

FUNCTIONAL STUDY OF CERTAIN GENES SELECTED FROM SSH LIBRARY
OF COMMON BEAN UNDER SALINITY STRESS

by

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ABSTRACT

FUNCTIONAL STUDY OF CERTAIN GENES SELECTED FROM SSH LIBRARY OF COMMON BEAN UNDER SALINITY STRESS

Considering that one third of Earth's arable lands are affected by soil salinity, understanding how plants respond to salinity stress and creation of stress tolerant crops is of outmost importance. *Phaseolus vulgaris* (common bean) is a major legume crop produced extensively around the world and in Turkey, but it is also one of the most salt susceptible crops. We aimed to functionally characterize candidate genes selected from subtractive cDNA libraries enriched in salt responsive genes and also from previous studies on candidate genes that are reported to confer enhanced tolerance upon overexpression. Seven candidate genes from the SSH library and eleven candidate genes from literature were selected. Seven candidate genes from literature and three candidate genes from the SSH library were confirmed to be differentially expressed at a significant level in leaf tissues of Ispir and TR43477 varieties upon salt treatment. Three candidate genes from literature, Na⁺-H⁺ antiporter (*NHX*), superoxide dismutase (*SOD*), and plasma membrane integral protein (*PIP*) together with one gene from the SSH library, vacuolar H⁺-ATPase (*pvVHP*) were cloned from common bean and constitutively overexpressed in wild type (*Col-0*) *Arabidopsis thaliana* lines following floral dip transformation. Furthermore, T-DNA insertional knock out lines for predicted *Arabidopsis* orthologs were also transformed with the same four candidate genes (*pvNHX*, *pvSOD*, *pvPIP* and *pvVHP*). Salt stress treatments of knock out lines for all four of the candidate genes and overexpression lines for *NHX*, *SOD*, and *VHP* have not revealed any phenotypic difference in salinity tolerance compared to wild type. However, two independent *PIP* overexpression lines (*PIP412-8* and *PIP412-10*) have shown significantly greater fresh weight and leaf area only under control conditions but under none of the treatment conditions. Further experiments such as creation of overexpression lines with confirmed overexpression at the protein level and quantitative comparison of overexpressed transcript levels with respect to native orthologues are needed to draw firmer conclusions for functional effects of the analyzed candidate genes.

ÖZET

TUZ STRESİ ALTINDAKİ FASÜLYE BİTKİSİNE AİT SSH KÜTÜPHANESİNDEN SEÇİLEN BAZI GENLERİN FONKSİYONEL ANALİZİ

Dünyanın ekilebilir alanlarının yaklaşık üçte birinin toprak tuzluluğunun etkisi altında olduğu düşünüldüğünde, bitkilerin tuz stresine nasıl cevap verdiğinin anlaşılması ve strese dayanıklı bitkilerin üretilmesi yüksek önem taşımaktadır. *Phaseolus vulgaris* (fasülye) önemli bir baklagil olup Türkiye dahil Dünya'nın birçok ülkesinde yoğun bir şekilde üretilmektedir. Bu yaygınlığının yanında aynı zamanda tuz stresine en duyarlı bitkilerden bir tanesidir. Bu çalışmadaki amacımız tuza duyarlı genler bakımından zengin eksiltilmiş cDNA kütüphanelerinden (SSH) ve overekspresyonunun tuza dayanıklılık sağladığı bildirilen genler arasından seçilmiş fasülye aday genlerinin fonksiyonel karakterizasyonudur. SSH kütüphanesinden 7 aday gen ve literatürden 11 aday gen seçilmiştir. Literatürdeki çalışmalardan seçilen 11 aday gen arasından 7'sinin, ve SSH kütüphanesinden seçilen 7 aday gen arasından 3 tanesinin anlatımının tuz stresini uygulaması sonucu fasülye yaprak dokusunda anlamlı bir biçimde değiştiği belirlenmiştir. Literatürden seçilen, $\text{Na}^+\text{-H}^+$ Değiştiricisi (*NHX*), Superoksit Dismutaz (*SOD*), ve plazma membran dahili protein (*PIP*); SSH kütüphanesinden ise koful $\text{H}^+\text{-ATPaz}$ (*VHP*) fasülye yaprak dokusundan klonlanmış ve floral dip yöntemi ile daimi overekspresyon yapan *Arabidopsis thaliana* hatları oluşturulmuştur. Bunun yanında aday genlerin tahmini Arabidopsis ortologlarının T-DNA knock out mutant hatları elde edilmiştir. Dört aday genin nakavt hatları ve *NHX*, *SOD*, ve *VHP* overekspresyon hatlarının tuz stresini tolerans seviyelerinde transforme olmamış bitkilere göre anlamlı bir farklılık gözlenmemiştir. İki bağımsız *PIP* overekspresyon hattında (*PIP412-8* ve *PIP412-10*) tuz stresini uygulaması sonucunda sadece stressiz kontrol koşullarında transforme olmamış bitkilere göre anlamlı seviyede daha yüksek yaş ağırlık ve yaprak alanı ölçülmüştür. Tüm stres koşullarında ise anlamlı bir fark görülmemiştir. İncelenmiş olan aday genlerin tuz stresindeki fonksiyonel etkileri üzerine daha sağlam çıkarımlara ulaşmak için ilave deneyler gerekmektedir.

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LIST OF SYMBOLS

bp	Base Pairs
Cl ⁻	Chloride
g	Gravity
gr	Gram
K ⁺	Potassium
kb	Kilobase
kDa	Kilodalton
L	Liter
M	Molar
mA	Milliamper
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
ng	Nanogram
Na ⁺	Sodium
°C	Centigrade degree
s	Second
V	Volt
μg	Microgram
μl	Microliter

LIST OF ACRONYMS / ABBREVIATIONS

APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
CaCl ₂	Calcium chloride
cDNA	Complementary DNA
C-terminal	Carboxyl terminal
ddH ₂ O	Double-distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
ECL	Enhanced Chemiluminescent Solution
EDTA	Ethylenediaminetetraacetat
EtOH	Ethanol
gDNA	Genomic DNA
HRP	Horse radish peroxidase
LB	Luria-Bertani broth
MgCl ₂	Magnesium chloride
mRNA	Messenger RNA
N-terminal	Amino terminal
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TAE	Tris acetic acid

TBS	Tris-buffered saline
TBST	Tris Buffered Saline with Tween
T-DNA	Transfer DNA
TEMED	N,N',N',N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tween	Polysorbate
U	Units (enzyme units)
UV	Ultra Violet

1. INTRODUCTION

1.1. Abiotic Stress and *Phaseolus vulgaris* (Common Bean) as a Salt Sensitive Crop

By 2050, earth will host 9.1 billion people, which means a population increment of 34% compared to today. Projections show that to feed 9.1 billion people in 2050, the overall food production need to increase by 70%. Majority (90%) of this increase is expected to come from increases in crop yield and cropping intensity whereas only 10% from increases in arable land. Average annual crop yield is expected to continue growing at 0.8%, a slower rate than the historical average of 1.7%. Advances in agricultural research and development are key to achieving food security by 2050 (FAO, 2009). A chief obstacle in increasing crop yields is abiotic and biotic stresses. Generally, stress can be defined as all abiotic (salinity, drought, heat, acidity etc.) and biotic (virus, bacteria, fungus, herbivores etc) elements that cause a reduction in plants ability to convert energy to biomass. These factors may occur simultaneously with each other or in isolation at different periods of plant growth. It is predicted that only 3.5% of Earth's land is not affected by one of the abiotic stress factors (Cramer *et al.*, 2011).

Among abiotic stresses, soil salinity is defined as soil having an electrical conductivity (EC) of 4dS/m or 40mM NaCl and it affects around 380 million hectares of land, which comprises around one third of total arable land (Brown, 2008). Majority of soil salinity is due to natural factors, including salt accumulation over long time periods in arid and semiarid areas, weathering of rocks, oceanic salts carried by wind and rain (Munns and Tester, 2008). However, it is thought that 60 million hectares are affected by salinity directly as a result of over-irrigation, which causes the raised water tables to carry underground minerals, especially NaCl, to the surface (FAO, 2003).

There is great diversity in the level at which different crops are affected by soil salinity. A small group of plants called “halophytes”, comprising approximately 1% of all plant species, has the ability to grow on extremely saline conditions such as 300-

400mM NaCl (Garthwaite *et al.*, 2005). *Phaseolus vulgaris* (common bean), on the other hand, is a salt sensitive legume crop that is referred as a glycophyte and even at soils with an EC of 3.6 dS/m, 50 % decrease in yield occurs (Ayers and Westcot, 1994). Common bean plays a significant role in human nutrition being a major source of dietary protein and representing a rich source of minerals and certain vitamins. It is also a useful crop for reclamation of marginal lands since it increases soil nitrogen content due to the nitrogen fixation ability of legumes (Alexander, 1984). Therefore, common bean is one of the most extensively produced grain legume worldwide. According to FAOSTAT (2014), 23.5 million tons of dry bean and 20.7 million tons of green bean was produced in 2012 worldwide over 29.2 million ha and 1.5 million ha respectively. Turkey is among the top ten producers of common bean with an annual production of 200,000 tons of dry bean and 614,965 tons of green bean.

1.2. Molecular Mechanisms of Salinity Tolerance in Plants

Common observations in plants under salinity stress are reduction in water content, loss of turgor, closure of stomata, wilting, reduction in rate of photosynthesis, reduction in cell division and growth. Some of these observations, such as stomata closure, reduction in photosynthesis rate, and reduction in cell division and growth rate, are actually defensive measures initiated by the plant to prevent further damage, which also lead to reduced growth and yield. Wilting, loss of turgor, and reduction in water content and leaf area are direct consequences of the salinity stress (Cowan, 1982). The amount of stress the plants experience is usually expressed with respect to plant survival, CO₂ assimilation, water status, biomass, leaf area etc. (Grimes, 1977, Verslues *et al.*, 2006).

An important feature of salinity stress is that it exerts its detrimental effects on plant growth in two distinct manners: osmotic stress and ionic stress. When plants are administered NaCl, they are immediately exposed to an osmotic stress created by the osmotic potential of NaCl around the roots. Due to the water potential of the apoplastic region being smaller than the symplastic region, cellular dehydration occurs leading to reduced turgor and water capacity (Taiz and Zeiger, 2009). With the uptake of Na⁺ and

Cl^- in the transpiration stream, these ions start accumulating gradually in root and shoot tissues of the plant. Only after Na^+ and Cl^- accumulate in plant cells and reach toxic levels in the old leaves over a longer period of time, ionic phase of the salt stress begins. Furthermore, reduction of cellular water content cause concentrations of cellular ions to reach cytotoxic levels. The impact of osmotic stress on plant growth is greater than the ionic stress, especially at low to moderate salinity levels. Therefore, response of a plant species to salt stress is determined by its capacity to tolerate both the osmotic stress and the ionic stress (Munns and Tester, 2008).

Munns and Tester (2008) classified stress tolerance mechanisms in three distinct categories: tolerance to the initial osmotic stress, the ability to avoid Na^+ uptake by the roots, and preventing Na^+ ions from reaching cytotoxic levels once it starts accumulating in the cells.

The osmotic stress experienced by the plant causes a rapid reduction in the rate of cell expansion in root tips and young leaves in addition to closing of stomata to avoid water loss through transpiration (Passioura and Munns, 2000). These effects are manifested as a net reduction in cell elongation and growth, and leaf development. The master regulators of these responses are usually plant hormones, such as abscisic acid (ABA), gibberellins (GA), and salicylic acid (SA). ABA signaling was shown to be a regulator of growth rate, stomatal conductance, and overall state of stress. The net rate of photosynthesis in salt stressed plants usually remains unchanged. This seems odd considering the net reductions in leaf area, but it is made possible by increased chloroplast density per unit leaf area and increased leaf thickness (Shabala and Munns, 2012).

Another common response to osmotic stress, which is also caused by the ionic stress, is increased reactive species (ROS) formation. Some of the primary ROSs are hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radical ($\text{OH}^\cdot$). A certain level of ROS is required for cellular signaling and proper maintenance of metabolism, but overproduction could cause oxidative damage to cells, especially the photosystems (Sharma *et al.*, 2012). To counter this situation, production of ROS scavengers such as superoxide dismutases (SODs), catalases, glutathione peroxidases, and ascorbate peroxidases are

increased. Specifically, superoxide dismutases are responsible for conversion of the superoxide radical ($O_2^{\cdot -}$) into H_2O_2 , which is further processed into H_2O by catalase activity (Tuteja, 2007).

The plants also respond to osmotic stress by accumulating either ions or organic solutes. Even though extrusion of Na^+ ions from tissues is a common defensive response, it is also common that plants accumulate Na^+ ions in their tissues to equalize the osmotic capacity of the plant and the soil and prevent dehydration (Flowers *et al.*, 1977, Munns, 2005). Halophytes are well known for their tolerance to extreme saline conditions, which is made possible by either extremely efficient Na^+ secretion or succulence (Aslam *et al.*, 2011). Some halophyte species are even dependent on accumulation of high Na^+ concentrations in their tissues to survive (Flowers, *et al.*, 1977). Interestingly, barley, which is not a halophyte, can also tolerate 400mM Na^+ in leaf blades (James *et al.*, 2006b). The genes that were detected to be responsible for Na^+ accumulation in shoots are salt-overly-sensitive1 (*SOS1*) and high affinity K^+ transporter1 (*HKT1*) (Rubio *et al.*, 1995, Shi *et al.*, 2002). Functions of *SOS1* and *HKT1* have been discovered via *Arabidopsis* mutants with impaired salinity tolerance, expressional cloning and subsequent functional analyses (Rubio, *et al.*, 1995, Shi, *et al.*, 2002).

Once the Na^+ ions are taken into the root, they are either transported back into the soil by Na^+/H^+ antiporters or Na^+ -translocating ATPases such as sodium-hydrogen antiporter 1 (*NHA1*). The Na^+ ions that are not extruded can be sequestered into root vacuoles by combined actions of vacuolar Na^+/H^+ antiporters (*NHX*) and vacuolar H^+ -translocating pyrophosphatases (*VHP*), which create the electrochemical potential difference for H^+ energizing the Na^+ pumps (Dubcovsky *et al.*, 1996, Tester and Davenport, 2003). The Na^+ can also be retrieved back from the xylem before reaching the shoot, which is another determinant of final Na^+ levels in tissues (James *et al.*, 2006a).

After Na^+ ions reach the shoot, they can either be sequestered in vacuoles which causes the vacuoles to swell with water (succulence) or excreted from the leaves (excretion) by modified trichomes or modified epidermal cells called bladders. Intracellular Na^+ concentrations are usually within 10-30mM range and around 100mM, it starts in-

terfering with the functionality of most enzymes (Greenway and Osmond, 1972). *Thellungiella halophila*, a halophyte relative of *A.thaliana*, can tolerate extreme saline conditions as well as other abiotic stresses like heat stress and cold stress (Gong *et al.*, 2005). However, this mechanism is merely postponing the ionic toxicity at the cost of osmotic debt, which would impair the water status of the cytoplasm if not controlled. This is remedied by accumulation of compatible solutes, maintaining the osmotic balance within the cell. Proline, one of the most common compatible solutes, tends to accumulate in leaves of halophyte species at over 40mM on a tissue water basis and around 10mM in glycophytes. The amount of proline accumulated in the cells directly translates as increased capacity to hold water and prevent dehydration (Flowers, *et al.*, 1977, Saneoka *et al.*, 1995). Furthermore, it has been suggested that proline synthetase gene (*P5CS1*) is a salinity inducible gene responsible for proline synthesis and also for regulating activities of the ROS scavenging enzymes catalase (CAT) and ascorbate peroxidase (APX) (Razavizadeh and Ehsanpour, 2010). Another one of the compatible solutes is glycine betaine. In a study comparing near-isogenic maize lines for their ability to accumulate glycine betaine showed that the lines that were homozygous for the glycine betaine (*Bet1*) gene had around 10-20% increased biomass when subjected to salinity stress (Saneoka, *et al.*, 1995) It has been suggested that proline has a role in stabilizing the tertiary structure of proteins and osmoprotection (Rhodes *et al.*, 2002). However, synthesis of compatible solutes requires substantial energy input and thus have some reductive effects on growth itself. For example the ATP requirement for accumulating for one molecule of NaCl is 3.5, where ATP requirement of proline and glycine betaine is 41 and 50 respectively. (Rhodes, *et al.*, 2002) The energy spent of solute accumulation takes its toll on plant growth and yield. The most economical way of accumulating osmolytes therefore is accumulating NaCl. However this also brings about toxicity problems and nutrient imbalances, which requires further action by the plant to remedy.

1.3. Biotechnological Approaches in Studying Crop Tolerance to Abiotic Stresses

The most frequent strategies in creating crops tolerant to abiotic stresses have been genetic alteration of selected candidate genes.(Pardo, 2010, Peleg *et al.*, 2011) Candidate genes that are responsible for synthesis of molecules associated with regula-

tion of osmotic homeostasis in plants, such as polyamines, compatible solutes, proline synthetase, glycine betaine, and aquaporins, has been overexpressed in various crops successfully to create stress tolerant crops (Alcazar *et al.*, 2006, Bhatnagar-Mathur *et al.*, 2009, Lv *et al.*, 2007, Vendruscolo *et al.*, 2007). *P5CS1* gene, which is responsible for production of proline, from moth bean has been overexpressed in tobacco (Kishor *et al.*, 1995). Transgenic plants produced 14-fold more proline in leaves compared to wild type plants and performed significantly better under drought stress applied in soil with respect to fresh weight, osmotic potential, and root length. It has also been shown in many plant species that proline accumulates in response to various stress conditions (Kishor and Sreenivasulu, 2014). Furthermore, it has been reported that insertional knock out lines of *P5CS1* results in decreased salinity tolerance as indicated by root elongation assays on NaCl infused medium (Szekely *et al.*, 2008).

Genes coding for proteins that are responsible for detoxification of ROS have also been successfully utilized in creation of crops with greater tolerance. Earlier reports showed that overexpression of superoxide dismutases, ascorbate peroxidases, catalases, glutathione transferases have conferred enhanced stress tolerance (Wang *et al.*, 2006, Zhao F., 2006). A cytosolic Cu-ZnSOD and ascorbate peroxidase (APX) has been overexpressed in plums and it was reported that transgenic lines performed better under salinity stress applied in-vitro with respect to fresh weight and root length (Diaz-Vivancos *et al.*, 2013). In another study, a mitochondrial MnSOD has been overexpressed in *Arabidopsis* and performed salinity treatments were performed in vitro. Authors reported that transgenic lines had at least two fold increased MnSOD activity and also significantly higher activities of other antioxidant enzymes such as FeSOD, catalase (CAT), and peroxidase (POD). Moreover, the transgenic lines have been observed to perform significantly better than wild type with respect to a qualitative assessment of wilting (Wang *et al.*, 2004). However, with respect to individual roles of superoxide dismutases in salinity stress, it is difficult to draw firm conclusions due to the great redundancy in the plant antioxidant network (Mittler *et al.*, 2004). Another study involved creation of null mutant lines for copper chaperone for SOD (CCS), which is responsible for delivering copper to Cu-ZnSOD as co-factor. Authors reported that even though no measurable Cu-ZnSOD activity was seen in the knock out lines, there was no visible impairment in tolerance to oxidative stress induced by methyl viologen or high intensity light treatment

with regard to qualitative assessment and leaf area measurements. It was suggested that the redundancy in the antioxidant system could be masking the affects created by the lack of Cu-ZnSOD activity and increased activity in the xanthophyll cycle could be responsible for this situation (Cohu *et al.*, 2009).

Furthermore, ion transporter genes such as vacuolar $\text{Na}^+\text{-H}^+$ antiporters have also been targets of overexpression studies leading to enhanced stress tolerance in various crops (Apse *et al.*, 2003, He *et al.*, 2005, Li *et al.*, 2011a, Li *et al.*, 2011b). It has been reported that insertional knock-out Arabidopsis lines of *NHX1* show inferior growth and germination against 100mM NaCl supplied in vitro (Hanin *et al.*, 2004). Similar experiment with *NHX1* knock out lines performed by (Apse, *et al.*, 2003) revealed compromised growth with respect to wild type under NaCl stress applied both in soil and in medium as indicated by leaf area changes eports on overexpression of vacuolar $\text{Na}^+\text{-H}^+$ are not only limited to Arabidopsis. An NHX gene from cotton (*Gossypium hirsutum*) was overexpressed in tobacco (*Nicotiana tobaccum*) plants and it was reported that transgenic plants displayed enhanced growth under salinity stress applied in soil as indicated by dry weight and qualitative assessments (Wu *et al.*, 2004). Considering that vacuolar $\text{H}^+\text{-ATPases}$ help create the necessary proton gradient to drive the membrane ion transporters, it was logical to investigate their functional importance in salinity tolerance. In parallel with this line of thinking, a vacuolar $\text{H}^+\text{-ATPase}$ Subunit c (VHA-c) from *Limonium bicolor* was overexpressed in transgenic tobacco and it was reported enhanced salinity tolerance with respect to biomass and water potential (Xu, 2011). It was even reported that Na^+ exchange ability of *atNHX1* is dependant on V-ATPase activity and that simultaneous co-expression of two genes could act synergistically towards better salinity tolerance (Zhou *et al.*, 2011). Indeed, a Na^+ antiporter (*tnHXS1*) and a V-yrophosphatase (*TVP1*), a closely related driver of proton gradient, was co-expressed in tobacco plants in a later study and co-expressing transgenic plants displayed better salinity tolerance than both genes individually with respect to root elongation, dry weight, and fresh weight. Furthermore, co-expressing plants accumulated less Na^+ and more K^+ in their shoots, which was another indicator of tolerance to salt stress (Gouiaa *et al.*, 2012). While co-expression studies on salt tolerance is yet scarce, this study hints that gene pyramiding could potentially be a useful strategy towards creating salinity tolerant crops.

A family of plasma membrane proteins worth mentioning is the plasma membrane intrinsic protein family (PIP). The highly abundant protein family contains two subfamilies, namely PIP1 and PIP2. It has been shown that PIPs function as water channel proteins that have direct roles in several physiological processes such as cell elongation, abiotic stress resistance, and photosynthesis (Yang and Cui, 2009). While there is considerable functional redundancy among members of the PIP family, it has been suggested that some members may be specifically required for certain processes. For example PIP2-3 has been suggested to be playing a specific role in salinity tolerance since it was the only PIP2 family member whose knock-out impaired salinity tolerance in *Arabidopsis* T-DNA insertional lines (Ines, 2008). Furthermore, involvement of PIPs from various organisms in salinity tolerance has been indirectly deduced through several overexpression studies across different model and non-model organisms (Bhardwaj *et al.*, 2013).

Transcription factors have also been utilized as pathway regulators successfully to confer stress tolerance. Members of various transcription factor families such as ERF/AP2, NAC, bZIP, and WRKY have been overexpressed, leading to greater stress tolerance in different crops with respect to fresh weight and osmotic potential. (Kasuga *et al.*, 1999, Yamaguchi and Shinozaki, 2006). It has been suggested that manipulation of such key regulators, could be effective in providing tolerance to several different stress factors at the same time. It was reported that overexpression of dehydration response element 1A (*DREB1A*) driven by a stress inducible promoter resulted in transgenic plants with enhanced tolerance to drought, salt, and freezing stress as indicated by plant height, survival rate, and qualitative assessment. However when the *DREB1A* gene was driven by 35S CaMV constitutional promoter, severely retarded growth was reported under control conditions in transgenic plants. This has indicated that choice of promoter could be a determining factor in analyzing functional effects of a candidate gene under a given condition (Kasuga, *et al.*, 1999).

In functional studies availability of genome wide T-DNA insertional knock out model plant lines have been highly useful tools. Alonso *et al.* (2003) reported creation of over 225,000 independent *Agrobacterium tumefaciens* transformed lines of the model

plant *A.thaliana*. The sites of the T-DNA insertions were determined by sequencing of the T-DNA flanking genomic regions. The mutant Arabidopsis lines include an insertion in 21,799 of the 29,454 annotated genes in five chromosomes of *A.thaliana*. Presence of a line with a T-DNA insertion in the gene of interest could be determined by searching the sequence-indexed database (<http://signal.salk.edu/cgi-bin/tdnaexpress>). The seed stocks for a specific line of interest were made commercially available from seed stock centers such as European Arabidopsis Stock Centre (NASC) (<http://arabidopsis.info/>) or Arabidopsis Biological Resource Center (<http://abrc.osu.edu/>). With the advances in whole genome sequencing techniques in the last few decades, several other plant species also achieved model plant status. A family of legume plants such as *Medicago truncatula*, *Glycine max*, and *Lotus japonicus*, have been model organisms especially for legume species. Just as for Arabidopsis, several collections of mutant lines were also generated for these legume family plants through different approaches such as T-DNA insertional knock-out populations, TILLING (Targeting Induced Local Lesions in Genomes) populations, EMS (Ethyl Methane Sulfonate) populations, and RNAi constructs (Zhu *et al.*, 2005). With different choices available, the decision of which model to utilize depends on the researchers objectives, experimental design, and tools of which model organism are most readily available to the researcher.

2. PURPOSE

The purpose of this thesis was to investigate functional role of certain candidate genes selected from SSH library of common bean previously constructed under salinity stress in our laboratory. Several other candidate genes from literature that were suggested to have role in salinity tolerance were also included in our study as possible positive control group of genes. Functional analysis of all candidate genes were performed using *Arabidopsis thaliana* as a model organism. Following the validation of differential expression of all candidate genes, they were overexpressed in wild type *Arabidopsis* plants and orthologous T-DNA knock out lines. Physiological assessments of the transgenic plants were performed to evaluate the functional effects of the transgenes under salt stress treatments.

3. MATERIALS

3.1. General Kits, Enzymes and Reagents

Table 3.1. List of kits, enzymes and reagents.

BCA Protein Assay Kit	23227, Thermo Scientific, USA
Plasmid Miniprep Kit	740615 Nucleospin Plasmid Quickpure, Macherey-Nagel, Germany
First Strand cDNA Synthesis Kit	K-2049, AccuPower CycleScript Premix dN6, Bioneer, Korea K1622, Thermo Scientific (RevertAid), USA
Protein Molecular Weight Marker	26616 PAGERuler Prestained Protein Ladder, Thermo Scientific, USA
Chemiluminescent HRP Substrate	34077, SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, USA
DNA Ladder	SM0311, GeneRuler 1 kb DNA Ladder, Fermentas, USA
DNA Loading Dye	B7021S, Fermentas, USA
LR Clonase	11791, Gateway LR Clonase II Enzyme Mix, Life Technologies, USA
BP Clonase	11789, Gateway BP Clonase II Enzyme Mix, Life Technologies, USA
qRT-PCR Master Mix	K-6252 Accupower 2X GreenStar Master Mix, Bioneer, Korea
DNA Polymerase	EP0711, DreamTaq Green DNA Polymerase, Thermo Scientific, USA
Magnesium Chloride	25mM MgCl ₂ Solution (Promega, USA)
dNTPs	10mM PCR Nucleotide Mix (Promega, USA)
Genomic DNA Extraction Kit	69106 DNeasy Plant Mini Kit, Qiagen, USA

3.2. Biological Materials

3.2.1. Bacterial Strains

Escherichia coli, DH5 α strain, and *Agrobacterium tumefaciens*, GV3101, strain were used for transformation experiments.

3.2.2. Plant Seeds

Ispir and TR43477 varieties of *Phaseolus vulgaris* (common bean) were kindly provided by Prof. Yıldız Dasgan from Çukurova University. Columbia-0 variety of *Arabidopsis thaliana* were kindly provided by Dr. Giorgia Batelli, CNR-IGV, Italy.

3.2.3. Plasmids

pGWB411 and pGWB412 gateway binary vectors were kindly provided by Dr. Nakagawa T. from Shimane University, Japan. pMDC32 gateway binary vectors were kindly provided by Dr. Stefania Grillo from CNR-IGV, Italy.

3.2.4. Primers

Table 3.2. List of qRT-PCR primers for candidate genes selected from the SSH library.

Gene Abbreviation	Sequence (5'-3')	Colony number of Template Sequence from the SSH library	Annealing Temperature(°C)
pvVHP3-F	TGACCACCCATCTGTTTTAACCTCC	P1-FW-LIB.C03_11071513S5	56
pvVHP3-R	TGCTAAAGCCTGAAAATGCTGCCT		
pvAQP-F	TGGAGCCAAGAAGCTGTGCAATC	LEAVE_FWLIB_PL3_G09	62
pvAQP-R	GGCCATGTCAACCCTGCTGTGA		
pvABAR-F	CCACTCCTGGCACTACGAAC	LEAVE_FWLIB_PL3_B10	63
pvABAR-R	GTAAGTGTGGATGGACATGATTT		

Table 3.2. List of qRT-PCR primers for candidate genes selected from the SSH library
(cont.).

pvBZIP-F	TTGGTGTGGGATGAGGCACAGG	LEAVE_FWLIB_PL1_F09	62
pvBZIP-R	ACAACGGATGAACTCCACGAAGG		
pvIDP-F	CGGCGATACAAAGCGCGACA	LEAVE_FWLIB_PL4_E06	62
pvIDP-R	GCACGCAAATCGGCAACGTCAA		
pvTAU-F	AGCTGAAGACTGACGGCGGTTG	LEAVE_FWLIB_PL1_H09	62
pvTAU-R	CGTTGCATGTGAAGGTGCTCGTTTG		
pvTPK-F	TGCCCAGAGTTTCTTCTCTGGACTT	LEAVE_FWLIB_PL4_E08	62
pvTPK-R	AGGAGGAGACGGCACAATGGAGA		

Table 3.3. List of qRT-PCR primers for candidate genes selected from previous studies.

Gene Abbreviation	Sequence (5'-3')	NCBI Accession Number	Annealing Temperature (°C)
pvP5CS1-F	GTCCTCCAAGTGACCCAACTCAAA	FE898310.1	62
pvP5CS1-R	CTCTGCCCAATCTTGACTTGTCTC		
pvCLCa-F	TTTGTGCAGCTTTCCGGGCT	FE709655.1	62
pvCLCa-R	CCCTGAGAACCACCACCACCAC		
pvCCS-F	CCCGGCTAATTGGACAAGGAA	CV536137	62
pvCCS-R	TGAGCTAGGCGAACAACACCA		
pvSOD-F	TGGGCATCACGACTGCTCTTCT	JK043819.1	56
pvSOD-R	AGAACTGCCACAGCCTTCACCA		
pvPIP22-F	CGGCTACAAGAGACAGACCGACA	EF624001	62
pvPIP22-R	TGCCAGCGGTGCAGTAAACGA		
pvNIP-1-F	TGGAAGGACCCGAAAGAGGGACA	FE685846.1	62
pvNIP-1-R	TGCCCATTGAGTTGAGGTGTGCT		
pvPTR-1-F	GGGTCTTGATTTGGCATTCTGCT	HO864348.1	63
pvPTR-1-R	ATAGGGCTCCCGCCTGGTTT		
pvPP2CA-F	TCGCCATAGTCACGCCGAAA	FE898980	62
pvPP2CA-R	ATATCACGCGCCACCAGCA		
pvNCED-F	AACCATGCCTTCACCAGCTTCAAAAT	AF190462.1	62
pvNCED-R	AGGTTGTGGGAGAAGAAGATGTGGA		

Table 3.4. List of qRT-PCR primers for reference genes used in qRT-PCR.

Gene Abbreviation	Sequence (5'-3')	NCBI Accession Number	Annealing Temperature (°C)
Cyclophilin-F	GTGGTCTTCGGACAGGTCGT	X74403.1	62
Cyclophilin-R	CAGAGCCACCAGCACAGAGA		
Gapdhlit-F	GCGAGTGGAGCAAGGCAGTT	Pv-RHS24-15	62
Gapdhlit-R	GAAGGGTGGCGCTAAGAAGGT		

Table 3.5. List of first step attb primers used for PCR amplification of candidate genes and attb site addition.

Primers	Sequence (5'-3')	Annealing Temperature (°C)
pvNHX1_ORF_(attB1_attB2)_F	AAAAAGCAGGCTTCATGGTGTTCGAAATCAGTTC	56°C
pvNHX1_ORF_(attB1_attB2)_R	AAGAAAGCTGGGTCTCAACGCCATTGTTGGCCAT	
pvNHX1_ORF_(attB1_attB2)_R_nonstop	AAGAAAGCTGGGTCACGCCATTGTTGGCCAT	
pvPIP2-3_ORF_(attB1_attB2)_F	AAAAAGCAGGCTTCATGGCTAAGCATGATGCTGA	52°C
pvPIP2-3_ORF_(attB1_attB2)_R	AAGAAAGCTGGGTCTTAAGCATTGCTCCTGAATG	
pvPIP2-3_ORF_(attB1_attB2)_R_nonstop	AAGAAAGCTGGGTCAGCATTGCTCCTGAATG	
pvSOD_ORF_(attB1_attB2)_F	AAAAA-GCAGGCTTCATGGTGAAGGCTGTGGCAGT	50°C
pvSOD_ORF_(attB1_attB2)_R	AAGAAAGCTGGGTCTTATCCTTGCAGACCAATGA	
pvSOD_ORF_(attB1_attB2)_R_nonstop	AAGAAAGCTGGGTCTCCTTGCAGACCAATGA	
pvVHP_ORF_ORF_(attB1_attB2)_F	AAAAA-GCAGGCTTCATGGCTGGCTTCAGCGGTGA	50°C
pvVHP_ORF_ORF_(attB1_attB2)_R	AAGAAAGCTGGGTCTTAGTCGGCTCTAGACTGGC	
pvVHP_ORF_ORF_(attB1_attB2)_R_nonstop	AAGAAAGCTGGGTCTGTCGGCTTAGACTGGC	
PvP5CS1_ORF_(attB1_attB2)_F	AAAAA-GCAGGCTTCATGGAACCTCTGCAAAATGG	52°C
PvP5CS1_ORF_(attB1_attB2)_R	AAGAAA-GCTGGGTCTTATGCTTTTAGTGGCAATT	
PvP5CS1_ORF_(attB1_attB2)_R_nonstop	AAGAAAGCTGGGTCTGCTTTTAGTGGCAATT	

Table 3.6. List of universal second step attb primers.

Primer	Sequence (5'-3')	Annealing Temperature (°C)
attb2nd_step_universal_F	GGGGACAAGTTTGTACAAAAA-GCAGGCT	54°C
attb2nd_step_universal_R	GGGGACCACTTTGTACAAGAAA-GCTGGGTC	

Table 3.7. List of primers used for homozygosity screen in knock out lines.

SALK ID_Primer Name	Associated gene	Sequence (5'-3')	Tm	BP	BP+RP product size	LP-RP product size
SALK_063517C_LP	atP5CS1	CTCTGCAACTTCGTGATCCTC	60	LB1.3	517-817	1108
SALK_063517C_RP		TGATCACTGATGCAATTCCAG	60			
SAIL_439_G04_LP	atSOD1	AGTAGCCAGGCTGAGTTCATG	59.5	LB3	433-733	1096
SAIL_439_G04_RP		ACAAAGGCCAAGTAACAATGG	59			
WiscDsLox461-464O11_LP	atVHP	TTGTTGTCTGAAACCCAGTC	60	p745	519-819	1184
WiscDsLox461-464O11_RP		GTAGAATTCAACAGGCGTTGC	59.8			
SALK_117876C_LP	atPIP2-3	TCGCTAAATTGTGTTTGCCTC	60	LB1.3	485-785	1133
SALK_117876C_RP		GAAACCGGTCCGGTTTATTAC	56.5			
SALK_046597C_LP	atNHX1	TTTTGGTTTCATTGAGTTGCC	60	LB1.3	597-897	1130
SALK_117876C_RP		TTATCTTTCCGTGCTGTCCAC	60			

Table 3.8. List of universal primers used for homozygosity screening.

Knock out line	Sequence (5'-3')
LB3	TAGCATCTGAATTTTCATAACCAATCTCGATACAC-
LBb1.3	ATTTTGCCGATTTCGGAAC
p745	AACGTCCGCAATGTGTTATTAAGTTGTC

Table 3.9. List of primers used for plasmid sequencing.

Vector/Gene	Primer Name	Sequence (5'-3')
pGWB412	pGWB412-F	AGGAGCATCGTGGAAGAAA
	M13- R	GCGGATAACAATTTACACAGG
pGWB411	35Sb- F	AGTGGAAAAGGAAGGTGGCT
	M13- R	GCGGATAACAATTTACACAGG
pMDC32	M13-R	GCGGATAACAATTTACACAGG
	35Sb-R	AGTGGAAAAGGAAGGTGGCT
pDONR207	pDONR207-F	TCGCGTTAACGCTAGCATGGATCTC
	pDONR207-R	GTAACATCAGAGATTTTGAGACAC
pvNHX	pvNHX-F1	TTGTATTTGGGGAGGGTGTGT
	pvNHX-F2	CCCCTTATCCTTCATATCCAAC

3.3. Chemicals, Plastics and Glasswares

Chemicals were purchased from either Applichem (Germany), Merck (Germany), Duchefa (Netherlands) or Sigma-Aldrich (USA); sterile plates from Interlab (Germany), micropipette tips, 2ml and smaller tubes from Axygen (USA), 15ml and 50ml tubes from BD Biosciences (USA). Glasswares were also purchased from VWR (USA). For sterilization, all glassware, tips and tubes were autoclaved at 121 °C for 20 minutes before use.

3.4. Buffers and Solutions

Table 3.10. Buffers and solutions used in this study.

10X SDS Running Buffer	250 mM Tris-Base (pH: 8.3)
	1.90 M Glycine
	1% SDS
10X Transfer Buffer	1.90 M Glycine
	250 mM Tris-Base
10X TBS	200 mM Tris-Cl (pH: 7.6)
	1.5 M NaCl
1X TBST	1X TBS
	0.1% Tween-20
12% SDS-PAGE Gel (20ml)	8.6 ml ddH ₂ O
	6 ml 40% Acrylamide mix
	5ml Tris (1.5 M, pH: 8.8)
	200 µl 10% SDS
	200 µl 10% APS
	20 µl TEMED
5% Blocking Buffer	5% BSA (w/v) in TBST
4X Sample Buffer (Laemmli)	250 mM Tris-Cl (pH: 6.8)
	8% SDS
	40% Glycerol
	20% β-mercaptoethanol
	0.008% Bromophenol Blue

Table 3.10. Buffers and solutions used in this study (cont.).

LB Medium	10 g/L Tryptone
	5 g/L NaCl
	5 g/L Yeast Extract
LB Agar	10 g/L Tryptone
	5 g/L NaCl
	5 g/L Yeast Extract
	15g/L Agar
YEP	10 g/L Peptone
	10g/L Yeast Extract
	5g/L NaCl
	10g/L Bactoagar (Optional)
1000X Ampicillin	100 mg/ml in ddH ₂ O
Germination Medium for Arabidopsis (GM) (1X)	4.4g/L Murashige Skoog Basal Medium (M5519)
	10g/L Agar (A4550)
	3% Sucrose (S0389)
Modified Hoaglands Macronutrient Stock Solution (100X)	0.14M MgSO ₄ ·7H ₂ O
	20mM KH ₂ PO ₄
	0.28M CaNO ₃
	0.18M KNO ₃
Modified Hoaglands Micronutrient Stock Solution (2000X)	20mM H ₃ BO ₃
	2.2mM MnSO ₄
	0.4mM CuSO ₄ ·5H ₂ O
	3.85e-05 M NaMoO ₄ ·2dH ₂ O
	1mM ZnSO ₄ ·7H ₂ O
Modified Hoaglands Ferric EDDHA Solution (100X)	10mM C ₁₈ H ₁₆ FeN ₂ O ₆ ·Na
1X Modified Hoaglands Working Solution (1L)	10ml Macronutrient Solution
	10ml Ferric EDDHA Solution
	0.5ml Micronutrient Solution

3.5. Antibodies

Table 3.11. Antibodies used in this study.

Name	Species	Dilution	Source
Anti-Flag	Mouse	1:2000	MA-91878, ThermoScientific
Anti-Actin	Mouse	1:1000	MA1-744, ThermoScientific
Anti-Flag	Goat	1:1000	#2368, Cell Signaling
Anti-rabbit IgG, HRP-Linked Secondary Antibody	Goat	1:2000	#7074, Cell Signaling
Goat Anti-mouse IgG Secondary Antibody	Mouse	1:20000	#31430, ThermoScientific

3.6. Equipments

Table 3.12. List of equipment used in this study.

Agarose Gel Electrophoresis	MINICELL PRIMO EC320 Electrophoretic Gel System, Thermo Scientific, USA
Autoclaves	Model MAC-601, Eyela, Japan
	Model ASB260T, Astell, UK
Balances	AY123, Satorius, Germany
Centrifuges	5453000 MiniSpin Plus Benchtop Centrifuge, Eppendorf, Germany
	Allegra X-22, Beckman, USA
	J2-MC Centrifuge, Beckman, USA
	J2-21 Centrifuge, Beckman, USA
Chemiluminescence Imaging System	Stella 2000, Raytest, Germany
Deep Freezers (-20°C)	Arçelik, TURKEY
Deep Freezers (-80°C)	Thermo Scientific, USA
Ice Machine	Scotsman Inc. AF20, ITALY
Magnetic Stirrer	0004810000 IKA RCT basic Safety Control, Germany
Scanner	EPSON GT-20000 Scanner

Table 3.12. List of equipment used in this study (cont.).

Thermal Cyclers	C1000 Thermal Cycler, Bio Rad, USA
	Runik Thermal Cycler, Sacem Life Technologies, TURKEY
Incubator	EN500 Nüve, TURKEY
Microwave	MD55I, Arçelik, TURKEY
Gel documentation system	Gel Doc XR System, Bio Rad, USA
Micro-centrifuge	Himac CT15RE, Hitachi Koki, Japan
Microplate Reader	680, Bio Rad, USA
Oven	Gallenkamp 300, UK
pH Meter	Hanna, USA
Pipettes	Pipetman, Gilson, USA
Power Supply	PowerPac Basic, Bio Rad, USA
	EC250-90 Thermo Scientific, USA
Refrigerator (4°C)	MFAA1, Hotpoint Ariston, Italy
Rotors	Beckman JS-7.5, USA
	Beckman JA-14, USA
SDS-PAGE Transfer System	Mini Protean Electrophoresis System, Biorad, USA
Spectrophotometer	NanoDrop1000, Thermo Scientific, USA
Vortex	NM110, Nüve, Turkey
Water Bath	Memmert, Germany
Gartengold Torf	SAB (Germany)
Plant Growth Chamber	JSR JSPC-960

4. METHODS

4.1. Selection of Candidate Genes

Candidate genes that were reported to be functionally involved in salinity tolerance, were selected from either the results of the Suppression Subtractive Hybridization (SSH) library prepared by Mahmut Can Hız within the scope of the TUBITAK project 110T922 or the literature. The SSH library was prepared by using mRNA from control group plants of salinity tolerant Ispir variety common bean as tester and mRNA from treatment group plants as driver. The ESTs that had annotations with genes already known or suspected to play a role in abiotic stress responses were chosen to form an initial pool. Several candidate genes from literature were also selected as a potential positive control group to see whether the bean orthologues of genes from other plant species that were shown to provide tolerance upon overexpression would have the same effect on salinity tolerance.

4.2. Bioinformatic Analyses

4.2.1. Sequence similarity analyses

Blast modules of CLC Main Workbench were used with default parameters for homology analyses of candidate gene sequences obtained from SSH library, RNA-Seq database and NCBI sequence databases.

4.2.2. Prediction of open reading frames (ORFs) of *Phaseolus vulgaris* candidate genes

At the time this analysis was performed the reference genome for common bean was not available and the EST sequences for candidate genes selected from SSH library

did not contain full-length sequences of the ORFs. Serendipitously, whole transcriptome sequencing of Ispir variety common bean under salinity stress had just been performed by our lab, which allowed us to make use of this transcriptome database to discover the ORF sequences. Therefore, EST sequences from the SSH library were blasted against the All-Unigene transcriptome database using default parameters. Closest match (>90% identity) was used in ORF prediction tool in CLC Main Workbench, which searches for start and stop codons that are in-frame with each other. Predicted ORFs were blasted against refseq mRNA database for confirmation of ORFs and if the predicted ORF had at least 90% identity and size similarity with the coding sequence from closely related *Medicago truncatula*, *Oryza sativa* or *Glycine max* species, the ORF was counted as a match. For the prediction of Arabidopsis orthologues of the candidate genes from the SSH library, closest transcriptome match were blasted against Arabidopsis refseq mRNA database and the closest match with a similar annotation was considered to be a putative orthologue.

For candidate genes selected from the literature, the Arabidopsis thaliana genes were preferred as being the first model plant to be sequenced in a genomic scale and are the most rigorously analyzed and accurately identified genes among the other plant model organisms. Selected candidate genes were blasted against All-Unigene database of Ispir bean variety. Those that produced at least 40% similarity were further blasted against refseq mRNA database. If the query returned a hit from closely related species *Medicago truncatula*, *Oryza sativa* or *Glycine max* with the same annotation, that unigene was accepted as a potential orthologue and ORF prediction was performed as described above.

4.2.3. Determination and procurement of *Arabidopsis thaliana* knock out lines

After the determination of potential Arabidopsis orthologues of the common bean candidate genes, the Arabidopsis Information Resource (TAIR) web page (<https://www.arabidopsis.org>) was searched for the presence of T-DNA insertional mutant lines with respect to the orthologue genes. The site of insertion was checked from

the sequence browser module and the mutant lines that had exonal insertions were obtained from National Arabidopsis Stock Center (NASC) (<https://arabidopsis.info>).

4.3. Plant Growth and NaCl treatment in hydroponic system

Salinity treatment of *P.vulgaris* varieties Ispir and TR43477 was performed in a hydroponic system which was fashioned out of black tupperware boxes connected to an air pump to continuously oxygenate the nutrient solution. Six equidistant holes were drilled on the cap of the tupperware box to house the plantlets. Common bean seeds were germinated in vermiculite and grown until emergence of second set of true leaves. Eight plantlets each from Ispir (salt tolerant) and TR43477 (salt susceptible) varieties were transferred to hydroponic system filled with 1X modified Hoagland nutrient solution (Table 3.10) after being wrapping above the roots with long strips of regular sponge several times over to prevent mechanical damage. There were four plantlets in each control and treatment group. NaCl concentrations were raised gradually so that the plants were exposed to 50mM, 100mM and 125mM NaCl at first, second, and the third day respectively and maintained at 125mM NaCl for 3 days for the treatment group. Nutrient solutions were renewed daily. At the end of the 3rd day, the plants were sacrificed. The root and leaf tissues were flash frozen in liquid nitrogen and stored in -80°C.

4.4. Validation of Candidate Genes by Quantitative Reverse Transcription PCR

4.4.1. RNA isolation from plant tissues

All of the common bean leaf samples (control and treated plants of both Ispir and TR43477) were homogenized using mortar and pestle after being flash-frozen in liquid nitrogen. Total RNA was isolated from 100 mg of homogenized leaf tissue of *Phaseolus* samples according to Onate-Sanchez *et al.* (2008). Isolated samples were quantified using Nanodrop 1000, Thermo Scientific, USA. Quality of the RNA samples was checked by agarose gel electrophoresis and subsequent visualization under UV.

4.4.2. cDNA synthesis by reverse transcription

Ten µg of total RNA was DNase treated using Turbo DNA-free™ Kit (Ambion Life Technologies) according to manufacturers instructions. cDNA was synthesized using Accupower® Cyclescript RT Premix (dN6) kit, (Bioneer) following CT-RT1 cycling conditions indicated in the instructions with annealing at 20°C and cDNA synthesis at 43°C.

4.4.3. Primer Design Parameters

Primers for qRT-PCR were designed according to parameters indicated in CLC Main Workbench. The parameters were set to ensure the following requirements; (i) the length of the primers between 19-25 base pairs, (ii) produced amplicon lengths between 100-200 base pairs, (iii) the melting temperature of the reaction between 55-63°C, (iv) the GC content of the primers between 40-60%, (v) maximum 5°C melting temperature difference between the forward and reverse pairs, (vi) maximum 8 hydrogen bonds between pair ends and (vii) exact complementarity to the template sequence.

4.4.4. qRT-PCR reactions

qRT-PCR reactions were performed using SYBR green based Accupower 2X GreenStar Master Mix (Bioneer, Korea). A total of 20µl volume mixture containing 20ng cDNA was prepared according to manufacturers instructions. The sequences of the primers used in qRT-PCR experiments were given in Table 3.3. Three biological replicates (three individual plants) and three technical replicates (three reactions with same cDNA for each plant) were used. Statistical analyses was performed using $2^{\Delta\Delta C_q}$ method (Livak and Schmittgen, 2001) and significance was calculated using students *t-test* (Student, 1908).

4.5. Gateway Cloning of Candidate Genes

4.5.1. Plasmid Selection

Improved Gateway Binary vectors (pGWB) kindly provided by T. Nakagawa (Graduate School of Life and Environmental Sciences, Osaka Prefecture University) were used for Gateway cloning experiments (Nakagawa *et al.*, 2007). Both of the pGWB412 and pGWB411 vectors contain two copies of 35S cauliflower mosaic virus (CMV) constitutive promoter, spectinomycin resistance gene for bacterial transformant selection and kanamycin resistance gene for transformant plant selection, the only difference being the location of the FLAG tag. While the pGWB411 vector has C-terminal end FLAG tag, the pGWB412 vector adds FLAG tag to the N-terminal of the expressed proteins. The third vector pMDC32 was chosen as a no-tag expression vector, which also contains dual 35S CMV promoter, but it contains kanamycin resistance gene for bacterial selection and hygromycin resistance gene for transformant plant selection (Curtis *et al.*, 2003).

4.5.2. PCR amplification of candidate gene ORFs

Attb sites were added to the ORFs of candidate genes through a 2 step PCR amplification (Figure 4.1A, B). In the first step, ORFs were amplified with Phusion® High Fidelity Polymerase (Thermo Scientific), using 20 bases long primers that contain 12bp attb sites (attb1 and attb2) adjacent to start and stop codons in either side. The first step amplification comprised of two separate reactions. The first one was performed using first step attb primers consisting of at least 20 gene specific nucleotides and 12 nucleotides long part of the whole attb site. For the second reaction, the stop codon is removed from the reverse primer so that the C-terminal tag in the pGWB411 vector could be expressed together with the ORF. The amplification product from the second reaction is annotated by “nonstop” suffix and is abbreviated as “ns”. PCR products from the 1st amplification step were subjected to agarose gel electrophoresis to confirm amplification and the expected product size. The relevant bands were excised from the gel and gel purification was performed. Gel purified PCR product was used as template in the second

step reaction. The second step was performed in a 50µl reaction mix using a pair of universal primers complementary to the 12 nucleotides of the attb site, which are named attb2ndstep_universal_F and attb2ndstep_universal_R (Table 3.6).

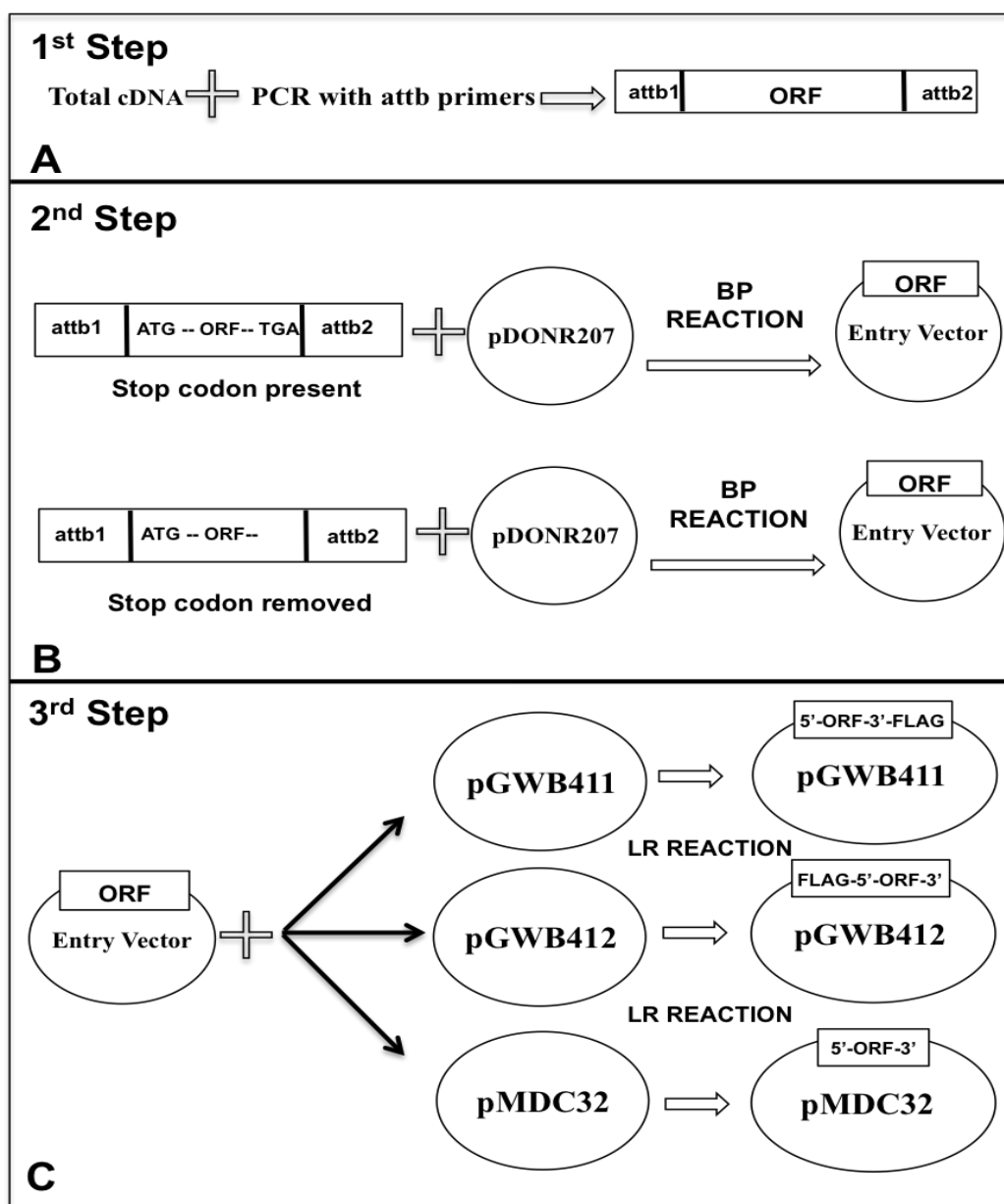


Figure 4.1. Flow chart of Gateway Cloning experiments. A) PCR with total cDNA and gene specific attb primers B) Entry vector creation with BP reaction C) Expression vector creation with different destination vectors carrying N-terminal FLAG tag (pGWB412), C-terminal FLAG tag (pGWB411), and no tag (pMDC32).

After amplification with the 2nd step attb primers, the PCR product contains attb1 and attb2 sites adjacent to the 5' and 3' ends of the complete ORF respectively. Amplification and product size was again confirmed by gel electrophoresis. Full sequences of the gene specific attb primers together with annealing temperatures and universal primers were provided in Table 3.5. Reaction cycles and reaction components were provided in Table 4.2 and Table 4.3.

Table 4.1. The sequences and orientation of attb1 and attb2 sites.

attb1:	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC – ATG (start codon of the gene)
attb2:	Stop codon - GACCCAGCTTTCTTGTACAAAGTGGTCCCC

Table 4.2. Reaction components for PCR amplification of ORFs.

Component	Final Concentration
H ₂ O	complete to 20µl
5x HF Buffer	1x
10 mM dNTPs	200 µM each
Forward primer	0.5 µM
Reverse primer	0.5 µM
<i>P.vulgaris</i> cDNA	2.5ng/µl
Phusion DNA Polymerase	0.2µl

Table 4.3. Cycling conditions for PCR amplification of ORFs.

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98°C	30s	1
Denaturation	98°C	10s	35
Annealing	Primer specific	30s	
Extension	72°C	30s/kb	

Table 4.3. Cycling conditions for PCR amplification of ORFs (cont.).

Final Extension	72°C	10min	1
Hold	4°C	Forever	1

4.5.3. Agarose Gel Extraction of PCR Products

PCR products were isolated from the agarose gel following electrophoresis to be used in LR and BR Clonase reactions as template. Pieces of agarose gel containing the relevant product were excised from the gel under UV light. Purification was performed using QIAquick Gel Extraction Kit according to manufacturer's instructions. Concentration of purified PCR products were measured on Nanodrop 1000.

4.5.4. BR and LR recombination reactions

Two entry vectors were generated by using pDONR207 as the entry vector using two separate PCR products as inserts in a BP clonase reactions, one of which contains the stop codon whereas the second doesn't (Figure 4.1.B). The removal of stop codon allowed the C-terminal flag tag in the expression vector to be transcribed in conjunction with the ORF before the transcription is stopped. The entry vector with the stop codon was named "Gene"207 and the one without the stop codon was named "Gene"_ns207. To generate the expression vector, three LR reactions were performed using pGWB411 (C-terminal tag), pGWB412 (N-terminal tag), and pMDC32 (No tag) as expression vectors (Figure 4.1.C). "Gene"207 entry vector was used for pGWB412 and pMDC32 whereas "Gene"_ns207 entry vector was used for pGWB411 in a 10ul reaction according to manufacturers instructions. 1ul of each reaction was used to transform competent *E.coli*.

4.5.5. Competent Bacteria Transformation and Plasmid Isolation

Vectors generated by Gateway cloning were propagated by transformation of competent bacteria and plasmids were isolated. *Escherichia coli* DH5 α strain competent cells were used for transformation. Fifty μ l aliquots of competent cells that were stored at -80°C were thawed on ice for 5 min. and 50-100ng of plasmids were added onto the competent cells prior to incubation on ice for 30 min. The cells were heat shocked at 42°C for 1 min. and incubated at 37°C for 1hr under constant agitation following the addition of 500 μ l of LB solutions. At the end of the incubation period, the cells were spread on selective nutrient mediums containing relevant antibiotics and incubated overnight at 37°C in an upside down fashion.

For plasmid isolation, LB medium of 5ml volume was inoculated with plasmid containing bacteria and grown overnight. Plasmid DNA was isolated with Nucleospin® Plasmid QuickPure, (Macherey-Nagel, Switzerland) according to manufacturer's instructions. In the last step of the protocol, the elutions of plasmid DNAs from the columns were performed with 50 μ l elution buffer volume.

4.5.6. Colony PCR

Maximum five colonies from a selection medium were picked with a sterile tip and dipped separately into microtubes containing 5 μ l dH₂O. One μ l from this mixture was used as template in a PCR reaction with gene specific primers to verify the presence of inserts. PCR reaction was performed using DreamTaq Polymerase with 0.2mM of each primer, 0.5 units Taq polymerase, and 1X DreamTaq Buffer in a 20 μ l reaction. The rest of the mixture was stored at 37°C temporarily until positive colonies were determined. Those mixtures containing confirmed positive colonies were used to inoculate 5ml of LB medium overnight with constant agitation at 37 °C. Plasmid isolation was performed from the overnight culture.

4.5.7. Sequencing of constructed plasmids

Maximum five colonies that produced bands at the expected size were grown in LB overnight and plasmid isolation was performed for sequencing. Plasmids were Sanger sequenced by Macrogen (S. Korea) using vector specific primers. Full sequences of the sequencing primers are provided in Table 3.9.

The sequences were blasted against the *P.vulgaris* reference genome data in the NCBI database, which was published around the time the final experiments of this thesis were concluded (Schmutz *et al.*, 2014).

4.6. Construction of *Arabidopsis thaliana* stable overexpression lines

4.6.1. Seed sterilization

Seeds were sterilized with vapor-phase sterilization by addition of 6ml of fuming HCl into 400ml bleach (5% sodium hypochlorite) and incubation in a desiccator for 3 hours (Clough and Bent, 1996).

4.6.2. Cultivation and Harvesting

Arabidopsis plants were grown in 50ml vials containing SAB Gartengold Torf (Germany) in JSPC-960 plant growth chamber (JSR) at 22°C, 16/8 hours light-dark cycle. Plants were watered with ¼ strength Modified Hoaglands Nutrient Solution or Basal Nutrient Solution (Conn *et al.* 2013) twice in a week until emergence of flowers and watered with distilled water thereafter. Watering was stopped when %60-80 of the siliques were dry. The seeds were harvested after the plants were completely dry and crisp.

4.6.3. Floral dip transformation of *Arabidopsis* plants

Wild type *Arabidopsis* plants of *Col-0* background were grown for 3-4 weeks under regular conditions until the emergence of first bolts. First bolts were clipped to prevent apical dominance and encouraged to produce secondary bolts. Clipping was repeated to delay the flowering of the plants when needed. Plants with many immature flower clusters and least number of ciliques were selected for floral dip. Five ml of YEP medium containing 100 µg/ml rifampimicin, 30 µg/ml gentamycin, and the vector specific antibiotic was inoculated with *A.tumefaciens* bacterial culture carrying the relevant expression vector and grown at 28°C overnight under constant agitation. This 5ml starter culture was poured into 500ml of YEP containing 100 µg/ml rifampimicin and the vector specific antibiotic and incubated at 28°C overnight under constant agitation. The 500ml culture was spinned down at 5000rpm for 10min and supernatant was discarded. The pellet was resuspended in 200ml of transformation buffer containing 5% sucrose, 55nM benzylaminopurine (BAP), and 0.05% Silwet L-77 (can be substituted with 0.02% Tween 20). Readily formed ciliques on the plants were clipped and inflorescences were dipped in transformation buffer for 10-15 seconds with gentle agitation. Dipped plants were covered with cling film to form a humidity dome and kept in horizontal orientation for 24 hours (Clough and Bent, The plant journal 1998). Dipped plants were watered normally and seeds were collected. Harvested seeds were selected for transformants in germination medium (GM) containing 50µg/ml for pGWB vectors and 15µg/ml of hygromycin for pMDC32. Seedlings that did not discolor or die after 10-15 days in kanamycin medium, were transferred to soil for further screening and seed collection. Hygromycin plates were incubated for 4-6 h under white light, transferred to dark chamber for 2 days and grown under normal 16-8 lighting conditions. Seedlings with significantly longer hypocotyls were transferred to soil for further screening and seed collection. (Harrison *et al.* 2006)

Table 4.4. Antibiotics used for transformant selection in bacteria and plant.

Vector	<i>E.coli</i>	<i>A.tumefaciens</i>	<i>A.thaliana</i>
pGWB412	Spectinomycin (50µg/ml)	Spectinomycin (100µg/ml)	Kanamycin (50µg/ml)
pGWB411	Spectinomycin (50µg/ml)	Spectinomycin (100µg/ml)	Kanamycin (50µg/ml)
pMDC32	Kanamycin (50µg/ml)	Kanamycin (50µg/ml)	Hygromycin (15µg/ml)
pDONR207	Kanamycin (50µg/ml)	-	-

4.6.4. Transformant verification by PCR

Genomic DNA was extracted from leaf tissues of potential transformant plants recovered from the antibiotic selection medium. PCR was performed using DreamTaq DNA polymerase with 100ng genomic DNA, 0.2mM of each gene specific primers (Table 3.5) and 0.5u DreamTaq Polymerase in a 20µl reaction.

4.6.5. Selection of Homozygous T2 Generation

T1 plants were sown and seeds of T2 generation were collected individually from every plant. Seeds were germinated in antibiotic medium and the T2 plants whose seeds showed 100% germination rate were designated as homozygous line.

To verify that the expression of the gene containing the T-DNA insertion is indeed abolished, RT-PCR reaction was performed with cDNA obtained from the homozygous T2 plants using gene specific primers (Table 3.5) and compared with wild type.

4.6.6. RNA Isolation from Arabidopsis leaf samples

Arabidopsis leaf samples were homogenized using micropestles in 1.5ml eppendorf tubes after being frozen in liquid nitrogen. RNA isolation was performed as described in 4.4.1.

4.6.7. Confirmation of Overexpression with RT-PCR

cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit using total RNA extracted according to manufacturers instructions from the homozygous T3 plants using random hexamer primers in a 20µl reaction.

4.6.8. Confirmation of Overexpression with Western blotting

4.6.8.1. Sample Preparation. Between 100-200mg of 10-15 days old seedlings were collected in 2ml microtubes and flash frozen in liquid nitrogen. The plant tissue was homogenized using a micropestle. The homogenates were suspended in 1X SDS-Sample buffer (2ml buffer per 1g tissue) and incubated at 95°C for 5 min. The samples were centrifuged at 10.000 rpm for 5 min and the supernatant was used for SDS-PAGE gel electrophoresis. Samples were stored in -20°C.

4.6.8.2. SDS-PAGE Gel Preparation. Denaturing and reducing (%10) SDS-PAGE gel of 12ml volume was prepared in a glass beaker. The mixture was poured in between the glass plates of the gel casting system immediately following addition of TEMED. The 1.5mm combs were inserted into the system so that no bubbles remained near the wells. The comb was removed after complete polymerization of the gel and the gel was placed inside the gel electrophoresis system containing 1X Running buffer (Table 3.10).

4.6.8.3. Protein Gel Electrophoresis. Twentyfive µl cell lysate was loaded with 4X Sample buffer in to the wells of the gel (Table 3.10) along with PageRuler Prestained

Protein Ladder 10-170K. The samples were run in 10% SDS polyacrylamide gel electrophoresis at room temperature at 120V until relevant size bands of the protein ladder were clearly separated (45-90 min).

4.6.8.4. Transfer of Proteins to PVDF Membrane. The parts of the gel containing the band sizes of interest were isolated and wet transfer was performed to PVDF membrane. Prior to the transfer, the membrane was cut at the size of the gel and activated by absolute methanol for 15 seconds and washed in dH₂O for 1 min. Four pieces of pads and whatman papers at the size of the transfer cassettes were cut and wetted in 1X freshly prepared transfer buffer. The PVDF membrane was placed on top of the gel so that no bubbles remained in between and sandwiched between two sets of whatman papers and pads and placed inside the transfer cassette. An ice block was placed inside the tank to keep the temperature down and a magnetic rod was added to ensure homogenous heat and ion distribution. Proteins were transferred in 1X transfer buffer at 100V for as long as one minute per kilodalton on a magnetic stirrer.

4.6.8.5. Antibody Incubation. Following transfer, the membrane was washed three times in 1X TBST and blocked in TBST containing 5% BSA for 1 hr at room temperature. Primary antibody was diluted according to manufacturers instructions in TBST containing 5% BSA overnight at 4°C. Following primary incubation, the membrane was washed three times in 1X TBST and incubated for two hours in horseradish peroxidase (HRP) conjugated secondary antibody, which was diluted in TBST according to manufacturers instructions (Table 3.10).

4.6.8.6. Chemiluminescence Imaging of the Membrane. Following secondary incubation, the membrane was washed three times in 1X TBST. The chemiluminescent substrate solution was prepared by mixing one part stable peroxide solution and 1 part substrate solution. Substrate solution was put on the membrane drop by drop so that all parts of the membrane was in contact with the solution. The membrane was placed on a regular glass plate prior to analyze in the Xstella imaging system by exposing in increments of 30 seconds.

4.7. Homozygosity Screening of T-DNA Knock Out Lines

4.7.1. Genomic DNA Extraction From Knock Out Lines

After the seed stocks were purchased from NASC, the plants were propagated by collecting seeds from 10-20 individually grown plants. Leaf samples from each individual plants were collected and genomic DNA was extracted using DNeasy Plant Mini Kit according to manufacturer's instructions. Quality of the extracted genomic DNAs was checked by measuring concentrations in Nanodrop 1000 (Thermoscientific, USA). DNA samples were run in agarose gel electrophoresis and visualized in gel documentation system.

4.7.2. Homozygosity screening with PCR

Homozygosity screening was performed by two separate PCR reactions using 3 different primers. Border primer (BP) is complementary to the T-DNA insertion. Left Primer (LP) and Right Primer (RP) are complementary to the left and right flanking regions of the insertion respectively (Figure 4.2). LP and RP primers are specific for every insertion but the BP primers are universal for a number of insertions since groups of T-DNA lines contain the same T-DNA insertion. First PCR reaction was performed using LP and RP primers and expected to yield a PCR product of 1-1.3kb in size if there was a wild type allele of the gene that does not carry the insertion. The second PCR reaction was performed using BP and RP and was expected to yield a product of 0.3-0.8kb in size. Presence of this band confirmed the insertion of T-DNA in the expected location. If both PCR reactions produce expected bands, the line was concluded to be heterozygous.

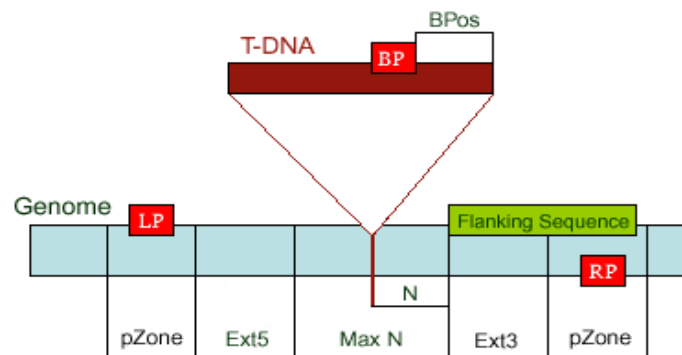


Figure 4.2. Diagram showing locations of LP, RP, and BP primers.

PCR reaction was performed as in “Colony PCR” except that 100ng of genomic DNA was used as template instead of picked colonies. Seeds obtained from plants that yield a PCR product from only BP+RP reaction were collected for further analyses as homozygous lines (Figure 4.3).

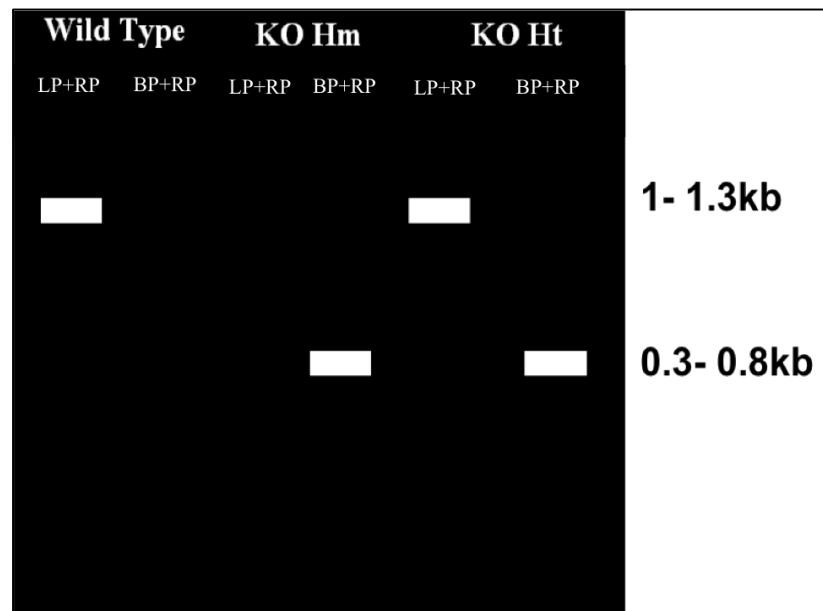


Figure 4.3. Representative agarose gel image from a homozygosity screen (“LP+RP”: PCR reaction performed with LP and RP primers, “BP+RP”: PCR Reaction performed with BP and RP primers, “KO Hm”: Knock out homozygous, “KO Ht”: Knock out heterozygous).

4.7.3. Agarose Gel Electrophoresis

Five µl of PCR products or around 600ng of plasmids were mixed with 6X DNA Loading Dye and loaded into the wells of 2% agarose gel containing 5% EtBr. If the analyzed product was below 1kb, TBE buffer was used to prepare the gel and for electrophoresis; however, TAE buffer was used if the product was bigger than 1kb. Electrophoresis was performed in 1X TAE or TBE buffer at 100V for 30 min. The gel was visualized under UV exposure in gel documentation system (BioRad, USA).

4.8. Salinity Stress Treatments

4.8.1. NaCl treatment in soil

The NaCl treatment of *Arabidopsis* plants was performed in soil. Seven-ten days old seedlings were transferred to pots filled with Gartengold Torf (SAB, Germany) that was soaked completely with 1X Basal Nutrient Solution (BNS) (Table 3.10). The pots were covered with cling film to preserve humidity and to aid in acclimatization. They were placed under 22°C and 16h-8h light-dark growth cycle. The cling films were removed after 2 days. The pots were watered by submersion in 1X BNS medium until the soil surface became thoroughly wet.

Salt stress (NaCl) application was performed in two separate manners. For “Gradual NaCl Treatment”, NaCl application started at 50mM at every watering until final NaCl concentrations of 100mM, 200mM, and 300mM were achieved. For “NaCl Shock Treatment”, NaCl application started at the final desired NaCl concentrations of 100mM and 200mM from the first day without gradual acclimation. Plants were sacrificed after 30 days of NaCl treatment. Above ground parts of the plants were collected for fresh weight and leaf area measurements.

4.8.2. Phenotype Evaluation

The above ground parts of the *Arabidopsis* plants were collected and immediately measured on microbalance to determine fresh weight. To determine leaf areas, all leaves of every single plant were plucked and placed on an acetate sheet and scanned on Epson scanner together with a ruler to serve as size reference. Leaf area calculations were performed using ImageJ v1.48r software (National Institutes of Health, accessed at 18 February 2014).

5. RESULTS

5.1. Candidate Gene Selection

5.1.1. Selection of candidate genes from SSH Library

Seven genes were initially selected as candidate genes from forward SSH library that was created by Mahmut Can Hiz for the studies conducted within the scope of Tubitak 110T922 project. Candidate genes were selected according to their homology with known salinity tolerance relevant proteins based on their provided nBLASTx results. Candidate genes majorly covered vacuolar membrane proteins, water or ion transporters, transcription factors implicated in regulation of abiotic stress responses, major stress hormones such as ABA or salt inducible proteins, a full list of which is provided in Table 5.1.

Among the selected candidate genes selected from SSH library, putative LRR transmembrane protein kinase (*pvTPK*) was included as a candidate gene due to its predicted kinase function since kinases are known to be commonly involved in regulation of diverse metabolic pathways (Golldack *et al.*, 2014). Osmotin-like protein (*pvTAU*) was reported to be responsive to salinity stress and overexpression enhanced tolerance to salinity and fungus infection (Rajam *et al.*, 2007). Intrinsically disordered protein LEA 1 was reported to be induced by cold in *Ammopiptanthus mongolicus* and had homology with late embryogenesis abundant class of proteins, which are known players in abiotic stress response formation (Hundertmark and Hinch, 2008, Liu *et al.*, 2005). bZIP transcription factor bZIP110 (*pvBZIP*) is a member of the well-studied bZIP transcription factor family in plants, members of which are known to be involved in regulation of abiotic and biotic stress response (Tuteja and Gill, 2013). ABA responsive protein (*pvABAR*) was reported to have homology with an ABA responsive protein in an in silico analysis and was suspected to be involved in ABA signaling (Jannoo *et al.*, 2007). Vacuolar H⁺-pumping ATPase 16 kDa proteolipid (*pvVHP*) is a vacuolar hydrogen pump

responsible for creating the proton gradient needed to drive the ion exchangers in the membrane (Tyerman *et al.*, 2002). Overexpression of a VHP was shown to confer enhanced salt tolerance in transgenic tobacco (Xu, 2011).

Table 5.1. Summary of BLASTx comparisons of ESTs from the SSH library against NCBI reference protein database (Mahmut Can Hız PhD Thesis Proposal).

BLAST Hit Description of EST	Gene Name	SSH Library Colony	BLASTx Hit Accession Number
Putative LRR trans-membrane protein kinase	<i>pvTPK</i>	LEAVE_FWLIB_PL4_E08	AAN05336.1
Osmotin-like protein	<i>pvTAU</i>	LEAVE_FWLIB_PL1_H09	CAE54084.1
Intrinsically disordered protein 1 LEA	<i>pvIDP</i>	LEAVE_FWLIB_PL4_E06	ACJ46652.1
bZIP transcription factor bZIP110	<i>pvBZIP</i>	LEAVE_FWLIB_PL1_F09	ABI34661.1
<i>Oryza sativa</i> protein similar to ABA-responsive protein	<i>pvABAR</i>	LEAVE_FWLIB_PL3_B10	NP_001152088.1
Water channel protein	<i>pvAQP</i>	LEAVE_FWLIB_PL3_G09	ACD12709.1
Vacuolar H ⁺ -pumping ATPase 16 kDa proteolipid	<i>pvVHP</i>	LEAVE_FWLIB_PL1_C03	AAB09472.1

5.1.2. Selection of genes from literature with known role in salt tolerance

Eleven genes that were known to play a role in salt response were selected from literature to act as control group of candidate genes (Table 5.2). The mRNA sequences of the selected genes were used as query in BLASTn analyses against the available *Phaseolus vulgaris* EST database in NCBI. The hits with greatest identity and *E*-value were chosen as putative orthologs of the candidate genes.

The candidate genes selected from the literature included chlorine channel protein (*atCLCa*) and $\text{Na}^+\text{-H}^+$ antiporter (*atNHX*), which are genes responsible for transport of Cl^- and Na^+ ions across membranes respectively (He, *et al.*, 2005, Zhou *et al.*, 2010). The plasma membrane integral protein 2-2 (*atPIP2-2*), 2-3 (*atPIP2-3*), and aquaporin NIP1 (*atNIP-1*) were aquaporins responsible for water transport (Ines, 2008, Kamiya *et al.*, 2009). Nine-cis-epoxycarotenoid dioxygenase 1 (*atNCED1*) and protein phosphatase 2c (*atPP2Ca*) were also selected as candidate genes due to their role for ABA signaling and biosynthesis (Iuchi *et al.*, 2001, Kuhn *et al.*, 2006, Nishimura *et al.*, 2009). Superoxide dismutase (*atSOD*) and copper chaperone for superoxide dismutase (*atCCS*) were selected since they are responsible for detoxification of superoxides and delivering copper to SODs for proper functioning (Chu *et al.*, 2005, Diaz-Vivancos, *et al.*, 2013, Wang, *et al.*, 2004). Peptide transporter 1 (*atPTR-1*) was included in the list of candidate genes due to its suggested involvement in stress response formation as indicated by root elongation assays (Komarova *et al.*, 2008).

Table 5.2. Summary of BLASTn comparisons of candidate genes selected from literature against NCBI *Phaseolus vulgaris* EST database.

Gene Description	Gene Name	Organism	Query Accession Number	Phaseolus vulgaris EST Accession Number	Identity	E value
Proline synthetase	<i>atP5CS1-1</i>	Arabidopsis thaliana	NM_001202785.1	FE898310.1	77%	2.00E-112
Chlorine Channel Protein	<i>atCLCa</i>	Arabidopsis thaliana	NM_113631	FE709655.1	75%	1.00E-164
CCS copper chaperone for SOD1	<i>atCCS</i>	Arabidopsis thaliana	NM_001035952.2	CV536137.1	75%	1.00E-83
Copper/Zinc Superoxide Dismutase 1	<i>atSOD1</i>	Arabidopsis thaliana	NM_100757.3	EX303781.1	75%	4.00E-95

Table 5.2. Summary of BLASTn comparisons of candidate genes selected from literature against NCBI *Phaseolus vulgaris* EST database (cont.).

Plasma Membrane Integral Protein-2;2	<i>atPIP2-2</i>	Arabidopsis thaliana	NM_129273	EF624001.1	75%	5.00E-156
Aquaporin NIP-1	<i>atNIP-1</i>	Arabidopsis thaliana	-	FE685846.1	81%	0.00E+00
Peptide transporter	<i>atPTR-1</i>	Arabidopsis thaliana	NM_126265.3	HO864348.1	69%	1.00E-84
PP2CA(aba responsive gene)	<i>atPP2Ca</i>	Arabidopsis thaliana	NM111974.3	FE898980.1	76%	7.00E-76
Na ⁺ -H ⁺ Antiporter	<i>atNHX-F</i>	Arabidopsis thaliana	AY685183.1	CX129689	78%	1.00E-91
ABA Biosynthesis	<i>atNCED1</i>	Arabidopsis thaliana	NM_116217.2	AF190462.1	74%	2.00E-84
Plasma Membrane Integral Protein-2;3	<i>atPIP2-3</i>	Arabidopsis thaliana	NM_129274	FE682762.1	76%	2.00E-171

5.2. NaCl treatment of *Phaseolus vulgaris* in Hydroponic System

Eight seedlings of each Ispir (salt tolerant) and TR43477 (salt susceptible) common bean varieties were treated with NaCl in an incremental fashion in hydroponic system. The plants were photographed just before sacrifice and leaf tissues were harvested.

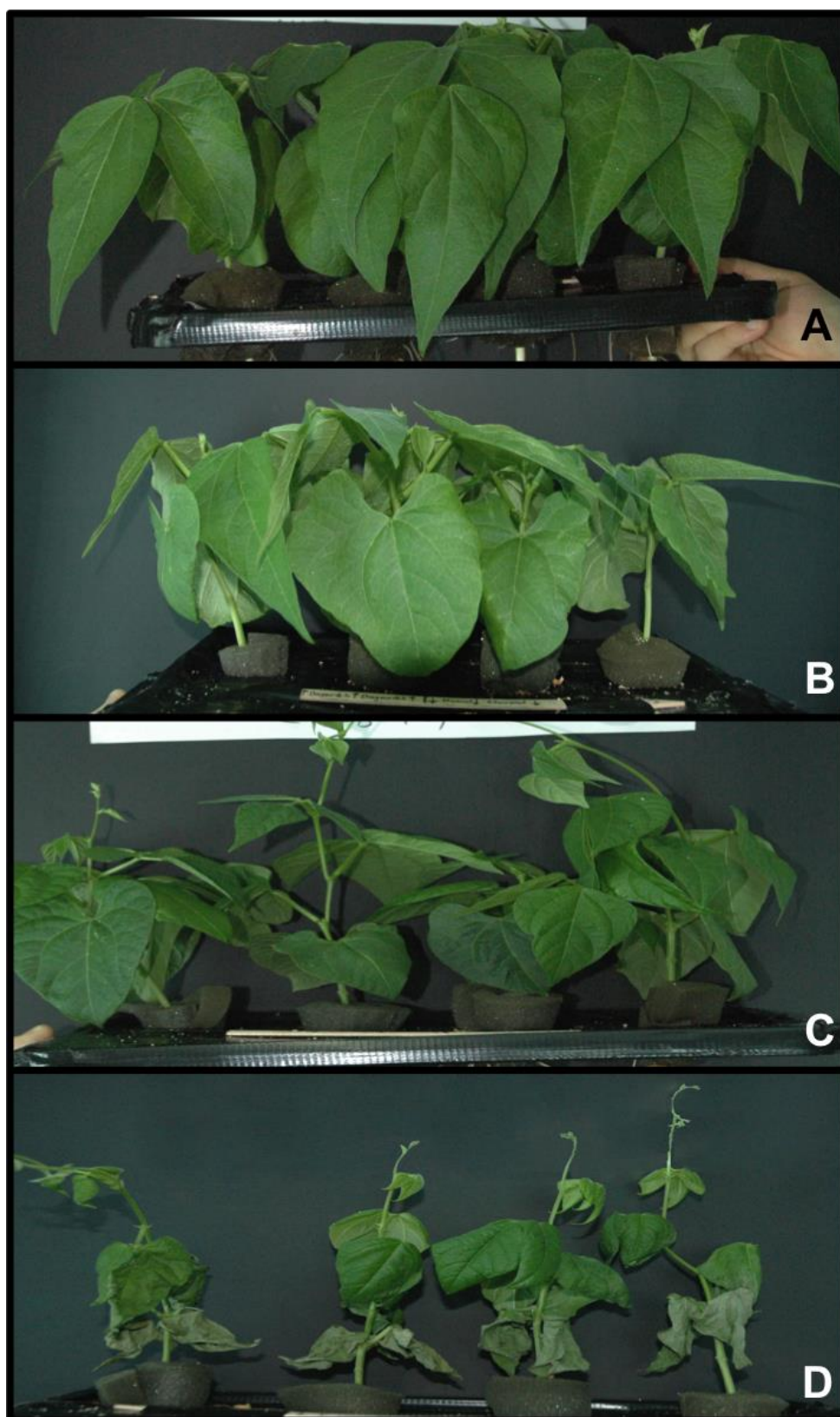


Figure 5.1. Common bean plants before sacrifice A) Ispir control B) NaCl treated Ispir C) TR43477 control D) NaCl treated TR43477.

5.3. Validation of differential expression of candidate genes by qRT-PCR

qRT-PCR analyses was performed for the cDNA obtained from NaCl treated bean leaf tissues and statistical analyses was performed. qRT-PCR was performed for 7 and 11 candidate genes from SSH library and literature respectively. Among candidate genes from the literature, 7 out of 11 genes (*P5CS1*, *SOD1*, *PIP2-2*, *PP2Ca*, *NHX*, *NCED* and *PIP2-3*) from the literature and 3 out of 7 genes (*IDP*, *ABAR*, and *VHP*) from the SSH library were differentially expressed at a significant level upon NaCl treatment between control and treated tissues. *NIP*, *PTR*, *CLCa*, and *CCS* from the literature and *TPK*, *TAU*, *BZIP*, and *AQP* from the SSH library were not differentially expressed at a significant level upon NaCl treatment between control and treated tissues. The differential regulation of candidate genes were the same for both Ispir and TR43477 samples with the exception of *IDP*, which was differentially expressed for TR43477 but not for Ispir samples (Figure 5.2, Figure 5.3, Figure 5.4).

The expression data for rarely expressed candidate genes are difficult to visually discern when viewed in the same scale as abundantly expressed genes. Therefore, the expression data for rarely (Figure 5.3) and abundantly (Figure 5.4) expressed genes were further compiled into two separate charts, which indicates the fold differences compared to Ispir control group expression data.

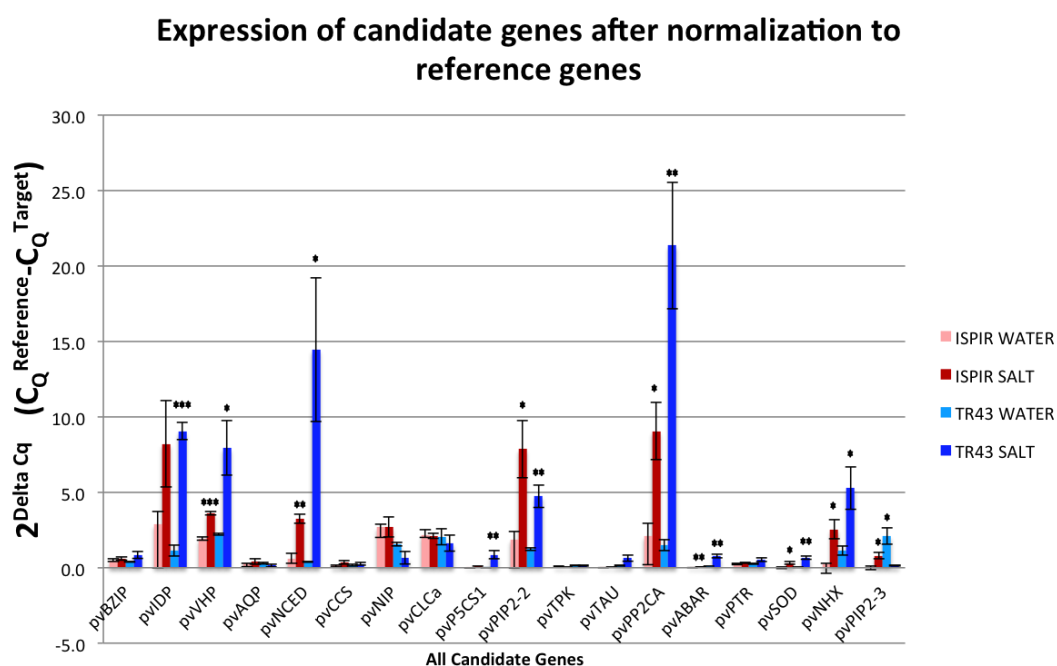


Figure 5.2. $2^{\Delta Cq}$ ($\Delta Cq = C_{Q \text{ Reference}} - C_{Q \text{ Target}}$) values for candidate genes for control and treatment groups of Ispir and TR43477 samples (“*”: $p < 0.05$; “***”: $p < 0.01$, “****”: $p < 0.001$).

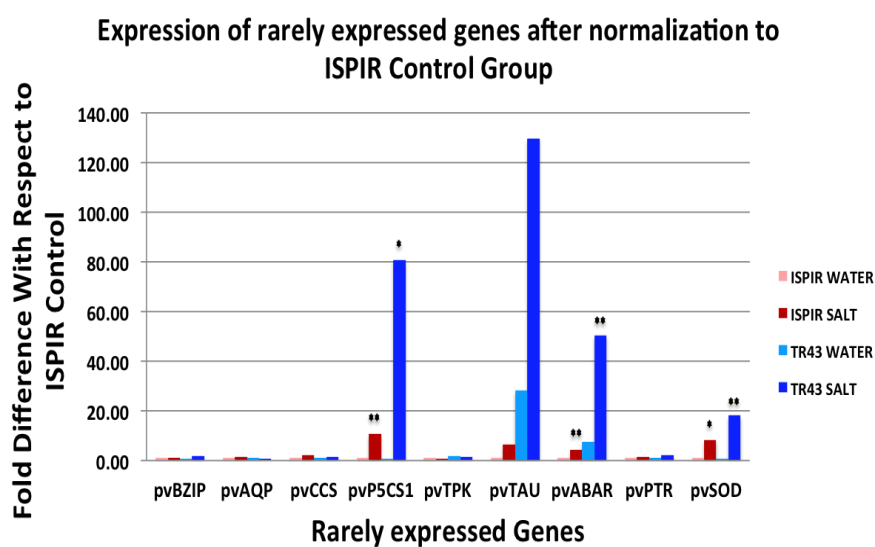


Figure 5.3. Fold difference of rarely expressed candidate genes normalized to Ispir control group (“*”: $p < 0.05$; “***”: $p < 0.01$, “****”: $p < 0.001$).

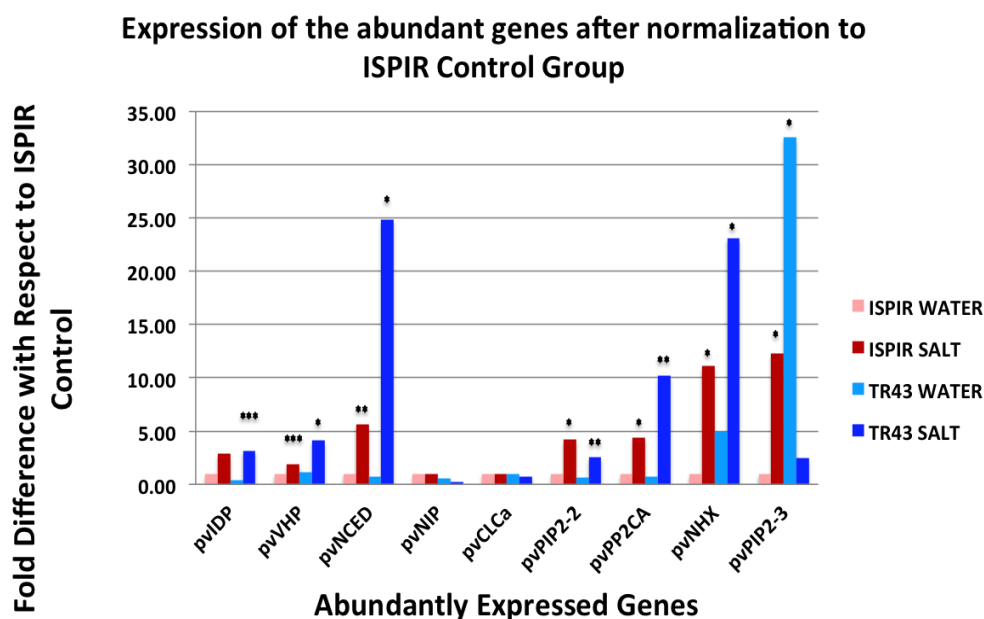


Figure 5.4. Fold difference of abundantly expressed candidate genes normalized to Ispir control group (“*”: $p < 0.05$; “**”: $p < 0.01$, “***”: $p < 0.001$).

5.4. Bioinformatic Analyses

5.4.1. Predicted open reading frames of candidate genes

Open reading frame (ORF) prediction was performed using ORF prediction tool of CLC Main Workbench. The ORF prediction tool produced a hit if a start codon (ATG) and a stop codon (TGA, TAA or TAG) were in frame with each other, which is the required case for a functional ORF. For all candidate genes except *IDP* and *ABAR*, there was a BLAST hit with 100% identity from the RNA-Seq database and the ORFs could be determined from these hits with confidence Table 5.3. For *IDP* and *ABAR*, the predicted ORFs no BLAST hits were found even among the ORFs of genes from closely related legumes such as *Glycine max* or *Medicago truncatula*. As a result, only one candidate gene (*VHP*) were left from the SSH library and there were 7 candidate genes (*P5CS1*, *SOD1*, *PIP2-2*, *PP2Ca*, *NHX*, *NCED* and *PIP2-3*) left from the literature. Summary of Blastn comparisons of predicted ORFs from all candidate genes against

ORFs from closely related species in NCBI Refseq mRNA database were provided in Table 5.4 and the sequences of the predicted ORFs were provided in the Appendix A.

During prediction analyses of *PIP2-2*, we came across a study reporting at *PIP2-2* T-DNA insertional knock out lines have not produced an altered phenotype in salinity stress tolerance, so we have excluded *PIP2-2* from further analyses (Ines, 2008).

Table 5.3. Open reading frame discovery status of all candidate genes.

Gene Abbreviation	ORF Discovery Status	Source of Candidate Gene
<i>pvP5CS1</i>	ORF determined	Literature
<i>pvSOD</i>	ORF determined	Literature
<i>pvPIP2-2</i>	ORF determined	Literature
<i>pvNHX</i>	ORF determined	Literature
<i>pvPP2CA</i>	ORF determined	Literature
<i>pvNCED</i>	ORF determined	Literature
<i>pvPIP2-3</i>	ORF determined	Literature
<i>pvIDP</i>	Not determined	SSH library
<i>pvABAR</i>	Not determined	SSH library
<i>pvVHP</i>	ORF determined	SSH library

Table 5.4. Summary of Blastn comparisons of predicted ORFs from all candidate genes against ORFs from closely related species in NCBI Refseq mRNA database.

Gene Abbreviation	Closest match from RNA-Seq Database	BLAST hit Accession Number	BLAST Hit Gene Name	Predicted ORF Size	BLAST HIT ORF Size	IDENTITY
<i>P5CS1</i>	CL1421.Contig4_All	XM_003516842	<i>Glycine max</i> P5CS1-like transcript variant	2256	2268	91%
<i>SOD</i>	Unigene29999_All	XM_003626314	<i>Medicago truncatula</i> Superoxide Dismutase	459	459	89%

Table 5.4. Summary of Blastn comparisons of predicted ORFs from all candidate genes against ORFs from closely related species in NCBI Refseq mRNA database (cont.).

<i>PIP2-2</i>	CL700.Contig5_All	XM_003540128	<i>Glycine max</i> PIP2-aquaporin-like	864	864	91%
<i>NHX</i>	CL4908.Contig3_All	NM_001253274	<i>Glycine max</i> sodium/hydrogen exchanger 2-like	1629	1641	93%
<i>PP2CA</i>	CL466.Contig1_All	XM_003519309	<i>Glycine max</i> protein phosphatase 2C 37-like	1200	1190	84%
<i>NCED1</i>	CL3137.Contig1_All	NM_102749	<i>Vitis vinifera</i> NCED1 chloroplastic-like	1848	1830	76%
<i>PIP2-3</i>	CL700.Contig1_All	NM_001253110	<i>Glycine max</i> PIP2-2 like	858	858	91%
<i>IDP</i>	-	No BLASTn hit	-	-	-	-
<i>ABAR</i>	-	No BLASTn hit	-	-	-	-
<i>VHP</i>	CL1746.Contig6_All	XM_003522775	<i>Glycine max</i> V-Type proton ATPase 16kDa proteolipid subunit like	495	495	96%

5.5. Gateway Cloning and Overexpression of Candidate Genes

In total, 7 candidate genes from literature and 1 candidate gene from the SSH library were available for Gateway recombinational cloning. Since this cloning system requires multiple steps and several construct formations for every candidate gene cloning, (Figure 4.1), due to time limitations, cloning of only 4 candidate genes were concluded. These four candidate genes consisted of 3 genes from the literature (*pvSOD*, *pvPIP2-3*, and *pvNHX*) and only 1 candidate gene from the SSH library (*pvVHP*).

5.5.1. Preparation of inserts for Entry Vectors

To create the inserts of entry vector constructs for 4 candidate genes, two sets of PCR reactions were set (Figure 4.1). First step reactions were performed using high fidelity polymerase with attb forward and attb reverse primers to amplify the whole ORFs. The second step reactions were performed with attb forward and attb reverse “nonstop” primer, from which the stop codon was removed to allow amplification of the ORF without the stop codon (Table 3.5). This prevented the transcription from stopping before the C-terminal could also be transcribed. The PCR products from these PCR reactions were visualized in gel documentation system after agarose gel electrophoresis to confirm the presence of expected band size. The expected size PCR products were purified from agarose gels and used as template in the third step PCR reactions, which were performed with universal attb primers. The PCR products were also visualized in gel documentation system after agarose gel electrophoresis. For *pvSOD*, *pvVHP*, *pvPIP*, and *pvNHX* genes the first step and the second step PCRs produced the expected bands at the 500bp, 550bp, 910bp, and 1690bp levels respectively (Figure 5.5, Figure 5.6, Figure 5.7 Figure 5.8.).

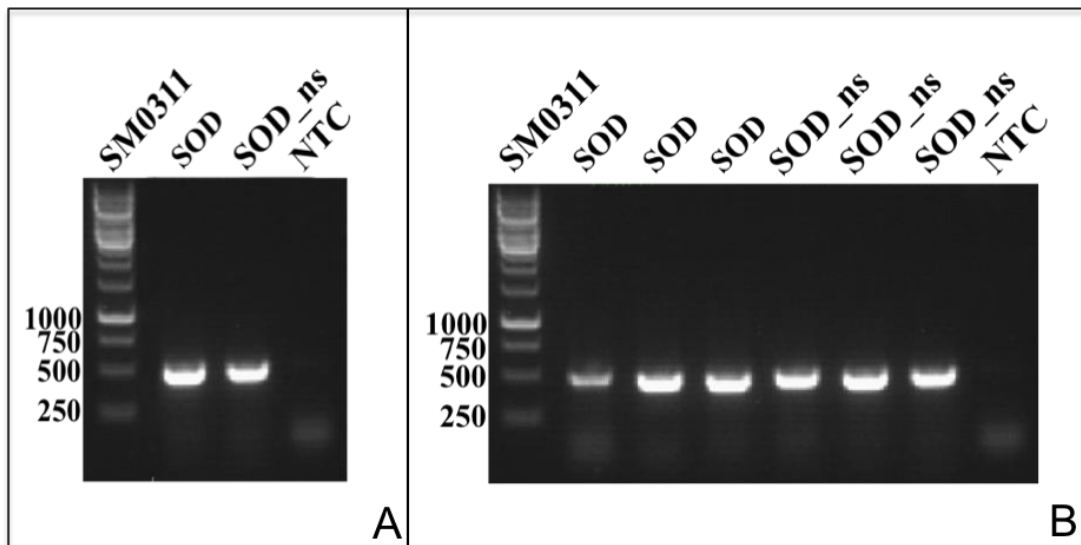


Figure 5.5. A) First step PCR amplifications of *pvSOD* with gene specific attB primers
 B) Second step PCR amplification of *pvSOD* with universal attB primers (SM0311: 1kb ladder; NTC: No template control).

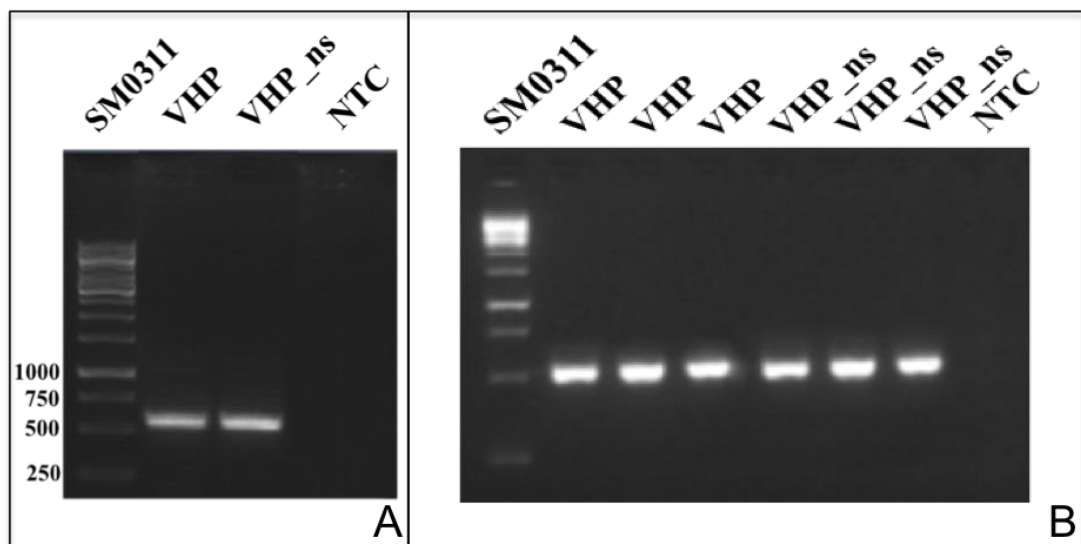


Figure 5.6. A) First step PCR amplifications of *pvVHP* with gene specific attB primers
 B) Second step PCR amplification of *pvVHP* with universal attB primers (SM0311: 1kb ladder; NTC: No template control).

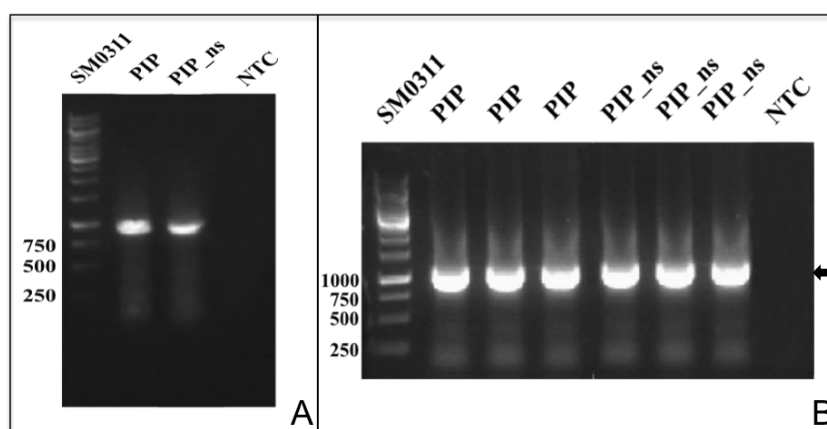


Figure 5.7. A) First step PCR amplifications of *pvPIP* with gene specific attb primers B) Second step PCR amplification of *pvPIP* with universal attb primers (SM0311: 1kb ladder; NTC: No template control).

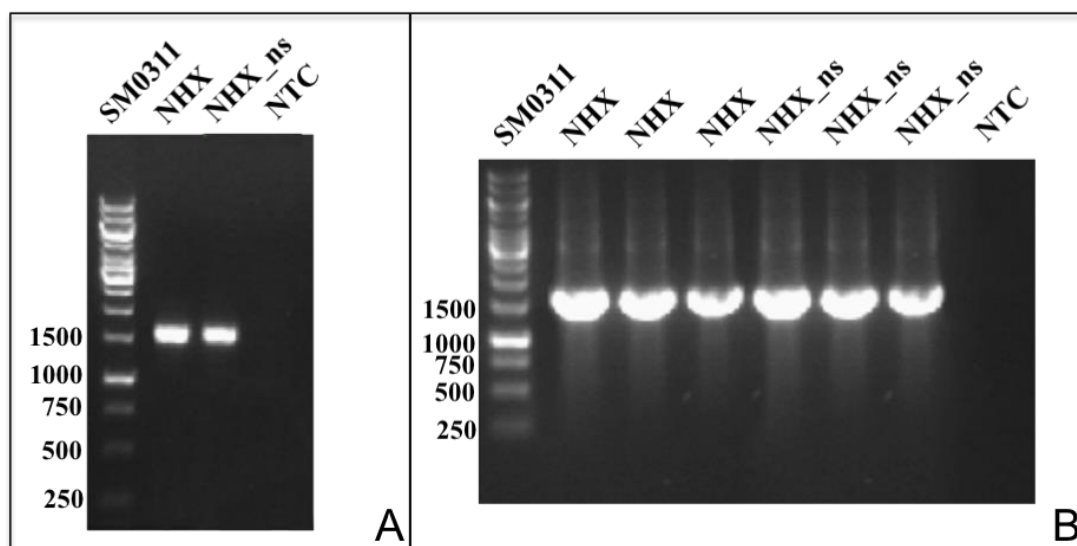


Figure 5.8. A) First step PCR amplifications of *pvNHX* with attb primers B) Second step PCR amplification of *pvNHX* with universal attb primers (SM0311: 1kb ladder; NTC: No template control).

5.5.2. Creation of Entry Vectors

BP clonase reactions were performed using the two entry vectors created for each candidate gene: one with the stop codon and one without. Transformation of competent *E.coli* cells produced multiple colonies on selective medium for all recombinant vectors. Selected colonies were colony PCR'ed using universal attb primers. Positive colonies were Sanger sequenced and compared with the expected ORF sequences.

Colony PCR from candidate genes *pvSOD*, *pvVHP*, and *pvNHX* produced bands at the expected sizes of 500 bp, 550 bp, and 1690 bp respectively (Figure 5.9a, Figure 5.9b, Figure 5.9c). Furthermore, Sequence analysis of the plasmids has shown 100% identity with the expected ORF sequences for both entry vector constructs of *pvSOD*, *pvVHP*, and *pvNHX*.

Blastn analyses were performed to compare the obtained Sanger sequences from the entry vector constructs to the newly released common bean reference genome sequence. For *pvSOD*, the analysis revealed that all 459 nucleotides of SOD207c1 matched perfectly to the long ORF sequence of XM_007147050 *P.vulgaris* hypothetical protein. Similarly, for *pvNHX* gene 1629 nucleotide long ORF sequence of NHX207c1 was also a perfect match to same size long ORF sequence of XM_007144720 *P.vulgaris* hypothetical protein.

In the case of *pvVHP*, while Blastn analysis of VHP207c1 to the common bean reference genome sequence revealed that 495 out of 495 nucleotides of VHP207c1 matched perfectly to the last 495 nucleotides of the 636 nucleotide long ORF sequence of XM_007135850 *P.vulgaris* hypothetical protein. The second best BLASTn hit was obtained with 93% match to 495 nucleotide long ORF of *P.vulgaris* XM_007131684 *P.vulgaris* hypothetical protein, which had a 495 nucleotide long ORF, similar to VHP207c1 (Figure 5.10). The identity between two sequences was 93% (462 out of 495 nucleotides).

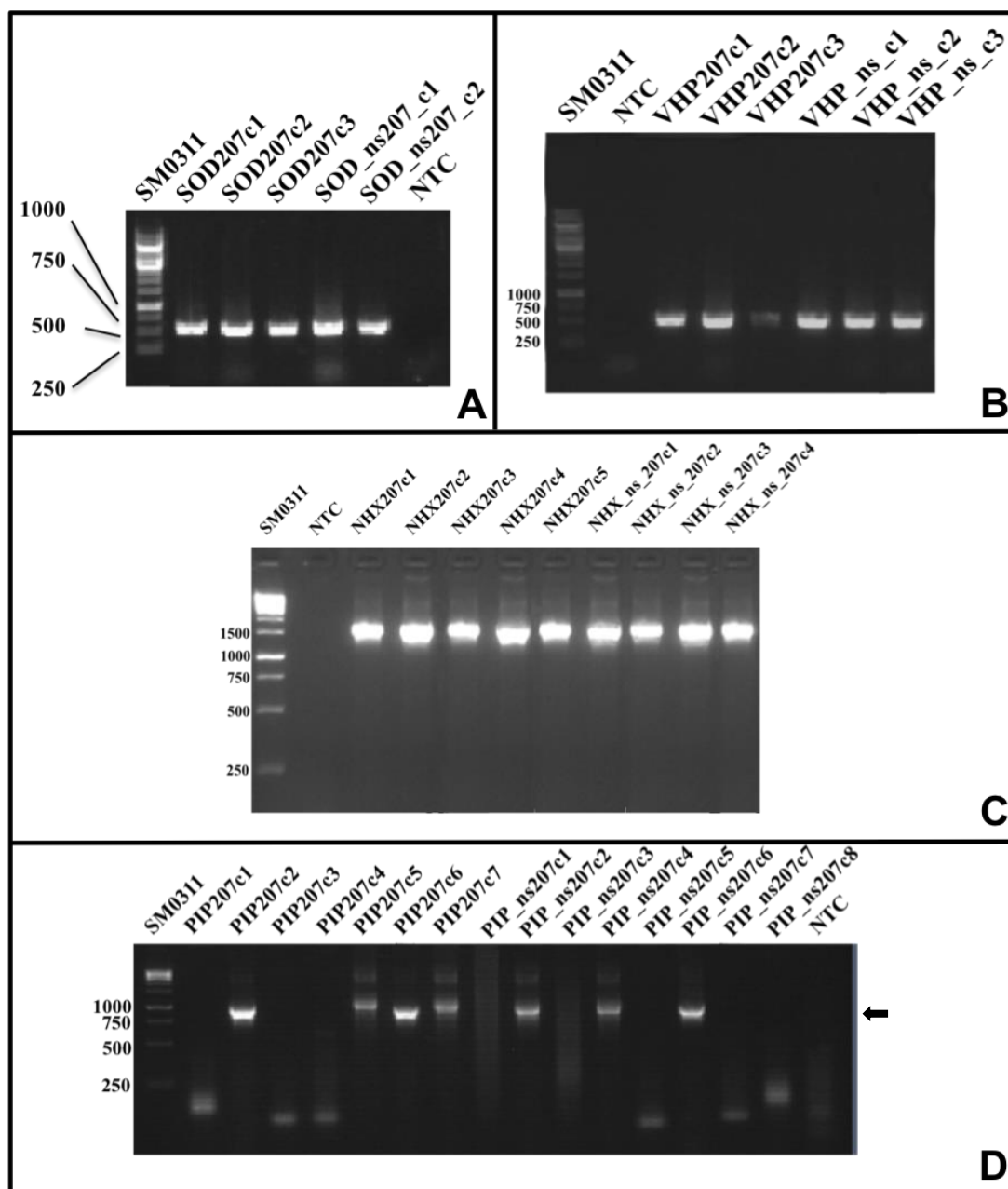


Figure 5.9. Colony PCR with gene specific primers after BP reaction A) SOD207 and SOD_ns207 B) VHP207 and VHP_ns207 C) NHX207 and NHX_ns207 D) PIP207 and PIP_ns207 (SM0311: 1kb ladder; NTC: No template control).

			20		
VHP207c1	ATG-----	-----	-----	3	
XM_007135850_ORF	ATG CACGTGG	TTATCCCCTG	GTCATGGCCC	30	
XM_007131684_ORF	ATG-----	-----	-----	3	
	40		60		
VHP207c1	-----	-----	-----	3	
XM_007135850_ORF	TCTCATGGGT	CGTATCTGTC	CCAGGGTCTC	60	
XM_007131684_ORF	-----	-----	-----	3	
		80			
VHP207c1	-----	-----	-----	3	
XM_007135850_ORF	CCAGCAGTGG	TGTGGATACT	TCACGAGTCA	90	
XM_007131684_ORF	-----	-----	-----	3	
	100		120		
VHP207c1	-----	-----	-----	3	
XM_007135850_ORF	GGTTTACCCC	CTACTGATCT	ATCCGACGTC	120	
XM_007131684_ORF	-----	-----	-----	3	
		140			
VHP207c1	-----	-----	-----GCTGGC	9	
XM_007135850_ORF	GTTGCAGATC	CATCAACGGT	CATGGCTGGC	150	
XM_007131684_ORF	-----	-----	-----GCT TCT	9	
	160		180		
VHP207c1	TTCAGCGGTG	ATGAAACGGC	ACCGTTTTTC	39	
XM_007135850_ORF	TTCAGCGGTG	ATGAAACGGC	ACCGTTTTTC	180	
XM_007131684_ORF	TTCAGCGG CG	ATGA G ACGGC	ACCGTTTTTC	39	
		200			
VHP207c1	GGCTTCCTCG	GAGCCGCAGC	TGCCCTCGTC	69	
XM_007135850_ORF	GGCTTCCTCG	GAGCCGCAGC	TGCCCTCGTC	210	
XM_007131684_ORF	GGCTTCCT TG	GAGCCGC TGC	TGC G CTCGTC	69	
	220		240		
VHP207c1	TTCTCCTGCA	TGGGAGCTGC	CTATGGAACA	99	
XM_007135850_ORF	TTCTCCTGCA	TGGGAGCTGC	CTATGGAACA	240	
XM_007131684_ORF	TTCTCCTG TA	TGGGAGCTGC	CTATGG C ACA	99	
		260			
VHP207c1	GCAAAGAGTG	GTGTGGGCGT	GGCTTCCATG	129	
XM_007135850_ORF	GCAAAGAGTG	GTGTGGGCGT	GGCTTCCATG	270	
XM_007131684_ORF	GCAAAGAGTG	GTGTGGGCGT	GGCTTC G ATG	129	
	280		300		
VHP207c1	GGTGTAATGA	GACCTGAGCT	AGTGATGAAG	159	
XM_007135850_ORF	GGTGTAATGA	GACCTGAGCT	AGTGATGAAG	300	
XM_007131684_ORF	GGTGT G ATGA	GACC G GAGCT	G GTGATGAAG	159	

Figure 5.10. Sequence alignment of VHP207c1, XM_007135850_ORF, and XM_007131684_ORF.

			320	
VHP207c1	TCTATTGTTT	CAGTTGTTAT	GGCTGGAGTT	189
XM_007135850_ORF	TCTATTGTTT	CAGTTGTTAT	GGCTGGAGTT	330
XM_007131684_ORF	TCTATTGTTT	CTGTTGTTAT	GGCTGGAGTT	189
		340		360
VHP207c1	TTGGGTATTT	ATGGCCTTAT	TATTGCTGTT	219
XM_007135850_ORF	TTGGGTATTT	ATGGCCTTAT	TATTGCTGTT	360
XM_007131684_ORF	TTGGGTATTT	ATGGTCTTAT	TATTGCTGTT	219
		380		
VHP207c1	ATTATTAGTA	CCGGTATTAA	TCCAAAGGCC	249
XM_007135850_ORF	ATTATTAGTA	CCGGTATTAA	TCCAAAGGCC	390
XM_007131684_ORF	ATTATCAGTA	CTGGCATTAA	TCCCAAGGCC	249
		400		420
VHP207c1	AAATCTTATT	ACCTCTTTGA	TGGTTACGCA	279
XM_007135850_ORF	AAATCTTATT	ACCTCTTTGA	TGGTTACGCA	420
XM_007131684_ORF	AAATCTTATT	ACCTCTTTGA	TGGTTATGCA	279
		440		
VHP207c1	CATCTCTCCT	CTGGTCTTGC	CTGTGGCTTG	309
XM_007135850_ORF	CATCTCTCCT	CTGGTCTTGC	CTGTGGCTTG	450
XM_007131684_ORF	CATCTCTCCT	CTGGTCTTGC	CTGTGGGCTG	309
		460		480
VHP207c1	GCTGGGCTGT	CTGCTGGCAT	GGCTATTGGT	339
XM_007135850_ORF	GCTGGGCTGT	CTGCTGGCAT	GGCTATTGGT	480
XM_007131684_ORF	GCTGGGCTCT	CTGCTGGCAT	GGCTATTGGC	339
		500		
VHP207c1	ATTGTTGGTG	ATGCTGGTGT	TAGAGCCAAT	369
XM_007135850_ORF	ATTGTTGGTG	ATGCTGGTGT	TAGAGCCAAT	510
XM_007131684_ORF	ATTGTTGGTG	ATGCTGGTGT	TAGAGCCAAC	369
		520		540
VHP207c1	GCACAGCAAC	CAAAACTTTT	TGTTGGAATG	399
XM_007135850_ORF	GCACAGCAAC	CAAAACTTTT	TGTTGGAATG	540
XM_007131684_ORF	GCACAGCAGC	CAAAAGCTTTT	TGTTGGAATG	399
		560		
VHP207c1	ATTCTCATCC	TCATTTTTC	TGAAGCTCTT	429
XM_007135850_ORF	ATTCTCATCC	TCATTTTTC	TGAAGCTCTT	570
XM_007131684_ORF	ATTCTCATCC	TCATCTTTC	TGAAGCTCTT	429
		580		600
VHP207c1	GCCCTCTATG	GTCTCATTGT	TGGTATTATC	459
XM_007135850_ORF	GCCCTCTATG	GTCTCATTGT	TGGTATTATC	600
XM_007131684_ORF	GCCCTCTATG	GTCTCATTGT	TGGTATTATC	459

Figure 5.11. Sequence alignment of VHP207c1, XM_007135850_ORF, and XM_007131684_ORF.

```

                                620
                                |
VHP207c1 CTGTCTTCTC GTGCTGGCCA GTCTAGAGCC 489
XM_007135850_ORF CTGTCTTCTC GTGCTGGCCA GTCTAGAGCC 630
XM_007131684_ORF CTGTCCTCTC GTGCTGGTCA GTCTAGAGCT 489

VHP207c1 GACTAA 495
XM_007135850_ORF GACTAA 636
XM_007131684_ORF GATTAA 495

```

Figure 5.12. Sequence alignment of VHP207c1, XM_007135850_ORF, and XM_007131684_ORF.

In the case of *pVPIP*, colony PCR was performed on seven colonies from the PIP207 plate and eight colonies from the PIP_ns207 plate using second step universal attb primers. Colonies PIP207c2, PIP207c5, PIP207c6, PIP207c7, PIP_ns207c2, PIP_ns207c4, and PIP_ns207c6 produced the expected 913 bp bands just below the 1kb marker band but with minute differences in size among themselves. Other colonies have not produced bands around the expected size (Figure 5.9d).

Plasmid isolation was performed from colonies PIP207c4, PIP207c7, PIP_ns207c2, PIP_ns207c4, and PIP_ns207c6 and plasmids were submitted for Sanger sequencing. Both forward and reverse reads of colonies PIP_ns207c4 and PIP_ns207c6 produced 204 bp long reads that had no homology with the expected sequence or among each other. BLASTn analyses of sequences from colonies PIP207c7, PIP_ns207c2, and PIP_ns207c4 against expected ORF sequences produced 96%, 95%, and 94% sequence identity respectively. Alignment of sequences from three colonies and expected ORF sequence is provided in Figure 5.15. PIP207c7 construct was chosen as the entry vector for following experiments since it had the highest sequence similarity (96%) to the expected ORF.

			20			40	
PIP2-3 ORF	ATGGCTAAGC	ATGATGCTG	A	GGGTGGTTCC	TTCTCTGCAA	40	
PIP_ns207c2	ATGGCTAAGC	ATGATGCTG	A	GGGTGGTTCC	TTCTCTGCAA	40	
PIP207c7	ATGGCTAAGC	ATGATGCTG	C	GGGTGGTTCC	TTCTCTGCAA	40	
PIP_ns207c4	ATGGCTAAGC	ATGATGCTG	C	TGGTGGTTCC	TTCTCTGCAA	40	
			60			80	
PIP2-3 ORF	AGGACTACC-	ATGACCCTCC	T	CCAGCACC	CTTGATTGAT	78	
PIP_ns207c2	AGGACTACC	ATGACCCTCC	T	CCAGCACC	CTTGATTGAT	80	
PIP207c7	AGGACTACC-	ATGACCCTCC	T	CCAGCACC	CTTGATTGAT	78	
PIP_ns207c4	AGGACTACC-	ATGACCCTCC	T	CCAGCACC	CTTGATTGAT	79	
			100			120	
PIP2-3 ORF	GCTCAGGAAC	TCACACAGTG		GTCCTTCTAC	AGGGCCTTGA	118	
PIP_ns207c2	GCTCAGGAAC	TCACACA	ATG	GTCCTTCTAC	AGGGCCTTGA	120	
PIP207c7	GCTCAGGAAC	TCACACAGTG		GTCCTTCTAC	AGGGCCTTGA	118	
PIP_ns207c4	GCTCAGGAAC	TCACACAGTG		GTCCTTCTAC	AGGGCCTTGA	119	
			140			160	
PIP2-3 ORF	TTGCTGAGTT	CATTGCCACA		CTGCTCTTCC	TTTACATCAC	158	
PIP_ns207c2	TTGCTGAGTT	CATTGCCACA		CTGCTCTTCC	TTTACATCAC	160	
PIP207c7	TTGCTGAGTT	CATTGCCACA		CTGCTCTTCC	TTTACATCAC	158	
PIP_ns207c4	TTGCTGAGTT	CATTGCCACA		CTGCTCTTCC	TTTACATCAC	159	
			180			200	
PIP2-3 ORF	AGTGCTCACC	GTCATTGGTT		ACAAGAGCCA	GAGTGATGTC	198	
PIP_ns207c2	AGTGCTCACC	GTCATTGGTT		ACAAGAGCCA	GAGTG	TGTC	200
PIP207c7	AGTGCTCACC	GTCATTGGTT		ACAAGAGCCA	GAGTGATGTC	198	
PIP_ns207c4	AGTGCTCAC	T	GTCATTGG	C	ACAAGAGCCA	GAGTGATGTC	199
			220			240	
PIP2-3 ORF	AAAGCTGGGG	GTGATCTCTG		TGGTGGGGTT	GGCATTCTTG	238	
PIP_ns207c2	AAAGCTGGGG	GTGATCTCTG		GGGTGGGGTT	GGCATTCTTG	240	
PIP207c7	AAAGCTGGGG	GTGATCTCTG		TGGTGGGGTT	GGCATTCTTG	238	
PIP_ns207c4	AA	G	GCTGGGG	GTGATCTCTG	TGGTGGGGTT	GGCATTCTTG	239
			260			280	
PIP2-3 ORF	GCATTGCTTG	GGCCTTCGGT		GGCATGATCT	TCATCCTTGT	278	
PIP_ns207c2	GCATTGCTTG	GGCCTTCGGT		GGCATGATCT	TCATCCTTGT	280	
PIP207c7	GCATTGCTTG	GGCCTTCGGT		GGCATGATCT	TCATCCTTGT	278	
PIP_ns207c4	GCATTGCTTG	GGCCTTCGGT		GGCATGATCT	TCATCCTTGT	279	
			300			320	
PIP2-3 ORF	TTACTGCACT	GCTGGAATCT		CAGGAGGTCA	CATAAACCCA	318	
PIP_ns207c2	TTACTGCACT	GCTGGAATCT		CAGGAGGTCA	CATAAACCCA	320	
PIP207c7	TTACTGCACT	GCTGGAATCT		CAGGAGGTCA	CATAAACCCA	318	
PIP_ns207c4	TTACTGCACT	GCTGGAATCT		CAGGAGGTCA	CATAAACCCA	319	
			340			360	
PIP2-3 ORF	GCAGTGACTT	TTGGACTCTT		CTTGGCTCGG	AAGGTGTCTT	358	
PIP_ns207c2	GCAGTGACTT	TTGGACTCTT		CTTGGCTCGG	AAGGTGTCTT	360	
PIP207c7	GCAGTGACTT	TTGGACTCTT		CTTGGCTCGG	AAGGTGTCTT	358	
PIP_ns207c4	G	T	AGTGACTT	TTGGACTCTT	CTTGGCTCGG	AAGGTGTCTT	359
			380			400	
PIP2-3 ORF	TGATAAGGGC	TATAATGTAC		ATGGTGGCTC	AGTGCTTGGG	398	
PIP_ns207c2	TGATAAGGGC	TATAATGTAC		ATGGTGGCTC	AGTGCTTGGG	400	
PIP207c7	TGATAAGGGC	TATAATGTAC		ATGGTGGCTC	AGTGCTTGGG	398	
PIP_ns207c4	TGATAAGGGC	TATAATGTAC		ATGGTGGCTC	AGTGCTTGGG	399	

Figure 5.13. Alignment of predicted PIP2-3 ORF sequence and sequences obtained from colonies PIP207c7, PIP_ns207c2, and PIP_ns207c4. Mismatching residues are highlighted.

		420		440	
PIP2-3 ORF	GGCCATATGT	GGAGTTGGGT	TGGTTAAGGC	CTTCCAAAAG	438
PIP_ns207c2	GGCCATATGT	GGAGTTGGGT	TGGTTAAGGC	CTTCCAAAAG	440
PIP207c7	GGCCATATGT	GGAGTTGGGT	TGGTTAAGGC	CTTCCAAAAG	438
PIP_ns207c4	GGCCATATGT	GGAGTTGGGT	TGGTTAAGGC	CTTCCAAAAG	439
		460		480	
PIP2-3 ORF	GCTTACTACA	ACAAGTATGG	TGGTGGGGCC	AATGAGCTGA	478
PIP_ns207c2	GCTTACTACA	ACAAGTATGG	TGGTGGGGCC	AATGAGCTGA	480
PIP207c7	GCTTACTACA	ACAAGTATGG	TGGTGGGGCC	AATGAGCTGA	478
PIP_ns207c4	GCTTACTACA	CAAGTATGG	TGGTGGGGCC	AATGAGCTA	479
		500		520	
PIP2-3 ORF	GTGATGGGTA	CAGCACAGGT	GTTGGATTGG	GTGCTGAGAT	518
PIP_ns207c2	GTGATGGGTA	CAGCACAGGT	GTTGGATTGG	GTGCTGAGAT	520
PIP207c7	GTGATGGGTA	CAGCACAGGT	GTTGGATTGG	GTGCTGAGAT	518
PIP_ns207c4	GTGAAGGGTA	CAGCACAGGT	GTTGGATTGG	GTGCTGAGAT	519
		540		560	
PIP2-3 ORF	CATTGGAACC	TTCGTTTTGG	TGTACACTGT	CTTCTCTGCT	558
PIP_ns207c2	CATTGGAACC	TTCGTTTTGG	TGTACACTGT	CTTCTCTGCT	560
PIP207c7	CATTGGAACC	TTCGTTTTGG	TGTACACTGT	CTTCTCTGCT	558
PIP_ns207c4	CATTGGAACC	TTCGTTTTGG	TGTACACTGT	CTTCTCTGCT	559
		580		600	
PIP2-3 ORF	ACCGACCCCA	AGAGGAATGC	TAGAGATTCT	CATGTCCCGG	598
PIP_ns207c2	ACCGACCCCA	AGAGGAATGC	TAGAGATTCT	CATGTCCCGG	600
PIP207c7	ACCGACCCCA	AGAGGAATGC	TAGAGATTCT	CATGTCCCGG	598
PIP_ns207c4	ACCGACCCCA	AGAGGAAGC	TAGAGATTCT	CATGTACCGG	599
		620		640	
PIP2-3 ORF	TTCTGGCTCC	ACTGCCAATT	GGGTTTGCTG	TGTTTCATGGT	638
PIP_ns207c2	TTCTGGCTCC	ACTGCCAATT	GGGTTTGCTG	TGTTTCATGGT	640
PIP207c7	TTCTGGCTCC	ACTGCCAATT	GGGTTTGCTG	TGTTTCATGGT	638
PIP_ns207c4	TTTGGCTCC	ACTGCCAATT	GGGTTTGCTG	TGTTTCATGGT	639
		660		680	
PIP2-3 ORF	TCACTTGGCT	ACAATCCCAG	TACTGGCAC	TGGCATTAAAC	678
PIP_ns207c2	TCACTTGGCA	ACCATCCCAG	TGACTGGCAC	TGGTATTAAC	680
PIP207c7	TCACTTGGCA	ACCATCCCAG	TGACTGGCAC	TGGTATTAAC	678
PIP_ns207c4	TCACTTGGCA	ACCATCCCAG	TGACTGGCAC	TGGTATTAAC	679
		700		720	
PIP2-3 ORF	CCAGCAAGGA	GTTTTGGAGC	AGCTGTAATC	TACAACAATG	718
PIP_ns207c2	CCTGCTAGAA	GTCTTGGAGC	TGCTGTCAAG	TACAACAAAG	720
PIP207c7	CCTGCTAGAA	GTCTTGGAGC	TGCTGTCAATA	TACAACAAAG	718
PIP_ns207c4	CCTGCTAGAA	GTCTTGGAGC	TGCTGTCAATA	TACAGCAAAG	719
		740		760	
PIP2-3 ORF	AAAAAATCTG	GGATGATCAG	TGGATTTTCT	GGGTTGGACC	758
PIP_ns207c2	AGAAGGTCTG	GGATGACCAA	TGGATCTTTT	GGGTTGGACC	760
PIP207c7	AGAAGGCCTG	GGATGACCAA	TGGATCTTTT	GGGTTGGACC	758
PIP_ns207c4	AGAAGGCCTG	GGATGACCAA	TGGATCTTTT	GGGTTGGACC	759
		780		800	
PIP2-3 ORF	AATTATTGGA	GCAGCAGTGG	CAGCAATTCTA	CCACCAATAC	798
PIP_ns207c2	ATTCATTGGA	GCAGCCATTG	CAGCCTTCTA	CCACCAATTC	800
PIP207c7	ATTCATTGGA	GCAGCCATTG	CAGCCTTCTA	CCACCAATTC	798
PIP_ns207c4	ATTCATTGGA	GTAGCCATTAA	CAGCCTTCTA	CCACCAATTC	799

Figure 5.14. Alignment of predicted PIP2-3 ORF sequence and sequences obtained from colonies PIP207c7, PIP_ns207c2, and PIP_ns207c4. Mismatching residues are highlighted.

			780			800	
PIP2-3 ORF	AAT	TATTGGA	GCAGCAGTGG	CAGCA	TTCTA	CCACCAATAC	798
PIP_ns207c2	ATTCATTGGA	GCAGCCATTG	CAGCCTTCTA	CCACCAATTC			800
PIP207c7	ATTCATTGGA	GCAGCCATTG	CAGCCTTCTA	CCACCAATTC			798
PIP_ns207c4	ATTCATTGGA	GTAGCCATT	A	CAGCCTTCTA	CCACCAATTC		799
			820			840	
PIP2-3 ORF	ATCCTTAGAG	CAGCTGCTAT	CAAGGC	ACTT	GGATCATTCA		838
PIP_ns207c2	ATCTTGAGAG	CGGTGCTGC	TAAGGCTCTT	GGATCATTCA			840
PIP207c7	ATCTTGAGAG	CAGGTGCTGC	TAAGGCTCTT	GGATCATTCA			838
PIP_ns207c4	ATCTTGAGAG	CAGGTGCTGC	TAAGGCTCTT	GGATCATTCA			839
PIP2-3 ORF	GGAGCAATGC	TTAA					852
PIP_ns207c2	GGAGCAATGC	T--					851
PIP207c7	GGAGCAATGC	TTAA					852
PIP_ns207c4	GGAGCAATGC	T--					850

Figure 5.15. Alignment of predicted PIP2-3 ORF sequence and sequences obtained from colonies PIP207c7, PIP_ns207c2, and PIP_ns207c4. Mismatching residues are highlighted.

With regard to the comparison of PIP207c to the common bean reference genome, BLASTn analysis revealed that 842 out of 845 nucleotides ORF sequence of PIP207c7 matched to the 848 nucleotide long ORF sequence of XM_007151810 *P.vulgaris* hypothetical protein. Sequence alignment of two sequences is provided in Figure 5.17.

			20			40
PIP207c7	ATGGCTAAGC	ATGATGCTG	C	GGGTGGTTCC	TTCTCTGCAA	40
XM_007151810_ORF	ATGGCTAAGC	ATGATGCTG	A	GGGTGGTTCC	TTCTCTGCAA	40
			60			80
PIP207c7	AGGACTACCA	TGACCCTCCT		CCAGCACCCCT	TGATTGATGC	80
XM_007151810_ORF	AGGACTACCA	TGACCCTCCT		CCAGCACCCCT	TGATTGATGC	80
			100			120
PIP207c7	TCAGGAACTC	ACACAGTGGT		CCTTCTACAG	GGCCTTGATT	120
XM_007151810_ORF	TCAGGAACTC	ACACAGTGGT		CCTTCTACAG	GGCCTTGATT	120
			140			160
PIP207c7	GCTGAGTTCA	TTGCCACACT		GCTCTTCCTT	TACATCACAG	160
XM_007151810_ORF	GCTGAGTTCA	TTGCCACACT		GCTCTTCCTT	TACATCACAG	160
			180			200
PIP207c7	TGCTCACCGT	CATTGGTTAC		AAGAGCCAGA	GTGATGTCAA	200
XM_007151810_ORF	TGCTCACCGT	CATTGGTTAC		AAGAGCCAGA	GTGATGTCAA	200
			220			240
PIP207c7	AGCTGGGGGT	GATCTCTGTG		GTGGGGTTGG	CATTCTTGGC	240
XM_007151810_ORF	AGCTGGGGGT	GATCTCTGTG		GTGGGGTTGG	CATTCTTGGC	240
			260			280
PIP207c7	ATTGCTTGGG	CCTTCGGTGG		CATGATCTTC	ATCCTTGTTT	280
XM_007151810_ORF	ATTGCTTGGG	CCTTCGGTGG		CATGATCTTC	ATCCTTGTTT	280
			300			320
PIP207c7	ACTGCACTGC	TGGAATCTCA		GGAGGTCACA	TAAACCCAGC	320
XM_007151810_ORF	ACTGCACTGC	TGGAATCTCA		GGAGGTCACA	TAAACCCATG	320
			340			360
PIP207c7	AGTGACTTTT	GGACTCTTCT		TGGCTCGGAA	GGTGTCTTTG	360
XM_007151810_ORF	AGTGACTTTT	GGACTCTTCT		TGGCTCGGAA	GGTGTCTTTG	360
			380			400
PIP207c7	ATAAGGGCTA	TAATGTACAT		GGTGGCTCAG	TGCTTGGGGG	400
XM_007151810_ORF	ATAAGGGCTA	TAATGTACAT		GGTGGCTCAG	TGCTTGGGGG	400
			420			440
PIP207c7	CCATATGTGG	AGTTGGGTTG		GTTAAGGCCT	TCCAAAAGGC	440
XM_007151810_ORF	CCATATGTGG	AGTTGGGTTG		GTTAAGGCCT	TCCAAAAGGC	440
			460			480
PIP207c7	TTACTACAAC	AAGTATGGTG		GTGGGGCCAA	TGAGCTGAGT	480
XM_007151810_ORF	TTACTACAAC	AAGTATGGTG		GTGGGGCCAA	TGAGCTGAGT	480
			500			520
PIP207c7	GATGGGTACA	GCACAGGTGT		TGGATTGGGT	GCTGAGATCA	520
XM_007151810_ORF	GATGGGTACA	GCACAGGTGT		TGGATTGGGT	GCTGAGATCA	520
			540			560
PIP207c7	TTGGAACCTT	CGTTTTGGTG		TACACAGTCT	TCTCTGCTAC	560
XM_007151810_ORF	TTGGAACCTT	CGTTTTGGTG		TACACAGTCT	TCTCTGCTAC	560
			580			600
PIP207c7	CGACCCCAAG	AGGAATGCTA		GAGATTCTCA	TGTCCCGGTT	600
XM_007151810_ORF	CGACCCCAAG	AGGAATGCTA		GAGATTCTCA	TGTCCCGGTT	600
			620			640
PIP207c7	CTGGCTCCAC	TGCCAATTGG		GTTTGCTGTG	TTCATGGTTC	640
XM_007151810_ORF	CTGGCTCCAC	TGCCAATTGG		GTTTGCTGTG	TTCATGGTTC	640
			660			680
PIP207c7	ACTTGGCAAC	CATCCCAGTG		ACTGGCACTG	GTATTAACCC	680
XM_007151810_ORF	ACTTGGCAAC	CATCCCAGTG		ACTGGCACTG	GTATTAACCC	680

Figure 5.16. Sequence alignment of PIP207c7 construct and XM_007151810, the matching sequence from the recently published reference genome.

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                                660                                680
PIP207c7  ACTTGGCAAC  CATCCCAGTG  ACTGGCACTG  GTATTAACCC  680
XM_007151810_ORF  ACTTGGCAAC  CATCCCAGTG  ACTGGCACTG  GTATTAACCC  680

                                700                                720
PIP207c7  TGCTAGAAGT  CTTGGAGCTG  CTGTCATATA  CAACAAAGAG  720
XM_007151810_ORF  TGCTAGAAGT  CTTGGAGCTG  CTGTCATATA  CAACAAAGAG  720

                                740                                760
PIP207c7  AAGGCCTGGG  ATGACCAATG  GATCTTTTGG  GTTGGACCAT  760
XM_007151810_ORF  AAGGCCTGGG  ATGACCAATG  GATCTTTTGG  GTTGGACCAT  760

                                780                                800
PIP207c7  TCATTGGAGC  AGCCATTGCA  GCCTTCTACC  ACCAATTCAT  800
XM_007151810_ORF  TCATTGGAGC  AGCCATTGCA  GCCTTCTACC  ACCAATTCAT  800

                                820                                840
PIP207c7  CTTGAGAGCA  GGTGCTGCTA  AGGCTCTTGG  ATCATTTCAGG  840
XM_007151810_ORF  CTTGAGAGCA  GGTGCTGCTA  AGGCTCTTGG  ATCATTTCAGG  840

PIP207c7  AGCAA-----TGCTTAA  852
XM_007151810_ORF  AGCAACCCCACTGTTTGA  858

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Figure 5.17. Sequence alignment of PIP207c7 construct and XM_007151810, the matching sequence from the recently published reference genome.

5.5.3. Creation of Expression Vectors

LR reactions were performed using pGWB412, pGWB411 and pMDC32 destination vectors and the expression vector constructs created in competent *E.coli* cells. Colony PCR was performed on several colonies from each of the three plates. Colonies from recombinant expression vectors produced bands at the expected sizes, which were the same as that of the entry vectors. (Figure 5.18a, Figure 5.19a, Figure 5.20a, and Figure 5.21a). Positive colonies of each expression vector of all candidate genes were subjected to plasmid isolation. The Sanger sequencing of all expression vectors has confirmed the expected sequence and insert size for all cloned 4 candidate genes. These expression vectors containing the confirmed insert sequences were used to transform competent *Agrobacterium* cells. Finally the resulting colonies were screened with colony PCR using first step attb primers and the positive colonies were made into glycerol stock (Figure 5.18b, Figure 5.19b, Figure 5.20b, Figure 5.21b).

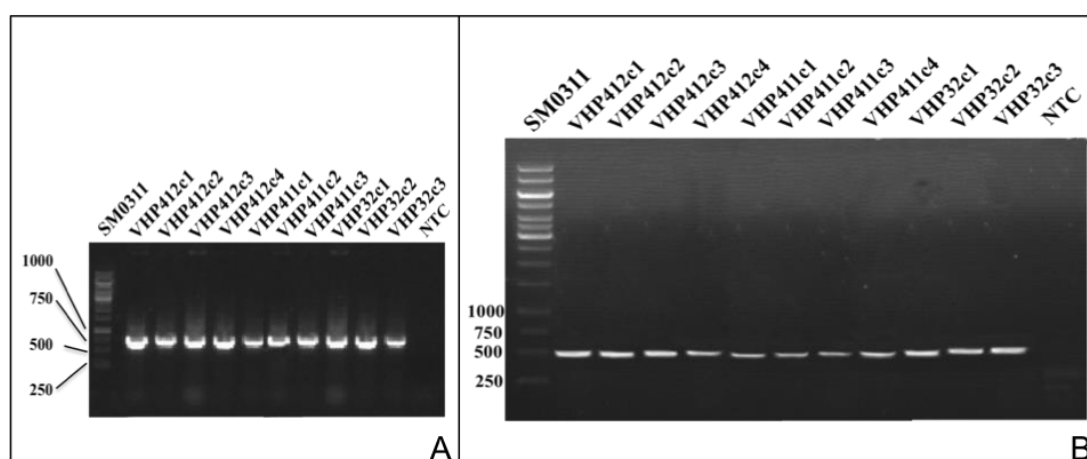


Figure 5.18. Colony PCR for VHP411, VHP412, and VHP32 constructs with gene specific primers (A) after LR reactions and (B) after Agrobacterium transformation (SM0311: 1kb ladder; NTC: No template control).

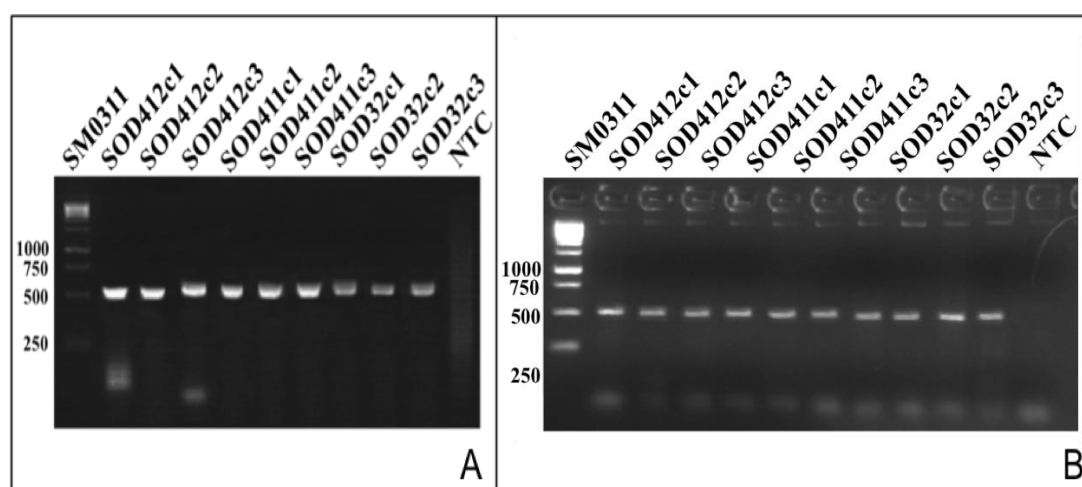


Figure 5.19. Colony PCR for SOD411, SOD412, and SOD32 constructs with gene specific primers (A) after LR reactions and (B) after agrobacterium transformation (SM0311: 1kb ladder; NTC: No template control).

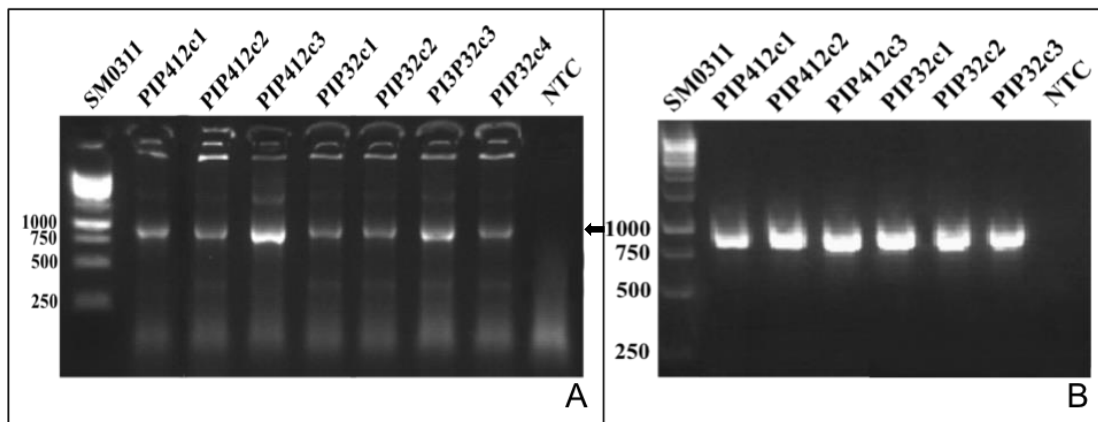


Figure 5.20. Colony PCR for PIP411 and PIP32 constructs with gene specific primers (A) after LR reactions and (B) after agrobacterium transformation (SM0311: 1kb ladder; NTC: No template control).

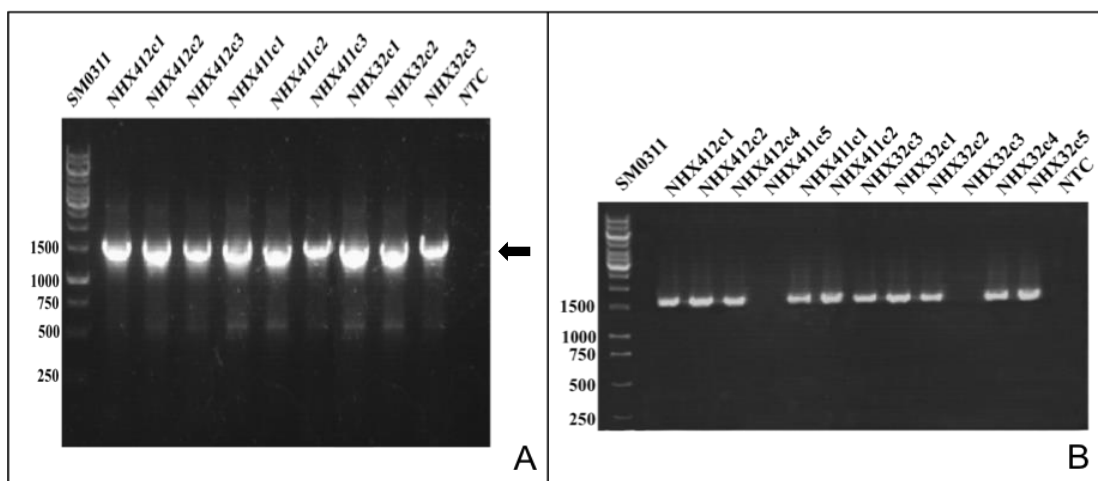


Figure 5.21. Colony PCR for NHX411, NHX412, and NHX32 constructs with gene specific primers (A) after LR reactions and (B) after agrobacterium transformation (SM0311: 1kb ladder; NTC: No template control).

5.6. Creation of Stable Arabidopsis Overexpression Lines

In the floral dip transformation of Arabidopsis wild type lines, all inflorescences of the Arabidopsis were exposed to the agrobacterium carrying the expression vector construct of interest. Each exposed inflorescence matures into a cilique carrying 10-20 seeds. Some of these seeds undergo transformation by Agrobacterium, which confers the ability to survive in selective antibiotic medium (Figure 5.22). Each of these surviving plants are called independent transformants or T1 generation, and each of them carry the insertion at a different position in their genome due to the random nature of the insertion. The phenotype analysis need to be performed on plants that are homozygous for the insertion. To this end, offspring from the independent lines were obtained and called the T2 generation, some of which were homozygous. To identify the homozygous T2 generation plants, T3 generation seeds were obtained and germinated on selective antibiotic medium. The T2 plants that produce seeds that all survive on the medium were designated as homozygous and the phenotype are performed on the plants grown from the T3 generation seeds. Moreover, in order to confidently reveal the functional effects of the transformation, multiple independent lines needed to be evaluated to prevent any functional bias from the site of insertion of the candidate gene. In addition, minimum of four T3 generation plants were phenotyped to achieve significance in the observed functional effects. Considering the presence of multiple independent transformant lines for every candidate gene and multiple plants assessed for every transformant lines, the number of plants to handle increases exponentially, which brings about space unavailability issues. Due to limited capacity of the growth chambers and large number of plants required to be phenotyped, floral dip transformations couldn't be performed with all of the expression vector constructs for each candidate genes.

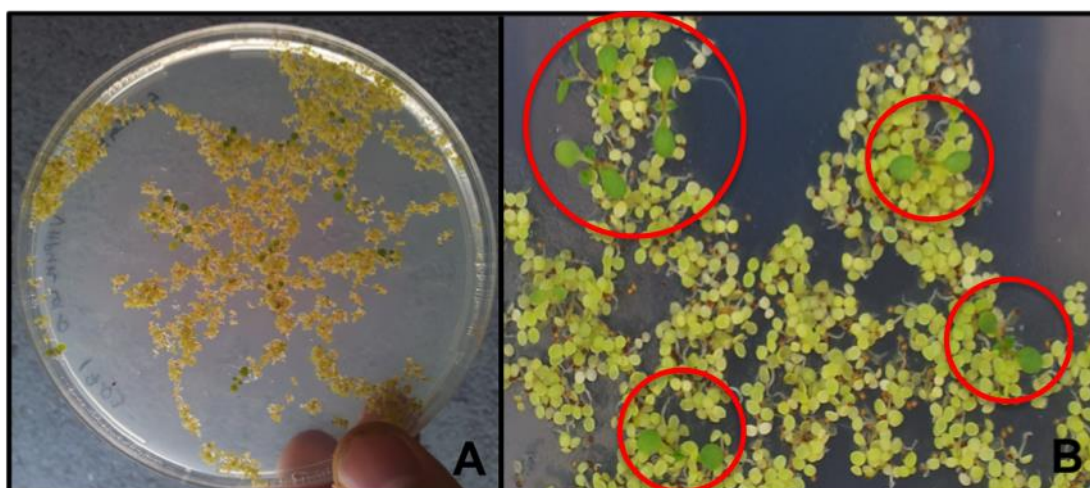


Figure 5.22. A) Transformant selection of seeds obtained from floral dip with VHP412 expression vector on antibiotic medium B) Close up of possible transformant T1 plants (Red circles indicate possible transformants).

For *pvSOD*, floral dip transformations were performed only with SOD412 and SOD411 constructs. A total of seven candidate transgenic plants from the SOD411 construct and two candidate transgenic plants from the SOD412 construct survived after antibiotic selection on medium (data not shown). PCR analysis with gene specific primers on genomic DNA of the transgenic plants indicated that SOD411-1,2,3,4,6,7 and SOD412-1,2 plants were positive for *pvSOD* (Figure 5.23a). Subsequent RT-PCR analysis on four of the transgenic lines, *pvSOD*411-1,6,7 and *pvSOD*412-2 have confirmed that the *pvSOD* gene was expressed at mRNA level (Figure 5.23b). In the end, homozygous T3 generation of SOD411-1, SOD411-6, and SOD412-2 lines were used for further phenotypic analyses.

In the case of *pvVHP*, floral dip transformations were performed only with VHP412 and VHP411 constructs. Five candidate transgenic plants from the VHP412 construct survived after antibiotic selection on medium (data not shown). Even though it was repeated twice, transformation with VHP411 constructs has not yielded any transgenic plants on antibiotic medium due to contamination problems in the hygromycin selection procedure. PCR analysis with gene specific primers on genomic DNA of the

candidate plants indicated that VHP412-1,2,3,4,5 plants were positive for *pvVHP* (Figure 5.24a). RT-PCR analysis on five of the transgenic lines, *pvVHP*412-1,2,3,4,5, have confirmed that the *pvVHP* gene is transcribed and is expressed at mRNA level (Figure 5.24b). For the phenotypic evaluation, homozygous T3 generation of *pvVHP*412-1,2,3,4 lines were used.

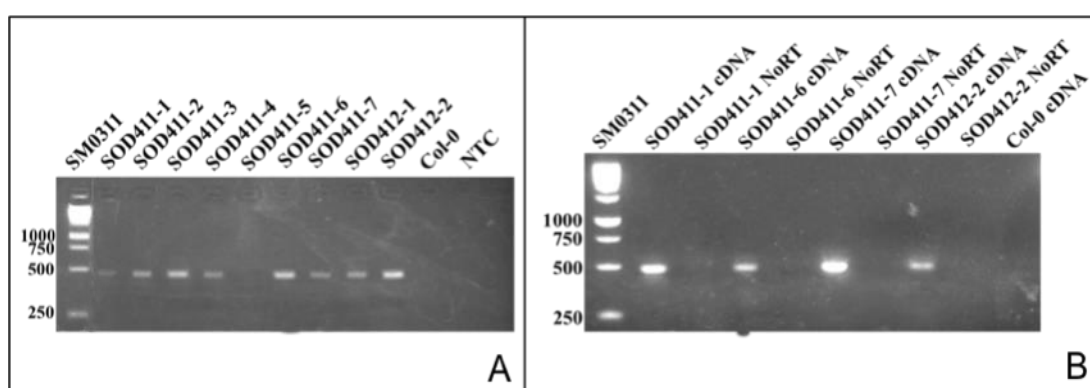


Figure 5.23. A) Transgene screening from genomic DNA of four *pvSOD* transgenic lines B) RT-PCR analyses of four *pvSOD* transgenic lines (SM0311: 1kb ladder; NTC: No template control).

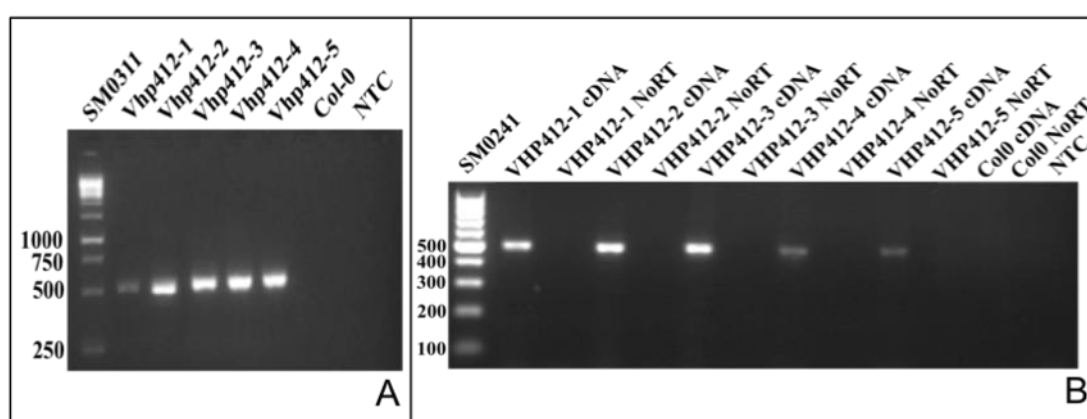


Figure 5.24. A) Transgene screening from genomic DNA of five candidate *pvVHP* transgenic plants B) RT-PCR analyses of five *pvVHP* transgenic lines (SM0311: 1kb ladder; NTC: No template control).

For the candidate gene *pvPIP*, floral dip transformation was performed only with PIP412 constructs. From the many seedlings that survived on the medium, eight transgenic plants from the PIP412 construct were selected for further study after antibiotic selection on medium (data not shown). PCR analysis with gene specific primers on genomic DNA of the candidate plants indicated that PIP412-1,4,5,6,8 plants were positive for *pvPIP* (Figure 5.25a). RT-PCR analyses on two of the transgenic lines, PIP412-8 and PIP412-10, have confirmed that the *pvPIP* gene was expressed at mRNA level (Figure 5.25b). Homozygous T3 generation of PIP412-8 and PIP412-10 were used for further phenotypic analyses.

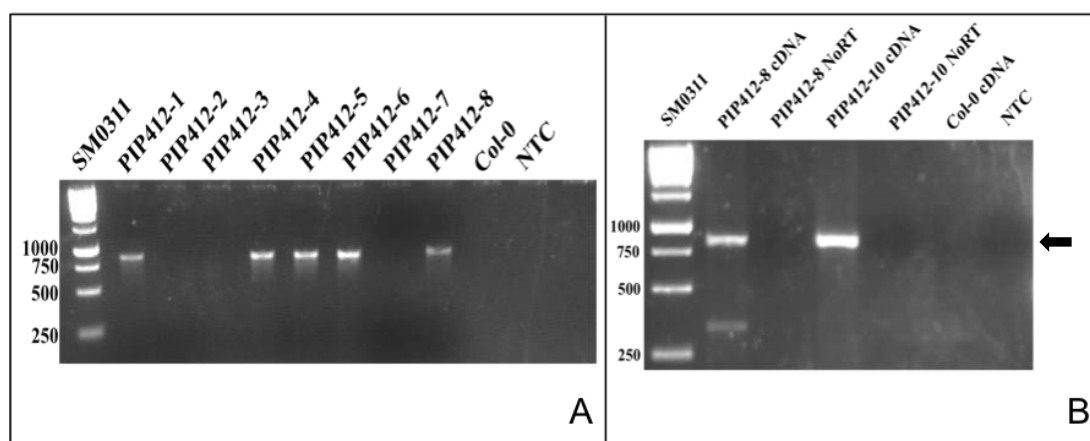


Figure 5.25. A) Transgene screening from genomic DNA of eight candidate *pvPIP* transgenic plants B) RT-PCR analyses of two *pvPIP* transgenic lines (SM0311: 1kb ladder; NTC: No template control).

In the case of *pvNHX*, floral dip transformations were performed only with NHX412 and NHX411 constructs. Only one transgenic plants from the NHX412 construct survived after antibiotic selection on medium (data not shown). Even though it was repeated twice, transformation with NHX411 construct has not yielded any transgenic plants on antibiotic medium due to contamination problems in the hygromycin selection procedure. Subsequent PCR analysis with gene specific primers on genomic DNA of the candidate plants indicated that NHX412-1 plant was positive for *pvNHX* (Figure 5.26a). RT-PCR analysis on transgenic line, NHX412-1, have confirmed that the

pvNHX gene was expressed at mRNA level (Figure 5.26b). NHX412-2 was not involved in RT-PCR analysis since all T2 generation was either heterozygous or non-transformant plants and thus, a homozygous T2 plant from NHX412-2 could not be obtained. This was an unexpected result since the theoretical allelic frequency for homozygous plants is 1:3. Eventually, homozygous T3 generation of NHX412-1 line was used for further phenotypic analyses.

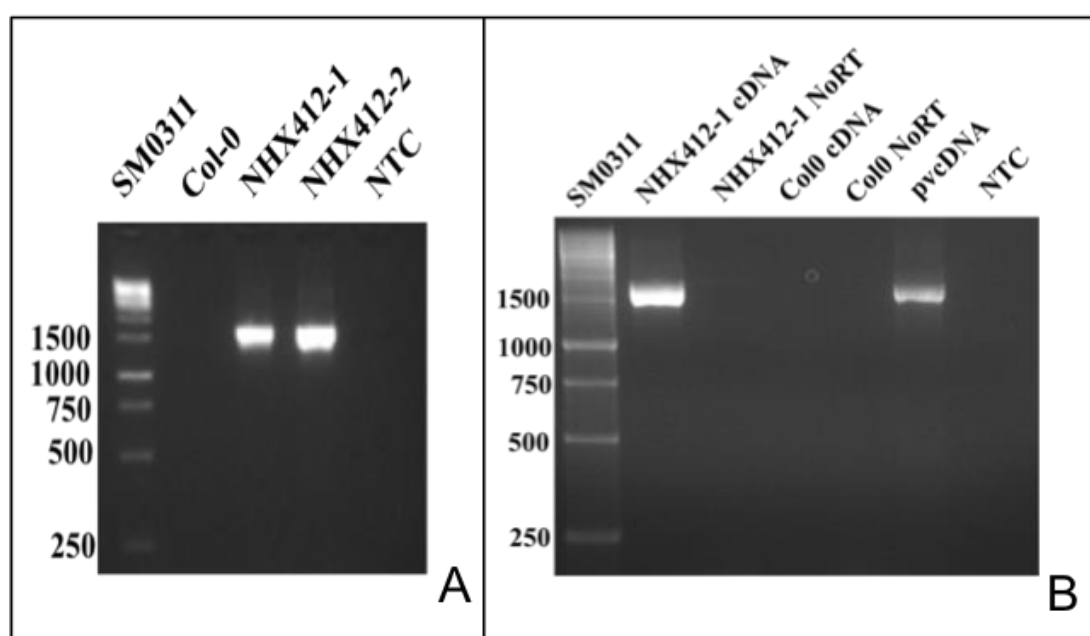


Figure 5.26. A) Transgene screening from genomic DNA of two candidate *pvNHX* transgenic plants B) RT-PCR analyses of NHX412-1 transgenic line (SM0311: 1kb ladder; NTC: No template control).

5.7. Western Blot Analyses of Overexpression lines

Transgenic plants were subjected to Western blot analysis to confirm the expression of transgenes at protein level. Since all expression vectors used in the floral dip transformations were flag tagged at the C-terminal, exposure of total protein extract of transgenic plants to anti-flag antibody were expected to produce a clear band at expected mass. The calculated molecular weights of *pvSOD*, *pvVHP*, *pvNHX* and *pvPIP* were 15,

16, 60, and 30 kDa respectively. Western blot analyses of VHP412-2, VHP412-4, VHP412-5, SOD411-1, SOD411-6, and SOD412-2 samples using monoclonal anti-flag antibody (Thermo Scientific) have produced non-specific banding around 15-35 kDa instead of clear bands at the 15 kDa and 16kDa (Figure 5.27). To eliminate the non-specific band formations, several efforts have been shown by using different blocking agents (non-fat milk powder and BSA) at different concentrations (1%-5%) and various primary antibody dilutions (1:1000 – 1:5000). Unfortunately, the results have produced similar non-specific binding patterns. Western blot analyses of NHX412-1 samples have also produced a non-specific band (around 70kDa), which was also present in the negative control wild type Col-0 sample (Figure 5.28). To eliminate the possibility of malfunction or non-specificity, together with the anti-flag antibody which was purchased from Thermo Scientific company, a different company anti-flag antibody (#2368, CSL cell signaling laboratory) was also tested on a flag tagged- *hsNLRP7* (Gene ID: 199713; 112kDa) overexpressing human cell line lysate (Figure 5.29). In this analysis, since only CSL anti-flag antibody produced expected 112kDa band, it was concluded that the Thermo Scientific anti-flag antibody was not functioning properly and CSL anti-flag antibody was used for further analyses.

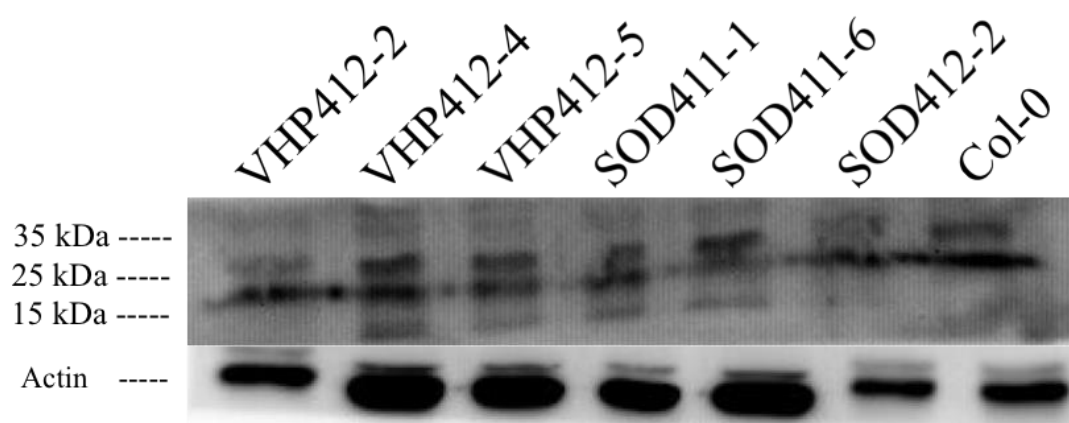


Figure 5.27. Western blot analyses of VHP412-2,4,5; SOD411-1,6; and SOD412-2 overexpression lines.

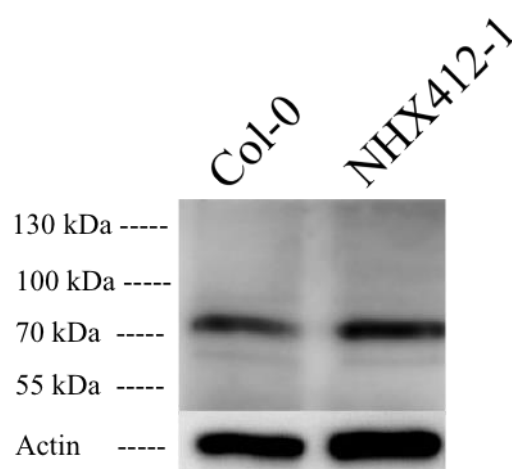


Figure 5.28. Western blot analyses of NHX412-1 overexpression line.

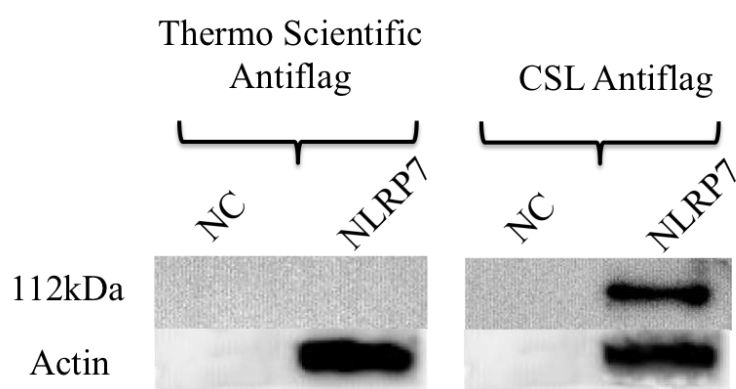


Figure 5.29. Western blot analyses of NLRP7 positive control using two different anti-flag antibodies: ThermoScientific (MA1-91878) and Cell Signaling Laboratory (#2368).

NC: negative control.

In order to confirm that the lack of specific bands in the western blotting experiments were not only due to the improperly functioning antibody, we consulted Yasin Dagdas of the Sainsbury Lab in Cambridge University about troubleshooting and repeat of the experiment in his laboratory. Dagdas kindly agreed to perform western blot experiments on the leaf samples of overexpression lines. Seeds of transgenic lines were shipped in 0.2 ml microtubes to Yasin Dagdas and germinated/harvested in the same germination

medium used in our study. From two weeks old seedlings, 100mg of tissue were homogenized in liquid nitrogen and boiled in sample loading buffer for loading sample preparation. The supernatant was recovered and used for SDS-PAGE. The samples were transferred to PVDF membrane and stained with Ponceau Red to confirm presence of protein on the membrane. Ponceau staining produced apparent banding at various sizes, which indicated proper transfer of total protein onto the membrane (Figure 5.30c). Membrane was blocked in 3% BSA in TBS-T for one hour and incubated overnight in 1:3000 diluted polyclonal rabbit anti-flag antibody in TBS-T (AB1162). Membrane was visualized using Pierce ECL Western Blotting Substrate (32106). Under normal exposure, flag positive control produced the expected band around 30 kDa whereas there were no visible bands at the expected 15-16kDa (for SOD and VHP), 30kDa (PIP), and 59kDa (NHX) (Figure 5.30a). Overexposure of the membrane produced non-specific banding in all samples without revealing any band specific to the overexpression samples even in Col-0 negative control (Figure 5.30b). These results suggest that the transgenic lines for all candidate genes did not contain the transgene proteins at a level detectable by the sensitivity of this assay.

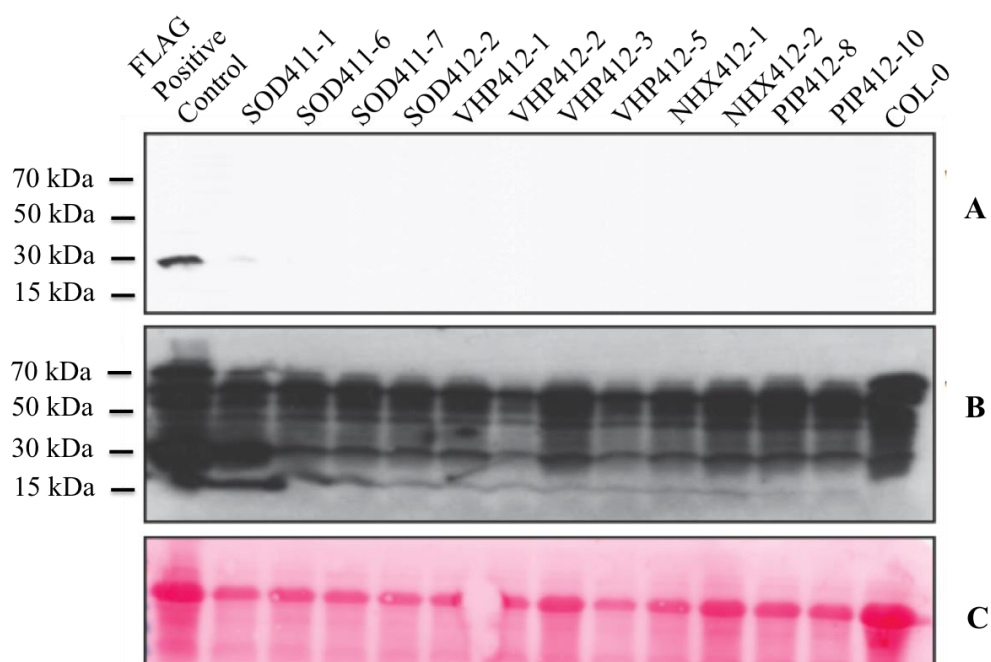


Figure 5.30. Western Blotting performed by Dr. Yasin Dagdas with polyclonal rabbit anti-flag antibody a) Normal exposure b) Overexposure c) Ponceau S Staining.

5.8. Homozygote Screening

5.8.1. Homozygote selection of the knock-out lines

Aside from the overexpression studies, another highly utilized method in functional studies is the use of knock out lines for the gene of interest. In order for the candidate gene to lose its function completely, all the alleles of that gene must be knocked out. Conveniently, T-DNA insertional mutant libraries are readily available as seed stocks from Nottingham Arabidopsis Stock Center (NASC) or Arabidopsis Biological Resource Center (ABRC). However, these seed stocks were not supplied prescreened for homozygosity. Therefore homozygosity screening were required for all Arabidopsis orthologous knock out lines acquired for all candidate genes prior to floral dip transformations.

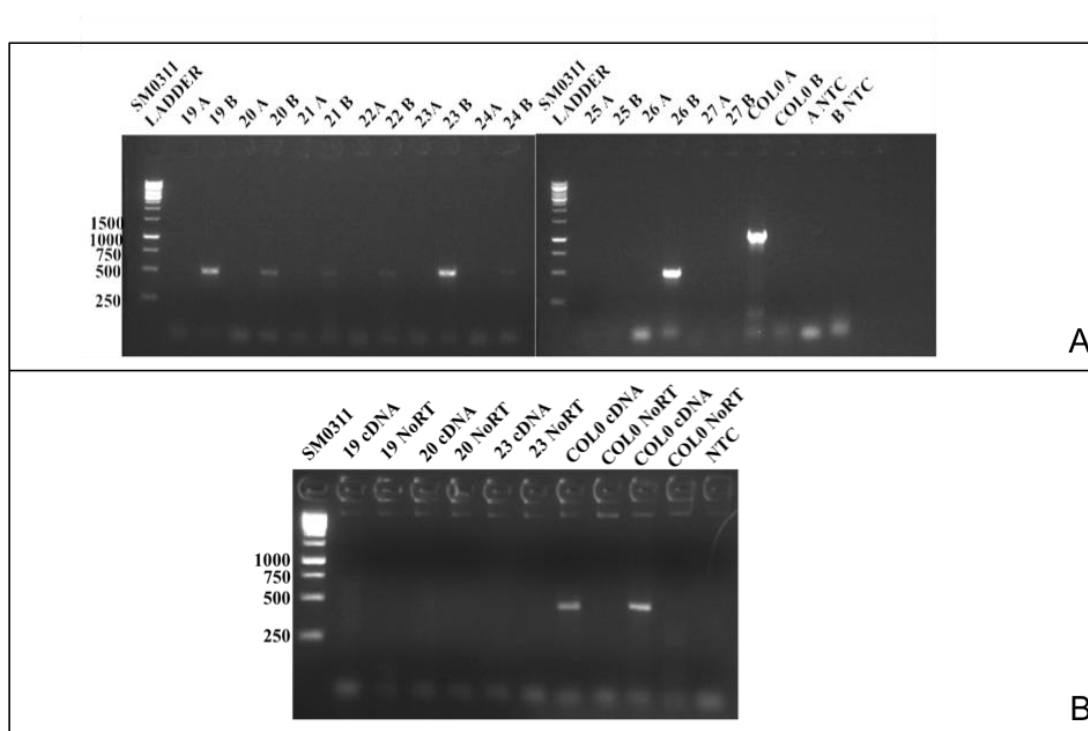


Figure 5.31. Homozygote screening on knock out lines of SAIL_439_G04 by (A) PCR reaction with LP+RP primers and PCR reaction with LB+RP primers (B) RT-PCR analyses of Plant#19,20,23 (SM0311: 1kb ladder; NTC: No template control).

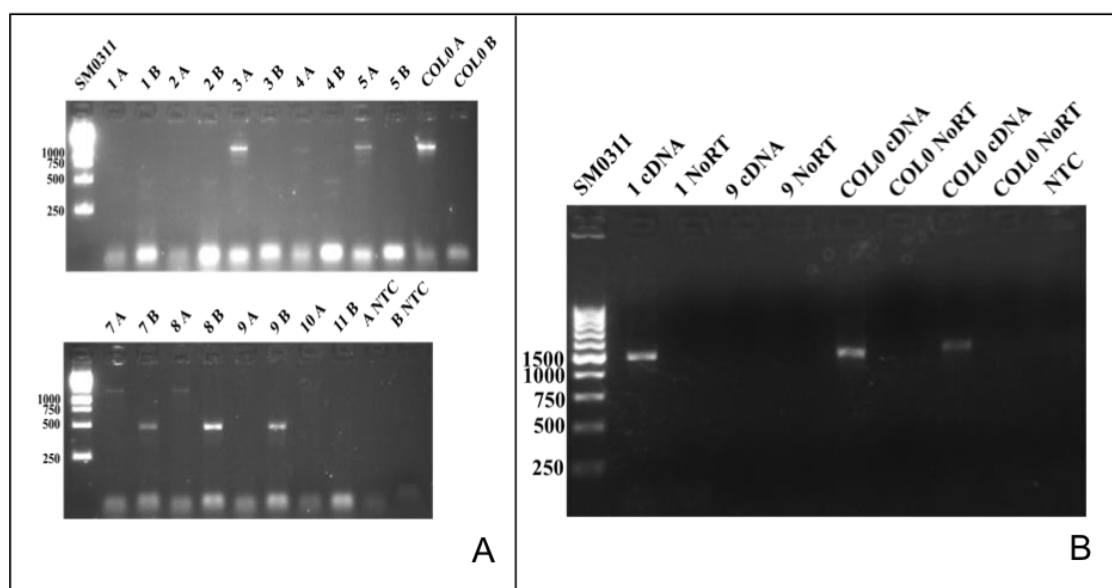


Figure 5.32. Homozygote screening knock out line WiscDsLox461-464O11 by (A) PCR reaction with LP+RP primers, and PCR reaction with LB+RP primers (B) RT-PCR analyses of Plant#1,9 (SM0311: 1kb ladder; NTC: No template control).

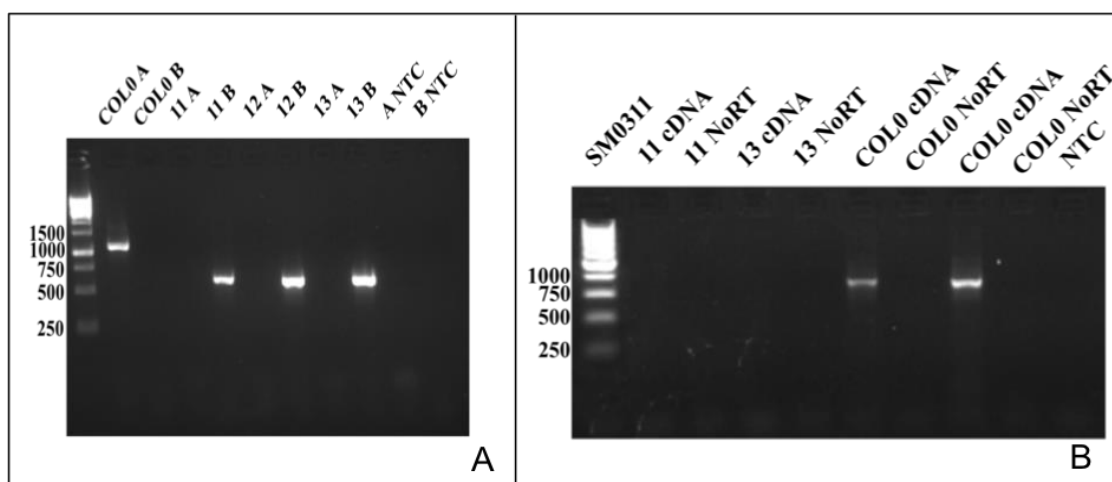


Figure 5.33. Homozygote screening on plants from T-DNA line SALK_117876C by (A) PCR reaction with LP+RP primers, and PCR reaction with LB+RP primers (B) RT-PCR analyses of Plant#11,13 (SM0311 1kb ladder; NTC: No template control).

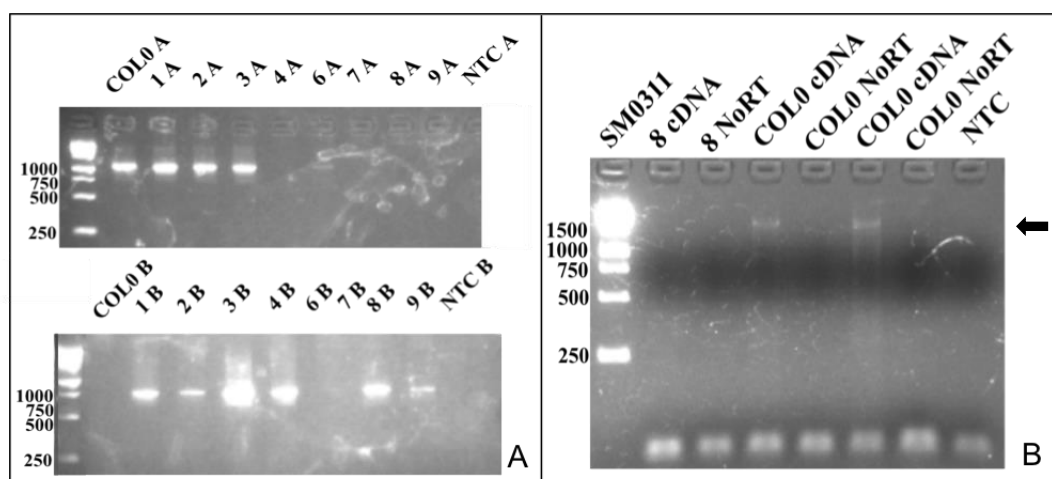


Figure 5.34. Homozygote screening on plants from T-DNA line SALK_046597C by (A) PCR reaction with LP+RP primers, and PCR reaction with LB+RP primers (B) RT-PCR analyses of Plant#8 (SM0311: 1kb ladder; NTC: No template control).

PCR analyses of genomic DNA (see Section 4.7.2) from leaf samples of knock out lines have shown that; all plants of SAIL_439_G04 line (*atSOD*) (Figure 5.31a), plant#9 from the WiscDsLox461-464O11 line (*atVHP*) (Figure 5.32a), plant#11,12,13 from the SALK_117876C line (*atPIP2-3*) (Figure 5.33a), and plant#4,8,9 from the SALK_46597C line (*atNHX*) (Figure 5.34a) were homozygous for T-DNA insertions and carried no wild type alleles. RT-PCR analyses showed that plant#19,20,23 from the *atSOD* knock out line (Figure 5.31b), plant#9 from the *atVHP* knock out line (Figure 5.32b), plant#11,13 from the *atPIP2-3* knock out line (Figure 5.33b), and plant#8 from the *atNHX* knock out line (Figure 5.34) did not contain detectable levels of the knocked out transcript. The plants used for phenotypic analyses were plant#23 for *atSOD*, plant#9 for *atVHP*, plant #13 for *atPIP2-3*, and plant#8 for *atNHX*.

5.9. Phenotype Screening for the Overexpression and Knock Outs on Tolerance to Salinity Stress

After the homozygous overexpression lines and knock out lines for all the candidate genes were obtained, they were screened for an alteration in their salinity profile

with respect to fresh weight and leaf area. An evident difference in salinity tolerance should be reflected in these two parameters as the growth rate of the plants decrease with increased stress exposure.

Another important parameter is the regime in which NaCl is applied. It has been reported that NaCl application creates differences in the amount and type of stress the plants are exposed to. Plants under salt shock are exposed largely to the osmotic stress whereas plants under the gradual salt treatment are exposed to ionic stress as well as the osmotic stress. (Shavrukov, 2013). To observe the functional effects of our candidate genes with respect to both regimes, transgenic lines were exposed to gradual NaCl and shock NaCl stresses.

5.9.1. Phenotype Analysis of Overexpression lines

Both mean leaf area and fresh weight decreased substantially with gradual NaCl treatment in both wild type and overexpression lines for all candidate genes tested (SOD411-1 (Figure 5.35a,b), SOD411-6 (Figure 5.35c,d), SOD412-2 (Figure 5.35d,e), VHP412-2 (Figure 5.36a,b), VHP412-4 (Figure 5.36c,d), VHP412-5 (Figure 5.36d,e), and NHX412-1 (Figure 5.37a,b)). The decrease in leaf area and fresh weight increased as the NaCl concentration increased. However, there were no significant differences between the transgenic line and the wild type plants with regard to leaf area and fresh weight for any of the independent transformant lines. Phenotype data couldn't be obtained from 300mM pots of SOD411-1 and SOD412-2 lines due to an accident with the pots during the experiment (Figure 5.35a-d).

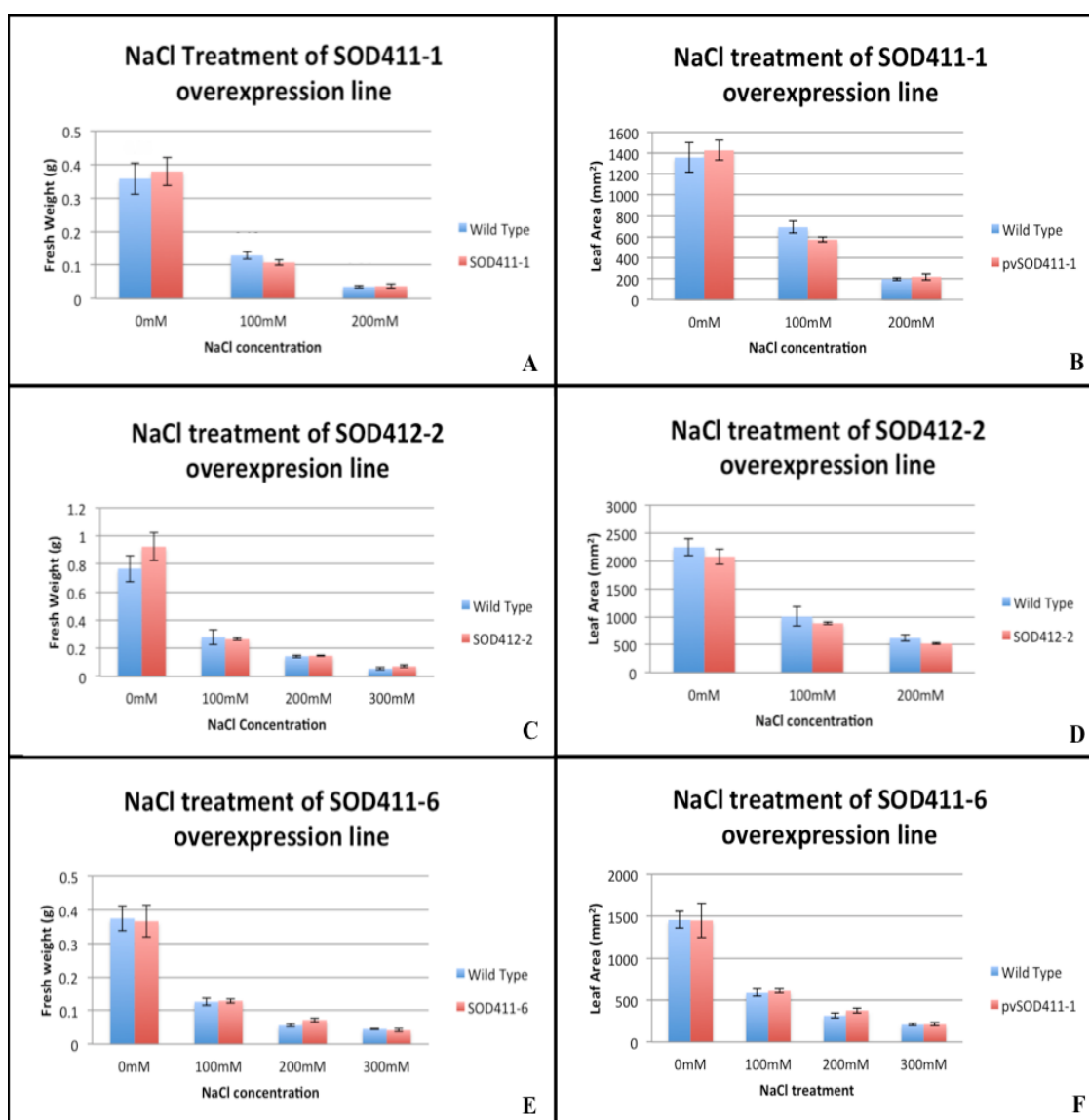


Figure 5.35. Fresh weight (panel A, C, E) and leaf area (panel B, D, F) measurements after gradual NaCl treatment of SOD411-1, SOD412-2 and SOD411-6 overexpression lines.

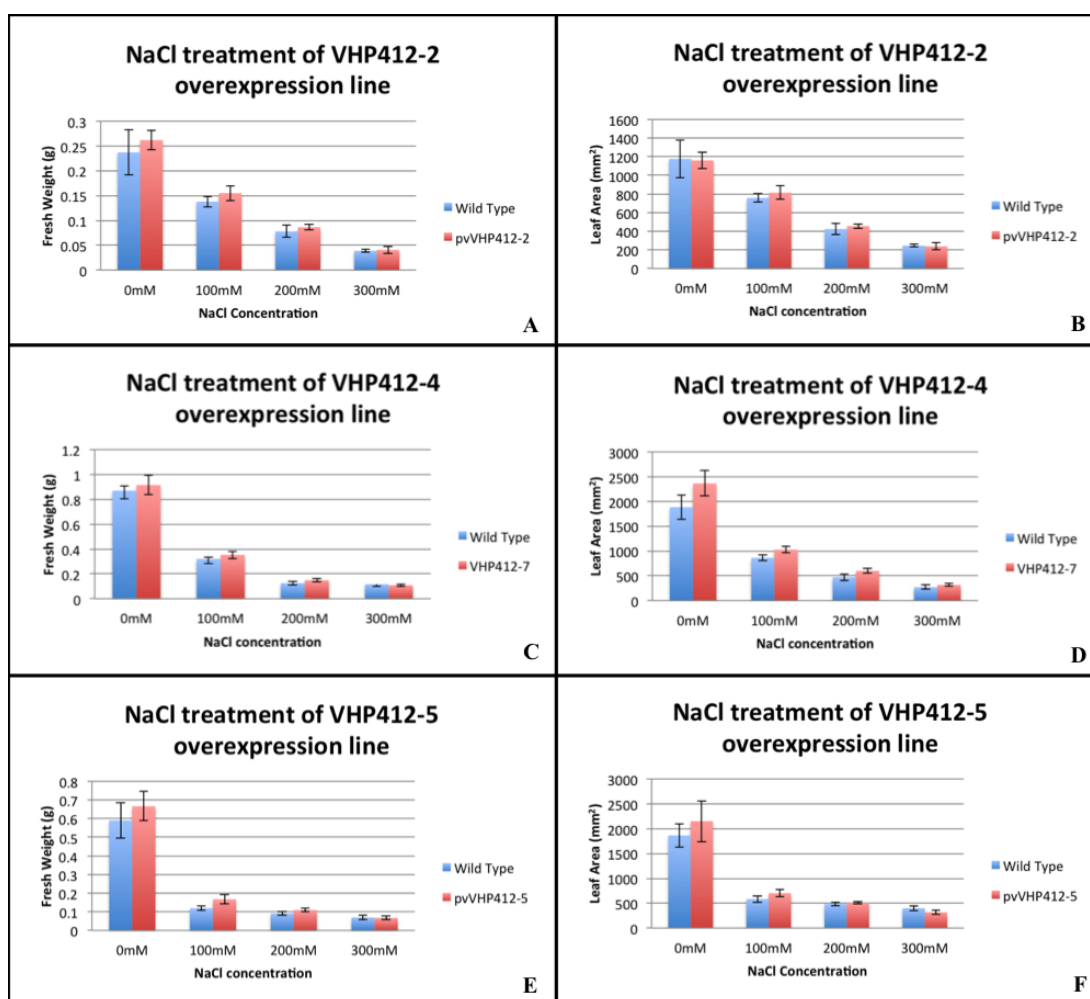


Figure 5.36. Fresh weight (panel A, C, E) and leaf area (panel B, D, F) measurements after gradual NaCl shock treatment of VHP412-2, VHP412-4, and VHP412-5 overexpression lines.

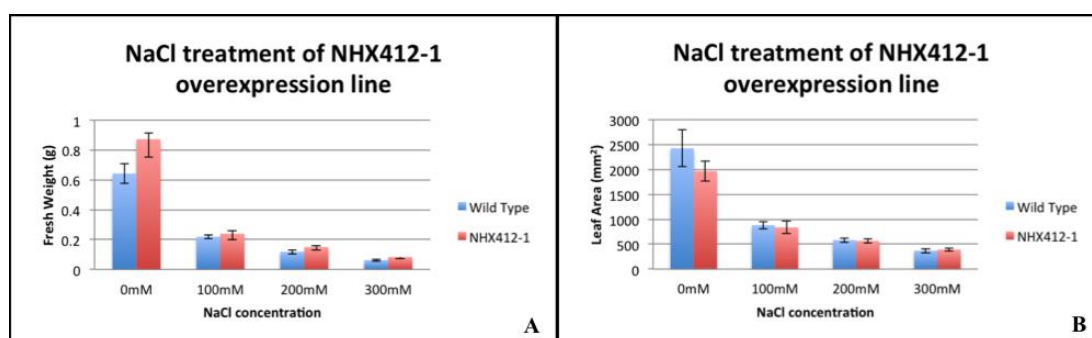


Figure 5.37. Phenotypic measurements after NaCl gradual and shock treatments of NHX412-1 overexpression line A) Fresh weight measurements B) Leaf area measurements.

Within the time frame of this study, NaCl shock treatment could be performed with overexpression lines for only *pvSOD* and *pvPIP*. The same lack of a significant phenotypic difference was evident for all three transformant lines of *pvSOD* under NaCl shock treatment (Figure 5.38a-e). Furthermore, it was not possible to directly compare the differential effects of gradual NaCl and NaCl shock treatments since were not performed synchronously.

For the two transformant lines of *pvPIP* however, gradual and shock treatments were performed synchronously. While both types of treatment caused decreases in fresh weight and wild type in all stress conditions, there were no significant differences between the wild type and transformants in any of the two parameters. Under normal conditions however, both transgenic lines had significantly higher in fresh weight and leaf area compared to wild type. Even though comparing the effects of shock and gradual treatment would potentially have some bias since two treatments were performed in two separate pots, it was evident that both transformant and wild type plants under NaCl shock had both lower fresh weight and leaf area compared to their counterparts receiving gradual NaCl treatment (Figure 5.39, Figure 5.40).

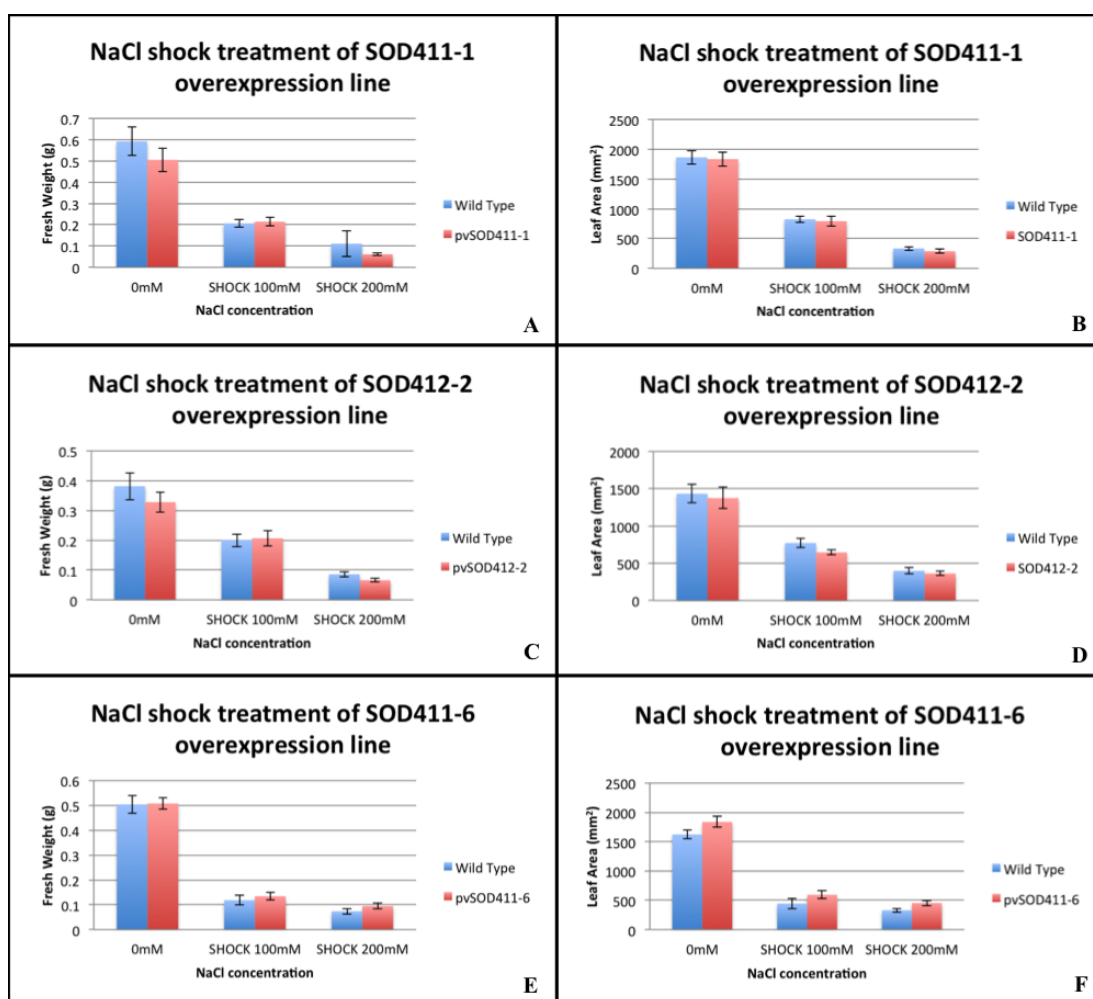


Figure 5.38. Fresh weight (panel A, C, E) and leaf area (panel B, D, F) measurements after NaCl shock treatment of SOD411-1, SOD412-2 and SOD411-6 overexpression lines.

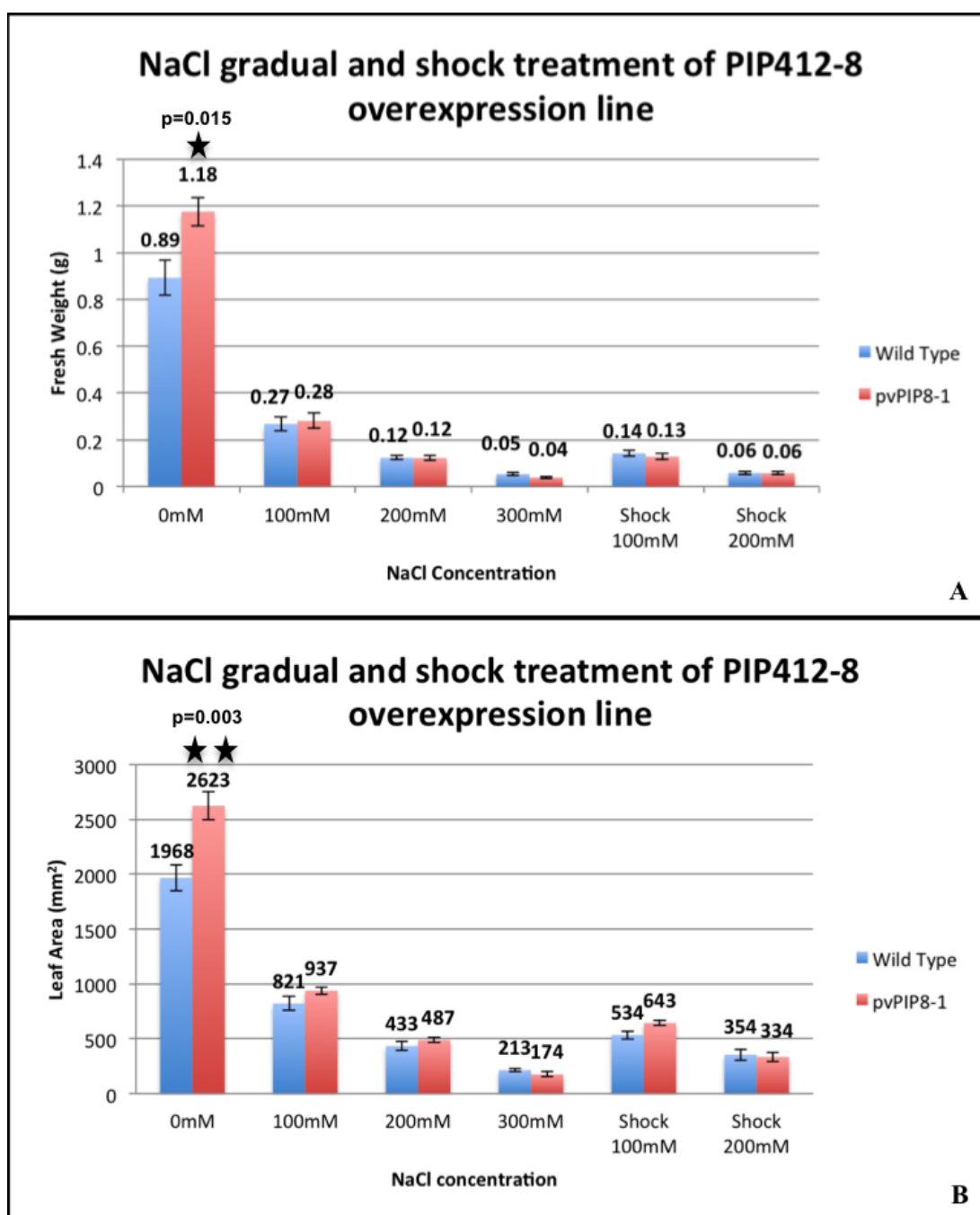


Figure 5.39. Phenotypic measurements after NaCl gradual and shock treatments of PIP412-8 overexpression line A) Fresh weight measurements B) Leaf area measurements (“*”: $p < 0.05$; “***”: $p < 0.01$, “****”: $p < 0.001$).

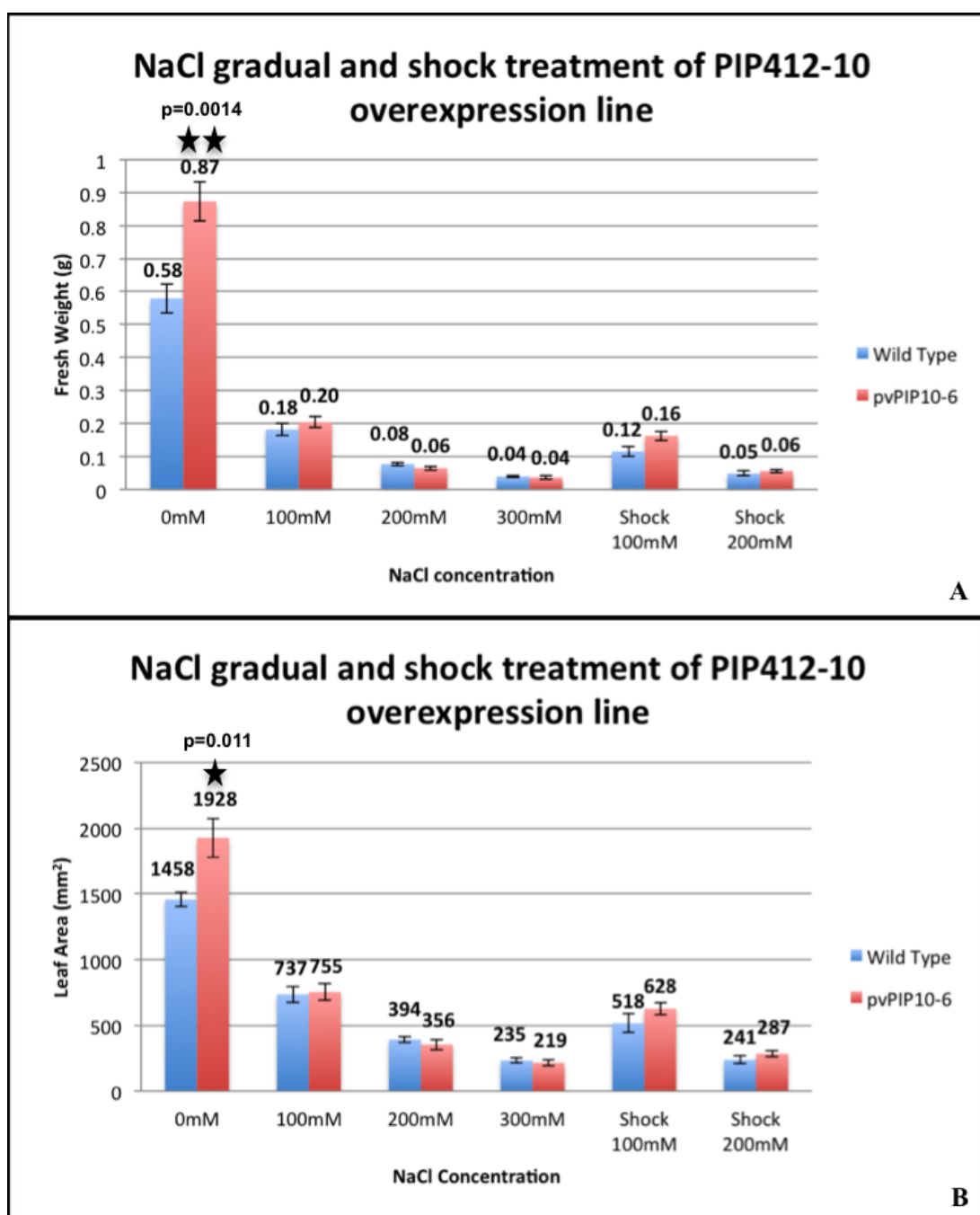


Figure 5.40. Phenotypic measurements after NaCl gradual and shock treatments of PIP412-10 overexpression line A) Fresh weight measurements after B) Leaf area measurements (“*”: $p<0.05$; “**”: $p<0.01$, “***”: $p<0.001$).

5.9.2. Phenotype Analysis of Knock-out Lines

Knock out lines for all four candidate genes were treated with NaCl in both gradual and shock regimes. Both mean leaf area and fresh weight decreased substantially with gradual NaCl treatment in both wild type and knock out lines of *atSOD* (Figure 5.41.a-d), *atVHP* (Figure 5.42a-d), *atPIP2-3* (Figure 5.43a-d), and *atNHX* (Figure 5.44a-d) genes in all NaCl concentrations tested for both gradual and shock NaCl treatments. The decrease in leaf area and fresh weight increased as the NaCl concentration increased both in the wild type and knock out plants. However, there was no significant difference between wild type and knock out line with regard to fresh weight or leaf area in any of the NaCl concentrations tested for neither of the gradual and shock NaCl treatments.

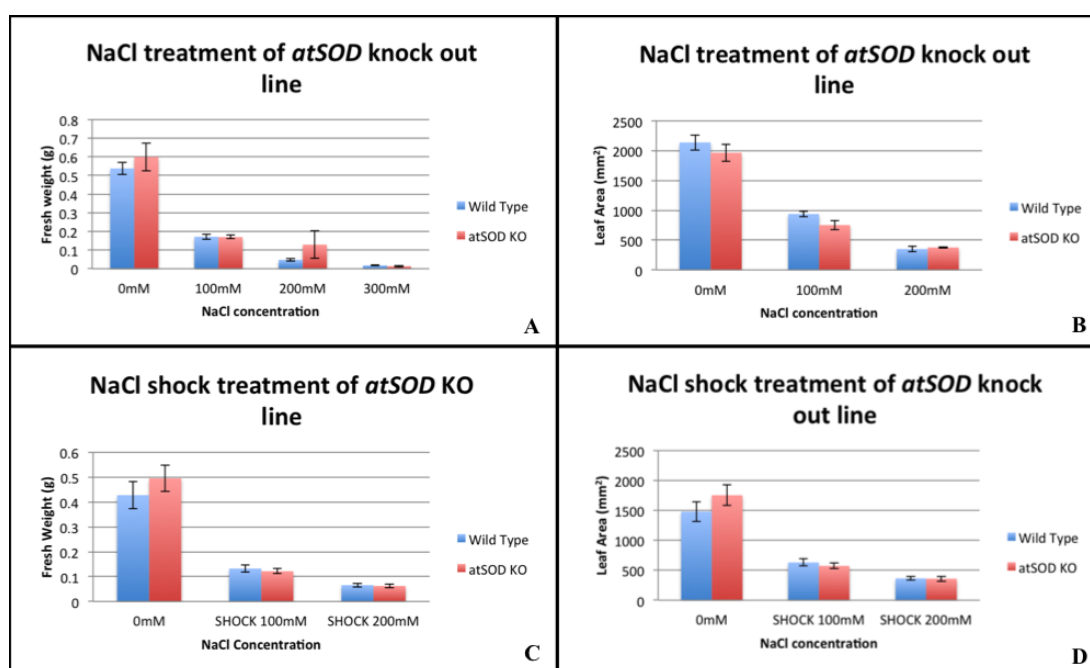


Figure 5.41. Fresh weight (A) and leaf area (B) measurements after NaCl gradual treatments of *atSOD* (SAIL_432G04) knock out line. Fresh weight (C) and leaf area (D) measurements after NaCl shock treatments of *atSOD* (SAIL_432G04) knock out line.

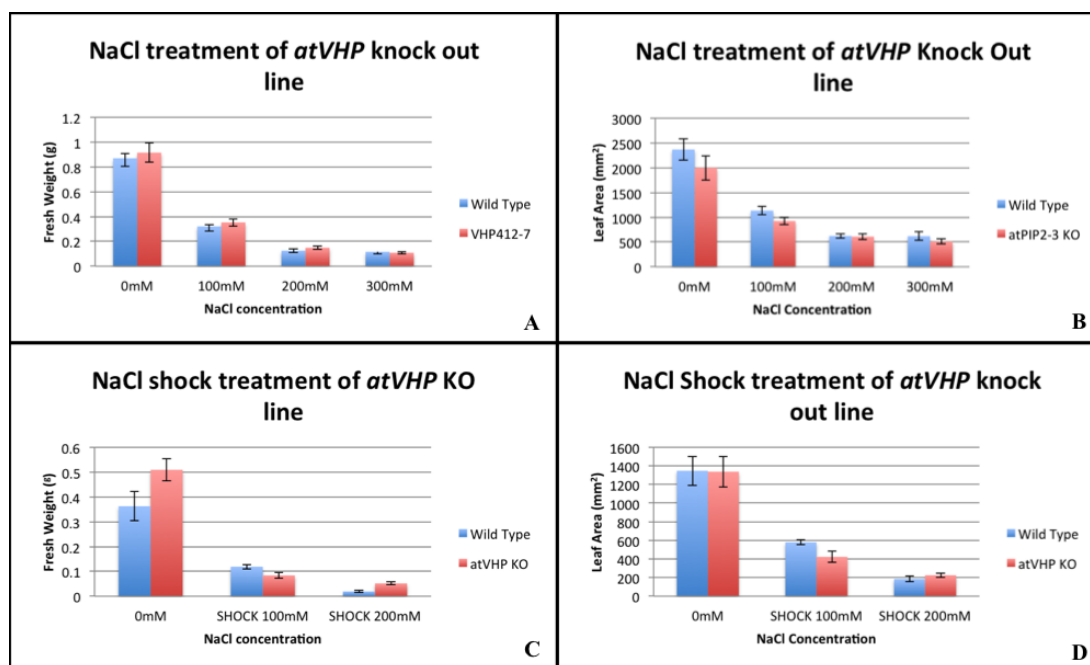


Figure 5.42. Fresh weight (A) and leaf area (B) measurements after gradual NaCl treatments of *atVHP* (WiscDsLox461-464O11_LP) knock out line. Fresh weight (C) and leaf area (D) measurements after NaCl shock treatments of *atVHP* (WiscDsLox461-464O11_LP) knock out line.

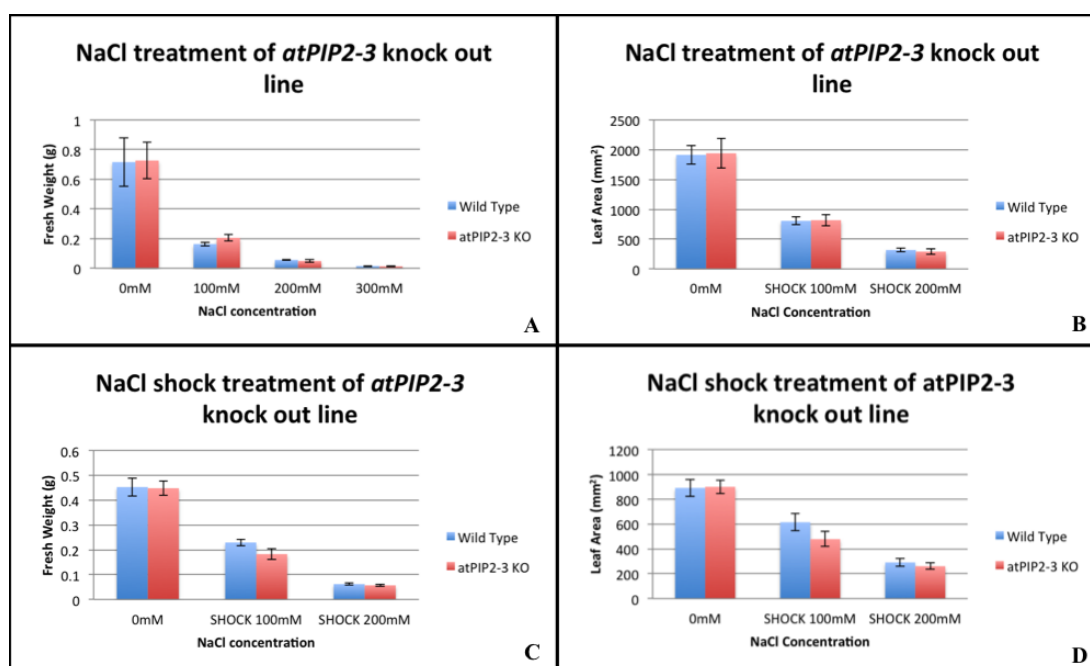


Figure 5.43. Fresh weight (A) and leaf area (B) measurements after gradual NaCl treatments of *atPIP2-3* (SALK_117876C) knock out line. Fresh weight (A) and leaf area (B) measurements after NaCl shock treatments of *atPIP2-3* (SALK_117876C) knock out line.

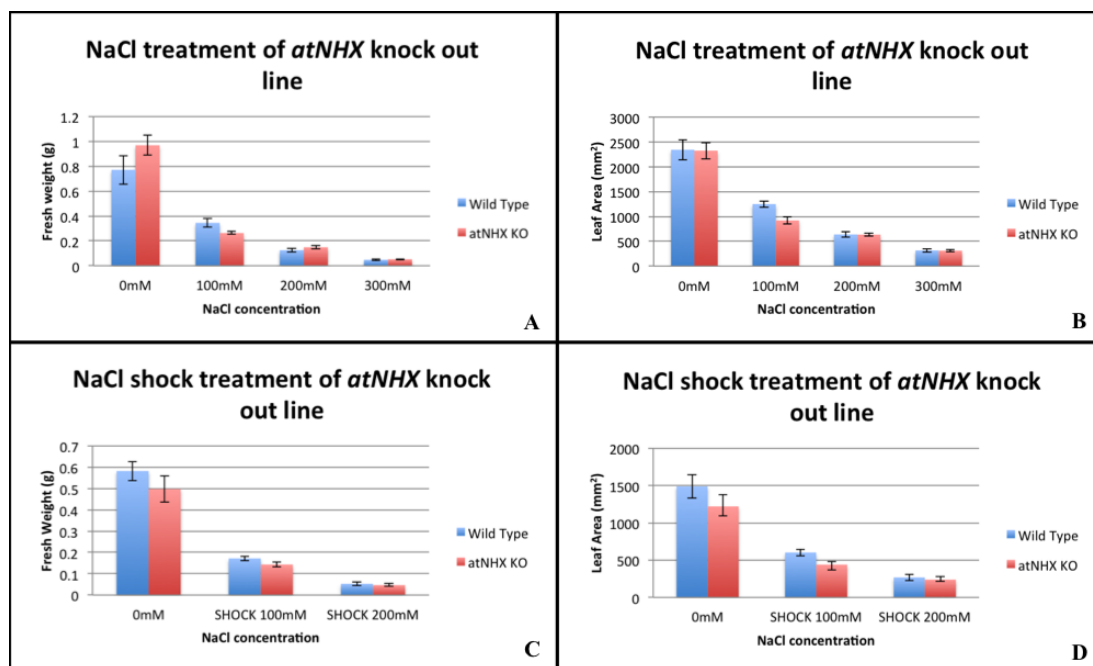


Figure 5.44. Fresh weight (A) and leaf area (B) measurements after gradual NaCl treatments of *atNHX* (SALK_46597C) knock out line C) Fresh weight (C) and leaf area (D) measurements after NaCl shock treatments of *atNHX* (SALK_46597C) knock out line.

6. DISCUSSION

One of the many strategies to increase crop tolerance to abiotic stresses is regulated expression of transgenes (Munns and Tester, 2008, Sharma *et al.*, 2000). To identify candidate genes for overexpression studies, we have selected 7 genes from the subtractive cDNA library prepared by Mahmut Can Hız and 11 genes that have been implicated in salinity tolerance in previous studies. The subtractive cDNA library represented genes differentially expressed in the NaCl treated tolerant Ispir variety and non-treated susceptible TR43477 variety. The candidate genes from the SSH library consisted of potential upstream regulators of the salinity response such as a transmembrane protein kinase (*pvTPK*), a transcription factor of the bZIP family (*pvBZIP*), and an ABA responsive protein (*pvABAR*) as well a vacuolar H⁺ pumping ATPase (*pvVHP*), osmotin-like protein (*pvTAU*), and intrinsically disordered protein LEA 1 (*pvIDP*) responsible for ion homeostasis, osmotic regulation, and protein stabilization respectively (Golldack, *et al.*, 2014, Hundertmark and Hinch, 2008, Jannoo, *et al.*, 2007, Liu, *et al.*, 2005, Rajam, *et al.*, 2007, Tuteja and Gill, 2013, Tyerman, *et al.*, 2002). From the literature, 11 candidate genes were selected such as chlorine channel protein (*atCLCa*) and Na⁺-H⁺ antiporter (*atNHX*) responsible for cellular ion homeostasis, plasma membrane integral proteins 2-2 (*atPIP2-2*) and 2-3 (*atPIP2-3*), and aquaporin (*atNIP-1*) responsible for water transport, 9-cis-epoxycarotenoid dioxygenase 1 (*atNCED1*) and protein phosphatase 2c (*atPP2Ca*) responsible for ABA signaling and biosynthesis, superoxide dismutase (*atSOD*) and copper chaperone for superoxide dismutase (*atCCS*) acting in detoxification of reactive oxygen species, and peptide transporter 1 (*atPTR-1*) responsible for detoxification and transport of dipeptides (Chu, *et al.*, 2005, Diaz-Vivancos, *et al.*, 2013, He, *et al.*, 2005, Ines, 2008, Iuchi, *et al.*, 2001, Kamiya, *et al.*, 2009, Komarova, *et al.*, 2008, Kuhn, *et al.*, 2006, Nishimura, *et al.*, 2009, Wang, *et al.*, 2004, Zhou, *et al.*, 2010). Since the reference genome of *P.vulgaris* was not published at the time of this analysis, the putative *P.vulgaris* orthologues of candidate genes selected from previous studies were determined for all selected genes based on homology analysis between the Arabidopsis genes and common bean EST database (Table 5.2).

In order to see the differential response of the candidate genes to NaCl treatment in two common bean varieties, the tolerant Ispir and the susceptible TR43477, we performed NaCl treatment in hydroponic system. qRT-PCR analysis of the leaf tissues of treated and non-treated leaves from both varieties was performed and expression data was analyzed. The candidate genes that were differentially expressed at a significant level upon NaCl treatment were *pvSOD*, *pvPIP2-2*, *pvPP2Ca*, *pvNHX*, *pvNCED* and *pvPIP2-3* from the literature. Differentially expressed candidate genes selected from the SSH library were *pvIDP*, *pvABAR*, and *pvVHP* (Figure 5.2). Four out of 7 candidate genes from the SSH library and 4 out of 11 candidate genes from the literature were not differentially expressed at a significant level. Regarding the nature of the SSH library it would be normal to expect that all candidate genes from the SSH library would be differentially expressed at a significant level. The fact that only 3 out of 7 candidate genes from the SSH library was differentially expressed could be due to a low level of difference in expression levels, which is sufficient to be represented in the subtractive library but insufficient to reach significance in qRT-PCR experiments. Furthermore, the fact that 4 out of 11 candidate genes from the literature was not differentially expressed would not be so surprising since a key component of a pathway could be activated without simultaneous increase in the abundance of its transcript, by common mechanisms such as phosphorylation. Moreover, it can be seen that 7 out of 9 genes among “abundantly expressed genes” were differentially expressed at a significant level where only 3 out of 9 genes were differentially expressed among “rarely expressed genes”. While this can be due to chance, it is also possible that the ability of qRT-PCR assay to detect a significant difference is lower for the rarely expressed genes compared to the abundant ones. It was reported that the efficiency of the reverse transcriptase utilized in the cDNA synthesis can be a limiting factor towards the sensitivity of the assay in detecting significant differences among low abundance transcripts (Levesque-Sergerie *et al.*, 2007).

Following in our study, the open reading frames (ORFs) were predicted with high confidence for all differentially expressed candidate genes except for *IDP* and *ABAR*. The inability to detect all candidate genes from the SSH library was due to the lack of a published reference genome for *P.vulgaris*, and the fact that ORF prediction had to be performed only by utilization of the current EST and the RNA-seq transcriptome of the common bean recently produced by our lab. Even though the EST database

and the RNA-seq transcriptome are indeed useful tools in ORF prediction, a reference genome allows better ORF prediction with higher accuracy and better confidence. Due to these circumstances, only one candidate gene, *VHP*, from the SSH library remained for further functional experiments (Table 5.3).

Determining the number of candidate genes that can be studied in a limited amount of time depends largely on the facilities available for controlled plant growth. For every candidate gene, there are three different expression vectors to eliminate any possible effects of the tag in functionality of the protein; N-terminal tag (pGWB412), C-terminal tag (pGWB411), and no tag (pMDC32). For every expression vector, at least three independent transformant *Arabidopsis* lines have to be created to prevent any bias from the location where candidate gene was inserted and its possible deleterious effects on other genes located in that region. Each of these transformant lines have to be phenotyped to confidently establish the functional effects of the candidate gene. Considering that 6-10 individual plants of the transformant line and of the wild type need to be phenotyped to draw any conclusions about that line, the number of plants needed to fully evaluate one candidate gene increases exponentially. Due to limited space available for plant growth, we have selected four candidate genes for further functional experiments: one candidate gene, *pvVHP*, from the SSH library, and three candidate genes, *pvNHX*, *pvPIP2-3*, and *pvSOD*, from previous studies.

Next, the candidate genes were cloned into pDONR207 entry vector and the presence of candidate genes was verified by colony PCR (Figure 5.9a-d). The colonies obtained following BP reaction and transformation were Sanger sequenced for verification. The sequences for *VHP*, *NHX* and *SOD* were exactly the same as predicted, whereas the sequences for *PIP* had differences compared to the expected sequence. Sequences from three colonies obtained after BP Clonase reaction had 96%, 95%, and 94% identity with the expected sequence (Figure 5.15). Most of the conflicting nucleotides resided within 640th and 850th base, which corresponds the 6th conserved transmembrane alpha helix of membrane integral proteins (Jung *et al.*, 1994). The difference between the cloned sequences could be due to cloning primers amplifying several different PIP family proteins in common bean that have similar 5' and 3' end sequences but present in

greater abundance. Sequencing of larger numbers of colonies after the creation of the entry vector could potentially allow discovery of the intended *PIP* transcript. Furthermore, comparison of sequences obtained from PIP207c7 entry vector, which was used for further cloning experiments, to the common bean reference genome revealed that 842 out of 845 nucleotides of PIP207c7 were identical to 848 nucleotide long reference sequence XM_00715180 (Figure 5.17). Due to this high similarity, we have chosen to go forward with the PIP207c7 entry vector in further cloning experiments instead of spending more time and resource to discover the expected sequence by sequencing many colonies. Comparison of sequences obtained from *NHX* and *SOD* revealed perfect matches to reference sequences XM_007144720 and XM_007147050 respectively. Sequence obtained from VHP entry vector showed perfect match to the last 495 nucleotides of a 636 nucleotide long reference sequence (XM_007135850) and 93% homology with a 495 nucleotide long reference sequence (XM_007131684) (Figure 5.12). Since the gene most similar to *VHP* in closely related organisms is a 495 nucleotide long *Glycine max* V-Type proton ATPase (Table 5.4), it is possible that predicted *VHP* sequence indeed exists and the sequence XM_007135850 in the reference genome was misassembled. This would not be surprising since this is the first release of common bean reference genome and it lacks the confidence of better-established older reference genomes such as that of *A.thaliana*. Another possibility is that both sequences exist but since de novo assembled RNAseq data was used for ORF prediction and de novo assemblies are very prone to errors, the predicted ORF sequence of *VHP* was misassembled.

Following cloning of candidate genes into entry vectors, the genes were cloned into pGWB411, pGWB412, and pMDC32 expression vectors and colony PCR was performed (Figure 5.18a, Figure 5.19a, Figure 5.20a, Figure 5.21a). After verification of the inserts in the expression vectors, they were also transformed into *Agrobacterium tumefaciens* to be used in floral dip experiments. The presence of candidate genes in *Agrobacterium* colonies was also verified by colony PCR (Figure 5.18b, Figure 5.19b, Figure 5.20b, Figure 5.21b). *PIP* was only cloned into pGWB412 and pMDC32 vectors since the available space in the plant growth cabinet would allow phenotype evaluations of plants transformed with only one or two of the constructs.

At this point, due to the plant growth space available at the time, we have decided to perform the floral dip experiments mostly with pGWB412 constructs of all candidate genes alone except for *PIP*, where pGWB412 and pMDC32 expression vectors were used. Floral dip experiments with pGWB411 vector yielded positive transformant plants for only *pvSOD* but not for other candidate genes due to contamination problems with hygromycin based selection procedure. Floral dip experiments with pGWB412 yielded positive transformant plants for all candidate genes. Presence of the candidate genes inside the genomic DNA was verified by PCR (Figure 5.23a, Figure 5.24a, Figure 5.25a, Figure 5.26a) and transcription of the genes into mRNA was verified by RT-PCR (Figure 5.23b, Figure 5.24b, Figure 5.25b, Figure 5.26b).

Confirmation of overexpression at protein level via western blot experiments in our lab were not successful due to non-specific binding of anti-flag antibody (Figure 5.29). Anti-flag antibody from another produced was ordered but the experiments could not be repeated within the time frame of this study. Dr. Yasin Dagdas from The Sainsbury Lab in University of Cambridge kindly agreed to perform the western blot experiments. Western blot was repeated using a different brand anti-FLAG antibody and the resulting data indicates that there is no flag tagged protein in all samples of all four candidate genes at a level detectable by this western blotting experiment. Lack of proper translation of the transgene mRNAs would be a logical conclusion if there was no phenotype in any of the candidate genes. However, both of the *PIP* overexpression lines displayed significantly increased fresh weight and leaf area under control conditions, which points to the presence of a transgene protein at a functionally relevant level. One explanation to this apparent incoherency is that all candidate gene mRNAs were translated at such a low level that was undetectable by the sensitivity of the western blot assay. It is possible that this low level of translation haven't allowed *pvSOD*, *pvVHP*, and *pvNHX* overexpression lines to display observable phenotype but was sufficient for the *pvPIP* overexpression lines to display enhanced growth under control conditions. This could be possible if there were mutations in the vector backbone, specifically inside the sequences required for efficient translation of the mRNA such as the Kozak sequence (Kozak, 1986). To confirm this situation, the vectors at hand could be Sanger sequenced to reveal any mutations in the vector backbone. Furthermore, another Gateway compatible destination vector system with another tag such as His (pGWB408-409) or Myc

(pGWB417-418) could be used to achieve recombinant protein with an alternative epitope.

In addition to overexpression studies, knock out experiments allow further functional characterization of candidate genes. In order to see the effects of absence of the candidate genes on salt tolerance phenotype, readily available T-DNA insertional knock out lines for the *A.thaliana* orthologues of the candidate genes were acquired from NASC. Homozygous plants from these lines were determined and seeds from these plants were used for phenotype analyses.

A recent study by Shavrukov *et al.* reports that most of the genes studied in salt stress studies are actually salt shock responsive genes specific to the osmotic phase and not the ionic phase of salt stress due to application of NaCl in a single step (NaCl shock) rather than gradually. The author suggests that the treatments should be performed in a gradual manner to study genes under ionic stress and not the osmotic stress (Shavrukov, 2013). To be able to evaluate functional effects of the candidate genes in not only gradually applied NaCl tolerance but also in NaCl shock tolerance, NaCl treatment was performed in both gradual and shock regimes. Gradual NaCl applications were performed for all knock out lines and overexpression lines for *pvVHP*, *pvSOD*, *pvPIP* and *pvNHX*. The NaCl shock treatments were performed only on transformant lines of *pvSOD* and *pvPIP* due to time and space limitations. The NaCl shock treatment was performed for *pvSOD* only after the gradual NaCl applications were finished. For *pvPIP*, gradual and shock NaCl treatments were performed synchronously to allow cross comparison of any differential effects between gradual and shock treatment.

There are four members of the vacuolar H⁺ pumping ATPase 16kDa proteolipid subunit c family in *A.thaliana*: AVA-P1, AVA-P2, AVA-P3, and AVA-P4. The knock out line analyzed in this study carried T-DNA insertion in the *AVA-P4* gene. RNAi mediated silencing of AVA-P1 and AVA-P3 has led to significantly decreased root length in 50mM NaCl containing medium (Padmanaban *et al.*, 2004). However, there have been no reports analyzing effects of AVA-P4 absence on stress tolerance. We observed that the knock out line performed no different from the wild type with respect to leaf ar-

ea and fresh weight in any of the NaCl concentrations applied in gradual or shock manner (Figure 5.44). While it is possible that AVA-P4 doesn't share the functional profile of AVA-P1 and AVA-P3, the difference could be due to difference in the treatment mediums and difference in stress indicators. The next step in the evaluation of AVA-P4 knock out line could be to assess root length under salt stress in parallel with AVA-P1 and AVA-P3 knock out lines to better establish functional characteristics of vacuolar H⁺ pumping ATPase 16kDa proteolipid subunit c family members of *A.thaliana*.

Xu *et al.* reported vacuolar H⁺-ATPase Subunit c overexpressing transgenic tobacco performs better under salt stress compared to wild type (Xu, 2011). Arabidopsis plants overexpressing a H⁺-ATPase Subunit c gene from wheat was previously shown to have better tolerance to salt stress (He *et al.*, 2014). We have created transgenic Arabidopsis plants overexpressing a vacuolar H⁺-ATPase Subunit c gene (*VHP*) from *Phaseolus vulgaris*. NaCl treatments of three independent transgenic lines have shown that transgenic plants do not perform better than wild type as indicated by fresh weight and leaf area in any NaCl concentration applied in gradual manner (Figure 5.36). Since there is no almost-identical hit of the predicted *pvVHP* ORF sequence in the reference genome, the lack of phenotype could be due to inaccurate ORF prediction, which would lead to a truncated or non-functional protein. To determine the ORF with higher confidence the upstream and downstream sequences of the predicted ORF should be determined by Random Amplification of cDNA Ends (RACE). Another possibility is that the absence of enhanced tolerance in transgenic plants is due to lack of functional homology between *A.thaliana* and *P.vulgaris* vacuolar H⁺-ATPase Subunit c genes. The lack of altered tolerance in Arabidopsis knock out mutants and overexpression lines could also mean that the activity of this specific member of vacuolar H⁺-ATPase Subunit c family is functionally less important for salinity tolerance compared to other members of the family. The very next step in further functional characterization of common bean *VHP* should be verification of the predicted ORF sequence. Further studies should involve creation of overexpression lines with an alternative destination vector. Moreover, verification of transgene mRNA levels quantitatively compared to native *VHP* genes and include overexpression and knock out experiments of *VHP* genes in *P.vulgaris* would be most helpful in order to concisely determine the functional roles of *VHP* genes in *P.vulgaris*.

So far, seven superoxide dismutases have been identified in the *Arabidopsis* genome. Three groups of superoxide dismutases according to their cofactors are Cu-ZnSODs (CSD1, CSD2, and CSD3), FeSODs (FSD1, FSD2, and FSD3), and MnSOD (MSD1) (Kliebenstein *et al.*, 1998). Cu-ZnSODs require copper to function, which is delivered by Copper Chaperone for SOD (CCS). Cohu *et al.* reported that a CCS null *Arabidopsis* mutant displaying no measurable SOD activity performed no different than wild type under high light stress (Cohu, *et al.*, 2009). CCS-KO plants exposed to oxidative stress in tissue culture medium displayed similar growth retardation with wild type. The authors suggest that there is considerable redundancy in the ROS scavenging systems that masks the effects created by the lack of Cu-ZnSOD activity. It was reported that common bean EMS mutants deficient in FeSOD or MnSOD shows normal growth under 50 μ M arsenic stress. Double mutants lacking both FeSOD and MnSOD showed formation of necrotic spots and ROS accumulation accompanied by down regulation of *Cu-ZnSOD* expression (Talukdar, 2013). In our experiments, CSD1-KO plants (*atSOD* KO) displayed similar decreases in leaf area and fresh weight compared to wild type under all NaCl concentrations applied in gradual or shock manner (Figure 5.41). This is in accordance with the results reported by Cohu *et al.* and further supports the notion of redundancy in ROS scavenging systems in *Arabidopsis*. To ascertain our conclusion, levels of Cu-ZnSOD activity in knock out plants should be measured with SOD activity gels and nitroblue tetrazolium staining experiments.

There have been numerous reports on enhanced tolerance to abiotic stresses in plants overexpressing different isoforms of SODs from various plants both in *Arabidopsis* and other organisms (Diaz-Vivancos, *et al.*, 2013, Faize *et al.*, 2011, Gill *et al.*, 2010, Prashanth *et al.*, 2008, Tanaka *et al.*, 1999, Wang, *et al.*, 2004). We have created transgenic *Arabidopsis* plants overexpressing a putative cytosolic Cu-ZnSOD from *P.vulgaris* and evaluated NaCl tolerance. Three independent transgenic lines showed comparable growth with wild type as indicated by leaf area and fresh weight at all NaCl concentrations applied in gradual or shock manner (Figure 5.35, Figure 5.38.). Our finding contrasts with many of the previous reports on enhanced tolerance upon overexpression of various SOD isoforms. While this effect could be due to differences in the functional activities of different SOD isoforms in *P.vulgaris*, it could also stem from the lack of transgenic SOD protein as suggested by the western blot experiments. Overexpression

lines with an alternative overexpression vector should be created to validate the outcomes obtained in this study. Furthermore, increased Cu-ZnSOD activity and expression levels of *Cu-ZnSOD* in transgenic lines with respect to native SODs need to be verified through qRT-PCR and SOD activity gels in order to draw conclusions about the functional roles of *P.vulgaris* Cu-ZnSOD with higher confidence.

Na^+/H^+ antiporters (NHXs) are evolutionarily conserved genes from yeast to higher plants. They can be located either in the plasma membranes or vacuolar membranes and they are responsible for extrusion of Na^+ from plasma membrane or vacuolar sequestration of Na^+ in vacuoles before intracellular Na concentrations reach toxic levels (Blumwald *et al.*, 2000).

A previous study reported that SALK T-DNA insertional mutant lines of *Arabidopsis NHX1*, carrying an insertion in the 5'-UTR region, display compromised growth and germination rate on MS medium supplemented with 100mM NaCl. The author reports that characterization efforts of another *NHX1* insertional mutant carrying an insertion in the ORF were ongoing (Hanin, *et al.*, 2004). In another study, compromised growth was reported in T-DNA insertional mutants of *NHX1* when treated with 100mM NaCl in both MS medium and soil as indicated by leaf area (Apse, *et al.*, 2003). Our experiments with T-DNA insertional mutants of *NHX1* carrying an exon insertion shows reduced growth similar with wild type under various NaCl concentrations applied in a gradual or shock manner. The reason for the contrasting results compared to previous studies could be due to differences in the experimental setups and the treatment conditions. The NaCl treatment of the *atNHX* knock out line could be repeated in vitro as well as in soil and more parameters indicating stress tolerance, such as root elongation, could be observed.

A number of studies have reported enhanced salinity tolerance upon overexpression of *NHX* genes from different organisms. (Apse *et al.*, 1999, Ohta *et al.*, 2002, Wu, *et al.*, 2004, Zhang and Blumwald, 2001). We have created one transgenic *Arabidopsis* line overexpressing a predicted *P.vulgaris NHX* gene. Transgenic plants displayed comparable growth with wild type plants at various NaCl concentrations administered in gradual

fashion. Our findings contrast with previous reports of tolerance conferred by *NHX* overexpression. While it is possible that the analyzed *P.vulgaris NHX* doesn't have the functional profile to confer increased tolerance, it is also possible that the lack of tolerance observed in our study is due to the gradual implementation of NaCl. It should be noted that NaCl administration was done in a single step without acclimation in most of the previous studies, which exerts a different stress profile on plants as discussed above. The next step in functional characterization of *P.vulgaris NHX* should be creation of overexpression lines using alternative destination vectors and evaluation of the functional effects with respect to those obtained in this study. Application of nutrient mediums with different K⁺ concentrations could also be revealing as in the case of knock out mutants. Furthermore, the lack of a phenotypical difference could be due to the possible disruptive effects of the candidate gene insertion on the genes located in the area of insertion. Since only one independent transformant line was evaluated, it was not possible to eliminate potential bias coming from the place of insertion. Creation of more than one independent transformant lines would make it possible to draw more confident conclusions with regard to the functional effects of the candidate gene irrespective of any bias from insertional effects.

Arabidopsis plasma membrane intrinsic proteins (PIPs) is a sub-member of membrane intrinsic family (MIP) proteins with 13 distinct members. The highly redundant PIP family is further divided into two groups, namely PIP1 and PIP2 proteins, where PIP1 subgroup have five and PIP2 subgroup have eight proteins (Wallace and Roberts, 2004). The PIP protein of interest in our study, PIP2-3, was shown to be a plasma aquaporin and possess water channel activity (Chaumont *et al.*, 1997).

A study by Ines *et al.* reported that T-DNA insertional mutant line SALK_117876 displayed reduced growth in MS medium containing 50-100mM NaCl with apparent chlorosis of the leaves. The author also states that the knock out line showed normal root growth in MS medium containing sucrose or mannitol as osmoticum and suggests that *PIP2-3* has an exclusive role in ionic stress tolerance but not in osmotic stress tolerance (Ines, 2008). In our study, NaCl treatment of the same T-DNA insertional mutant line of *PIP2-3,vSALK_117876*, have displayed no significant differences with wild type as in-

licated by fresh weight and leaf area in gradual or NaCl shock treatments (Figure 5.43). These results contrast with the expected based on what was reported by Olivier *et al.* especially considering that the very same T-DNA insertional mutant line was used (Ines, 2008). The difference between two results could be due to differences between salt stress application in nutrient medium and in soil or due to minor differences in the experimental setups. Further efforts in characterization of *PIP2-3* mutants should include simultaneous NaCl treatment in plates containing MS medium and in soil.

As in other candidate genes, there are several studies reporting enhanced tolerance to abiotic stresses upon overexpression of PIP1 and PIP2 proteins from various organisms in *Arabidopsis* or their donor organism (Hu *et al.*, 2012, Mosa *et al.*, 2012, Xu, 2011, Zhou *et al.*, 2012). To understand the functional profile of a PIP2-3 like *P.vulgaris* gene on salt tolerance, we have created two independent *A.thaliana* overexpression lines and verified transcript accumulation through RT-PCR. Simultaneous NaCl shock and gradual NaCl treatment of PIP412-10 and PIP412-8 overexpression lines revealed that both transgenic lines had significantly higher fresh weight and leaf area in control conditions compared to wild type but no difference was observed in all treatment groups of both gradual and shock NaCl treatment regime (Figure 5.39, Figure 5.40). This finding is similar to other PIP overexpression studies in the sense that PIP overexpression lines usually display enhanced growth performance also in control conditions, but differ with respect to the better growth performance also under stress conditions. The lack of a significant difference in tolerance under stress conditions could be due to weakness of the growth advantage provided by the overexpression, which is insufficient to make a significant difference under stress conditions, especially due to existence of the ionic stress factor imposed by NaCl in addition to the osmotic stress. It is also possible that the analyzed PIP protein has exclusive water channel activity but no solute transport activity as have been reported for some PIPs (Tyerman, *et al.*, 2002). In a particularly interesting previous study, it was reported that overexpression of *Arabidopsis* PIP1-2 in *Nicotiana tobaccum* conferred growth advantage under control conditions but not under salt stress conditions. However, the same transgenic lines displayed decreased growth performance and faster wilting under drought stress condition compared to wild type, even though they retained their superior cell membrane stability as observed in control conditions. The authors suggest that the compromised growth under stress conditions was simply

due to faster water loss (Aharon *et al.*, 2003). Thus, it is possible that the *pvPIP* overexpressing lines may display enhanced, normal, or even compromised growth under drought conditions. To address these possibilities the very next step in functional characterization of *pvPIP* gene should be drought stress experiments in parallel with NaCl stress experiments. Determining whether common bean PIP protein has solute transport activity besides water channel activity through transgene expression in *xenopus* oocytes and radioactive flux monitoring would also allow further insight into its functional properties.

In conclusion, this study serves as a good introductory investigation into functional properties of common bean genes, orthologues of which are intensely studied in other organisms, under salt stress. Functional properties of common bean candidate genes are still largely underexplored; especially considering it is one of the most consumed legumes in the world. Since three of the four candidate genes functionally analyzed in this study are aquaporins, this study has the potential to be a starting point for characterization of common bean aquaporins and determination of functional properties under salt stress. Validation of the vector backbone for intactness of the sequences essential for efficient translation and creation of overexpression lines using an alternative expression vector would substantially strengthen the conclusions drawn in this study. Furthermore, analysis of other parameters such as root elongation, water capacity, dry weight etc. as well as fresh weight and leaf area could provide a more complete picture about the salinity tolerance of the analyzed plants.

APPENDIX A: PREDICTED ORF SEQUENCES OF ALL ORF SEQUENCES

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      20      40      60
ATG GCTAAGC ATGATGCTGA GGGTGGTTCC TTCTCTGCAA AGGACTACCA TGACCCTCCT CCAGCACCCCT
      80     100     120     140
TGATTGATGC TCAGGAAGTC ACACAGTGGT CTTCTACAG GGCCTTGATT GCTGAGTTCA TTGCCACACT
      160     180     200
GCTCTTCCTT TACATCACAG TGCTCACCCT CATTGGTTAC AAGAGCCAGA GTGATGTCAA AGCTGGGGGT
      220     240     260     280
GATCTCTGTG GTGGGGTTGG CATTCTTGGC ATTGCTTGGG CTTTCGGTGG CATGATCTTC ATCCTTGTTT
      300     320     340
ACTGCACTGC TGAATCTCA GGAGTCACA TAAACCCAGC AGTGACTTTT GGACTCTTCT TGGCTCGGAA
      360     380     400     420
GGTGTCTTTG ATAAGGGCTA TAATGTACAT GGTGGCTCAG TGCTTGGGGG CCATATGTGG AGTTGGGTTG
      440     460     480
GTAAAGGCCT TCCAAAAGGC TTACTACAAC AAGTATGGTG GTGGGGCCAA TGAGCTGAGT GATGGGTACA
      500     520     540     560
GCACAGGTGT TGGATTGGGT GCTGAGATCA TTGGAACCTT CGTTTGGTG TAACTGTCT TCTCTGCTAC
      580     600     620
CGACCCCAAG AGGAATGCTA GAGATTCTCA TGTCCCGGTT CTGGCTCCAC TGCCAATTGG GTTTGCTGTG
      640     660     680     700
TTCATGGTTC ACTTGGCTAC AATCCCAGTT ACTGGCACTG GCATTAACCC AGCAAGGAGT TTTGGAGCAG
      720     740     760
CTGTAATCTA CAACAATGAA AAAATCTGGG ATGATCAGTG GATTTTCTGG GTTGGACCAA TTATTGGAGC
      780     800     820     840
AGCAGTGGCA GCATTCTACC ACCAATACAT CCTTAGAGCA GCTGCTATCA AGGCACTTGG ATCATTCAGG
AGCAATGCTT AA

```

Figure A.1. Predicted ORF of *pvPIP*. Green highlighted codon designates the start codon and red highlighted codon designates the stop codon.

20 40 60
ATGGTGTTTGAAATCAGTTCTGTTGTTTCAAATGCAAATGTTATCCACTTCGGATCAATTCCTCCGTT
 80 100 120
 GTCTCCATGAACTTATTTGTTGCCCTTCTTTGTGGTTGATTGTCCTTGGCCATCTTCTCGAGGAGAAT
 140 160 180 200
 CGGTGGATGAATGAGTCTATCACTGCCCTTTTGATTGGTGTACCCTGGCGTAGTCATTTTGCTGTTT
 220 240 260
 AGTGGTGGTAAAAGCTCACATATTCTTGTTCAGTGAAGATCTTTCTTCATATACCTTCTGCCACCT
 280 300 320 340
 ATAATATTCAATGCTGGGTTTCAGGTGAAAAAGAAACAGTTTTTTGTAACTTCATGACCATCATGTTG
 360 380 400
 TTTGGTGCTATTGGTACATTAATATCTTGTGCTGTCATAACTTTGGGTGCGACACAAATTTTAAGAAG
 420 440 460 480
 TTGGATATCGGTCCACTGGATTTAGGGGATTACCTAGCAATTGGTGCAATATTGCTGCGACAGATTCT
 500 520 540
 GTTTCACATTGCAGGTGCTAAATCAGGATGAGACACCTTTGCTGTACAGTCTTGTATTTGGGGAGGGT
 560 580 600 620
 GTTGTGAATGATGCTACATCAGTGGTGCTTTTCAATGCGATCCAAAGCTTTGACCTCAACCAAATAGAC
 640 660 680
 TCTTCAATAGCTTTGCACTTTTAGGCAATTTCTTGTATTTATTTATTGCAAGCACAATGCTTGGAGTG
 700 720 740
 TTGACAGGTCTACTCAGTGCTTATATTATCAAAAAGCTGTATATTGGAAGGCACTCTACAGATCGTGAG
 760 780 800 820
 GTTGCACCTTGTGATGTTAATGGCGTACCTATCCTACATGCTAGCTGAATTATTCTATCTAAGTGGCATC
 840 860 880
 CTCACCTGTATTTTTTGTGGAATTGTTATGTCTCATTATACCTGGCATAATGTGACTGAGAGTTCAAGA
 900 920 940 960
 ATCACTACCAAGCATTCTTTTGCAACCTTGTCAATTTGTTGCTGAGATCTTTATCTTCCTTTATGTTGGT
 980 1,000 1,020
 ATGGATGCCTTGGACATTGAAAAATGGAAATTCGTCACTGATAGCCCTGGAACATCTATAGCAGCTAGT
 1,040 1,060 1,080 1,100
 TCAGTATTGTTGGGTCTAATTCTTCTTGAAGAGCAGCTTTTGTTCCTTTATCCTTCATATCCAAC
 1,120 1,140 1,160
 TTGGCTAAAAAATCAACCAATGAGAAAATTAGCTTCAGACAGCAAGTGATTATTTGGTGGGCTGGTCTT
 1,180 1,200 1,220 1,240
 ATGAGGGGTGCTGTTTCAATGGCACTTGCATATAATCAGTTCACCATGTGCGGGGCATACTTCACTGCAA
 1,260 1,280 1,300
 AGCAATGCAATCATGATCACCAGCACCATCACTGTGGTACTCGTCAGCACAGTGGTGTGTTGGTTTGATG
 1,320 1,340 1,360 1,380
 ACTAAGCCACTCATAAGGCTTTTACTGCCTCATACTCCACATCCTAAAGAATTAAACATCGTGATAGCC
 1,400 1,420 1,440
 ACCGATCCATCTACTCCAAAATCAGCCACTGTCCCACTTCTTGGGAGTGCCAGGACTCAGAAGTCGAT
 1,460 1,480 1,500
 ATTGATTCAATCCATCGTCCAAGCAGCATCCGCGCATTGCTTAGAACTCCAACACACACTGTTTCATCGT
 1,520 1,540 1,560 1,580
 TTGTGGCGTAAGTTTCGATGATGCATTCATGCGTCCTGTTTTCCGTGGAAGGGTTTTGTTCTGTAGAA
 1,600 1,620
 CCTGGCTCTCCAACCTGAACGTAATGGCCAACAATGGCGT**TGA**

Figure A.2. Predicted ORF of *pvNHX*. Green highlighted codon designates the start codon and red highlighted codon designates the stop codon.

```

      20      40      60      80
      |      |      |      |
ATGGAAGTTC TGCAAAATGG CCACAAAACC AAACCCTCTG AAATACCCCA CGTCAATGGA ACCGCGTTAA CTCACCTCAA
      |      |      |      |
      100     120     140     160
CTCGCTCTTC CAAACTCACT TCTCCGGCAA CATCGATCCT TCCAGAGTTT TTCTCTCCA GGTCAAGCGT CTCATTGTAA
      |      |      |      |
      180     200     220     240
AGGTTGGAAC AGCTGTTGTT ACTAGAAGTG ATGGAAGATT AGCACTAGGT AGAATTGGAG CACTTTGTGA GCAGCTTAAA
      |      |      |      |
      260     280     300     320
GAACTTAGCT CTCAAGGATT TGAAGTCATA CTGGTAACCT CAGGTGCTGT TGGCCTTGGC AGGCAAAGAC TTAGATATCG
      |      |      |      |
      340     360     380     400
CAAATTGGCA AATAGCAGTT TTTCTGATCT TCAAAAACCA CAAGGTGAGC TTGATGGAAA AGCATGTGCA GCTGTTGGGC
      |      |      |      |
      420     440     460     480
AAAGTAGTCT CATGGCTCTC TATGATACCA TGTTCAAGCA GCTTGATGTG ACTTCTTCCC AACTTCTTGT GAATGATGGA
      |      |      |      |
      500     520     540     560
TTTTTTAGGG ATGCTGGCTT CAGAAAACAA CTTTCAGACA CAGTGAAGTC GTTGTTAGAT TTAAGGGTTA TCCCCATTTT
      |      |      |      |
      580     600     620     640
TAATGAAAAAT GATGCTGTTA GTAAGTAGGAA AGCACCATAT GAGGATTCTT CTGGTATTTT CTGGGACAAT GACAGTTTGG
      |      |      |      |
      660     680     700     720
CTGGACTATT AGCCTTAGAA CTTCAAGGCTG ATCTCCTTGT TTTATTGAGT GATGTTGAGG GCCTTTACAG TGGTCTCTCA
      |      |      |      |
      740     760     780     800
AGTGACCCAA ACTCAAAATT GATCCATACA TATGTAAAGG AGAAACATCA AGGAGAAATT ACTTTTGGAG ACAAGTCAAG
      |      |      |      |
      820     840     860     880
ATTGGGCAGA GGGGGTATGA CTGCTAAAGT TAATGCTGCT GTTTGTGCTG CTCATGCTGG TATCCCTGTC ATTATAACTA
      |      |      |      |
      900     920     940     960
GTGGCTATGC TACAACAAC ATCATACGTG TTCTTCAAGG GGAAAGAATA GGTACTGTCT TTCATAAAGA TGCTCATTTA
      |      |      |      |
      980    1,000    1,020    1,040
TGGAGCAATA TAAAGGAAGT GAGTGCACGT GAAATGGCAG TTTCAGCACG TGAGAGTTCT AGACGACTTC AGATCCTCAA
      |      |      |      |
    1,060    1,080    1,100    1,120
ATATGAAGAA AGAAGTAAAA TATTATTGGC AATTGCTGAT GCATTGGAGA AAAATGCAAG TCTAATAAGT CTTGAGAATG
      |      |      |      |
    1,140    1,160    1,180    1,200
AAGCTGATGT TGCTGAAGCT GTGATTGCTG GATATGAAAA TTCCTTGATA TCACGTTTAA CCCTGAAGCC TGAGAAGATC
      |      |      |      |
    1,220    1,240    1,260    1,280
TCTAAGCTTG CAAAGTCTGT GCGCATTTTG GCAGCTATGG AAGAACCAAT TGGTCAAAC TTAACAAAAA CAGAGATAGC
      |      |      |      |
    1,300    1,320    1,340    1,360
AGATAAACTA GTCCTGGAGA AAATTTCTTG TCCTTTGGGA GTCCTTCTGG TTATATTCGA GTCTCGACCT GATGCCCTTG
      |      |      |      |
    1,380    1,400    1,420    1,440
TTCAGATAGC TGCATTGGCA ATTCAAAGTG GGAATGGTTT ACTGCTTAAA GGTGGAAAGG AAGCCAGAAG ATCAATGCA
      |      |      |      |
    1,460    1,480    1,500    1,520
ATCTTGACAC AGGTCATTAC TTCGGTGATT CCAAATACTG TAGGTGACAA ACTTATTGGG CTTGTGAATT CAAGAGATGA
      |      |      |      |
    1,540    1,560    1,580    1,600
AATTCCATAC CTACTTAAGC TTGATGATGT GATAGATCTT GTGGTCCCAA GAGGCAGTAA CAAACTTGTT TCTCAATCA
      |      |      |      |
    1,620    1,640    1,660    1,680
AGGAATCAAC AAAAATTCCT GTTCTTGGAC ATGCTGATGG AATTTGTCAT GTATATGTCG ACAAATCAGC TAATATTGGT
      |      |      |      |
    1,700    1,720    1,740    1,760
ATGGCAAAAC AGATTGTTAG AGATGCAAAG ACTGATTATC CTGCAGCCTG CAATGCAATG GAAACTCTTC TTGTACACAA
      |      |      |      |
    1,780    1,800    1,820    1,840
GGATCTATCA AAAAATGGTG GACTTAATGA GCTTGTCCCT GAACTCCGAC GTGAAGGTGT TAAACTGTAC GGTGGACCAC
      |      |      |      |
    1,860    1,880    1,900    1,920
AAGCTAGTGA CTTACTAAAC ATTGATGAAA CAAGCTCTTT TCATCATGAG TATAGTTCAC TTGCTTGAC AGTTGAAATT
      |      |      |      |
    1,940    1,960    1,980    2,000
GTAGAAGATG TATTTGCTGC CATTGACCAC ATAAATCAAC ATGGAAGTGC TCATACTGAA TGCATTGTTA CAGAAGATTG
      |      |      |      |
    2,020    2,040    2,060    2,080
TGAAGTTGCT GAGACTTTCA TAAGTCAAGT TGACAGTGCT GCTGTTTTCC ACAATGCAAG TACACGATTT TGTGATGGAG
      |      |      |      |
    2,100    2,120    2,140    2,160
CACGATTTGG GCTTGGTGCA GAGGTTGGAA TTAGCACAAG TCGAATTCAT GCACGAGGTC CTGTGGGAGT AGAGGGTTTG
      |      |      |      |
    2,180    2,200    2,220    2,240
TTGACAAACA GATGGGTATT GAGAGGGAGT GGGCATGTGG TTAATGGAGA TAAAGGGGTC CATTACACTT ACAAAGAATT
GCCACTAAAA GCATATA

```

Figure A.3. Predicted ORF of *pvP5CS1*. Green highlighted codon designates the start codon and red highlighted codon designates the stop codon.

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      20      40      60
ATGCCTTCAC CAGCTTCAAA TACTTGATT AACACCACAC TCCCATCTTC TTGTTCTTCC CCCTTTAAAG
      80     100     120     140
ACCTGGCTTC CACATCTTCT TCTCCACAAA CTTACTTCC CTTCAAGAAA AGATCCTCTT CCAACACCAA
      160     180     200     220
CACCATCACA TGTCTCTTTC AAACACTCCA CTACCCCAAA CAGTACCAAC CAACATCCAC ATCCACAACC
      240     260     280
ACAACCCCAA CTCCGATTAA ACCCACCACC ACCACCACAA CCACCACCCC GCACAGGGAA ACCAAACCTC
      300     320     340
TCTCCGACAC CAAACAACCA TTTCCTCAAA AATGGAATT TCTGCAGAAA GCCGCTGCGA CAGGCTTGGA
      360     380     400     420
TATGGTGGAG ACGGCGCTGG TTTGCACGA GAGCAAACAC CCGTCCCCA AAACGGCGGA CCCGAAAGTC
      440     460     480
CAAATCGCCG GGAATTTCGC GCCGGTGCCG GAGCAGCCCG CGGATCAAGC CCTTCCGGTG GTCGAAAAAA
      500     520     540     560
TCCCCAAATG CATTGACGGC GTGTACGTGC GCAACGGCGC GAATCCGCTC TACGAGCCCC TCGCCGGGCA
      580     600     620
CCACTTCTTC GACGGCGACG GCATGGTCCA CGCCGTGAAG TTCACGAACG GCGCTGCCAG CTACGCGTGC
      640     660     680     700
CGCTTCACCG AAACACAGCG TCTCGCGCAA GAGAAATCTT TAGGCCGCC GGTGTTCCCG AAGGCTATCG
      720     740     760     780
GCGAACTCCA CGGCCACTCC GGCATAGCTC GCCTCTCTCT TTTCTACGCT CGCAGCCTCT TCCAGCTCGT
      800     820     840
TGATGGGTCC CACGGCATGG GGGTGGCGAA CGCCGGTCTC GTCTATTTCA ACAACCACCT CTTGGCCATG
      860     880     900
TCCGAAGACG ATTTACCATA CCACGTGAGA ATCACCTCTA ACGGCGACTT AACCACCGTT GGCCGTTACG
      920     940     960     980
ACTTCAACGG CCAGCTCAAC TCCACAATGA TCGCCACCCC GAAACTCGAC CCCGTCAACG GCGACCTCCA
      1,000     1,020     1,040
CGCGCTCAGC TACGACGTCG TTCAGAAGCC TTACCTCAAG TACTTCCGTT TCTCCGCTGA CGGCGTAAAG
      1,060     1,080     1,100     1,120
TCCCCCGACG TCGAAATCCC CCTGAAAGAG CCAACCATGA TGCACGATTT CGCAATAACG GAGAATTTCTG
      1,140     1,160     1,180
TCGTCGTCCC CGACCAGCAG GTGGTCTTCA AACTAACAGA GATGATCACC GGCGGCTCCC CCGTGGTCTA
      1,200     1,220     1,240     1,260
CGACAAGAAC AAAACCTCAC GGTGTTGGGAT TTTGGATAAG AATGCGAAGG ACGCGAATGC GATGCGGTGG
      1,280     1,300     1,320
ATCGACGCGC CGGAGTGTTT CTGCTTCCAT CTCTGGAACG CGTGGGAGGA GCCTGAAACC GACGAGATTG
      1,340     1,360     1,380     1,400
TGGTGATTGG GTCCTGCATG ACCCTGCGG ACTCCATTTT CAATGAATGC GATGAGAGTT TGAAGAGCGT
      1,420     1,440     1,460
CTTGCTGAG ATAAGGCTGA ACTTGAGGAC GGGCAAGTCG ACTCGCCGCC CCATTATCTC CGACGCCGAA
      1,480     1,500     1,520     1,540
CAAGTGAACC TTGAAGCCGG CATGGTGAAC AGAAACAAGC TAGGAAGGAA AACTCAGTTC GCTTATCTGG
      1,560     1,580     1,600
CTCTGGCCGA GCCCTGGCCC AAAGTCTCGG GCTTTGCGAA AGTTGATTTG TTCAGTGGGG AAGTGCAGAA
      1,620     1,640     1,660     1,680
GTACATGTAT GGAGAAGAAA AGTTCGGTGG GGAGCCTCTG TTTCTTCCCA ACGGCGAAGA AGAAGGTGAT
      1,700     1,720     1,740
GGGTATATTC TGGCATTTCG GCACGACGAG AAAGAGTGGA AATCCGAGCT GCAGATTGTG AATGCCCAAA
      1,760     1,780     1,800     1,820
ATTTAAAGCT CGAAGCTTCC ATCAAATCC CCTCTCGCGT TCCTATGGT TTTTATGGCA CTTTTATTCA
      1,840
TTCTAAGGAT TTGAGGAAGC AAGCTTGA

```

Figure A.4. Predicted ORF of *pvNCED*. Green highlighted codon designates the start codon and red highlighted codon designates the stop codon.

```

      20      40      60
ATGGCCAAAG ACGTTGAGCA GGTCACAGAG CAACCCGAAT ACTCCGCCAA GGACTACCAC GACCCACCTC
      80     100    120    140
CCGCGCCGTT GTTCGACCCC GATGAGCTCA CCAAGTGGTC CTTGTACAGA GCCGCCATAG CCGAATTCAT
      160    180    200    220
AGCCACCCTC CTCTTCCTTT ACATCACCGT CTTGACCATT ATCGGCTACA AGAGACAGAC CGACACCAGT
      240    260    280
ATTCCCGGTA ACACCGAGTG TGACGGCGTT GGCATTTTGG GCATCGCGTG GGCCTTCGGT GGCATGATCT
      300    320    340    360
TCATCCTCGT TTACTGCACC GCTGGCATCT CCGGGGGACA CATCAACCCT GCAGTCACAC TGGGGTCGTT
      380    400    420
CCTGGGGGCG AAGGTGTCGC TGGTGAGGGC GTTGTGTGAC ATAGTAGCAC AGTGCGCCGG TGCAATCTCC
      440    460    480    500
GGTGCTGGGT TGGCGAAGGC GTTCCAAAA TCCTTCTACA ACAGGTACGG TGGAGGAGCC AACACTGTGA
      520    540    560
GCGATGGCTA CAACAAAGGC ACTGCCTTGG GTGCTGAAAT CATCGGTACC TTTGTTCTTG TCTACACAGT
      580    600    620    640
TTTCTCCGCC ACCGATCCTA AGAGAAACGC TAGGGACTCT CATGTTCTTG TACTAGCACC CCTTCCCATT
      660    680    700
GGGTTTGCCG TCTTCATGGT TCACTTGGCT ACAATCCCTA TCACTGGAAC CGGCATTAAC CCGCAAGGA
      720    740    760    780
GTTTCGGACC AGCTGTGATC TTCAACAACG ACAAAGCCTG GGACGACCAA TGGATTACT GGGTTGGACC
      800    820    840
GTTTATTGGA GCTGCGGTGG CTGCATTTTA CCACCAGTAC ATTCTGAGAG CTGCAGCAAT AAAGGTTCTT
      860
GGATCCTTCA GGAGCAACGC ATAA

```

Figure A.5. Predicted ORF of *pvPIP2-2*. Green highlighted codon designates the start codon and red highlighted codon designates the stop codon.

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                20              40              60
pvSOD_ORF  ATGCTGAAGG CTGTGGCAGT TCTTGGCAGC AGTGAGGGTG TCACTGGAAC TGTTTACTTC AGTCAGGAGG
                80              100             120             140
pvSOD_ORF  GAAATGGTCC AACCACCGTA ACTGGCTCTC TTAGTGGTCT TAAGCCTGGT CACCATGGCT TCCATGTCCA
                160             180             200
pvSOD_ORF  TGCCTTGGGG GACACTACCA ATGGTTGCCT GTCAACTGGA CCACATTTC AATCCTAATGG CAAGGAGCAT
                220             240             260             280
pvSOD_ORF  GGTGCCCCTG AGGATGAGAG TCGCCATGCT GGTGATCTAG GGAATGTAAA TGTCGGTGAT GATGGTACCG
                300             320             340
pvSOD_ORF  CCAACTTCAC TCTTACTGAC AGCCAGATTC CTCTTACTGG ACCAACTCC ATCATTGGGA GAGCTGTTGT
                360             380             400             420
pvSOD_ORF  TGTCCATGCT GATCCTGATG ATCTTGGGAA AGGTGGTCAT GAGCTTAGCA AGAGTACTGG AAATGCTGGT
                440
pvSOD_ORF  GGCAGAGTAG CCTGTGGTAT CATTGGTCTG CAAGGATAA

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Figure A.6. Predicted ORF of *pvSOD*. Green highlighted codon designates the start codon and red highlighted codon designates the stop codon.

```

      20      40      60
ATGCGCAGGAA TTTGTTGCGG CGTTGTTGGA GAGGCCGGAG AGACTGCTTC TCCGACCAAA CCCACCTCGC
      80     100    120    140
GCCCTTTCTC TCGCCGAAGT TTGGACCAAC TGCCTTTGAA ATACATCGCC GGCGTGGCAA TCCCGGCGCC
      160    180    200    220
GGAGAACCCT CGGAAGCGCC AGAAGTTAGA TCAGCGTGCT TCTCCGCCGC GGGAGTGTCA AAACGCGGTT
      240    260    280
CAGAGTTCTG AATCGAAGCG AGTCGAGGCT GTCTCGCTTC CGAATAGTTC GAAGGTTCTT ACGTTGGACT
      300    320    340
CGACAATTGT TGTGGTCGAG GAGGAGAGTC CGAGGTATGG GGTAACCTCG GTTTGTGGCC GGAGGAGGGA
      360    380    400    420
CATGGAAGAC GCTGTTTCAG TGCATCCTTC GTTCTGTCTT GAAAATCGCG ATGAAGATCA GAAAGGCTTT
      440    460    480
CATTTCTTCG CTGTTTTTGA CGGCCACGGA TGCTCACACG TGGCGAGTAT GTGCAAGGAG AGGTTACACG
      500    520    540    560
AGATAGTGAA GGAGGAGATT CAAAAGCGA AAGAGAATTT GGAATGGGAA TCGATGATGA AGAAGAGTTT
      580    600    620
TGCTCGAATG GACCAAGAGG TCCAAAGTTG GAGCAGAACC AACGAGACAC GCACGTGCAG GTGCGAGCTT
      640    660    680    700
CAGACCCCGC GCTGCGACGC CGTTGGCTCC ACCGCGGTCT TCGCCATAGT CACGCCGGAA AAGATAATCG
      720    740    760
TGGCCAAC TG CGGCGACTCG CGCGCCGTGC TCTGCCGTAA CCGCGCACCC GTCCCACTCT CGGACGATCA
      780    800    820    840
CAAGCCGGAT CGACCCGACG AGTTGCTTCG GATCGAAGAT GCTGGTGGGC GCGTGATATA TTGGGACGGG
      860    880    900
CCTAGAGTGC TTGGGGTCTT GGCCATGTCA AGAGCCATAG GGGACAAC TA TCTGAAGCCG TATGTAATTT
      920    940    960    980
CAGAACCGGA GGTAACGGTA ACGGAGCGGA GTGAGGAGGA TGAGTGTGTTG ATACTAGGGA GTGACGGGCT
      1,000  1,020  1,040
TTGGGACACG GTGCAAAACG ACACCGCGTG TAAGGTGGCG CGGATGTGTC TGAACGGGCA GAAGGCGGCG
      1,060  1,080  1,100  1,120
TCTCCGGGGA GAGAGTTGGC GGTTGAGTGT TCCGACAAGG CGTGCTCCGA CGCCTCCATT CTGCTGACTA
      1,140  1,160  1,180
AGTTGGCGTT GGTGAGGCAC AGTTCCGACA ATGTGAGCGT GGTGGTGGTT GATTTGAGGA GAGATGATCG
      1,200
TGAACAATAA

```

Figure A.7. Predicted ORF of *pvPIP2-3*. Green highlighted codon designates the start codon and red highlighted codon designates the stop codon.

```

      20      40      60
ATG GCTGGCT TCAGCGGTGA TGAAACGGCA CCGTTTTTCG GCTTCCTCGG AGCCGCAGCT GCCCTCGTCT
      80     100    120    140
TCTCCTGCAT GGGAGCTGCC TATGGAACAG CAAAGAGTGG TGTGGGCGTG GCTTCCATGG GTGTAATGAG
      160    180    200
ACCTGAGCTA GTGATGAAGT CTATTGTTCC AGTTGTTATG GCTGGAGTTT TGGGTATTTA TGGCCTTATT
      220    240    260    280
ATTGCTGTTA TTATTAGTAC CGGTATTAAT CCAAAGGCCA AATCTTATTA CCTCTTTGAT GGTTACGCAC
      300    320    340
ATCTCTCCTC TGGTCTTGCC TGTGGCTTGG CTGGGCTGTC TGCTGGCATG GCTATTGGTA TTGTTGGTGA
      360    380    400    420
TGCTGGTGTT AGAGCCAATG CACAGCAACC AAAACTTTTT GTTGAATGA TTCTCATCCT CATTTTTGCT
      440    460    480
GAAGCTCTTG CCCTCTATGG TCTCATTGTT GGTATTATCC TGTCTTCTCG TGCTGGCCAG TCTAGAGCCG

ACTAA

```

Figure A.8. Predicted ORF of *pvVHP*. Green highlighted codon designates the start codon and red highlighted codon designates the stop codon.

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