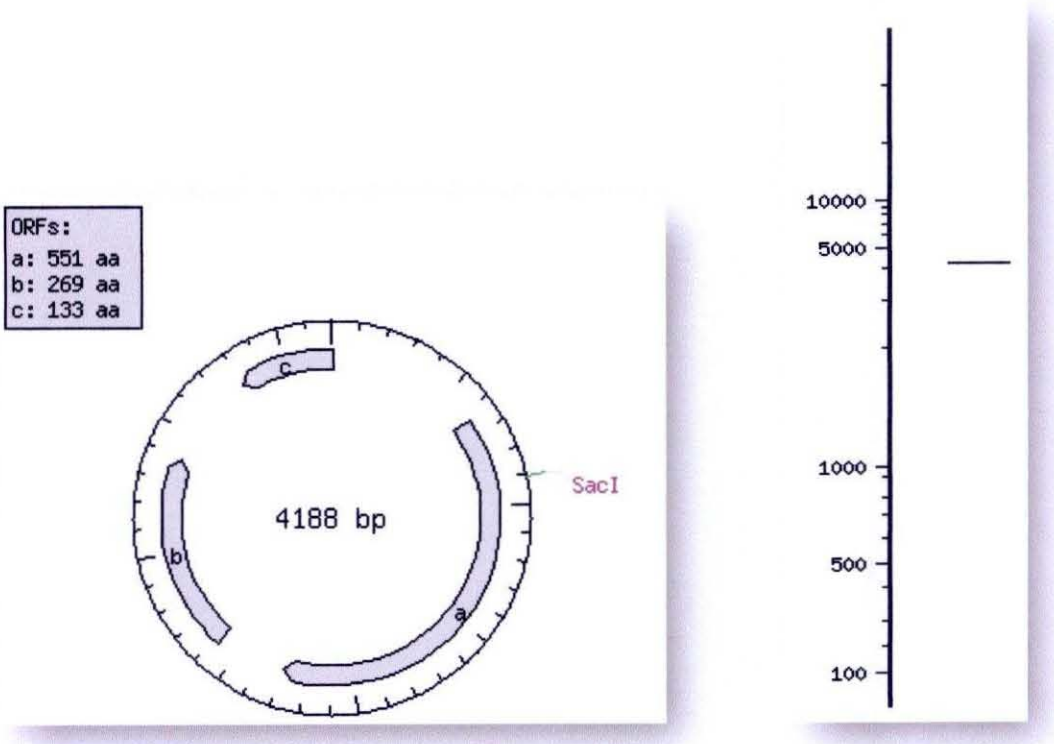
Figure 4.13: PCR with the M13 Forward and M13 Reverse primer; Band size is exactly as predicted with the insert size of 1608 bp.

4.2.7 Restriction Digestion with isolated plasmid (pENTR_OsNHX1_1.6):

After a successful amplification of the desired sequence , Restriction digestion with two different enzymes were done, one was EcoRV with two cutting site in which one was in the insert and another was in the vector and the other one was SacI with a single cutting site .

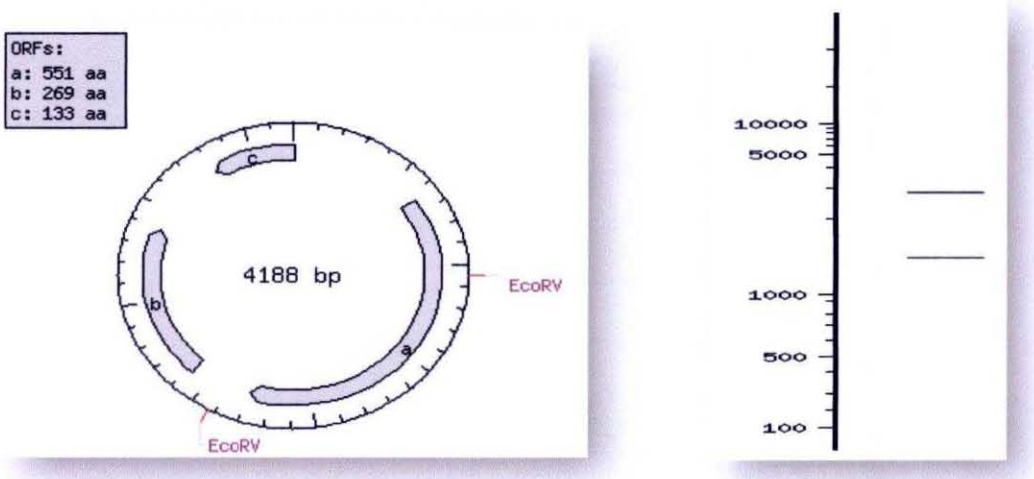
Result

4.2.7.1 Predicted result for pENTR_OsNHX1_1.6 with the restriction enzyme (SacI):



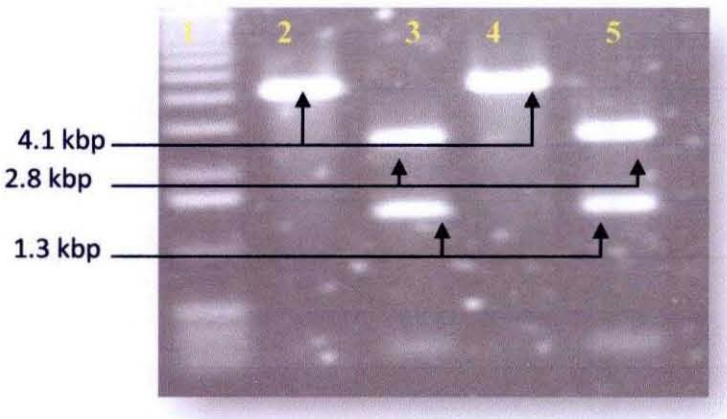
4.18 kbp

4.2.7.2 Predicted result for pENTR_OsNHX1_1.6 with the restriction enzyme (EcoRV):



2.8 kbp and 1.3 kbp

4.2.7.3 Digested photo with SacI and EcoRV respectively:



Lane 1: 1 kb Ladder

Lane 2, 4: Cut with SacI (Single cut)

Lane 3, 5: Cut with EcoRV (Double cut)

Figure 4.14: pENTR_OsNHX1_1.6 is digested with SacI and EcoRV, found bands exactly matched with the predicted band size.

4.2.8 Sequencing of the insert from pENTR_OsNHX1_1.6:

After confirmation through PCR ans Restriction Digestion, sequencing of the cloned pENTR was done by 1st Base Company applying four reactions , two was for the forward reaction and others were for the reverse reaction to check out the quality of the insert. Every corresponding forward and reverse primer made overlap and found sequenced bases were blasted in the NCBI Blast and found 100% similarities with the desired sequence (Section 3.2.3.8).

Table4.1:

Sequencing primers used to sequence OsNHX1 insert from pENTR_OsNHX1_1.6

Primer name	Sequence (gene specific)	Length (bp)	Tm	%GC
OsNHX1_F	CACCATGGGGATGGAGGTGGCG	22	61.5	%68.2
OsNHX1_R	TCATCTTCCTCCATGGCTCTGC	22	59.0	%54.5
Primer name	**Sequence(Vector specific)**	**Length (bp)**	**Tm**	**%GC**
M13_F	TGTAAAACGACGGCCAGT	18	54.4	50%
M13_R	CAGGAAACAGCTATGAC	17	47.0	47.1%

By using M13 primer, the complete sequence (1 to 1608 bp) of OsNHX1 was obtained and found an overlap between two primers of 5 bp.The insert specific primer also covered the whole sequences of the insert in which a overlap sequence of 557bp was observed. The results of aligning the sequencing run with that of the published sequence of OsNHX1 cDNA which are given in Appendix A.

Then the sequence similarity was analyzed with published NCBI sequence database using the BLAST tool. The BLAST result showed 100% similarities. As a result, the sequence of pENTR_OsNHX1_1.6 was accepted & further used for LR Recombination with Destination Vector (pH7WG2.0). Blast result of the obtained sequence through the sequencing has been provided in Appendix.

4.2.9 LR reaction and Molecular screening:

After successful confirmation of cloning into pENTR , LR reaction to transfer the target sequence into the Destination Vector (pH7WG2.0) was performed, features and vector map of the destination vector are discussed in the third chapter. Before performing LR reaction fresh plasmids from the both cloned pENTR (pENTR_OsNHX1_1.6) and Destination Vector (pH7WG2.0) were isolated using the isolation kit from promega. Then visualizing plasmids after running through 0.8% agarose gel and checking the quantitation of the plasmids, LR reaction was performed and kept the reaction for 16 hours at 28 degree celsius temperature (Section 3.2.4).

4.2.9.1 Plasmid isolation from the both Destination vector and the Entry vector:

Lane 1: 1 kb Ladder

Lane 2: Lambda DNA (100ng)

Lane 3, 5: pENTR_OsNHX1_1.6

Lane 4, 6: pH7WG2.0

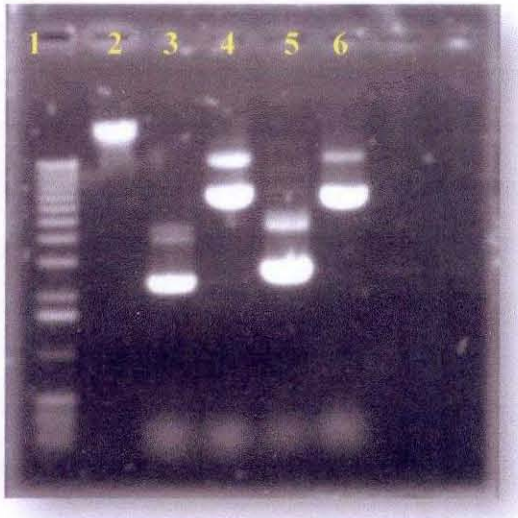


Figure 4.15: Plasmid isolated from both the Destination vector (pH7WG2.0) and the Entry vector (pENTR_OsNHX1_1.6)

4.2.9.2 Transformation into E.coli after the LR reaction:

Result

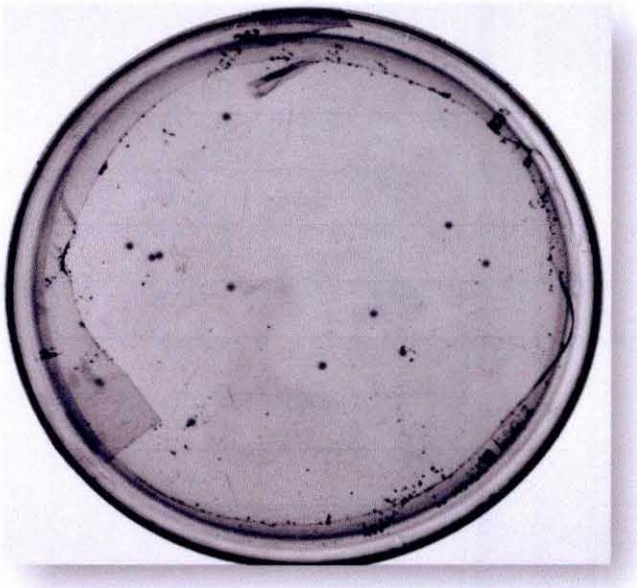


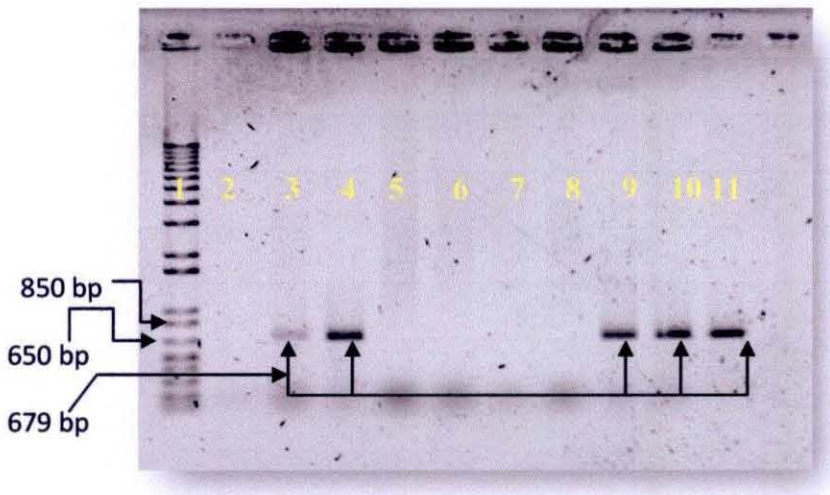
Figure 4.16: Colonies after LR reaction and transformation into *E.coli* in LB media containing Spectinomycin and streptomycin antibiotic

Then the reacted plasmids were transformed into *E.coli* and putative colonies were taken for further molecular screening.

4.2.9.3 Lysate PCR with obtained colonies:

Result

Obtained colonies after transformation were done with patch culture first and prepared the cell lysate from the patch culture and performed PCR with an internal primer of the whole insert (Section 3.2.3.1).



Lane 1: 1kb+ Ladder

Lane 2: Negative control

Lane 3-10: Lysate PCR from different colonies

Lane 11: Positive colony

Figure 4.17 :Lyaste PCR result shows confirmation of the insert in a few colonies

4.2.9.4 PCR and Restriction Digestion with the Lysate PCR confirmed colonies:

A few colonies gave positive result in the lysate PCR and that found colonies were then inoculated into the liquid media and plasmid was isolated and confirmed through PCR and Restriction Digestion.

Result

Lane 1: 1 kb Ladder

Lane 2: Isolated plasmid (pH7WG2.0_OsNHX1_1.6)

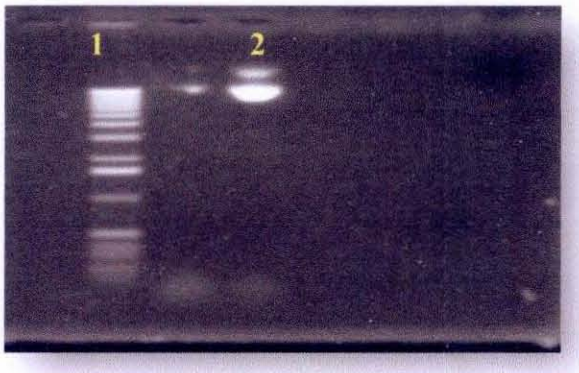
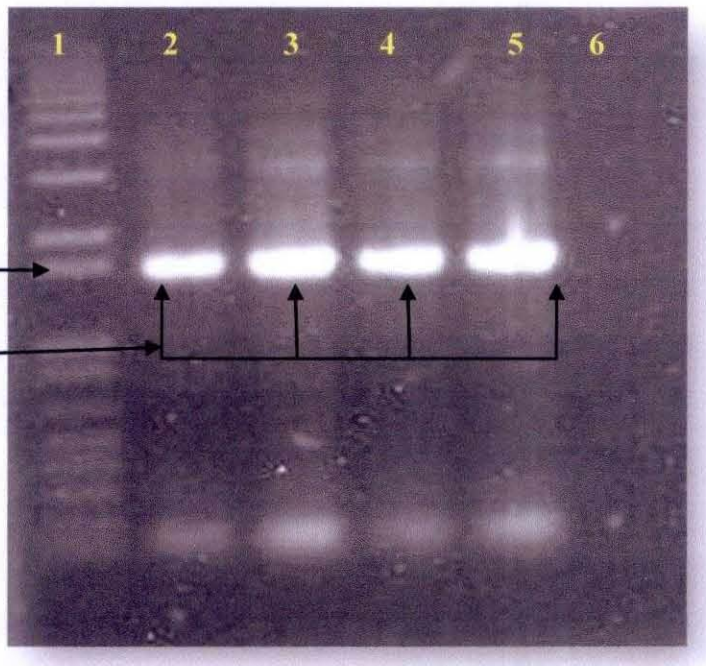


Figure 4.18: Isolated Plasmid of the Destination vector after doing Lysate PCR confirmation from the LR reacted transformed plate.

4.2.9.5 PCR Photo with the insert specific primers:



1.6 kbp (1kb+ Ladder)

1.6 kbp

Lane 1: 1 kb+ Ladder

Lane 2-5: Amplified OsNHX1 gene

Lane 6: Negative control

Figure 4.19: PCR confirmation with the insert specific primer from the isolated plasmid after doing LR reaction

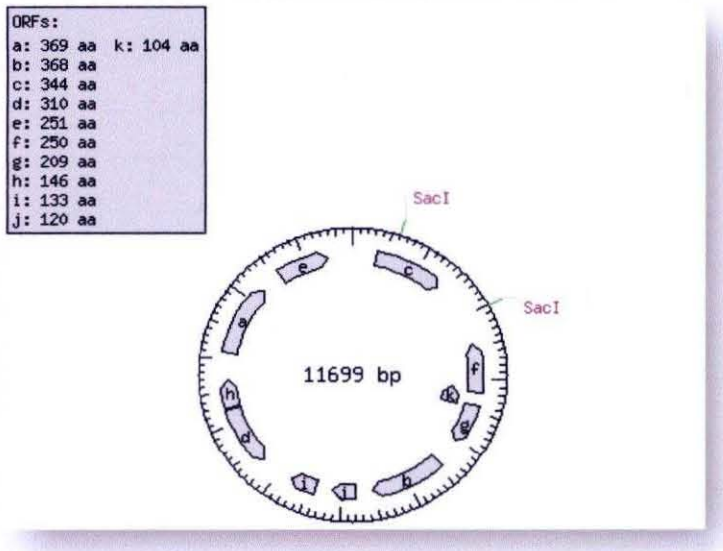
Result

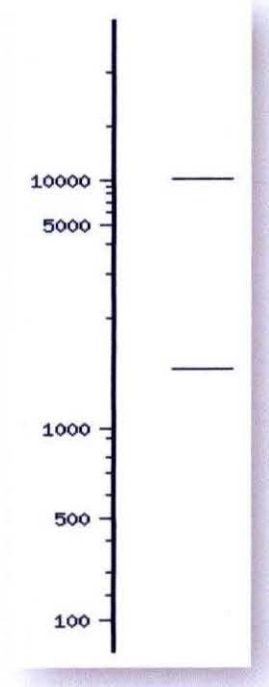
4.2.9.6 Restriction digestion of the destination vector with the insert:

4.2.9.6.1 Predicted result with the SacI and HindIII respectively:

SacI has got double cut if the insert properly gets incorporated with the destination vector. Among its two cutting site , one site is in the insert and other one is in the vector. On the other hand HindIII has got three cutting site in the empty destination vector (without insert) while if the insert get attached with the destination vector then there should be only one cutting side in the vector.

4.2.9.6.1.1 Predicted result with cutting site with SacI:

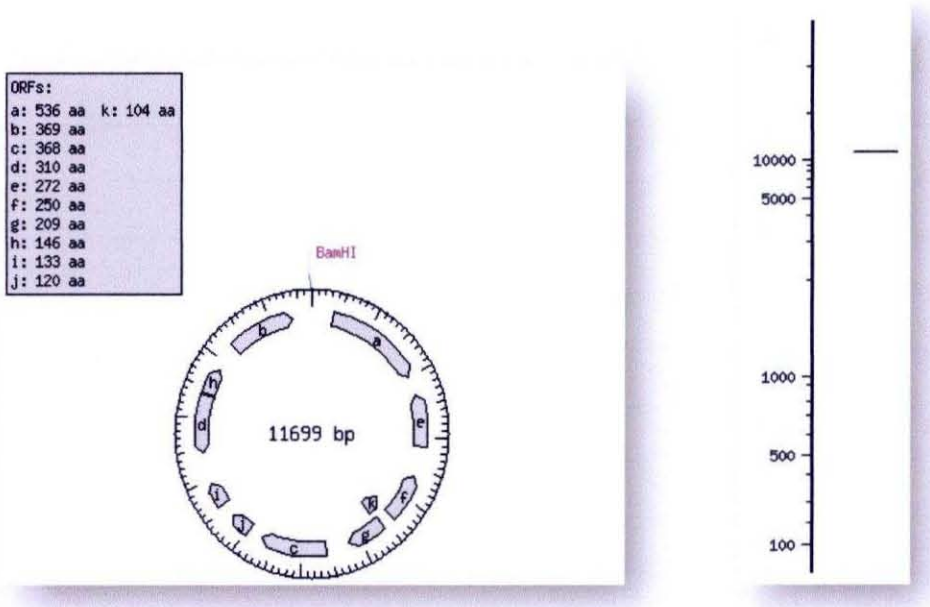




10.2 kbp and 1.4 kbp

4.2.9.6.3 Predicted cutting site with HindIII:

Result



11.6 kbp

4.2.9.6.4 Digested with HindIII and SacI respectively:

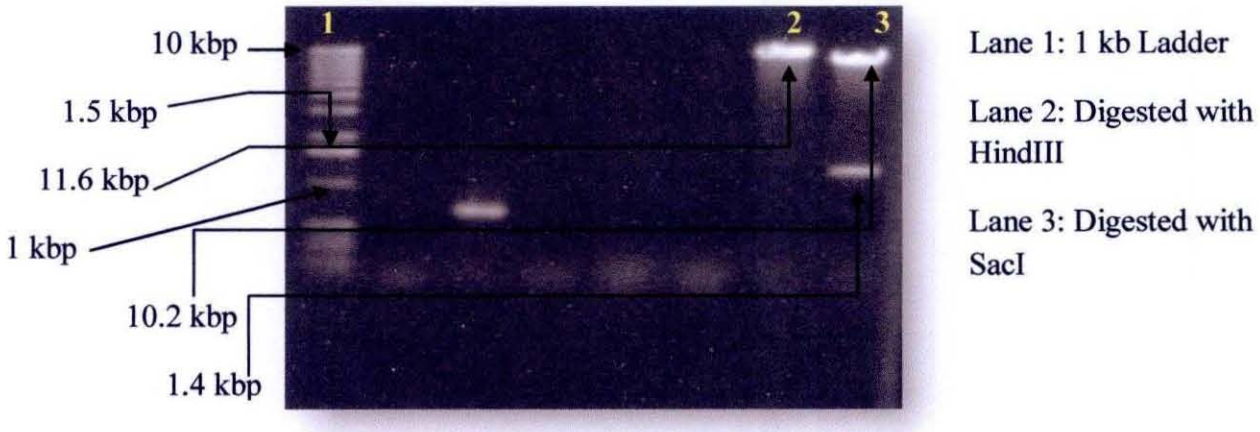


Figure 4.20: Digested with HindIII and SacI. Lane 3 is the cutting result with SacI that came up as predicted and then lane 2 is the result by HindIII.

4.2.10 Transformation into *Agrobacterium* and Molecular Screening:

After confirmation of successful recombination, the recombinnat plasmids were transfered into *Agrobacterium* applying the electroporation method. After 48 hours of incubation at 28 degree celsius temperature, obtained colonies were taken to do the molecular screening to confer the successful insertion of the recombinant plasmid into the *Agrobacterium*. Firstly, found colonies were checked with lysate PCR and performed the PCR for the whole insert later and finally performed restriction digestion for further confirmation (Section 3.2.3.6).

4.2.10.1 Trasformed plate after trasforming into *Agrobacterium*:

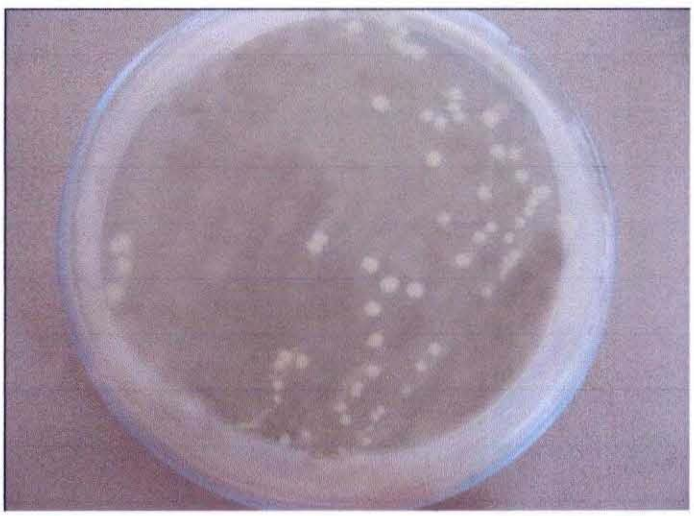


Figure 4.21: Trasformed plate in *Agrobacterium* with the recombinant vector pH7WG2.0_OsNHX1_1.6 in YM media containing Spectinomycin and streptomycin antibiotic

4.2.10.2 Isolated plasmid from the transformed *Agrobacterium*:

Lane 1: 1 kb Ladder

Lane 2-3: Isolated plasmid from Transformed *Agrobacterium*

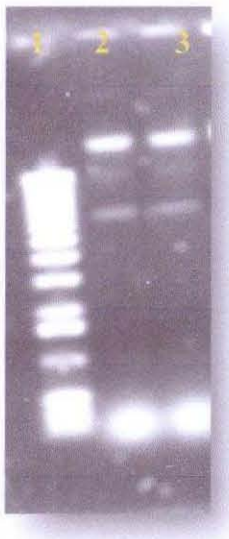


Figure 4.22: Plasmid isolated from the transofrmed *Agrobacterium*

4.2.10.3 PCR result with the whole insert primers:

After isolating plasmid from the transformed Agrobaterium, PCR for the whole insert was done and received confirmation of the insertion of the gene properly.

Lane 1: 1 kb+ Ladder

Lane 2: Positive control

Lane 3: Negative control

Lane 4, 6: Amplified OsNHX1 gene

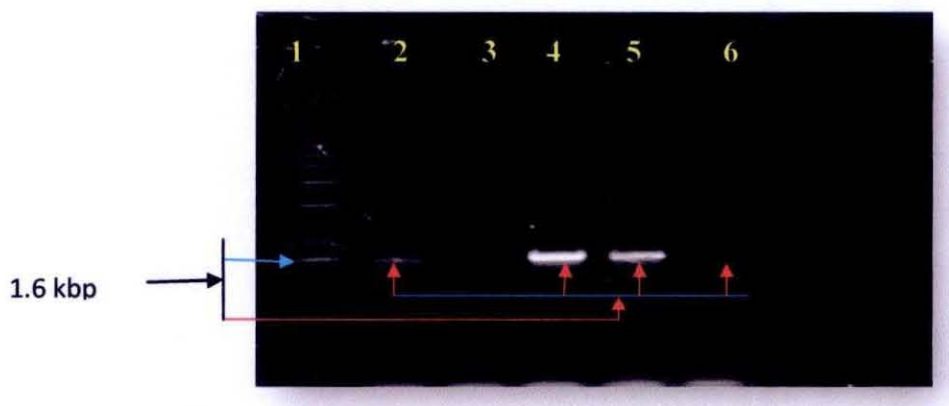


Figure 4.23: PCR confirmation for the target sequence form the isolated plasmid from *Agrobacterium*

CHAPTER 5 : *DISCUSSION*

Discussion:

Environmental stresses like salinity in the coastal region of Bangladesh has been a major problem for decreasing crop production. However, due to heavy pressure of population problem, it has been a fundamental requirement to establish an ideal structure of plant to face all the stresses and to secure food security. However, Ideal Plant Architecture (IPA), a new term has been introduced for ensuring food security by enhancing all required characteristics to face all challenges like biotic and abiotic stresses during the growth and development of the plants.

Salinity is now accounted as the most important issue of concern and a few approaches have already been initiated with some novel methods like molecular breeding and cloning to improve salt tolerance in plants. Sodium proton anti-porter (NHX), Hydrogen potassium transporter (HKT), osmolytes like Trehalose, mannitol, sorbitol production etc are among the candidates for molecular cloning approach.

Conferring salt tolerance in tomato by incorporating a vacuolar transporter from Arabidopsis has been reported in Zhang et al. Primary target of this study was to clone that same gene. However, there was a problem with the availability of *Arabidopsis* seeds at the beginning and later the cDNA preparation was also unsuccessful, so that the amplification of the desired sequence from target plant could not be performed. Instead, the available antiporter at PBT Lab, University of Dhaka (www.pbtlabdu.net) was taken as a possible source of the target gene for the cloning procedure. The available construct was cloned with an antiporter gene from rice and contained 5′ UTR. The UTR portion was proved not to play an important role for expressing the target gene. Then further studies with the nucleotide sequences of NHX1 gene from both rice and *Arabidopsis* were carried out to check the identities along with the qualities of the sequences. Then sequences were blasted applying NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast) and observed 73% of identities. Conversion of the nucleotide bases to protein was performed then using EBI Protein Conversion Tool (http://www.ebi.ac.uk/Tools/emboss/transeq). Converted amino acid bases were then again blasted and identities achieved to 96%. Then the presences of protein domains were checked by InterProScan tool (http://www.ebi.ac.uk/Tools/pfa/iprscan) and the predicted domains for both sequences were identical. So, it can be interpreted form all the queries is that the coding sequence of an antiporter gene from rice is almost similar in both

the structural and functional activities with the antiporter genes from *Arabidopsis* ,thus the gene from rice can be easily cloned and can be predicted as efficient as the genes from *Arabidopsis* (Section 4.1).

Moreover, Overexpression of vacuolar Na^+/H^+ antiporters (NHX) has been reported to confer salt tolerance in a transgenic approach to dicotyledonous plants like brassica and tomato. For example, the vacuolar Na^+/H^+ antiporter gene, OsNHX1, has been cloned from rice and found to play an important role to confer salt tolerance of rice (Fukuda et al. 1999). Constructs with the antiporter gene (OsNHX1) have been prepared in the Plant Biotechnology lab of Seraj, ZI (www.pbtlabdu.net), which have been be used to over express rice vacuolar antiporter OsNHX1 cDNA sequence after further manipulation (Rasul,2005). One such construct was pENTR_OsNHX1_1.9 containing the 5′ UTR with the coding sequence of the vacuolar antiporter gene.

Gateway cloning technology was applied to clone the target sequence. Firstly, amplified sequence was cloned into pENTR and confirmed through PCR, Restriction Digestion and Sequencing. Then target sequence was then recombined into a destination vector (pH7WG2.0).Finally, constructed vector was transformed into *Agrobacterium* and insertion was also confirmed applying PCR and Restriction Digestion.

CHAPTER 6 : *CONCLUSION*

Conclusion

- Cloning of the coding sequence of vacuolar antiporter gene into pENTR was a bit difficult. A few cloning reaction protocol needed to be followed. After a few hurdles cloning into pENTR was successful. Right clone was confirmed through the PCR, Restriction Digestion and sequencing technique. Finally, it can be concluded that the Directional TOPO cloning reaction was successful.

- The gene of interest was recombined from the pENTR to the Destination vector (pH7WG2.0). The recombination was also successfully screened by PCR and Restriction Digestion.

- Finally the recombinant vector was transformed into the *Agrobacterium* for further experiment. The transformation also confirmed by PCR and Restriction Digestion.

CHAPTER 7 : *REFERENCES*

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APPENDIX

Appendix

Neocleotide sequence of pENTR

>pENTR/D-TOPO, 2580 bp

CTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAG
TCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCG
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TTCTGCTTAGTTTGATGCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCACAACGTTCAAATCCGCTCCCGGCGGAT
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pENTR with OsNHX1 -1.9

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Appendix

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TGCAATAATGATCACCAGCACCATCACTGTCGTTCTTTTTAGCACTATGGTATTTGGGATGATGACAAAGCCATTGATCAGGCTGC
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Appendix

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Appendix

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cgaagtagcgcgtctgctgctccatacaagccaaccacggcctccagaagaagatgttggcgacctcgtattgggaa
tccccgaacatcgcctcgctccagtcaatgaccgctgttatgcggccattgtccgtcaggacattgttggagccgaa
tccgcgtgcacgagtgccggacttcggggcagtcctcgcccaaagcatcagctcatcgagagcctgcgcgacggacg
cactgacggtgtcgtccatcacagtttgccagtgatacacatggggatcagcaatcgcgcatatgaaatcacgccat
gtagtgtattgaccgattccttgcggtccgaatgggccgaacccgctcgtctggctaagatcggccgcagcgatagc
atccatagcctccgcgaccggctgaagaacagcgggcagttcggtttcaggcaggtcttgcaacgtgacaccctgtg
cacggcgggagatgcaataggtcaggctctcgctgaactccccaatgtcaagcacttccggaatcgggagcgcggcc
gatgcaaagtgccgataaacataacgatctttgtagaaaccatcggcgcagctatttacccgcaggacatatccacg
ccctcctacatcgaagctgaaagcacgagattcttcgccctccgagagctgcatcaggtcggagacgctgtcgaact
tttcgatcagaaacttctcgacagacgtcgcggtgagttcaggctttttcatagggggattcgagttgagagtgaa
tatgagactctaattggataccgaggggaatttatggaacgtcagtggagcatttttgacaagaaatatttgctagc
tgatagtgaccttaggcgacttttgaacgcgcaataatggtttctgacgtatgtgcttagctcattaaactccagaa

acccgcggctcagtggctccttcaacgttgcggttctgtcagttccaaacgtaaaacggcttgtcccgcgtcatcgg
cggggtcataacgtgactcccttaattctcatgtatgataattcgagggtacccggggatcctctagagggcc

Sequencing result of the pENTR_with OsNHX1 gene:

Coding seq of the antiporter OsNHX1 (1608 bp)

sequenced with the forward M13, Reverse M13, OsNHX1_F, OsNHX1_R

ATGGGGATGGAGGTGGCGGCGGCGCGGCTGGGGGCTCTGTACACGACCTCCGACTACGCGTCGGTGGTGTCCATCAACCTGTTCGT
CGCGCTGCTCTGCGCCTGCATCGTCCTCGGCCACCTCCTCGAGGAGAATCGCTGGGTCAATGAGTCCATCACCGCGCTCATCATCG
GGCTCTGCACCGGCGTGGTGATCTTGCTGATGACCAAAGGGAAGAGCTCGCACTTATTCGTCTTCAGTGAGGATCTCTTCTTCATC
TACCTCCTCCCTCCGATCATCTTCAATGCAGGTTTTCAGGTAAAGAAAAAGCAATTCTTCCGGAATTTCATGACGATCACATTATT
TGGAGCCGTCGGGACAATGATATCCTTTTTCACAATATCTATTGCTGCCATTGCAATATTCAGCAGAATGAACATTGGAACGCTGG
ATGTAGGAGATTTTCTTGCAATTGGAGCCATCTTTTCTGCGACAGATTCTGTCTGCACATTGCAGGTCCTCAATCAGGATGAGACA
CCCTTTTTGTACAGTCTGGTATTCGGTGAAGGTGTTGTGAACGATGCTACATCAATTGTGCTTTTCAACGCACTACAGAACTTTGA
TCTTGTCCACATAGATGCGGCTGTCGTTCTGAAATTCTTGGGGAACTTCTTTTATTTATTTTTGTCGAGCACCTTCCTTGGAGTAT
TTGCTGGATTGCTCAGTGCATACATAATCAAGAAGCTATACATTGGAAGGCATTCTACTGACCGTGAGGTTGCCCTTATGATGCTC
ATGGCTTACCTTTCATATATGCTGGCTGAGTTGCTAGATTTGAGCGGCATTCTCACCGTATTCTTCTGTGGTATTGTAATGTCACA
TTACACTTGGCATAACGTCACAGAGAGTTCAAGAGTTACAACAAAGCACGCATTTGCAACTCTGTCCTTCATTGCTGAGACTTTTC
TCTTCCTGTATGTTGGGATGGATGCATTGGATATTGAAAAATGGGAGTTTGCCAGTGACAGACCTGGCAAATCCATTGGGATAAGC
TCAATTTTGCTAGGATTGGTTCTGATTGGAAGAGCTGCTTTTGTATTCCCGCTGTCGTTCTTGTCGAACCTAACAAAGAAGGCACC
GAATGAAAAAATAACCTGGAGACAGCAAGTTGTAATATGGTGGGCTGGGCTGATGAGAGGAGCTGTGTCGATTGCTCTTGCTTACA
ATAAGTTTACAAGATCTGGCCATACTCAGCTGCACGGCAATGCAATAATGATCACCAGCACCATCACTGTCGTTCTTTTTAGCACT
ATGGTATTTGGGATGATGACAAAGCCATTGATCAGGCTGCTGCTACCGGCCTCAGGCCATCCTGTCACCTCTGAGCCTTCATCACC
AAAGTCCCTGCATTCTCCTCTCCTGACAAGCATGCAAGGTTCTGACCTCGAGAGTACAACCAACATTGTGAGGCCTTCCAGCCTCC
GGATGCTCCTCACCAAGCCGACCCACACTGTCCACTACTACTGGCGCAAGTTCGACGACGCGCTGATGCGACCGATGTTTGGCGGG
CGCGGGTTCGTGCCCTTCTCCCCTGGATCACCAACCGAGCAGAGCCATGGAGGAAGATGA

Sequence covered by M13F

ATGGGGATGGAGGTGGCGGCGGCGCGGCTGGGGGCTCTGTACACGACCTCCGACTACGCGTCGGTGGTGTCCATCAACCTGTTCGTCGCGC
TGCTCTGCGCCTGCATCGTCCTCGGCCACCTCCTCGAGGAGAATCGCTGGGTCAATGAGTCCATCACCGCGCTCATCATCGGGCTCTGCACCG
GCGTGGTGATCTTGCTGATGACCAAAGGGAAGAGCTCGCACTTATTCGTCTTCAGTGAGGATCTCTTCTTCATCTACCTCCTCCCTCCGATCAT
CTTCAATGCAGGTTTTCAGGTAAAGAAAAAGCAATTCTTCCGGAATTTCATGACGATCACATTATTTGGAGCCGTCGGGACAATGATATCCTTT
TTCACAATATCTATTGCTGCCATTGCAATATTCAGCAGAATGAACATTGGAACGCTGGATGTAGGAGATTTTCTTGCAATTGGAGCCATCTTTT
CTGCGACAGATTCTGTCTGCACATTGCAGGTCCTCAATCAGGATGAGACACCCTTTTTGTACAGTCTGGTATTCGGTGAAGGTGTTGTGAACG
ATGCTACATCAATTGTGCTTTTCAACGCACTACAGAACTTTGATCTTGTCCACATA

Sequence Covered by M13R

CCACATAGATGCGGCTGTCGTTCTGAAATTCTTGGGGAACTTCTTTTATTTATTTTTGTCGAGCACCTTCCTTGGAGTATTTGCTGGATTGCTCA
GTGCATACATAATCAAGAAGCTATACATTGGAAGGCATTCTACTGACCGTGAGGTTGCCCTTATGATGCTCATGGCTTACCTTTCATATATGCT
GGCTGAGTTGCTAGATTTGAGCGGCATTCTCACCGTATTCTTCTGTGGTATTGTAATGTCACATTACACTTGGCATAACGTCACAGAGAGTTCA
AGAGTTACAACAAAGCACGCATTTGCAACTCTGTCCTTCATTGCTGAGACTTTTCTCTTCCTGTATGTTGGGATGGATGCATTGGATATTGAAA
AATGGGAGTTTGCCAGTGACAGACCTGGCAAATCCATTGGGATAAGCTCAATTTTGCTAGGATTGGTTCTGATTGGAAGAGCTGCTTTTGTAT
TCCCGCTGTCGTTCTTGTCGAACCTAACAAAGAAGGCACCGAATGAAAAAATAACCTGGAGACAGCAAGTTGTAATATGGTGGGCTGGGCTG
ATGAGAGGAGCTGTGTCGATTGCTCTTGCTTACAATAAGTTTACAAGATCTGGCCATACTCAGCTGCACGGCAATGCAATAATGATCACCAGC
ACCATCACTGTCGTTCTTTTTAGCACTATGGTATTTGGGATGATGACAAAGCCATTGATCAGGCTGCTGCTACCGGCCTCAGGCCATCCTGTCA
CCTCTGAGCCTTCATCACCAAAGTCCCTGCATTCTCCTCTCCTGACAAGCATGCAAGGTTCTGACCTCGAGAGTACAACCAACATTGTGAGGCC

TTCCAGCCTCCGGATGCTCCTCACCAAGCCGACCCACACTGTCCACTACTACTGGCGCAAGTTCGACGACGCGCTGATGCGACCGATGTTTGG
CGGGCGCGGGTTCGTGCCCTTCTCCCCTGGATCACCAACCGAGCAGAGCCATGGAGGAAGATGA

M13 primers OVERLAP

CCACAT

Sequence covered by OsNHX1 Forward

ACGACCTCCGACTACGCGTCGGTGGTGTCCATCAACCTGTTCGTCGCGCTGCTCTGCGCCTGCATCGTCCTCGGCCACCTCCTCGAGGAGAAT
CGCTGGGTCAATGAGTCCATCACCGCGCTCATCATCGGGCTCTGCACCGGCGTGGTGATCTTGCTGATGACCAAAGGGAAGAGCTCGCACTT
ATTCGTCTTCAGTGAGGATCTCTTCTTCATCTACCTCCTCCCTCCGATCATCTTCAATGCAGGTTTTCAGGTAAAGAAAAAGCAATTCTTCCGGA
ATTTCATGACGATCACATTATTTGGAGCCGTCGGGACAATGATATCCTTTTTCACAATATCTATTGCTGCCATTGCAATATTCAGCAGAATGAA
CATTGGAACGCTGGATGTAGGAGATTTTCTTGCAATTGGAGCCATCTTTTCTGCGACAGATTCTGTCTGCACATTGCAGGTCCTCAATCAGGAT
GAGACACCCTTTTTGTACAGTCTGGTATTCGGTGAAGGTGTTGTGAACGATGCTACATCAATTGTGCTTTTCAACGCACTACAGAACTTTGATC
TTGTCCACATAGATGCGGCTGTCGTTCTGAAATTCTTGGGGAACTTCTTTTATTTATTTTTGTCGAGCACCTTCCTTGGAGTATTTGCTGGATTG
CTCAGTGCATACATAATCAAGAAGCTATACATTGGAAGGCATTCTACTGACCGTGAGGTTGCCCTTATGATGCTCATGGCTTACCTTTCATATA
TGCTGGCTGAGTTGCTAGATTTGAGCGGCATTCTCACCGTATTCTTCTGTGGTATTGTAATGTCACATTACACTTGGCATAACGTCACAGAGAG
TTCAAGAGTTACAACAAAGCACGCATTTGCAACTCTGTCCTTCATTGCTGAGACTTTTCTCTTCCTGTATGTTGGGATGGATGCATTGGATATT
GAAAAATGGGAGTTTGCCAGTGACAGACCTGGCAAATCCATTGGGATAAGCTCAATTTTGCTAGGATTGGTTCTGATTGGAA

Sequence covered by OsNHX1 Reverse

TGCACATTGCAGGTCCTCAATCAGGATGAGACACCCTTTTTGTACAGTCTGGTATTCGGTGAAGGTGTTGTGAACGATGCTACATCAATTGTG
CTTTTCAACGCACTACAGAACTTTGATCTTGTCCACATAGATGCGGCTGTCGTTCTGAAATTCTTGGGGAACTTCTTTTATTTATTTTTGTCGAG
CACCTTCCTTGGAGTATTTGCTGGATTGCTCAGTGCATACATAATCAAGAAGCTATACATTGGAAGGCATTCTACTGACCGTGAGGTTGCCCTT
ATGATGCTCATGGCTTACCTTTCATATATGCTGGCTGAGTTGCTAGATTTGAGCGGCATTCTCACCGTATTCTTCTGTGGTATTGTAATGTCACA
TTACACTTGGCATAACGTCACAGAGAGTTCAAGAGTTACAACAAAGCACGCATTTGCAACTCTGTCCTTCATTGCTGAGACTTTTCTCTTCCTGT
ATGTTGGGATGGATGCATTGGATATTGAAAAATGGGAGTTTGCCAGTGACAGACCTGGCAAATCCATTGGGATAAGCTCAATTTT

OVERLAP between the OsNHX1 Primers

TGCACATTGCAGGTCCTCAATCAGGATGAGACACCCTTTTTGTACAGTCTGGTATTCGGTGAAGGTGTTGTGAACGATGCTACATCAATTGTG
CTTTTCAACGCACTACAGAACTTTGATCTTGTCCACATAGATGCGGCTGTCGTTCTGAAATTCTTGGGGAACTTCTTTTATTTATTTTTGTCGAG
CACCTTCCTTGGAGTATTTGCTGGATTGCTCAGTGCATACATAATCAAGAAGCTATACATTGGAAGGCATTCTACTGACCGTGAGGTTGCCCTT
ATGATGCTCATGGCTTACCTTTCATATATGCTGGCTGAGTTGCTAGATTTGAGCGGCATTCTCACCGTATTCTTCTGTGGTATTGTAATGTCACA
TTACACTTGGCATAACGTCACAGAGAGTTCAAGAGTTACAACAAAGCACGCATTTGCAACTCTGTCCTTCATTGCTGAGACTTTTCTCTTCCTGT
ATGTTGGGATGGATGCATTGGATATTGAAAAATGGGAGTTTGCCAGTGACAGACCTGGCAAATCCATTGGGATAAGCTCAATTTT

BLAST Result with the found Sequences