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Overexpression of a Plasma Membrane Protein Gene, SaPMP3, from Spartina alterniflora L. Enhances Salinity Tolerance in Rice (Oryza sativa L.)

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OVEREXPRESSION OF A PLASMA MEMBRANE PROTEIN GENE, *SAPMP3*, FROM *SPARTINA ALTERNIFLORA* L. ENHANCES SALINITY TOLERANCE IN RICE (*ORYZA SATIVA* L.)

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This dissertation is dedicated in memory of my late grandparents. They helped me learn how to identify medicinal plants, grow crops, and select the best plants for seed production. My curiosity and passion for crop improvement started with them, and has gotten me as far as I am today.

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CaMV35S	Cauliflower Mosaic Virus 35S Promoter
Chla	Chlorophyll-a
Chlb	Chlorophyll-b
Chlt	Chlorophyll-total
DMSO	Dimethyl Sulfoxide
dNTPs	deoxy Ribonucleoside Tri-Phosphates
DO	Drop Out
dsDNA	double stranded Deoxyribonucleic Acid
dSm^{-1}	deciSiemens per meter
DW	Dry Weight
EBI	European Bioinformatics Institute
EDTA	Ethylene Di-amine Tetra Acetic acid
FW	Fresh Weight
GFP	Green Fluorescent Protein
HPTII	Hygromycin B phosphotransferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
LiAc	Lithium Acetate
M	Molar
Min	Minute
mL	Milliliter
mM	Milli Molar
MS2D	Murashige and Skoog medium with 2% 2,4-D
NAA	Naphthalene Acetic Acid
NCBI	National Center for Biotechnology Information
NPT	Neomycin Phosphotransferase
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PMP3	Plasma Membrane Protein3
QTL	Quantitative Trait Loci
RAP-DB	The Rice Annotation Project Database
RB	Right Border
RdRp	RNA dependent RNA polymerase
RE	Restriction Endonuclease
RNA	Ribonucleic Acid
RNAi	RNA interference
RPM	Rotation per Minute

RT	Room Temperature
RWC	Relative Water Content
SD	Synthetic Drop out medium
SDS	Sodium Dodecyl Sulphate
sec	Second
SSC	Saline Sodium Citrate buffer
Taq	*Thermus aquaticus*
TE	Tris EDTA
TMD	Trans Membrane Domain
TW	Turgid Weight
U	Unit
V	Volt
WT	Wild Type
YP	Yeast Peptone
YPD	Yeast Peptone Dextrose

ABSTRACT

Salinity continues to be a major abiotic stress limiting crop productivity. As rice is staple food for nearly half of the world population, improvement in its salt tolerance will have a major impact on global food security. Compared to rice and other field crops, halophytes have evolved special physiological mechanisms to withstand high salinity. The overall goal of this study was to characterize plasma membrane protein 3 genes, *SaPMP3-2 and SaPMP3-1,* from a halophyte, *Spartina alterniflora* L., and evaluate their potential in single gene as well as pyramided transgenic plants in combination with the vacuolar ATPase subunit c1 (*SaVHAc1*) gene in improving salt tolerance in cv. Cocodrie background.

Both genes, *SaPMP3-2* and *SaPMP3-1,* enhanced the ability of *E. coli* to survive at 600 mM NaCl. Genetic complementation of the mutant yeast strain and enhanced salt tolerance in wild type yeast strain by *SaPMP3-2* indicated its conserved functional role in salt tolerance. Subsequently, enhanced salt tolerance in transgenic rice plants was demonstrated through overexpression of *SaPMP3-2* and *SaPMP3-1* independently as well as the combination of *SaPMP3-1* and *SaVHAc1.* Chlorophyll retention and relative water content were higher in transgenic plants compared to Cocodrie under salt stress during the vegetative stage. The transgenic plants survived wilting and drying symptoms with enhanced growth and higher K^+/Na^+ ratio at 100 mM NaCl stress during early seedling stage in hydroponic conditions. Salt stress screening during reproductive stage revealed that the single gene and the pyramided transgenic plants had better grain filling whereas only the pyramided plants showed significantly higher grain yield per plant and higher test weight compared to Cocodrie.

The improvement in salt tolerance in transgenic rice plants could be due to the role played by *SaPMP3-2* and *SaPMP3-1* through maintenance of ion homoestasis by restricting uptake of salts. The impact of *SaPMP3* gene was further amplified when combined with *SaVHAc1* in pyramided transgenic plants, which showed better growth, vigor, and enhanced salt tolerance at all stages of crop growth compared with Cocodrie. Our study provided evidence that *S. alterniflora* could be a potential source for mining genes to enhance salt tolerance in rice and other cereal crops.

CHAPTER 1. INTRODUCTION

Rice (*Oryza sativa* L.) is the second most important cereal crop, which serves as a staple food for nearly half of the world's population. Approximately 3 billion people, predominantly in developing countries, are dependent on rice for their calories (FAO 2004). Productivity of rice and other food crops is severely affected by abiotic stresses such as drought, salinity, flooding, temperature extremes, and nutrient deficiency or toxicity world-wide. Among these, soil salinity is one of the age old problems and a major constraint for growing agricultural crops. Moreover, the salinity problem is aggravated due to intensive use of fertilizers and indiscriminate use of bad quality irrigation water. The severity is more prominent in arid and semiarid rice growing environments. At global level, salt-affected land constitutes about 20% of the irrigated land (Pitman and Läuchli 2004) or approximately 830 million hectares of cultivated lands (Hillel 2000). Plant growth and development are impaired due to salinity. The severity of the effects depends on various factors: species type, genotypes, plant growth stage, ionic strength, and composition of the soil solutes. The most common harmful mineral solutes are various proportions of dissociated cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) and anions (Cl^-, SO_4^{2-}, NO_3^-, HCO_3^-, and CO_3^{2-}). In addition to these, the presence of other elements such as B, Se, Sr, Li, Si, Rb, F, Mo, Mn, Ba, and Al in hyper saline water may also cause toxicity to the crop plants (Tanji 1990).

The responses to salinity vary with the phenology of the plants. Plants show characteristic temporal differences to salinity from early vegetative stage to maturity (Munns 2002). Generally, the physiological response to salinity can be recognized in two phases: osmotic effects and salt-specific effects (Munns 2005). The osmotic effects are the immediate consequences of salinity observed during the initial stages of salt stress due to the impairment of water uptake in the rhizosphere resulting in growth retardation. There is very narrow genetic variation among plants

for osmotic stress (Kaddah 1963; Lutts et al. 1995; Pearson and Bernstein 1959). The second phase of response, which manifests slowly, may take from a few days to months for the plants exposed to continued salinity to manifest. Generally, older leaves show the symptoms of salt toxicity when Na^+ and Cl^- content exceed the salt compartmentation capacity of the vacuoles. Eventually, salt toxicity leads to the death of leaves and net reduction in the photosynthetic efficiency (Munns 2002). Plants show marked genotypic differences in their ability to withstand salinity during the second phase and the salt-specific effects are lacking during the first phase. This phenomenon is ascribed to the capacity of salt tolerant genotypes to prevent the accumulation of salts to toxic levels in leaves (Munns 2005; Munns et al. 2006). Studies on physiological and biochemical mechanisms of salt tolerance in model organisms and halophytes indicated that salt tolerance can be enhanced by introgression of genes for tolerance to hyper-osmotic stress and Na^+ toxicity (Gong et al. 1999; Zhao et al. 2006).

It is possible to increase the global food production if salt tolerance of the crop plants is enhanced. Once salt tolerant plants are made available, even saline water can be efficiently utilized for crop production (Bernstein 1974). Therefore, development of salt tolerant varieties is a logical option to enhance crop yield under saline environments. During last few decades, considerable efforts have been made through classical breeding to introgress genes from salt tolerant donor parents as well as wild relatives. Commercial varieties have been improved through introgression of salt tolerance genes from promising landraces like Nona Bokra and Pokkali (Gregorio et al. 2002). However, the progress was slow due to the transfer of many undesirable characters along with the targeted salt tolerance genes (Thomson et al. 2010). Advances in quantitative trait loci (QTL) mapping and genomics approaches has led to better understanding of salt tolerance mechanisms and development of molecular breeding tools. QTLs

have been identified using mutant (Zhang et al. 1995) as well as $F_{2:3}$ populations (Koyama et al. 2001; Lin et al. 2004). Likewise, recombinant inbred lines (RILs) and near-isogenic lines (NILs) were used to characterize the effect of the *Saltol* locus of a salt tolerant donor Pokkali (Thomson et al. 2010). Further, one salt stress related QTL *SKC1* was isolated by map-based cloning to understand the molecular and physiological basis of salt tolerance (Ren et al. 2005). As a result, potential application of marker assisted selection to improve salt tolerance of widely adopted varieties was envisaged.

It is clear from the QTL mapping studies that salt tolerance is a complex trait controlled by many genes and influenced by environmental interactions (Flowers et al. 2000; Koyama et al. 2001; Lin et al. 2004). Since multiple QTLs are involved, developing new varieties through marker assisted selection will be time consuming. Extensive phenotyping and genotypic screening are needed for mapping and introgression of QTLs into elite cultivars. Given the future challenges to meet the food requirement over the next 20 years, the progress in developing new varieties for unfavorable environments is not encouraging.

Transgenic approach offers plant breeders an opportunity to utilize the salt tolerant genes from halophytes and other living organisms for genetic improvement. Desirable traits can be incorporated into existing varieties by overcoming the limitations of hybridization and resources needed for subsequent selection procedures. It also helps to understand the physiological functions of the gene. Therefore, transgenic approach can supplement the conventional breeding programs to improve salt tolerance of crop plants in less time.

Halophytes have evolved physiological mechanisms to survive under natural saline conditions. Plasma membrane is the primary structure, which helps to maintain the intracellular ion homeostasis and selectively allows the exchange of other organic compounds. For example,

smooth cordgrass (*Spartina alterniflora* L.), a halophyte found in salt marshes can grow well at 0.6 M NaCl (Vasquez et al. 2006). Previous study (Baisakh et al. 2008) indicated enhanced expression of plasma membrane protein3 (*SaPMP3*) under salt stress. The PMP3 protein from *Saccharomyces cerevisiae* was first reported to be involved in salt tolerance (Navarre and Goffeau 2000) followed by discovery of *RCI2A*, an *Arabidopsis* gene homologous to *PMP3* involved in salt stress (Mitsuya et al. 2005). Similarly, *AcPMP3-1* in a monocotyledonous wild halophyte, sheep grass (*Aneurolepidium chinense*), was reported to restrict excessive uptake of N^+ ions (Inada et al. 2005). Based on these findings, two members of the *SaPMP3* gene, *SaPMP3-2* and *SaPMP3-1,* were used for rice transformation and to investigate their role in enhancing the salt tolerance of rice.

1.1 Research objectives

The overall goal of this study was to characterize plasma membrane protein 3 genes, *SaPMP3-2 and SaPMP3-1,* from a halophyte, *Spartina alterniflora* L, and evaluate their potential in improving salt tolerance in transgenic plants overexpressing either gene or the combination of *SaPMP3-1* and the vacuolar ATPase subunit c1 (*SaVHAc1*) in cv. Cocodrie background. The specific objectives of this study were: 1) to characterize the expression of plasma membrane protein3 genes in *Escherichia coli and* yeast; 2) to produce transgenic rice lines in the background of an elite Louisiana rice cultivar, Cocodrie, with salt tolerant genes *SaPMP3-2* and *SaPMP3-1* from *Spartina alterniflora* L.; 3) to pyramid the salt tolerance genes by crossing the *SaPMP3-1* plants with the previously developed transgenic plants overexpressing vacuolar ATPase Subunit c1 gene (*SaVHAc1*) from *Spartina alterniflora* L. (Baisakh et al. 2012); and 4) to evaluate the single gene transgenic as well as pyramided transgenic rice lines for salt stress at various stages of crop growth.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Importance of rice and salt tolerance

Rice (*Oryza sativa* L.) is the staple food for nearly half of the world population. Nearly ninety percent of the rice is grown and consumed in Asia. According to International Rice Research Institute report, 85% of total rice production goes to human consumption as compared to 72% for wheat and 19% for maize. At global level, it supplies 21% of energy and 15% of protein on per capita basis (IRRI 2007).

In the United States, rice is grown in six states: Arkansas, California, Louisiana, Mississippi, Missouri, and Texas. In 2011, rice acreage in the USA was estimated at 2.69 million acres with an estimated production of 185 million cwt (USDA 2012). Nearly 50 percent of the production is consumed domestically and the remaining 50 percent is exported. In the global rice trade, United States ranks among the top five rice exporting nations.

Crop production is hampered by many biotic and abiotic factors. Among abiotic stresses, drought, salinity, flooding, temperature extremes, and nutrient deficiency or toxicity cause maximum yield loss both under irrigated and rainfed conditions. Salinity is the second most important abiotic constraint after drought in many rice growing countries of the world. Soil salinity is increasing in irrigated rice growing areas due to indiscriminate use of fertilizers and bad quality water. Increase in area under salinity is more prominent in irrigated arid and semiarid rice growing regions of the world. Plants vary in the level of salinity tolerance and the extent of stress damage is influenced by the growth stage. Based on field experiments, it is reported that the salinity tolerance threshold of rice is around 1.9 to 3.0 dSm^{-1} (Grattan et al. 2002). Experimental results have shown that the rice plant is negatively affected when exposed to salinity levels above 1.9 dSm^{-1}. Salinity had a negative impact on a number of yield components

including stand establishment, panicles/plant, tillers/plant, spikelets/plant, sterility, individual grain size, grain yield, and even delayed heading (Grattan et al. 2002).

2.2 Effect of salinity on different growth stages in rice

Rice is considered more salt sensitive than wheat and maize (Munns and Tester 2008). Generally, the extent of salt stress on rice depends on different stages of growth and amount and type of salts present in the rhizosphere. Generally, early seedling stage is more vulnerable than the reproductive stage. Therefore, understanding the level of salt tolerance at different stages is necessary for developing salt tolerant varieties.

2.2.1 Seedling stage

Seedling stage in rice is most sensitive to salinity. Significant reduction in seedling growth soon after planting was reported with different concentrations of NaCl and duration of salt stress (Zeng and Shannon 2000). Their results from a hydroponic experiment using a salt sensitive variety M-202 indicated significant reduction in shoot dry weight as well as survival rates at salt concentration as low as 1.9 and 3.4 dSm^{-1}. The extent of reduction was influenced by both level of salt concentration and duration of salt exposure. Longer duration of salt stress of about 15 to 17 days at low NaCl concentration (1.9 and 3.4 dSm^{-1}) was more detrimental than higher salt levels for short duration.

Experiments have been conducted to determine the varietal differences for salt tolerance at the seedling stage (Flowers and Yeo 1981). Seven day old seedlings of various varieties and breeding lines were grown under untreated condition for 7 d before imposition of salt stress using 50 mM KCl or NaCl. Half of the seedlings were dead after 7 d of salt stress when 7 days old seedlings were given stress. But 14 days old seedlings survived up to 25 days of stress and 35 days old seedlings survived stress for further 36 days of stress. There was considerable amount

of variability in the survival ability of different rice genotypes. Similarly, genotypes showed large variability for sodium uptake under saline condition than potassium uptake. Moreover, the plants accumulated more potassium under saline condition. There was a negative correlation between survival ability and sodium ion content. Genetic variability for salt tolerance at the early seedling stage, which allows the stable establishment of seedlings, was considered as an important factor in determining salt tolerance (Kaddah 1963; Lutts et al. 1995; Pearson and Bernstein 1959). Their findings highlighted that the effect of soil salinity was less on later vegetative growth stage than early seedling stage.

Rice is comparatively more salt tolerant during germination than early seedling stage (Pearson et al. 1966). Salt screening of different rice indicated no change in percentage germination, but there was delay in germination. Salt tolerance during germination does not have much impact in most of the rice growing areas in the world as transplanting of 20 to 40 d old nursery grown seedlings is a common practice (Kaddah and Fakhry 1961; Pearson and Bernstein 1959). However, salt tolerance during germination is more important in salt affected areas where the crop is direct seeded. Deposition of salt after water evaporation near soil surface causes failure of germination and poor crop stand. Recombinant inbred lines (RILs) were screened under 100 mM NaCl concentration for 10 days to identify seed germination ability of rice (Wang et al. 2010). Significant differences were detected for six germination traits: imbibition rate, germination rate, germination index, root length, shoot length, and vigor index. Further, results from this study showed involvement of two or three major genes for each of the six germination traits. Results from different types of genetic models showed the presence of major genes with additive effect, which accounted for 12.5-99.0% of the total phenotypic variation (Wang et al. 2010).

An RIL population derived from a cross between a salt-tolerant variety Jiucaiqing and a salt-sensitive variety IR26 was used to understand the genetic mechanism of salt tolerance (Wang et al. 2012). The seedling traits such as seedling height (SH), dry shoot weight (DSW), dry root weight (DRW) and Na^+/K^+ ratios (Na^+/K^+) in roots after 10 days of salt stress were studied under three salt treatments (0.0, 0.5 and 0.7 % NaCl). Eleven main effect QTLs and 11 epistatic QTLs were identified for the salt tolerance indices with phenotypic variation of 7.8 to 23.9 % and 13.3 to 73.7 %, respectively. Five of the identified main effects QTLs were novel when compared with previous reports. Five salt tolerant RILs had six to eight positive alleles of main effect QTLs indicating their utility in breeding program.

Kanawapee et al. (2012) evaluated 106 rice genotypes under salt stress condition in a hydroponic experiment to identify salt tolerant genotypes. Multivariate cluster analysis results based on salinity tolerance scores (ST), survival percentage, and Na^+/K^+ ratio indicated negative correlation between ST scores with chlorophyll concentration under saline condition but positively correlated with Na^+/K^+ ratio and proline content. Based on ST scores, rice genotypes were classified into five different salt tolerance groups. Similarly, Na^+/K^+ ratio and proline content were significantly different among the five tolerant groups indicating their reliability in classification. In contrast, chlorophyll content did not indicate any direct relationship with the salt tolerance. Multivariate analysis was useful in identifying new salt tolerant varieties, which were comparable to a known salt-tolerant donor Pokkali.

2.2.2 Reproductive stage and yield

Although all stages of growth and development are affected by salinity, salt stress at early seedling stage and reproductive stage, particularly during the formation of panicles and flowering, results in reduced yield.

The effects of NaCl stress on the growth and development of different *indica* and *japonica* rice varieties and elite breeding lines were evaluated to study the interrelationship between salt tolerance at vegetative stage and reproductive stage (Lutts et al. 1995). There was no correlation between the variability in salt tolerance of different genotypes during the vegetative and reproductive phases of development. Tall *indica* varieties, Nona Bokra, Buhra Rata, Panwell, and Pokkali showed stable salt tolerance during the entire vegetative stage while *japonica* varieties I Kong Pao (IKP) and Tainung 67 and other breeding lines (IR 4630, IR 2153, and IR 31785) showed variations in their level of salt tolerance. Only Panwell, IKP, IR 4630, and IR 31785 showed salt tolerance during booting, heading, and grain maturation. Some varieties showed better tolerance during the vegetative stage while others showed tolerance at reproductive stage. Salt tolerance seems to be both variety specific as well as growth stage specific. Thus, their findings emphasized the identification of genotypes tolerance to salt stress at specific developmental stages.

Effect of salt stress on grain maturation and yield were studied by many researchers (Kaddah 1963; Kaddah and Fakhry 1961; Pearson and Bernstein 1959). Based on their results, salt stress at reproductive stages reduced the number of panicles per plant, percentage of filled grains, and 1000-grain weight.

2.3 Salt tolerance mechanisms

Several adaptive mechanisms contribute to salt tolerance in plants. In general, salt tolerance mechanisms are distinguishable into osmotic stress and salt-specific effects at physiological and molecular levels (Munns 2005). The presence of excess salts in the soil solutes immediately reduces the plants ability to absorb water at root surface and results in slower growth. This indirect effect of water deficiency is termed as osmotic effect. It is primarily due to

excess salt present outside the plant. At this point Na^+ and Cl^- ions have not reached toxic level in the growing tissues. Entry of salt into the plant system through the transpiration stream and its accumulation leads to toxicity and injury to cells. The presence of toxic levels of salt within the plant system causes reduction in growth and development, which are known as salt specific effects. Through these adaptive mechanisms plants reduce the uptake of excess salts from root surface and partition the salts at cellular and tissue level to maintain the normal growth and development.

2.3.1 Mechanisms involved in osmotic stress tolerance

High levels of salts present at the interface of plant root and soil create increased osmotic strength of the soil solution. This leads to decrease in the osmotic potential of the soil solution and plant's access to soil water. As a result, rice plants experience water stress and show reduction in growth during initial stages of salt stress.

The impact of osmotic effect on plant growth was studied by Munns et al. (1995) in wheat and barley. All 15 genotypes from wheat and barley showed decreases in the rate of leaf elongation under 250 mM NaCl.

Yeo et al. (1991) reported no significant short term effect on leaf growth, turgor pressure in the growing zone, and photosynthetic gas exchange in rice. There was no immediate effect on length of leaves, which were developing at the time of salt application or shortly after salt stress. However, prolonged salt stress resulted in reduction in leaf length. This growth reduction was due to osmotic stress caused by salinization, which is different from long-term effects of salt accumulation in expanded leaves.

A great effort has been made to find the similarity in response to water and salt stress in *Arabidopsis*, rice, wheat, and barley (Duan and Cai 2012; Munns 2002). Overexpression of late

embryogenesis abundant (LEA) protein *OsLEA3-2* from rice in yeast, *Arabidopsis*, and rice implied their induction by abiotic stresses (Duan and Cai 2012). Transformed yeast cells displayed improved growth compared to the control strain at 1.2 M NaCl concentration. The lag phase was shorter (24 hours) than that of the control strain (60 hours) under salt stress of 1.2 M NaCl and 1.2 M KCl and also under osmotic stress of 2.0 M sorbitol. The growth of transgenic *Arabidopsis* plants was significantly stronger than WT plants on MS media supplemented with 150 mM mannitol or 100 mM NaCl immediately after germination. Similarly, growth of transgenic rice was stronger under salinity or osmotic stress after 5 and 14 days. When one month old rice plants were screened for drought stress, there was a complete inhibition of growth in both transgenic and control plants 20 days after imposition of stress. However, transgenic plants produced new leaves after withdrawal of drought stress and set seeds. In vitro freezing tests indicated the role of *OsLEA3-2* in protecting the lactate dehydrogenase (LDH) aggregation. These results clearly indicated role of *OsLEA3-2* in abiotic stress tolerance (Duan and Cai 2012).

Alteration in cell water relationship within few minutes of salt stress and transient reductions in leaf expansion rates in salt treated plants was observed in wheat, barley, and maize (Neumann 1993; Passioura and Munns 2000; Yeo et al. 1991). These initial responses were seen when plants were treated with other osmotic agents similar to NaCl such as KCl, mannitol, or polyethylene glycol (PEG). These findings implicated the symptoms of osmotic stress rather salt-specific responses during early stages of stress and parallelism between salt stress and drought stress.

Plants have evolved numerous physiological and biochemical strategies to manage the initial phase of osmotic stress caused by salinity. They perceive the dehydration stresses and stress signals activate the expression of stress related genes and synthesis of proteins.

Transcription factors were thought to control the expression of various stress responsive genes (Singh et al. 2002). Several families of transcription factors such as DREB, MYB, and bZIP have been critically analyzed for their role in stress response (Hu et al. 2008; Mukhopadhyay et al. 2004).

Hu et al. (2008) have successfully isolated and characterized NAC gene *SNAC1* (*Stress-Responsive Nac 1*) from upland rice IRA109 (*Oryza sativa* L. ssp *japonica*), which encode NAM, ATAF, and CUC (NAC) transcription factor. Transgenic rice plants overexpressing *SNAC1* showed enhanced drought tolerance and higher seed setting (22–34%) than control plants. These transgenic rice plants showed significant salt tolerance at the vegetative stage. More than 80% of transgenic seedlings survived 12 d exposure to 200 mM NaCl, whereas almost all of the control seedlings died. Similarly, transgenic rice seedlings were significantly more sensitive to ABA treatment by closing their stomata under drought stress. This was confirmed by the strong localized expression of *SNAC1* in guard cells under drought stress. Moreover, there was a significant closure of stomata pores in transgenic rice than in the WT under both normal and drought-stressed conditions. Thus, rice plants overexpressing *SNAC1* gene enhanced both drought and salinity tolerance.

Mukhopadhyay et al. (2004) isolated and characterized a stress-associated protein 1 gene, *OSISAP1* transcription factor from rice, which was induced by excess cold, desiccation, salt, submergence, heavy metals, and ABA. Transgenic tobacco seedlings gained more fresh weight than control plants after 4 d of 250 mM NaCl stress. *OSISAP1*-overexpressing lines showed increase in germination percentage after 8 d of dehydration stress with 0.3M mannitol. Similarly, transgenic seedlings gained 67-92% fresh weight than untransformed (WT) plants (35%) after

recovery from cold stress. This revealed the complexity of genes involved and overlapping of responses to different stresses.

Prolonged exposure to salinity over several weeks and months leads to salt-specific injuries in salt-sensitive species. Susceptible plants begin to show yellowing of leaf tips and gradual death of older leaves. The extent of salt injury is inversely proportional to the ability of salt exclusion at rhizosphere and the amount of salt compartmentalization inside vacuoles. Salt tolerant species have the ability to maintain the ion homeostasis by preventing excess salt build up in the cytoplasm. This greatly helps to maintain regular developmental and metabolic activities under higher soil salinity conditions. To understand the cross talk between different abiotic-stress responses at the gene expression level, transcriptome profile of rice seedlings was studied by Rabbani et al. (2003). Microarray and RNA gel-blot analysis revealed up-regulation of 73 genes after exposure to four kinds of abiotic stresses such as drought, salinity, cold, and ABA. Fifty six genes were induced by both drought and high salinity and 15 genes were induced in common by all four stresses. These results indicated the likely cross talk between different abiotic stress tolerance factors through overlapping stress signaling pathway.

2.3.2 Mechanisms involved in salt-specific tolerance

The second phase of growth reduction, in response to excess salt, was attributed to the salt specific response caused by over accumulation of salts within plants. At the phenotypic level, plants display marked reduction in growth rate when sodium ion concentration reaches to toxic level. Higher sodium concentration in cell is deleterious to many biochemical processes in the cytoplasm. Basic functions necessary for growth and development are affected in glycophytes. Inhibition of critical enzymes involved in photosynthesis, respiration, and protein synthesis were reported (Hall and Flowers 1973; Schubert and Läuchli 1990).

It is well known fact that essential nutrients are taken up by terrestrial plants along with water. The evapotranspiration pull exerted by atmosphere through stomata is the major force for the movement of water from soil into plant system. Generally, it helps to uptake essential nutrients to the shoots under normal growing conditions. However, under saline conditions the excess salts present in soil solute move into the plant system through transpiration pull. To cope with the increased salt levels in the leaves, salt tolerant plants have evolved two physiological adaptive mechanisms. The first one is the Na^+ exclusion from leaves and the second is tissue tolerance (Munns and Tester 2008).

2.3.2.1 Salt exclusion

Exclusion of Na^+ entry at the root surface has been regarded as an important mechanism to minimize damages to the plants under saline condition. The high unidirectional influx of Na^+ from the soil solution into the root cortical cells, which occurs in a passive way, is poorly understood (Schubert and Läuchli 1990).

Regulation of Na^+ uptake at the rhizosphere level was considered as an important check point to prevent the entry and excess accumulation of salt in shoots. One maize cultivar, Pioneer 3906, accumulated less sodium than DeKalb XL75 (Schubert and Läuchli 1990; Tyerman et al. 1997). They measured the influx and efflux ratios to assess the relative salt tolerance. The reduced influx rates observed in Pioneer 3906 cultivar indicated difference in sodium exclusion at the root surface. Their findings emphasized the low passive Na^+ permeability of the epidermal and cortex plasmalemma.

Salt tolerance in plants is strongly associated with the ability of plant to exclude Na^+ by both root and shoot, whereby plants maintain low cellular Na^+/K^+ ratio (Martínez et al. 2007; Schubert and Läuchli 1990; Tyerman et al. 1997). OsSOS1, a functional homolog of *Arabidopsis*

salt overly sensitive 1 (SOS1) protein (Shi et al. 2002) was identified in rice (Martínez et al. 2007). When the rice transporter, OsSOS1, was expressed in yeast strains (AXT3K) lacking sodium transporters, cells could grow in a medium containing up to 100 mM NaCl. Similarly, *OsSOS1* restored the function of *Arabidopsis* mutant *sos1-1* when mutant plants were transformed with *OsSOS1*. Transgenic *Arabidopsis* plants could grow at 65 mm NaCl whereas mutants failed to perform well in terms of seedling growth and fresh weight. *OsSOS1* transcript level was increased in transgenic *Arabidopsis* plants in response to salt stress. In addition, phosphorylation assays showed that rice OsSOS1 protein was recognized as a substrate by the *Arabidopsis* SOS2/SOS3 protein kinase complex like AtSOS1. Their finding demonstrated high degree of structural conservation among the SOS proteins from dicots and monocots, and Na^+ exclusion mechanisms to prevent the Na^+ concentration to reach the toxic level.

Eight QTLs responsible for the variations in their K^+ or Na^+ content were mapped in a BC_2F_2 population developed from the cross between a salt-tolerant *indica* variety, Nona Bokra and a susceptible elite *japonica* variety, Koshihikari (Lin et al. 2004). In a follow up research, *SKC1* gene encoding a member of HKT-type transporters was isolated by map-based cloning (Ren et al. 2005). The gene expression was localized to the parenchyma cells surrounding the xylem vessels. Physiological analysis, K^+ and Na^+ contents in rice, and functional analysis of *SKC1* in oocytes indicated its function as a selective Na^+ transporter. Like *AtHKT1*, it also mediates both influx and efflux of Na^+ across the plasma membrane in a K^+-independent manner and regulates K^+/Na^+ homeostasis under salt stress condition.

The underlying transport proteins involved in ion flux were broadly classified into three major groups such as pumps, carriers, and channels, based on energy needed during their transport across the plasma membrane (Sussman and Harper 1989). Pumps utilize energy in the

form of ATP or light for vertical transport of ions. The carriers operate due to electrochemical ion gradients across the membrane. As two ions couple and move through carriers, they are further classified as symporter and antiporter, which transport ions in the same direction or opposite direction, respectively (Niu et al. 1995; Sussman and Harper 1989). In the case of channels, ions move down a free energy gradient in a passive mode.

Experimental results from several authors implicated the Na^+ permeable transporters in Na^+ influx into root cells, which leads to Na^+ toxicity due to over accumulation in shoots. Generally, the cation channels and transporters associated with the Ca^{2+}, K^+, and Na^+ uptake (Amtmann et al. 1997; Tyerman et al. 1997). Demidchik and Tester (2002) and Tester and Davenport (2003) suggested the role of nonselective cation channels (NSCCs), a distinct group of plant ion channels mediating high unidirectional influx of Na^+ to the cell.

Although exact mechanisms of Na^+ influx are not completely understood, it is known to act as a competitor of K^+ uptake (Niu et al. 1995; Watad et al. 1991). For example, plant roots acquire K^+ either in high affinity or low affinity from soil solutions depending on the K^+ concentration. Watad et al. (1991) categorized the K^+ uptake mechanism into two systems namely, system 1 and 2. System 1 operates when K^+ concentration is low in soil solution, where uptake occurs through high affinity manner and is not inhibited by Na^+. System 2 is involved under high soil saline condition, when K^+ concentration is high due to presence of NaCl in soil. Eventually K^+ uptake occurs via low-affinity manner and as a consequence more Na^+ influx (Niu et al. 1995; Rains and Epstein 1967).

Schachtman and Schroeder (1994) commented that system 1 reflected the K^+-H^+ symporter, which is an active transporter. The higher concentration of NaCl in soil solutes will have rate limiting effect on K^+ uptake mediated by high affinity mode. As a result, plant's

intracellular K^+ accumulation was affected, and complex physiopathology symptoms were noticed. Since K^+ is the most important macronutrient in plant system, vital functions related to growth, tropism, enzyme homeostasis, and osmoregulation were affected (Epstein 1966; Rains and Epstein 1967).

Physiological experiment conducted by Watad et al. (1991) in tobacco (*Nicotiana tabacum/gossii*) cell cultures indicated 3.5 times more potassium uptake into NaCI adapted tobacco cells than control cells under 160 mM NaCI and 1.5 times more under control without NaCl. As K^+ leaching to the medium was experimentally controlled, the total K^+ estimated from the NaCl adapted cells was certainly due to enhanced entry. It seems certain that salt tolerant varieties maintain a higher potassium-sodium ratio compared to that in non-tolerant variety.

2.3.2.2 Tissue tolerance

The ability of mesophyll cells in leaf tissues to tolerate the overaccumulation of Na^+ and Cl^- without adverse effects is another significant tolerance mechanism. Tissue tolerance involves the modification of cell anatomy by compartmentalizing excess Na^+ and Cl^- to maintain the normal cellular functions by preventing them to reach toxic level. The importance of maintaining low Na^+ and high K^+ in cytoplasm for the optimum functions of enzymes and protein synthesis was reported under salt stress in wheat and barley (Flowers and Dalmond 1992; James et al. 2006).

Two major anatomical adaptations reported in dicotyledons are succulence and salt glands or bladders (Flowers et al. 1986; Munns and Tester 2008). Increased vacuole and cell size under salt stress condition leads to succulence, which dilutes the effect of excess salt within the cell. Succulence is rarely noticed in monocotyledons, but presence of salt glands is more common in monocotyledonous halophytes (James et al. 2006).

The importance of compartmentalizing excess Na^+ and Cl^- was studied in salt-sensitive durum wheat and salt tolerant barley seedlings under varying concentrations of salt stress. The photosynthetic efficiency and cellular as well as subcellular Na^+, K^+ and Cl^- distribution was assessed to find their effect on salt tolerance level (James et al. 2006). The results indicated that Na^+ accumulated linearly in the vacuoles of mesophyll cells with increasing leaf Na^+ concentration, which was higher than the vacuoles of epidermal cells in both wheat and barley at leaf Na^+ concentrations greater than 250 mM. However, vacuolar Na^+ concentrations of barley was 10% more in mesophyll cells than the whole leaf and same in wheat. Similarly, K^+ accumulated to much higher concentrations in the mesophyll compared to the epidermis in both genotypes and vacuolar K^+ concentrations in the mesophyll cells were not significantly different between barley and wheat. Likewise, vacuolar Cl^- concentrations in both mesophyll and epidermis increased linearly with increasing leaf Cl^- concentration in both.

2.4 Transgenic approach to improve salinity tolerance in rice

Transgenic approach is considered as an essential tool for functional characterization of the genes. Successful transfer and expression of foreign genes involved in different salt tolerance mechanisms such as osmotic stress tolerance, Na^+ exclusion, and tissue tolerance have been demonstrated in rice and other plants. With the identification of new genes from gene expression studies, several salt tolerance genes involved in regulation of carbohydrate, nitrogen and energy metabolism, ROS scavenging, detoxification, signal transduction, and post translation modifications have been systematically transformed with satisfactory results.

Several crop species have shown increased salt tolerance following transformation with the Na^+/H^+ antiporter gene, *NHX1*, from different plants (Apse and Blumwald 2002; Fukuda et al. 1999; Fukuda et al. 2004). Fukuda et al. (1999) isolated a cDNA clone of Na^+/H^+ exchanger

from rice (*OsNHX1*), which catalyzes the counter transport of Na^+ and H^+ across membranes. The amino acid sequence of *OsNHX1* showed high similarity with *NHX1* from *Saccharomyces cerevisiae*. The increased expression of the gene under salt stress condition indicated its potential role in salt tolerance of rice.

Similarly, the vacuolar-type Na^+/H^+ antiporter gene from a halophytic plant, *Atriplex gmelini* (*AgNHX1*) was transformed into a salt-sensitive rice cultivar (*Oryza sativa* cv. Kinuhikari) (Ohta et al. 2002). Seedling screening results at 300 mM NaCl for 3 days showed survival of transgenic plants, while the wild-type rice plants started drying. There was eight-fold increase in the activity of the vacuolar-type Na^+/H^+ antiporter in the transgenic rice plants than wild-type rice plants. Overexpression of *AgNHX1* from *Atriplex gmelini* significantly improved the salt tolerance in rice plants.

Rice plants were transformed with a gene encoding catalase, *kat*E, from *Escherichia coli,* which decomposes H_2O_2, one of the reactive oxygen species produced by saline stress. The transgenic rice plants of *indica* cultivar BR5 and *japonica* cultivar Nipponbare (Nagamiya et al. 2007) could grow in 100 mM NaCl for 14 days. On the other hand, non-transformed plants of both cultivars did not grow in 50mM and higher concentrations of NaCl. Similarly, these transgenic plants showed 1.5 to 2.5 fold higher catalase activity than the wild-type rice plants. Higher rate of survival was reported in transgenic plants compared with the non-transgenic plants at 100 mM NaCl stress conditions. When 250 mM NaCl solution was applied during the reproductive stage, transgenic plants could set the seeds.

Prolonged salinity during reproductive stage affects the development and viability of reproductive organs in rice and other crops (Munns and Rawson 1999; Sultana et al. 2012). Sultana et al. (2012) transformed rice with monodehydroascorbate reductase (MDHAR), an

enzyme associated with the ascorbate-glutathione cycle, which scavenges reactive oxygen species (ROS). They found no significant difference in tillers numbers and length of panicle between transgenic and control plants under control as well as 100 mM NaCl stress for 15 days. However, seed sterility was low in one of the transgenic plant MT24 (40%) compared to wild type (70%). Moreover, 100 mM NaCl stress for 30 days resulted in 100% seed sterility in both transgenic and WT plants.

Transgenic rice plants were developed with *MDHAR* gene from the mangrove plant *Acanthus ebracteatu.* *MDHAR* is known to scavenge reactive oxygen species (ROS) produced due to cellular dehydration under salt stress. The ROS generated by superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) can cause oxidation of lipids, proteins, and nucleic acids. Transgenic plants overexpressing *MDHAR* showed higher MDHAR enzyme activity compared to untransformed plants under both NaCl and control conditions. Growth parameters of transgenic plants revealed salt tolerance at germination and seedling stages. These plants showed less seed sterility and more test weight than control plants during reproductive stage stress.

A novel wheat gene, *TaRSG*, expressed in cDNA microarray analyses after 12h of salt treatment, was studied both by overexpression in *Arabidopsis* and its silencing in rice (He et al. 2011). Overexpression in WT and functional complementation in *Arabidopsis* mutant showed enhanced salt tolerance of transgenic plants compared to salt sensitive reaction in wild type and mutant plants. Similar tolerance trend was observed after ABA treatment in *Arabidopsis*. Under salt stress condition, these transgenic plants contained a lower level of Na^+, higher ratio of K^+/Na^+, and higher proline content whereas the SALK mutant had a high level of Na^+, a lower ratio of K^+/Na^+, and low proline content. Results from silencing the homologous gene in

transgenic rice revealed suppression of *OsSRG* expression by more than 50% and also increased salt sensitivity. Moreover, there was significant decrease in their survival rate and leaf chlorophyll content when compared to the WT control. The results from overexpression and mutant complementation in *Arabidopsis* and homologous gene silencing in rice clearly demonstrated the function of *TaSRG* in salt tolerance mechanisms.

Most of the functional characterization studies are based on the gene expression at transcription level and very little effort has been made to account for the post-transcriptional changes under salt stress condition. Since gene expression at mRNA level was not very well correlated with the protein expression, characterization of post-transcriptional and post-translational regulatory mechanisms were studied to understand the crucial molecular mechanisms governing plant adaptation under salt stress (Sultana et al. 2012). They have tested the role of protein kinases function of *OsRK1*, which is a key regulator of salt stress and ABA signaling in plants. Comparative proteomic analysis of transgenic rice root results indicated the expression of proteins involved in diverse metabolic pathways by over-expression of *OsRK1* in rice roots. Protein phosphorylation activity was significantly increased in transgenic plants when compared to wild type after 3 h or 7 h of salt treatment. The 2-DE gel results showed difference in the abundance of 52 early salt-responsive protein spots. Further analysis revealed the constitutive expression of these proteins upon overexpression of *OsRK1* in rice roots. This implies that *OsRK1* may act as an upstream regulator of the salt stress response by regulating enzymes involved in glycolysis, detoxification, branched amino acid catabolism, GA-responsive proteins, signaling, and protein turn over.

Improvement in stress tolerance has been achieved in plants by transfer of single stress responsive gene using transgenic approach (Karan and Subudhi 2012a, 2012b). For both genes,

PMP3 and *VHAc1*, transfer of individual gene from multiple sources resulted in improved abiotic stress tolerance in both model and crop plants (Baisakh et al. 2012; Chang-Qing et al. 2008; Fu et al. 2012; Inada et al. 2005; Medina et al. 2001). Due to complexity associated with abiotic stress tolerance, stacking of multiple stress responsive genes involved in different stress responsive pathways in a single genotype might result in greater stress tolerance (Samis et al. 2001). Qiu et al. (2007) reported that salinity tolerance in halophyte *Suaeda salsa* might be due to coordinated upregulation of two genes V-H^+ATPase and vacuolar Na^+/H^+ antioporter, which help in sequestering the Na^+ into the vacuoles. Pyramiding of two transgenes, *SsNHX1* from *S. salsa* and Arabidopsis *AVP1* conferred increased salt tolerance in rice than the *SsNHX1* alone and non-transformed controls (Zhao et al. 2006). Significant increase of *AVP1* activity was observed in the pyramided transgenics in comparison to single gene transgenic and non-transgenic plants upon salt stress imposition, which was mainly due to the overexpression of *AVP1*. Its activity was higher in 3-week-old seedlings than in 5-week-old seedlings, which was ascribed to the reduced synthesis of macromolecules and less PPi generation resulting in reduced availability of PPi substrate for pyrophosphatase hydrolysis. In addition, transgenic plants exhibited higher level of root *P-ATPase* activity, which could be due to the increased activity of vacuolar Na^+/H^+ antiporter, and *AVP1*, and more Mg^{2+} in SA-transgenics might protect P-ATPase proteins from salt stress. Measurements of ion levels indicated higher level of photosynthesis, K^+/ Na^+ ratio in their shoots, and root proton exportation capacity coupled with reduced H_2O_2 generation in transgenic plants than in non-transformed controls. Similarly, enhanced abiotic stress tolerance in pyramided transgenic tobacco plants expressing both betA (encoding choline dehydrogenase) and *AtNHX1* genes (Duan et al. 2009) and transgenic maize plants stacked with both *betA* (encoding choline dehydrogenase from *E. coli*) and *TsVP*

(encoding *V-H*$^{+}$*-PPase* from *Thellungiella halophila* (Wei et al. 2011). These studies clearly highlighted the advantage of pyramiding salt stress related genes in transgenic plants to improve salt tolerance. Since both *PMP3* and *VHAc1* have role in ion homoeostasis, pyramiding of both genes in single genotype is expected to improve salt tolerance

2.5 *Spartina alterniflora* L. as a source of salt tolerance genes

Some organisms have evolved efficient physiological mechanisms to survive under harsh environments. For example, halophytes have evolved the capacity to withstand high salt stress. Smooth cordgrass *(Spartina alterniflora* L.), a halophyte found in salt marshes can grow well at 0.6 M NaCl concentration (Vasquez et al. 2006). The greater salt tolerance of *S. alterniflora* is mainly due to the excretion of excess salt through salt glands present on its leaves (Bradley and Morris 1991), its ability to use Na^{+} for osmotic adjustment in the shoots (Vasquez et al. 2006), and ion exclusion at their roots (Bradley and Morris 1991). In addition to these physiological defense mechanisms, synthesis of osmoprotective compound, 3-Dimethylsulfoniopropionate (DMSP) is also implicated for its salt tolerance (McNeil et al. 1999). Since it is known to harbor these valuable salt tolerance genes, their possible use in genetic engineering has been explored in the present study.

In order to study the stress tolerance of *S. alterniflora* at the transcription level, differential gene expression studies were carried out, and cDNAs with role in various salt tolerance mechanisms were isolated (Baisakh et al. 2006; Baisakh et al. 2008). Fourteen tissue specific transcript derived fragments (TDFs) were analyzed for their expression pattern in the leaf and root tissues under salt stress. There was noticeable difference among these TDFs with respect to the quantitative expression pattern in both leaf and root tissues at different stages of

stress. Vacuolar H^+-ATPase (*SaVHAc1*), which showed increased expression after imposition of salt stress, improved salt tolerance in transgenic rice plants (Baisakh et al. 2012).

Apart from their role in the salt tolerance mechanism, eight genes and nine transcription factors were assessed for their possible involvement in heat stress tolerance (Baisakh and Subudhi 2009). Expression analysis at mRNA level revealed temporal and tissue specific expression patterns. The leaves showed higher level of *PMP3* accumulation compared to other genes at all the stages of stress; however, the expression was more conspicuous at 1 h than other time points. Similarly, root tissues showed a constant level of mRNA expression for *PMP3*, but the amount of expression was low compared to the leaf samples. Real-time reverse transcription PCR results confirmed the quantitative difference in the expression of the *PMP3* gene between leaf and root tissues under heat stress (Baisakh and Subudhi 2009). These results suggested *S. alterniflora* as a potential source for isolating abiotic stress tolerance genes (Subudhi and Baisakh 2011). Due to higher level of expression in leaf and root tissues, *PMP3* gene was used in the present study to investigate its possible role in salt tolerance. In addition to *PMP3,* a vacuolar *H^+-ATPase* (*SaVHAc1*) transgenic plants already developed in Cocodrie background (Baisakh et al. 2012) was used for pyramiding both genes..

Several authors studied the special adaptive mechanisms and importance of certain physiological responses of *S. alterniflora* to salinity. However, the quantitative evidences indicating the relative importance of individual tolerance mechanisms was not comprehensive until Bradley and Morris (1991), who studied the ion exclusion at rhizospere and secretion of ion from shoots. Growth characteristics under salt stress indicated that ion exclusion at the roots was most important tolerance mechanism and it accounted for 91-97% of the total ion uptake by the plant. Quantitative evaluation showed that secretion of ion from shoots was approximately half

of total ion uptake. Among the different mechanisms, ion exclusion by the root was more important than tissue accumulation and leaf secretion and absorption of K^+ was more than Na^+ compared to the availability of these ions in the soil solution.

Vasquez et al. (2006) evaluated the salt tolerance of two halophytes, *Phragmites australis* and *Spartina alterniflora*, under varying concentrations of salt in greenhouse. Normal growth and salt tolerance up to 0.6 M NaCl stress was observed in *S. alterniflora* whereas *P. australis* did not grow well above 0.2 M NaCl. Salt tolerance in *S. alterniflora* was attributed to the osmotic adjustment in the shoots. *P. australis* was considered as an excluder as it restricts the entry of excess Na^+ into the shoots.

The physiological/biochemical responses to salinity stress were studied in salt-tolerant populations of *Panicum hemitomon*, *Spartina patens*, and *Spartina alterniflora* to identify potential indicators of salt tolerance (Hester et al. 2001). Based on growth responses, high CO_2 assimilation and the proportion of dead above ground biomass showed promising indicators of salt tolerance. There was accumulation of significantly more glycine betaine and lower leaf Na^+/K^+ ratios in salt-tolerant populations of *S. alterniflora* than *S. patens* and *P. hemitomon* under salt stress condition. Elevated leaf proline concentration was noticed in all three populations under salt stress over short duration of 1 to 5 weeks.

The accumulation of free proline content in response to imposed environmental stresses such as drought, high salinity, and low temperature has been studied in some plants (Handa et al. 1986; Nanjo et al. 1999). Proline plays an important role in osmotic adjustment, stabilization of subcellular structures, scavenger of free radicals, and mechanical support to cells (Handa et al. 1986; Karan and Subudhi 2012; Miller et al. 2010; Nanjo et al. 1999; Yonamine et al. 2004).

2.6 The role of plasma membrane in salt tolerance

The major adaptive mechanism of plants is to minimize the uptake of excess salts from the roots (Munns 2005) and to maintain the intracellular ion homeostasis (Hasegawa et al. 2000; Niu et al. 1995). The lipid bilayer found around the plant cells and vacuoles is the primary structure, which helps to maintain the intracellular ion homeostasis and selectively allow the exchange of other organic compounds.

The functions of the plasma membrane in transport of solutes, both into and out of each cell, and their role in signal transduction by sensing and initiating the cellular response to changing environmental conditions is vital under salt stress condition (Sussman and Harper 1989). The advances made in the understanding of physiology and biochemical responses to salt stress helped to dissect the flux of ions across the plasma membrane and tonoplast via transport proteins. The role of transport proteins in the passive and active transport of ions, down and against the thermodynamic gradient ($\Delta\mu$), was proposed long ago (Niu et al. 1995; Nobel 1991). Major advances in research contributing to the understanding of transport proteins and their potential application in the enhancement of resistance to hyper-osmotic stress came from the basic research studies in yeast (Farcasanu et al. 1995; Inada et al. 2005; Nakamura et al. 1993; Navarre and Goffeau 2000), *Arabidopsis thaliana* (Apse et al. 1999; Mitsuya et al. 2005; Shi et al. 2003). Plasma membrane protein3 (*PMP3*) was first purified from the proteolipidic fraction of *Saccharomyces cerevisiae* plasma membrane (Navarre and Goffeau 2000). A deletion mutant of *PMP3* lacking the major Na^+ efflux systems showed hypersensitive reaction to more than 1M NaCl concentration than the *PMP3* wild-type strain. The *pmp3*Δ mutant cells accumulated excess Na^+ than wild types and intracellular Na^+ concentration in mutants was nearly double the amount found in wild types. To investigate the possible role of *PMP3* in plasma membrane Na^+-ATPases and Na^+/H^+ antiporter activity, combinations of mutants deficient in the plasma membrane Na^+-

ATPases (*Enap/Pmr2p*), Na^+/H^+ antiporter (*Nha1p*), were exposed to different levels of salt stress (125, 200, and 300 mM NaCl). The mutant combinations used were *enapΔ,/pmr2pΔ,/pmp3Δ*, and *enapΔ,/pmr2pΔ,/PMP* for Na^+-ATPases and *Nha1p*/*pmp3Δ*, and *Nha1p*/*PMP3* for Na^+/H^+ antiporter. The salt tolerance pattern observed in mutants indicated that *PMP3* does not interfere with the functioning of the two plasma membrane Na^+ efflux transporters. It was concluded that the hypersensitive reaction displayed by the mutants (*pmp3Δ)* might be associated with the increased uptake and accumulation of Na^+ under salt stress. Interestingly, the deletion of *PMP3* partially restored the growth of a strain lacking the K^+ transporters (Trk1p and Trk2p) and also protected from low pH. This indicated that the N^+ uptake and the Trk1p and Trk2p transporters mediated uptake of K^+ work independently in yeast.

Subsequently, the functional similarity between PMP3 and *Arabidopsis thaliana* RCI2A protein were evaluated under the control of the PMP3 transcription promoter in yeast. The expression results from yeast clearly showed that functionally both genes are homologous and their deletion caused increased intracellular Na^+ accumulation (Navarre and Goffeau 2000). Following these outcomes, Mitsuya et al. (2005) tested the physiological role of *RCI2A* in a salt sensitive *Arabidopsis.* As expected from the previous reports in yeast, there was no difference between the growths of *rci2a* mutants and wild type under normal conditions; however, when salt stress was imposed, growth of mutant plants was weak due to increased accumulation of Na^+ than the wild type. These results confirmed the function of *RCI2A* in restricting excess uptake of N^+ ions in higher plants.

Mitsuya et al. (2005) observed significant reduction in shoot chlorophyll content in wild-type plants than the transformed *RCI2A* phenotypes under salt stress condition. The decrease in chlorophyll content was in tune with the increasing concentration of salt stress. When photo

oxidative damages was assessed, based on the malondialdehyde (MDA) content, wild type plants showed an increase in MDA content than transgenics at induced salt stress (200 and 300 mM). Similar trend was also observed in H_2O_2 production using 3, 3-diaminobenzidine (DAB) polymerization. These results indicated that, apart from limiting the N^+ uptake, there was a significant reduction in photooxidative damages in *RCI2A* phenotypes.

The ability of the *PMP3* to regulate the cellular Na^+ and K^+ accumulation under salt stress was further investigated in sheep grass (*Aneurolepidium chinense*), a monocotyledonous wild halophyte plant found in the semi-arid region of Inner Mongolia (Inada et al. 2005). The functional characterization of two homologous genes, *AcPMP3-1* and *AcPMP3-2,* revealed similar results like the preceding authors. Initial higher expression was observed in response to the 500 mM salt stress. In addition to salt stress, exposure to cold, drought, ABA, H_2O_2, and salicylic acid triggered expression of *AcPMP3* genes. Initially, based on the presence of two hydrophobic segments in *PMP3*, it was predicted to be a part of plasma membrane. The cellular localization results of AcPMP3-1-GFP fusion protein showed that it was localized to the plasma membrane in yeast cells. Similarly, when tested in *A. chinense*, it was found to be localized in the root cap and epidermal cells, which are in direct contact with the soil.

To understand the abiotic stress tolerance during various developmental stages, *Arabidopsis* plants were transformed with promoters of *RCI2A* and *RCI2B* fused to the *uidA* (*GUS*)-coding sequence (Medina et al. 2001). Interestingly, when these transformed plants were exposed to salt, drought, and cold, strong *GUS* expression was noticed during the first stages of seed development and one day after germination, but the expression disappeared after 4 days of germination. However, there was a noticeable GUS expression in 7-day-old seedlings in the guard cells of cotyledons. There was a difference between *RCI2A* and *RCI2B* expression, which

was considered to be specific to the developmental stages. These results highlighted that the promoters of *RCI2A* and *RCI2B* contain all the cis-acting elements involved in their up-regulation in response to abiotic stresses and their expression was regulated at transcription stage.

Recent accomplishments helped to understand the physiological functions of PMP3 in plants, but progress toward isolation and characterization of the proteins is still lacking. A comprehensive understanding of the cellular role played by *PMP3* and its interaction with other proteins can make it a potential candidate gene for improving salt tolerance in crops.

CHAPTER 3. MATERIALS AND METHODS

3.1 Plant material

Cocodrie, a high yielding long grain rice variety released by the Louisiana State University Agricultural Experiment Station in 1998, was used for the *Agrobacterium* transformation. For pyramiding salt tolerance genes, previously developed transgenic lines containing a vacuolar ATPase subunit c1 (*SaVHAc1*) gene from *Spartina alterniflora* L. (Baisakh et al. 2012) were crossed with Sa*PMP3-1* transgenic plants. Hybrids and progenies were confirmed by the PCR amplification of both genes.

3.2 Analysis of PMP3: Alignment and phylogeny

An expressed sequence tag (EST) sequence from *Spartina alterniflora L*. (GenBank Acc. No. EH277593), highly expressed under salt stress condition (Baisakh et al. 2008), was used to characterize its potential role in salt tolerance. The gene is hereafter referred to as *SaPMP3-2*, since the amino acid sequences showed similarity to Plasma Membrane Protein 3 (PMP3) from different species. The ORF was determined using ORF Finder in the NCBI website (http://www.ncbi.nlm.nih.gov). The homologous nucleotide and amino acid sequences showing similarity to SaPMP3-2 were retrieved from the NCBI database. BLAST search was also made in The Rice Annotation Project Database (RAP-DB) (http://rapdb.dna.affrc.go.jp/) to compare the distribution of *SaPMP3-2* related sequences on different chromosomes. The retrieved amino acid sequences from fifteen different species were used in phylogenetic analysis to determine the evolutionary relationships between them. The amino acid sequences from different organisms were used for Multiple Sequence Alignment (MSA) in Geneious Pro 5.5.6 (http://www.geneious.com/) and dendrogram construction using ClustalW2 program (Thompson et al. 1994) from The European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk). In

addition, protein domain was predicted using SMART program (http://smart.embl-heidelberg.de) (Schultz et al. 1998) to determine the possible structural and functional units of SaPMP3-2. The amino acid sequence was also used for the prediction of secondary and three-dimensional (3D) structures of SaPMP3-2 proteins using Phyre2 program (http://www.sbg.bio.ic.ac.uk) (Kelley and Sternberg 2009).

Another EST sequence from *Spartina alterniflora L*. (GenBank Acc. No. EH277327) was isolated and cloned to study its effect on salt tolerance. Sequence analysis was performed in similar manner as described earlier. The deduced amino acid sequences showed close similarity to the PMP3 protein from different species and therefore here after the gene is referred to as *SaPMP3-1*.

3.3 Overexpression of *SaPMP3-2* and *SaPMP3-1* in *Escherichia coli*

3.3.1 Construction of *E. coli* expression vector

For *E. coli* overexpression, blunt-end PCR products from the complete Open Reading Frame (ORF) of *SaPMP3-2* and *SaPMP3-1* were directionally cloned by adding four bases to the forward primer (CACC) into pET101/D-TOPO® vector (Life Technologies, Grand Island, NY). The primers sequences used for cloning are shown in Appendix 1. The resulting recombinant vectors were called as pET-SaPMP3-2 and pET-SaPMP3-1. The gene was cloned after T7 promoter, which was induced in response to IPTG.

3.3.2 Overexpression of *SaPMP3-2* and *SaPMP3-1*

The recombinant plasmids, pET-SaPMP3-2 and pET-SaPMP3-1 as well as negative control plasmid pET-C without the gene of interest, were transformed into competent *E. coli* strain BL21 (DE3). Transformed cells were inoculated in 3 mL LB medium containing kanamycin (50 mg/L) and allowed to grow overnight in a shaker. Secondary culture was initiated

in 20 mL LB medium containing kanamycin (50 mg/L) with equal concentration of each transformed cells at 37 ^{o}C with constant shaking (250 rpm). In order to induce the protein synthesis in BL21 (DE3) cells, IPTG (100 mM) was added to the media when the OD_{600} value reached 0.5. For salt tolerance screening, BL21 (DE3) cells transformed with expression control, *SaPMP3,-2* and *SaPMP3-1* were streaked on selection media plates with 200, 400, 600, 800, 1000 mM NaCl and incubated at 37 ^{o}C. Based on the relative growth pattern, threshold NaCl concentration was determined to differentiate the growth of expression between control and recombinant cells.

3.3.3 Liquid growth assay

For liquid growth assay **s**secondary culture was initiated in 50 mL LB medium containing kanamycin (50 mg/L). The media were inoculated with an equal concentration (0.1 OD_{600}) of each transformed cells at 37 °C with constant shaking at 250 rpm. IPTG (100 mM) was added to the media to induce the protein expression when the OD_{600} value reached to 0.5. At this point, 600 mM NaCl was added to the LB broth in one flask but not to the other flask. The experiment was conducted in three replications and the optical density of the culture was noted after regular time interval (1, 4, 8, 16 h) to construct the growth curve.

3.4 Expression of *SaPMP3-2* in *Saccharomyces cerevisiae*

3.4.1 Yeast strains and expression vector

For yeast expression experiment, two yeast strains, BY4741 (wild type) and Y03635 (mutant YDR276c::kanMX4) from the Euroscarf collection (Entian and Kötter 1998) were used. Complete open reading frame (ORF) of *SaPMP3-2* was PCR amplified by gene specific primers (Appendix 1) and cloned into pYES2 vector (Life Technologies, Grand Island, NY) under the control of the GAL1 promoter at *Hind*III and *Bam*HI restriction endonuclease (RE) sites. The

recombinant vector, pYES2-SaPMP3-2, was transformed into competent *E. coli* DH5α cells and transformants were selected on LB plates containing 100 µg/mL ampicillin. Plasmid DNA isolated from 10 colonies were analyzed by PCR amplification and sequencing to confirm the presence of insert.

3.4.2 Preparation of competent yeast cells

Single colony from wild type (BY4741) and mutant (Y03635) strains were inoculated in 3 mL of YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose] and incubated overnight at 30 °C with shaking at 250 rpm. The following day, 10 µl of this culture was inoculated into 50 mL of YPD and incubated until the OD600 reached 0.4-0.5 at 30 °C with constant shaking at 250 rpm. The culture was then centrifuged at 3000 rpm for 5 min, and the pellet was resuspended in 30 mL sterile water. Cells were centrifuged at 3000 rpm for 5 min and the pellet was resuspended in 1.5 mL of 1.1X TE/LiAc. The cells were again centrifuged briefly and resuspended in 600 µl of 1.1X TE/LiAc for transformation.

3.4.3 Yeast transformation

Expression plasmid, *pYES2-SaPMP3-2*, was transformed into wild type (BY4741) and mutant (Y03635) strains by lithium acetate method (Becker and Lundblad 2001). For transformation, 200 µl LiAc treated competent cells were mixed with 200 µg carrier DNA and 100 µg *pYES2-SaPMP3-2* DNA in a 1.5-mL microcentrifuge tube. To this, 1.2 mL of freshly prepared 50% PEG 3350 solution was added and incubated at 30 °C for 30 min. The cells were given heat shock for 15 min at 42 ^{o}C and centrifuged briefly. The cells were resuspended in 250 µl of 1X TE buffer and spread onto Ura^{-} dropout plates. The plates were incubated at 30 °C for 2-3 days to observe the transformants.

3.4.4 Screening of yeast cells for NaCl sensitivity

Transformed yeast strains (mutant + vector control, mutant + *SaPMP3-2*, wild type + vector, wild type + *SaPMP3-2)* and control strains (mutant Y03635 and wild type BY4741) were grown on suitable YPD or SD Ura^- dropout media [0.67% (w/v) yeast nitrogen base with 2% (w/v) glucose] (Difco Lab., Detroit, MI). For induction of SaPMP3-2 and salt tolerance tests, 2% (w/v) Gal was added to the YPD or SD Ura^- dropout media in petri plates and liquid assay. For salt sensitivity assay, these yeast strains were grown in liquid YPD medium to saturation. The OD_{600} value of each strain was normalized to 0.5 and six serial dilutions (S1 > S2 >S3 >S4 >S5 >S6) of 5 fold dilutions were plated on YPD or SD Ura^- dropout plates containing NaCl concentrations (0, 0.5, 1.0, 1.2 and 1.5 M) following a slightly modified procedure as described by Nylander et al. (2001). The modifications were made in serial dilution and NaCl concentration levels as mentioned above. Yeast cells were incubated for 3 days at 30 ^oC, and differences in their growth pattern under different NaCl concentrations were noted.

For liquid growth assay**,** 50 mLYP media were inoculated with an equal concentration of each transformed cells from S3 serial dilution at 30 °C with constant shaking at 250 rpm. 2% (w/v) Gal was added to the media to induce the protein expression when the OD_{600} value reached 0.1. At this point 600 mM NaCl was also added to the YP media to one flask and no salt was added to the other flask. The experiment was conducted in three replications, and the optical density of the culture was noted at regular time interval (2, 3, 4, 6, 10, 14, 23, 44, and 50 h) to construct the growth curve.

3.5 Development of transgenic rice plants

3.5.1 Plasmid construction and *Agrobacterium* strains

The pCAMBIA 1305.2 (CAMBIA, Australia), a binary plasmid vector showing stable replication in *E.coli* and *Agrobacterium tumefaciens,* was used to deliver the *SaPMP3-2* gene

into embryonic calli. The plasmid contains the hygromycin B phosphotransferase antibiotic resistance gene (*hptII*) to screen the positive plants as well as it also harbors kanamycin (*nptIII*) resistance gene for *E. coli* and *A. tumefaciens* selection (Broothaerts et al. 2005). The *A. tumefaciens* strain, EHA105 (kindly provided by Norimoto Murai, Louisiana State University, Baton Rouge, LA), harboring pTOK233 plasmid and the virulence loci necessary for T-DNA transfer was used for callus infection (Hiei et al. 1994). Full-length cDNA clone of *SaPMP3-2* was cloned under the control of CaMV *35* promoter in pCAMBIA 1305.2 vector at *Bgl*II and *Pml*I restriction endonucleases sites. Sequences of the primers used for cloning are shown in Appendix 1. The plant expression vector pC-SaPMP3-2 was confirmed by both restriction analysis and DNA sequencing.

The complete Open Reading Frame (ORF) of *SaPMP3-1* was PCR amplified from the EST clone using gene specific primers (Appendix 1). The full-length ORF was cloned into a binary plant expression vector, pCAMBIA 1305.2 at *Bgl*II and *Bst*EII restriction endonucleases sites following the above procedure. The resulting recombinant vector, pC-SaPMP3-1 was confirmed by PCR analysis and DNA sequencing.

3.5.2 Gel extraction of vector and insert DNA

Restriction enzyme digested products were separated on 1.4% agarose gel and lanes containing DNA of proper sizes were excised with a sharp blade. DNA purification was performed using QIAquick PCR purification kit (QIAGEN, Valencia, CA) following the manufacturer's instructions.

3.5.4 Ligation of DNA fragments

Purified cohesive and blunt end DNA fragments were ligated to generate the appropriate recombinant DNA constructs using T4 DNA ligase (Life Technologies, Grand Island, NY).

DNA concentrations of insert fragments and vector backbones were quantified in Nanodrop spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA). Approximately 3:1 and 10:1 molar ratio of insert to vector was mixed for sticky-end restriction fragments and blunt-end fragments, respectively. Four microliters of T4 ligase buffer, 1µl of ligase, sterile water and appropriate ratios of insert and vector DNA were added to make 20µl volume. The ligation reaction was incubated overnight at 14 °C. The next day, 2 µl of ligation product was used for the transformation of chemically competent *E. coli* strain DH5α and *A. tumefaciens* strain LBA4404 (An et al. 1998).

3.5.5 Transformation of *E.coli*, plasmid DNA isolation, restriction analysis, and DNA sequencing

Competent *E. coli* cells were transformed following standard heat shock method (Hanahan et al. 1991; Sambrook and Russell 2001). Recombinant colonies containing gene insert were selected on the LB selection media the following day.

Ten transformed colonies were inoculated into 5 mL liquid LB medium with 50 mg/L kanamycin and incubated over night to harvest *E. coli* cells for plasmid DNA isolation using GeneJET plasmid miniprep kit (Fermentas, USA), following manufacturer's instructions. The DNA bound to the membrane was eluted with 30 µl of nuclease free water for further restriction analysis and sequencing purpose.

Plasmid DNA was digested with *Bgl*II and *Pml*I enzymes to confirm the true recombinants (Goranson and Erbe 2003; Sambrook and Russell 2001). Restriction digestion mixture consisted of plasmid DNA (8 µl), 10X buffer (2µl), *Bgl*II and *Pml*I enzymes (1µl each), and sterile water (8 µl) to make 20µl final volume. The reaction was mixed gently, centrifuged, and incubated at 37 °C for one hour. After digestion, the cut and uncut samples were electrophoretically separated on 1.4% gel at 80 volts for 90 minutes. Plasmid clone containing

correct insert was identified by PCR amplification of *SaPMP3-2* insert and 30% glycerol stock was stored in -80 °C freezer for further use. Similarly, true pC-SaPMP3-1 recombinants were confirmed by PCR amplification and glycerol stocks were made.

The recombinant vectors, pC-SaPMP3-2 and pC-SaPMP3-1, were sequenced to confirm the correct orientation of two genes and part of upstream CaMV *35* promoter region. DNA sequencing was done at the GeneLab of the LSU School of Veterinary Medicine, Baton Rouge, USA.

3.5.6 *Agrobacterium* transformation

Dehusked seeds were surface sterilized with 70% ethanol for two minutes followed by 50% Chlorox treatment for 20 min with gentle shaking at 150 rpm. Seeds were then rinsed with autoclaved distilled water for 3-4 times, excess water was removed, and seeds were placed on MS medium (Murashige and Skoog 1962) supplemented with 2 mg/L 2,4-D (MS2D) for callus induction at 26 ^{o}C under dark condition for 10 days. Subculturing was performed for 3-4 times after 10 d interval to facilitate the growth of healthy calli. Four to six weeks old light yellowish and compact embryogenic calli were used for *Agrobacterium* transformation. After *Agrobacterium* cocultivation, calli were placed on selection media for 3-4 cycles of hygromycin selection. Subsequently, these calli were transferred to regeneration media (Appendix 2) and then into the rooting medium (Appendix 2). After 5 days of root regeneration, small seedlings of 3 to 8 cm height were initially grown in hydroponics under control conditions of light with six 40W Sylvania cool white fluorescent bulbs (~100 μE m^{-2} s^{-1}) at 26 °C in 1/4th strength Yoshida media (Yoshida et al. 1976). The hydroponic nutrient media was changed after every 7 days. Transgenic plants were identified by PCR amplification of target gene as well as hygromycin B phosphotransferase gene. The positive seedlings were grown in a greenhouse, and self-pollinated

to advance the generation. The greenhouse growing condition was set at 13:11 hours (day:night) photoperiod with temperature setting of at 28 °C/25 °C (day/night) and a light intensity of 350µE m^{-2} s^{-1}. Five seedlings were allowed to grow per 1 gallon pot of 6.25 inch depth x 6.5 inch diameter size.

3.6 Molecular analysis of transgenic plants

3.6.1 PCR confirmation of transgenic plants

Regenerated individual plants from hydroponic media at 4-5 leaf stage were analyzed to confirm the successful transformation. As an initial step in screening, putative transgenic plants were identified by PCR amplification of *SaPMP3-2*, *SaPMP3-1* and hygromycin B phosphotransferase genes by specific primers (Appendix 1). The positive transgenic plants were then transferred to greenhouse and selfed for two successive generations to get the homozygous plants. Segregation of transgene (s) in successive generations of each individual plant was recorded.

3.6.2 PCR reaction and amplification conditions

All PCR amplification reactions consisted approximately 100 ng of template DNA, 50 ng of each of forward and reverse primers (Integrated DNA Technologies, Inc., USA), 200µM dNTPs, 2.0 mM $MgCl_2$, 1U *Taq* DNA polymerase (Promega, Madison, WI) and 1X PCR buffer in a total reaction volume of 25µl. Amplification was carried out on a Bio-Rad C1000 Thermal Cycler. The amplification profile was as follows: Initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. Final cycle of primer extension was carried out at 72 °C for 7 min.

3.6.3 Southern blot analysis

Transgenic rice plants identified from PCR analysis were further confirmed through southern hybridization (Sambrook and Russell 2001). Genomic DNA from transgenic plants was isolated from independent positive plants as well as untransformed control plants. Ten micrograms of genomic DNA was digested with *Hind*III restriction enzyme (Fermentas, USA), which cuts at a single restriction site within the T-DNA region (pCAMBIA 1305.2). Subsequently, the digested DNA was separated by gel electrophoresis (1% agarose gel) at low voltage of 25 mV for 8 hours and visualized under UV light after ethidium bromide staining. The gel was briefly depurinated for 20 minutes (250 mM HCl), denatured in alkaline solution (1.5 M NaCl, 0.5 N NaOH) for 45 minutes, and neutralized (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4) for 30 minutes with constant agitation. The separated DNA on agarose gel was transferred to Hybond^{TM} N^+ membrane (GE Healthcare Biosciences, Piscataway, NJ) by capillary transfer method in 10 X SSC (1.5 M NaCl, 0.15 M sodium citrate pH 7.0) for overnight (Sambrook and Russell 2001). The blot was removed carefully the next day and DNA on blot was fixed by UV cross-linking.

Hybridization buffer was prepared by adding 0.5 M NaCl and 5% (w/v) blocking agent and mixed thoroughly by stirring for an hour followed by heating for 30 minutes. The membrane was prehybridized for 1 hour at 42 ^{o}C with gentle agitation. Transformed PMP3 genes, *SaPMP3-2* and *SaPMP3-1*, present on T-DNA were used as a probe to detect the transgenic plants following the ECL direct nucleic acid labeling and detection system (GE Healthcare Biosciences, Piscataway, NJ). The blots were kept in the film cassette and exposed to autoradiography film (Hyperfilm ECL, GE Healthcare, Piscataway, NJ) for 5 hours to visualize the gene specific bands.

3.6.4 Reverse transcription PCR (RT-PCR)

Total RNA was isolated from T_2 homozygous rice seedlings using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) following manufacturer's instructions. The total RNA was eluted in RNase-free water for gel analysis and RT-PCR. First strand complementary DNA (cDNA) was synthesized from total RNA using the qScript™ Flex cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD). The cDNA synthesis was performed as follows: Pre-incubation for 5 min at 65 °C in a 15 µL reaction mixture containing 1 µg of RNA, 2.0 µL oligo(dT) primer, and nuclease free water, followed by 60min incubation at 42 °C after adding 4.0 µL qScript Flex Reaction Mix (5X) and 1.0 µL qScript reverse transcriptase to the above 5µL pre-incubated reaction mixture, and final incubation of 5 min at 85 °C. The cDNA was subsequently used as a template for PCR with gene specific primers (Appendix 1). In addition the amplification of elongation factor 1-α gene (ef1α) was used as an internal control for comparing gene expression in wild type and transgenic rice plants. The amplified products were separated on 1.2% agarose gel for comparison of gene expression.

3.7 Phenotypic characterization of transgenic plants

Transgenic plants were grown in 1-gallon plastic pots with 5 plants per pot under greenhouse conditions. The greenhouse growing condition was set at 13:11 hours (day: night) photoperiod with temperature setting of at 28 °C/25 °C (day/night) and a light intensity of 350 µE m^{-2} s^{-1}. The relative humidity was maintained at 60% and soil moisture was maintained by keeping the pots in trays with two inches of water. Phenotypic traits such as plant height, root length, grain yield per plant, test weight, and seed sterility percentage were recorded during different phases of the experiment.

3.7.1 Salinization treatments

For seedling screening, rice seeds were germinated in petri plates and five days old seedlings were transferred to Yoshida nutrient media (Yoshida et al. 1976) under a 14 hour light and 10 hours dark at 25^{o} C. The seedlings were supported on a styrofoam sheet fabricated with supporting sponge or nylon mesh at the bottom. Seedlings were allowed to grow under normal condition for ten days. Salt stress was imposed to 12 d old seedlings by adding 100 and 150 mM NaCl to the nutrient solution and a moderate acidic condition of pH 5.2 was maintained. On the contrary, control plants were not subjected to salt stress. Plant growth related observations were taken before and after 12 d of stress treatment. For vegetative stage screening, plants were exposed to 60 and 80 mM NaCl 45 days after planting and physiological observations were recorded after 12 d of salt stress under greenhouse condition. Likewise, plants were also screened at reproductive stage, under greenhouse conditions, by adding 60 and 80 mM NaCl at the time of boot leaf emergence. During vegetative and reproductive stage screening, fresh salt solution was added regularly at 5 days interval to maintain the desired salt stress and 5.2 pH was maintained. For reproductive stage screening, salt stress and pH was maintained till harvesting. Leaf and root fresh weights (FWs) were taken immediately after harvesting the plants and dry weights were determined after samples were dried in an oven at 70 °C for 2 days.

3.7.2 Leaf disc assay

Leaf injury due to leakage of ion and damage to the photosynthetic unit was visually evaluated by floating leaf disk assay. During vegetative stage, fully expanded leaf sections of 2 cm length from one leaf below the flag leaf were used for comparison. Leaf discs were floated in control and NaCl concentrations of 100 mM and 150 mM for 72 h. After 72 h, phenotypic difference in chlorophyll retention was visually compared (Baisakh et al. 2012; Munns and James 2003).

3.7.3 Measurement of chlorophyll content

As salt stress is known to reduce the photosynthetic ability and overall growth of the plants, chlorophyll content was measured to see the possible differences between transgenic plants and control plants. Leaf samples from vegetative stage screening were used for comparing the variation in total chlorophyll content. Fresh leaf weight was measured and chlorophyll was extracted with 80% acetone from individual transgenic and control plants. The absorbance of the supernatant was measured at 645 and 663 nm. The concentration of chlorophyll a (Chl-a), chlorophyll b (Chl-b), and total chlorophyll (Chl-t) were estimated using the extinction coefficient equations described by Arnon (1949). Total chlorophyll content in leaf sample was expressed as milligram per gram of fresh leaf weight (mg/g).

$$\text{Chla} = 12.72\ A_{663} - 2.59A_{645}$$

$$\text{Chlb} = 22.9\ A_{645} - 4.67A_{663}$$

$$\text{Chlt} = 20.31\ A_{645} + 8.05A_{663}$$

3.7.4 Relative Water Content (RWC)

A leaf section of 2 cm length from one leaf below the flag leaf was used to determine the RWC during vegetative stage. Ten leaf sections of 2 cm length from 5 cm below the leaf tip from each plant were used and fresh weights were taken. The leaf sections were then transferred to fresh Yoshida nutrient solution (Yoshida 1976) for about 3 hours at room temperature under dark condition and turgid weight was taken after 3 h. Leaf samples were dried in oven at 70 °C overnight and dry weights were taken. The RWC was calculated using the following formula.

$$\text{RWC}\ (\%) = \frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})} \text{X}100$$

Where FW is fresh weight, DW is dry weight, and TW is turgid weight.

3.7.5 Proline estimation

Proline accumulation in response to hyperosmotic stress is well documented in *Arabidopsis* (Toka et al. 2010) and other plants. The amount of accumulated proline in transgenic and control plants was quantified during vegetative stage at 60 mM salt stress and control conditions following the procedure of Bates et al. (1973). Known quantity of leaf samples were ground and homogenized in 3% 5-sulfosalicylic acid. The amount of proline content in each sample was quantified by Ninhydrin labeling. Total amount of proline content in leaf sample was expressed as microgram per gram of fresh leaf weight (µg/g).

3.7.6 Measurement of Na^+ and K^+ content

Total Na^+ and K^+ content in shoot were measured under stress and control conditions during seedling stage salt stress. Leaf samples were collected and rinsed thoroughly in distilled water to ensure the exclusion of external salt deposition. Samples were completely dried at 80 ^oC for 2 days and powdered. One hundred micrograms of powdered samples were incubated with 5 mL of 4M HCl at 37 ^oC overnight to extract the Na^+ and K^+ (Zhao et al. 2007). The supernatant was filtered and diluted samples were used to determine the total Na^+ and K^+ by Flame photometer (Jenway model PFP7, Bibby Scientific Limited, UK). Total concentration of Na^+ and K^+ in plant samples were estimated based on the concentration determined in NaCl and KCl standard curve and K^+/Na^+ ratio was used for comparing the transgenic and control plants.

3.7.7 Statistical analysis

The effect of salt stress on various morphological and physiological traits was analyzed by one way ANOVA using the mean observations from replicated experiment separately for salt stress and control condition. The level of significance was tested at 0.05 probability using F-test. Differences in mean values of traits for transgenic and control plants under contrast growing

conditions were analyzed using paired t-test. Post hoc comparison was made using Dunnett's test, when one-way ANOVA results showed difference in genotypic mean values at 0.05 probability level. All statistical analyses were performed using SAS version 9.1.3 (SAS Institute Inc. 2006).

CHAPTER 4. RESULTS

4.1 Bioinformatic analysis of *SaPMP3-2* and *SaPMP3-1*

4.1.1 DNA sequence analysis: Alignment and phylogeny

An expressed sequence tag (EST) clone (GenBank Acc. No. EH277593), isolated from the salt stressed cDNA library of *Spartina alterniflora* (Baisakh et al. 2008), containing *SaPMP3-2* was used for analyzing the Open Reading Frame (ORF) and comparing the nucleotide sequences. The ORF of *SaPMP3-2* was 174 bp long (Figure 4.1). The extent of sequence conservation in higher plants was investigated using the BLASTN option at the NCBI website. Homology to several DNA sequences from vascular plants was observed (Table 4.1). Highest sequence similarity was with *Cleistogenes songorica* (96%*)* followed by barley (94%), rice (93%), Chinese lyme grass (*Leymus chinensis*) (89%), and sorghum (89%). The presence of high level of similarity at nucleotide level demonstrated that *PMP3* sequences are highly conserved during the evolution of higher plants.

a)

SaPMP3-2 GenBank Acc. No. EH277593

AGATCGAGAGCTTCAGCTTTGCTCAAGCAACGATGGCGGACGAAGG
GACGGCCAACTGTATCGACATCATCATCGCCATCCTCCTCCCGCCGC
TCGGCGTCTTCCTCAAGTTCGGATGCGGGCACGAGTTCTGGATCTGC
CTGCTGCTCACTTTCCTCGGCTACATCCCCGGGATCATCTACGCCGTC
TACGCCATCACCAAATAAGCTACGCGTATACAGCTGGTGCCCGGTGG
TGTGATGTGATGAACTTTGCATGAGATCTTTGTCTGAGTGCTCCGT

b)

M A D E G T A N C I D I I I A I L L P P L G V F L K F G C G H E F W I C L
L L T F L G Y I P G I I Y A V Y A I T K S

Figure 4.1 The complete sequence of *SaPMP3-2* gene isolated from *Spartina alterniflora* L. (a) The ORF is underlined and start codon is highlighted with green color and stop codon with red color. (b) Sequence of 58 amino acids in SaPMP3-2 protein.

Table 4.1 Results from the BLASTN search of *SaPMP3-2* against NCBI nr database.

Accession No.	Description	Maxima score	Total score	Query coverage (%)	E-value	Maxima identity (%)
FJ972825.1	*Cleistogenes songorica* stress-induced hydrophobic peptide mRNA, complete cds	276	276	97	4.00E-71	96
AK372180.1	*Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein, complete cds, clone: NIASHv2147M03	237	237	90	2.00E-59	94
AK248603.1	*Hordeum vulgare* subsp. *vulgare* cDNA clone: FLbaf127j22, mRNA sequence	237	237	90	2.00E-59	94
BT132082.1	*Oryza sativa* clone RRlibD00967 mRNA sequence	233	233	93	3.00E-58	93
AK288109.1	*Oryza sativa* Japonica Group cDNA, clone: J075198O10, full insert sequence	233	233	93	3.00E-58	93
CU406420.1	*Oryza rufipogon* (W1943) cDNA clone: ORW1943C006E13, full insert sequence	233	233	93	3.00E-58	93
CT828904.1	*Oryza sativa* (indica cultivar-group) cDNA clone:OSIGCSA031E19, full insert sequence	233	233	93	3.00E-58	93
AB161677.1	*Leymus chinensis AcPMP3-2* mRNA for plasma membrane protein 3, complete cds	200	200	93	3.00E-48	89
XM_002440512.1	*Sorghum bicolor* hypothetical protein, mRNA	193	193	90	4.00E-46	89
XM_002440511.1	*Sorghum bicolor* hypothetical protein, mRNA	169	169	88	7.00E-39	89

Since BLASTN results showed the presence of *PMP3* homologous sequences in rice cDNA, we further investigated the extent of similarity at nucleotide level using BLASTN search in the Rice Annotation Project Database (RAP-DB). The results indicated the presence of *PMP3* related sequences on six rice chromosomes (Table 4.2).

Table 4.2 Results from the BLASTN search of *SaPMP3-2* against *Oryza sativa* L. ssp. *japonica* cultivar Nipponbare sequences in the RAP-DB.

Chromosome number	Number of HSPs	Bit score	E-value
1	4	111	2.00E-23
3	4	86	7.00E-16
5	4	127	2.00E-28
6	4	80.6	3.00E-14
7	2	102	9.00E-21
9	4	68	2.00E-10

Likewise, EST sequence from the cDNA library (GenBank Acc. No. EH277327) was used for initial gene annotation and amino acid sequence prediction. The complete ORF is made up of 162bp and the protein is 54 amino acids long (Figure 4.2).

Figure 4.2 Nucleotide and amino acid sequence of *SaPMP3-1* (a) Nucleotide sequence of SaPMP3-1 gene from the *Spartina alterniflora* L. The transcriptional start and stop sites are indicated in green and red color, respectively. (b) The deduced amino acid sequences of the SaPMP3-1.

Among the homologous DNA sequences of higher plants, some were very similar to the results of *SaPMP3-2*. Highest sequence similarity was observed with rice (95%), followed by *Cleistogenes songorica* (95%), *Brachypodium distachyon* (94%), sorghum (94%), maize (94%), and *Cupressus sempervirens* (94%) (Table 4.3). The presence of more than 90% sequence relatedness at nucleotide level demonstrated that *SaPMP3-1* sequences were highly conserved during the evolution of higher plants. The results from BLASTN search in the RAP-DB indicated the presence of similar nucleotide sequences on six rice chromosomes like *SaPMP3-2* (Table 4.2).

4.1.2 Amino acid sequence analysis: Alignment and phylogeny

The amino acid sequences showing homology to *SaPMP3-2* were retrieved from NCBI BLASTP server to compare the similarity and make phylogenetic analysis (Table 4.4). Multiple Sequence Alignment (MSA) results revealed highly conserved amino acid sequences among all the species. Dark green color indicated 100% homology of a particular amino acid (Figure 4.3). Fourteen amino acids out of 58 (25%) were identical across the species. These conserved regions were located mainly in two regions. The first conserved region was located between 15 to 25 amino acid residues and the second conserved region was located between 35-50 amino acid residues. There was a wide range of similarity values between SaPMP3-2 and its homologs from different organisms. Maximum similarity was observed with *Cleistogenes songorica* (93%), which is a grass halophyte and minimum similarity with *Saccharomyces cerevisiae* (36%), a single cell microorganism (Figures 4.4 and 4.5).

The details of pairwise similarity matrix between each species generated from MSA presented in heat maps (Figure 4.4). The darkest shade along the diagonal and right bottom shows highest similarity between two species under comparison.

Table 4.3 Results from the BLASTN search of *SaPMP3-1* against NCBI nr database.

Accession No.	Description	Maxima score	Total score	Query coverage (%)	E-value	Maxima identity (%)
BT132082.1	*Oryza sativa* clone RRlibD00967 mRNA sequence	141	141	56	1.00E-58	95
AK288109.1	*Oryza sativa* Japonica Group cDNA, clone: J075198O10, full insert sequence	141	141	56	1.00E-58	95
CU406420.1	*Oryza rufipogon* (W1943) cDNA clone: ORW1943C006E13, full insert sequence	141	141	56	1.00E-58	95
CT828904.1	*Oryza sativa* (indica cultivar-group) cDNA clone:OSIGCSA031E19, full insert sequence	141	141	56	1.00E-58	95
FJ972825.1	*Cleistogenes songorica* stress-induced hydrophobic peptide mRNA, complete cds	139	139	53	5.00E-71	95
XM_003568926.1	Predicted: *Brachypodium distachyon* hydrophobic protein LTI6B-like (LOC100838178), mRNA	132	132	53	9.00E-28	94
XM_002440512.1	*Sorghum bicolor* hypothetical protein, mRNA	132	132	53	9.00E-46	94
XM_002440511.1	*Sorghum bicolor* hypothetical protein, mRNA	132	132	53	9.00E-46	94
BT062152.1	*Zea mays* full-length cDNA clone ZM_BFb0221E15 mRNA, complete cds	132	132	53	9.00E-46	94
FJ379991.1	*Cupressus sempervirens* isolate cyplp109 putative early drought-induced protein mRNA, partial cds	132	132	53	9.00E-46	94

Table 4.4 Results from the BLASTP search of *SaPMP3-2* against NCBI nr database.

Accession No.	Description	Maxima score	Total score	Query coverage (%)	E-value	Maxima identity (%)
ADC45381.1	stress-induced hydrophobic peptide [*Cleistogenes songorica*]	110	110	0.98	2E-30	0.93
XP_002440557.1	hypothetical protein SORBIDRAFT_09g003060 [*Sorghum bicolor*] >gb|EES18987.1| hypothetical protein SORBIDRAFT_09g003060 [*Sorghum bicolor*]	105	105	0.96	1E-28	0.88
ACN26849.1	unknown [*Zea mays*] >gb|AFW57543.1| naCl stress protein1 [*Zea mays*]	105	105	0.96	1E-28	0.88
NP_001107634.1	LOC100135422 [*Zea mays]* >gb|ABY71210.1| early drought induced protein [*Zea mays*] >gb|ACG26762.1| hydrophobic protein LTI6B [*Zea mays*] >gb|ACG33537.1| hydrophobic protein LTI6B [*Zea mays*]	105	105	0.96	1E-28	0.88
EAY96485.1	hypothetical protein OsI_18385 [*Oryza sativa Indica* Group] >gb|EEE62260.1| hypothetical protein OsJ_17047 [*Oryza sativa Japonica* Group]	105	105	0.93	4E-28	0.93
NP_001054591.1	Os05g0138300 [*Oryza sativa Japonica* Group] >sp|Q0DKW8.1|LTI6B_ORYSJ RecName: Full=Hydrophobic protein LTI6B;	103	103	0.93	9E-28	0.93
XP_003568974.1	Predicted: hydrophobic protein LTI6B-like [*Brachypodium distachyon*] >dbj|BAK03378.1| predicted protein [*Hordeum vulgare* subsp. *vulgare*]	103	103	0.93	9E-28	0.93

Continued on next page

(Table 4.4 Continued)

Accession No.	Description	Maxima score	Total score	Query coverage (%)	E-value	Maxima identity (%)
BAD34658.1	plasma membrane protein 3 [*Leymus chinensis*]	103	103	0.91	1E-27	0.94
XP_002329990.1	stress-induced hydrophobic peptide [*Populus trichocarpa*] >gb|EEF08546.1| stress-induced hydrophobic peptide [*Populus trichocarpa*]	103	103	0.98	2E-27	0.86
ACA66247.1	cold-induced plasma membrane protein [*Musa* ABB Group]	101	101	0.98	7E-27	0.86
BAG54793.1	plasma membrance protein3 [*Puccinellia tenuiflora*] >dbj|BAJ88867.1| predicted protein [*Hordeum vulgare* subsp. *vulgare*]	100	100	0.98	2E-26	0.84
XP_003562607.1	Predicted: hydrophobic protein LTI6A-like [*Brachypodium distachyon*]	99.8	99.8	0.98	4E-26	0.82
XP_002440556.1	hypothetical protein SORBIDRAFT_09g003050 [*Sorghum bicolor*] >gb|EES18986.1| hypothetical protein SORBIDRAFT_09g003050 [*Sorghum bicolor*]	99.4	99.4	0.89	5E-26	0.88
ACV50425.1	cold induced plasma membrane protein [*Jatropha curcas*]	97.8	97.8	0.98	2E-25	0.82
NP_001151840.1	hydrophobic protein LTI6B [*Zea mays*] >ref|NP_001152948.1| hydrophobic protein LTI6B [*Zea mays*] >gb|ACG43609.1| hydrophobic protein LTI6B [*Zea mays*] >gb|ACG44549.1| hydrophobic protein LTI6B [*Zea mays*] >tpg|DAA54255.1| TPA: hydrophobic protein LTI6B [*Zea mays*]	97.1	97.1	0.96	4E-25	0.8

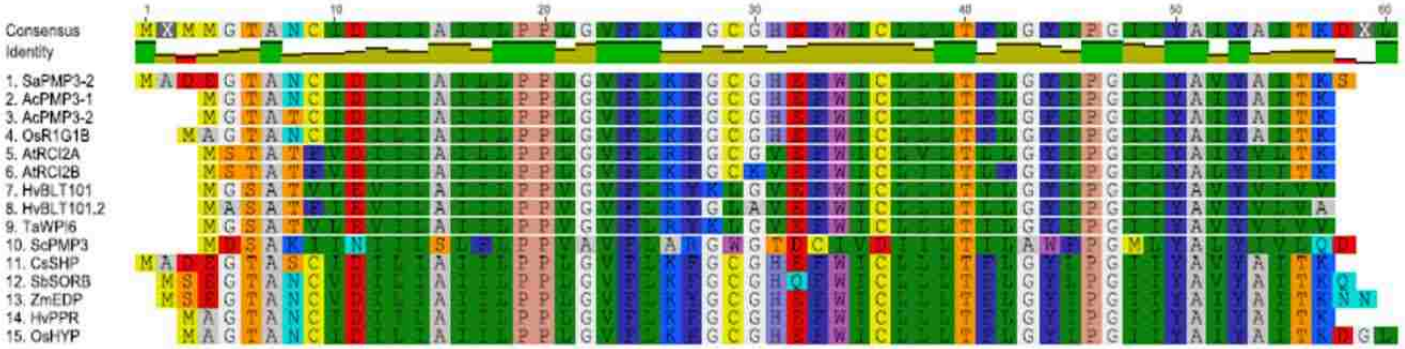

Figure 4.3 Multiple sequence alignment of SaPMP3-2 homologs from different species. Dark green color indicates 100% homology of a particular amino acid across the species. The species names are abbreviated by first two letters followed by PMP3 homolog: AcPMP3-1 (*Aneurolepidium chinensis* Trin.or *Leymus chinensis* Trin.), AtRCI2A (*Arabidopsis thaliana* L.), CsSHP (*Cleistogenes songorica*), HvBLT101 (*Hordeum vulgare* L.), OsR1G1B (*Oryza sativa* L.), SaPMP3-2(*Spartina alterniflora* L.), SbSORB (*Sorghum bicolor* (L.) Moench), ScPMP3 (*Saccharomyces cerevisiae*), TaWPI6 (*Triticum aestivum*), ZmEDP (*Zea mays*).

	ScPMP3	HvBLT101	TaWPI6	HvBLT101.2	AtRCI2B	AtRCI2A	SbSORB	CsSHP	SaPMP3-2	ZmEDP	AcPMP3-2	OsR1G1B	OsHYP	HvPPR	AcPMP3-1
ScPMP3		40.7%	40.7%	40.7%	42.6%	37.0%	32.7%	35.2%	36.4%	32.7%	38.9%	35.2%	36.4%	38.9%	40.7%
HvBLT101	40.7%		100%	87.0%	61.1%	68.5%	59.3%	61.1%	63.0%	63.0%	64.8%	61.1%	61.1%	64.8%	66.7%
TaWPI6	40.7%	100%		87.0%	61.1%	68.5%	59.3%	61.1%	63.0%	63.0%	64.8%	61.1%	61.1%	64.8%	66.7%
HvBLT101.2	40.7%	87.0%	87.0%		66.7%	72.2%	57.4%	61.1%	63.0%	61.1%	64.8%	61.1%	61.1%	64.8%	66.7%
AtRCI2B	42.6%	61.1%	61.1%	66.7%		77.8%	70.4%	70.4%	68.5%	68.5%	72.2%	68.5%	68.5%	72.2%	74.1%
AtRCI2A	37.0%	68.5%	68.5%	72.2%	77.8%		72.2%	72.2%	77.8%	75.9%	79.6%	75.9%	75.9%	75.9%	77.8%
SbSORB	32.7%	59.3%	59.3%	57.4%	70.4%	72.2%		91.1%	86.0%	91.2%	85.2%	89.1%	87.5%	85.5%	87.0%
CsSHP	35.2%	61.1%	61.1%	61.1%	70.4%	72.2%	91.1%		93.0%	87.5%	88.9%	90.9%	90.9%	87.3%	88.9%
SaPMP3-2	36.4%	63.0%	63.0%	63.0%	68.5%	77.8%	86.0%	93.0%		86.0%	90.7%	90.9%	89.3%	90.9%	92.6%
ZmEDP	32.7%	63.0%	63.0%	61.1%	68.5%	75.9%	91.2%	87.5%	86.0%		88.9%	92.7%	89.5%	89.1%	90.7%
AcPMP3-2	38.9%	64.8%	64.8%	64.8%	72.2%	79.6%	85.2%	88.9%	90.7%	88.9%		92.6%	92.6%	92.6%	94.4%
OsR1G1B	35.2%	61.1%	61.1%	61.1%	68.5%	75.9%	89.1%	90.9%	90.9%	92.7%	92.6%		100%	96.4%	94.4%
OsHYP	36.4%	61.1%	61.1%	61.1%	68.5%	75.9%	87.5%	90.9%	89.3%	89.5%	92.6%	100%		96.4%	94.4%
HvPPR	38.9%	64.8%	64.8%	64.8%	72.2%	75.9%	85.5%	87.3%	90.9%	89.1%	92.6%	96.4%	96.4%		98.1%
AcPMP3-1	40.7%	66.7%	66.7%	66.7%	74.1%	77.8%	87.0%	88.9%	92.6%	90.7%	94.4%	94.4%	94.4%	98.1%	

Figure 4.4 Heat map for the similarity matrix generated by the MSA of SaPMP3-2 homologs from different species. The pair-wise amino acid similarity between different species is indicated by the intensity of the dark color. AcPMP3-1 (*Aneurolepidium chinensis* Trin. AtRCI2A (*Arabidopsis thaliana* L.), CsSHP (*Cleistogenes songorica*), HvBLT101 (*Hordeum vulgare* L.), OsR1G1B (*Oryza sativa* L.), SaPMP3-2(*Spartina alterniflora* L.), SbSORB (*Sorghum bicolor* (L.) Moench), ScPMP3 (*Saccharomyces cerevisiae*), TaWPI6 (*Triticum aestivum*), ZmEDP (*Zea mays*).

The degree of amino acid similarity between different species increases as we go along the diagonal from left to right. In addition, phylogenetic tree was constructed to understand the evolutionary relationships among the 15 SaPMP3-2 homologs. Phylogenetic tree indicated close evolutionary relationship among the cereals (Figure 4.5). Close amino acid similarity was evident among the monocots, dicots, C3, and C4 plant species.

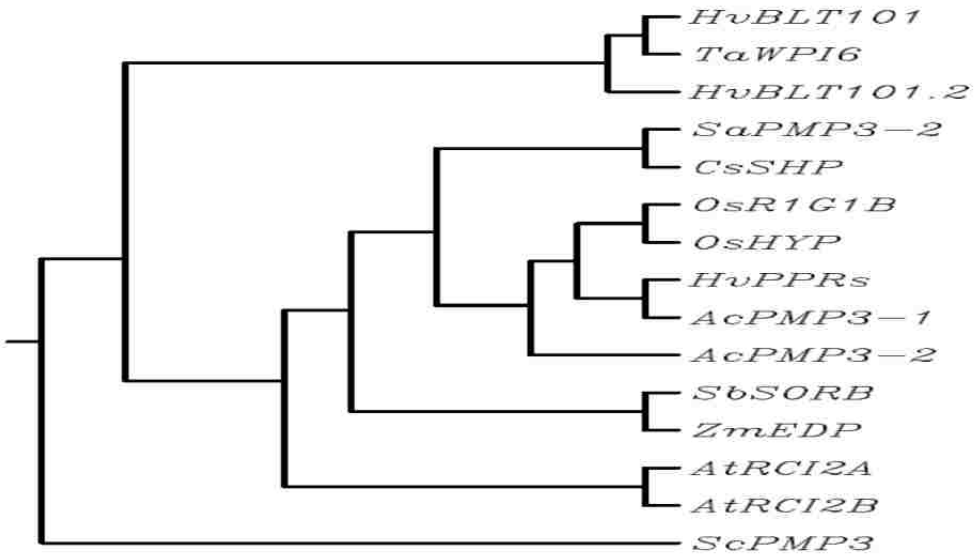

Figure 4.5 The evolutionary tree diagram of 15 PMP3 homologs from different species. The amino acid sequences from different species were used to construct phylogenetic tree.

Similarly, multiple sequence alignment results revealed conservation of amino acid sequences among the selected species for SaPMP3-1 (Figure 4.6). However, only 11 to 15 percent sequence homology was observed between SaPMP3-1 and other PMP3 homologs. Phylogenetic tree showed close evolutionary relationship among AtRCI2A (*Arabidopsis thaliana* L.), SaPMP3-2 (*Spartina alterniflora L.*), LcPMP3 (*Leymus chinensis* (Trin.), and ScPMP3 (*Saccharomyces cerevisiae)*. These results illustrated the underlying evolutionary relationships between different species for the *PMP3* gene.

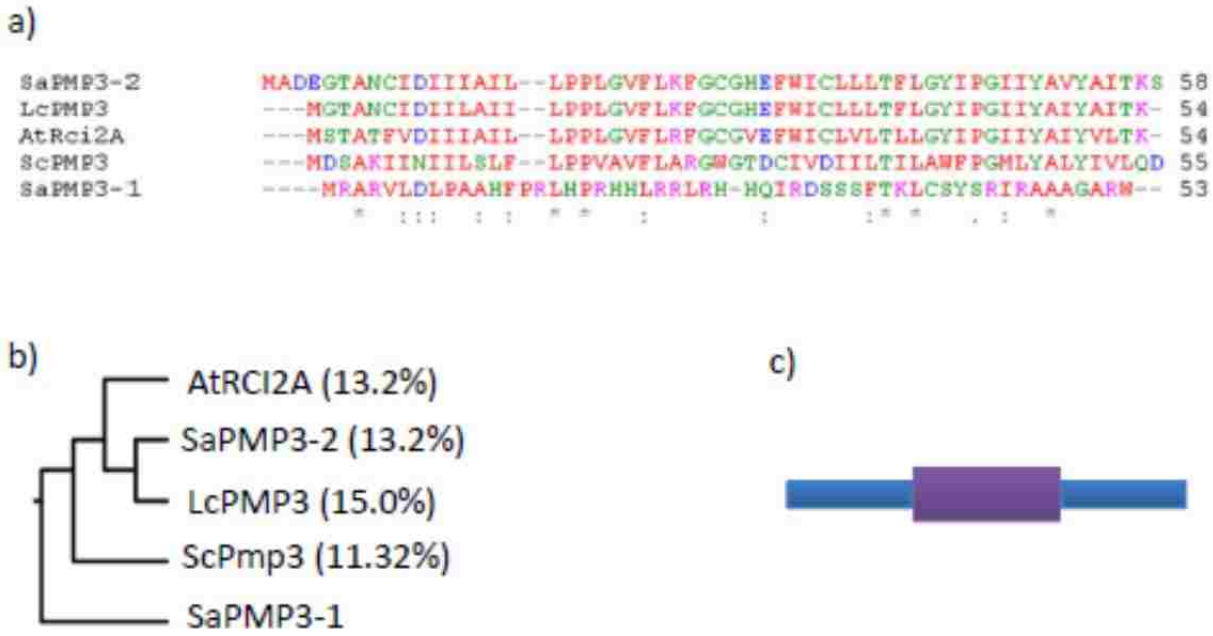

Figure 4.6 Amino acid analysis of SaPMP3-1 (a) Multiple sequence alignment of SaPMP3-1 amino acids and its homologous amino acid sequences from different species. (b) Phylogenetic tree of SaPMP3-1 amino acids using neighbor-joining and maximum likelihood analysis with amino acid sequences from different species AtRCI2A (*Arabidopsis thaliana* L.), SaPMP3-2 and SaPMP3-1 (*Spartina alterniflora* L.), LcPMP3 (*Leymus chinensis* (Trin.)), and ScPMP3 (*Saccharomyces cerevisiae*). (c) Protein domain prediction from SMART program. The purple box indicates the presence of single transmembrane domain from 14th amino acid to 28th amino acid.

4.1.3 The structure of SaPMP3-2 and SaPMP3-1 protein.

The complete amino acid sequence of SaPMP3-2 was used to predict the protein domains. There were two transmembrane domains (TMDs) in SaPMP3-2. The first transmembrane (TMD1) was made up of 18 amino acids from 10th amino acid to 27th and the second transmembrane domain (TMD2) consisted of 23 amino acids from 34 to 56 amino acid residues (Figure 4.7). The composition of amino acids indicated presence of more hydrophobic residues. Twenty seven amino acids were highly hydrophobic and 21 were less hydrophobic.

Since the folding of a protein chain determines the shape of the protein, secondary structure was determined for these amino acid sequences. As shown in Figure 4.8, the predicted

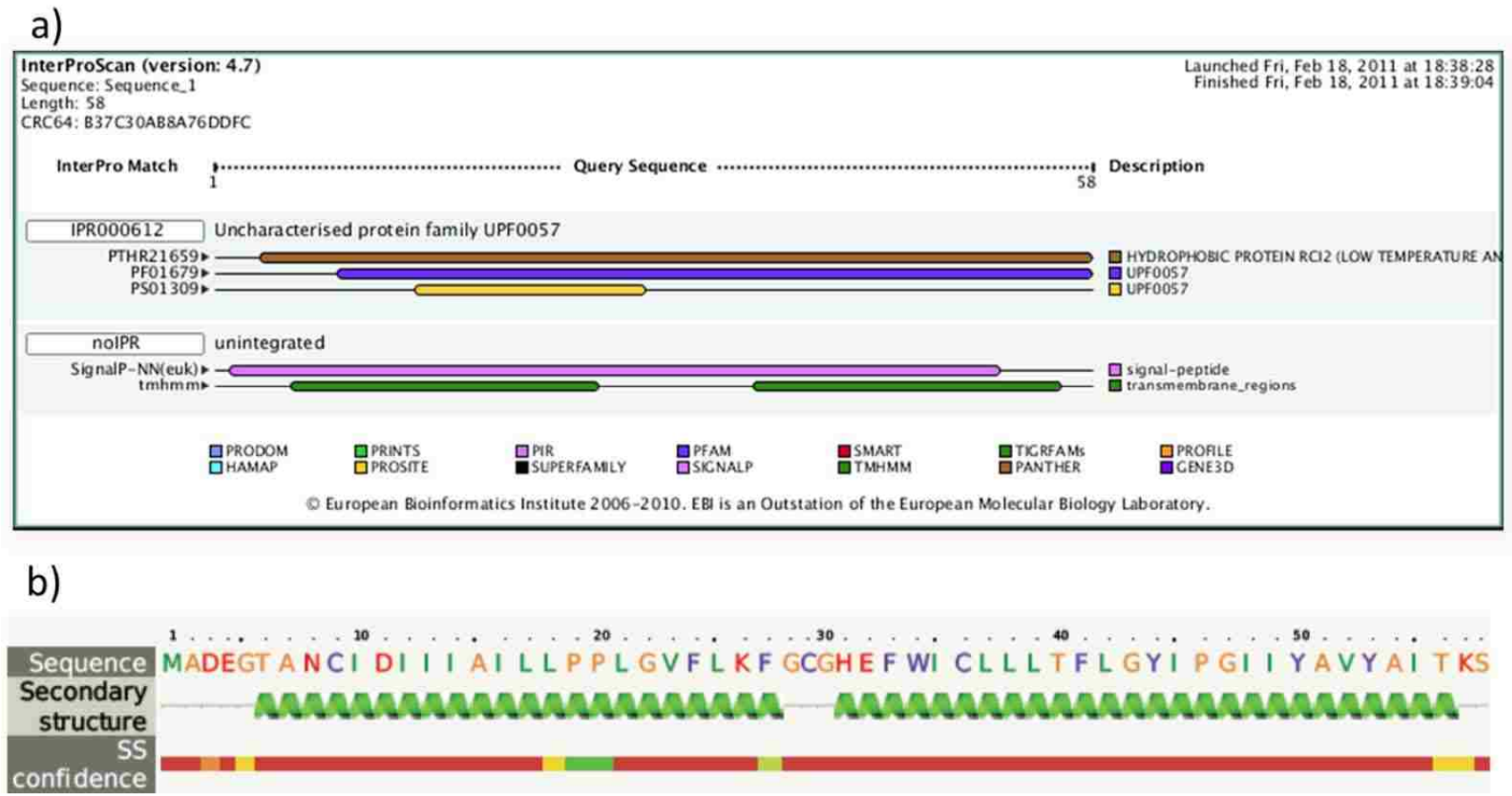

Figure 4.7 Secondary structure prediction of SaPMP3-2 (a) Predicted protein domain results indicating two transmembrane domains in SaPMP3-2. First transmembrane domain corresponds to 10-27 amino acid residues and second transmembrane domain corresponds to 34-56 amino acid residues. (b) Secondary structure prediction results showing two α helix structure

secondary structure was composed of two α helix structures. Two α helix structures constituted the two predicted trnasmembrane domains. The three-dimensional structure was determined to understand the conserved functions. Amino acids from 10 to 20 residues formed a single transport protein, which covered 17% of the amino acids of SaPMP3-2 (Figure 4.8a). Similarly there were two different 3D structures formed by 21 to 56 amino acids residues. The 36 amino acids in 2nd transmembrane domain (TMD2) can form either a single transmembrane helix or Calponin homology actin-binding domain (or CH domain)-like protein (Figure 4.8b and 4.8c). Therefore, the shared sequence similarity as well as similarity of 3D structure among the PMP family members across different organisms indicated the most likely conserved functions in salt tolerance.

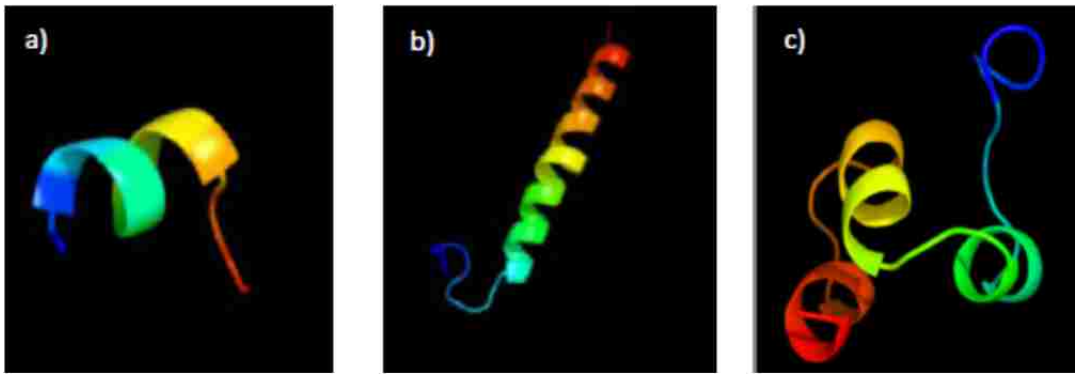

Figure 4.8 Predicted three-dimensional (3D) structures of the SaPMP3-2 protein. The helical ribbon represents the overall topographical progression of the polypeptide chain (colored blue to red in rainbow colors along the chain, N-terminal to C-terminal). (a) Transport protein, (b) Single transmembrane helix, and (c) CH domain-like.

Similarly, amino acid sequence of SaPMP3-1 was used for secondary structure prediction. Initial analysis indicated that 44 out of 53amino acids, which accounts for 83 percent, were hydrophobic in SaPMP3-1. The pattern is very much similar to the second transmembrane domain (TMD 2) of SaPMP3-2. However, a single hydrophobic transmembrane domain (TMD) was present in SaPMP3-1 from 14 to 28th amino acids (Figures 4.6c and 4.9b). This was in contrast to the PMP3 homologous proteins, which had two transmembrane domains. The

presence of a single TMD indicated the possibility of unique PMP evolution to form an uncharacterized but crucial protein to survive under abiotic stress environments. The secondary structure prediction indicated the presence of one prominent α helix structure and very small size β strand as well (Figure 4.9a). Further, the three-dimensional (3D) structure prediction result showed the formation of DNA/RNA-binding 3-helical bundle, which belongs to "Winged helix" DNA-binding domain superfamily (Figure 4.9b).

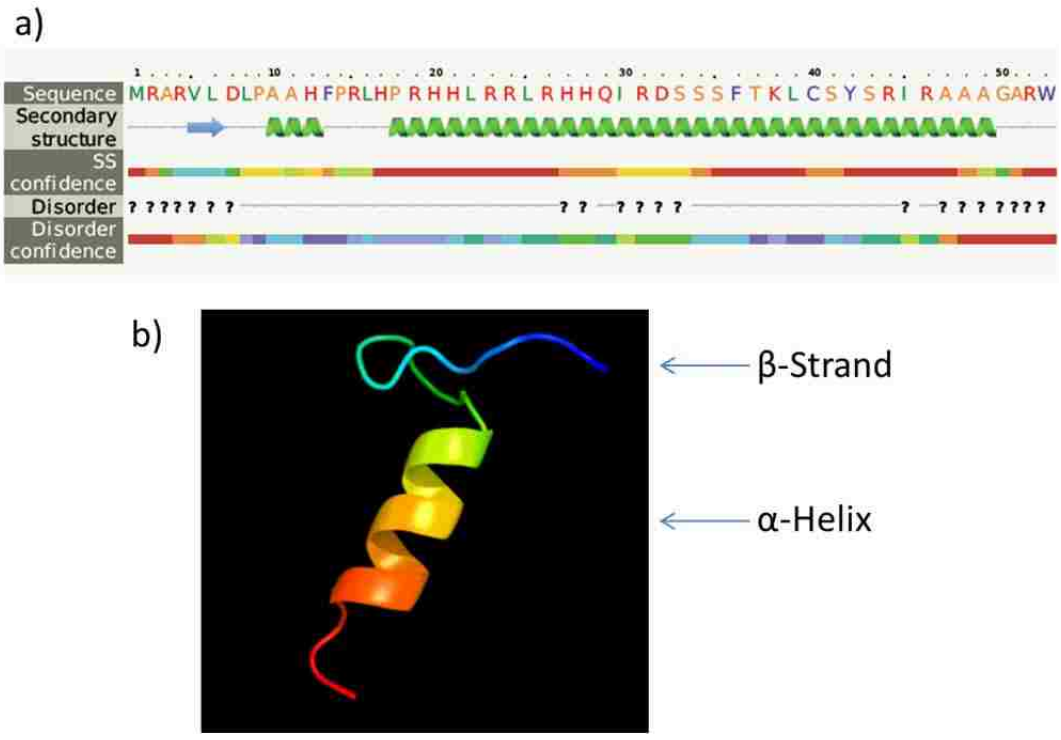

Figure 4.9 The Schematic structure of SaPMP3-1 protein (a) Secondary structure prediction (b) 3D protein structure model of SaPMP3-1.

4.2 Overexpression of *SaPMP3* in *E. coli* cells

4.2.1 Cloning of *SaPMP3-2* and *SaPMP3-1* into *E. coli* expression vector

For expression in *E. coli*, *SaPMP-2* and *SaPMP3-1* genes were amplified using *SaPMP-2* and *SaPMP3-1* specific primers (Appendix 1) and cloned into pET101/D-TOPO® vector (Life Technologies, Grand Island, NY) (Figure 4.10a) The resulting recombinant vectors, pET-

SaPMP3-2 and pET-SaPMP3-1, were transformed into BL21 (DE3) cells and the transformed BL21 (DE3) cells were confirmed by PCR amplification (Figures 4.10b and 4.10c).

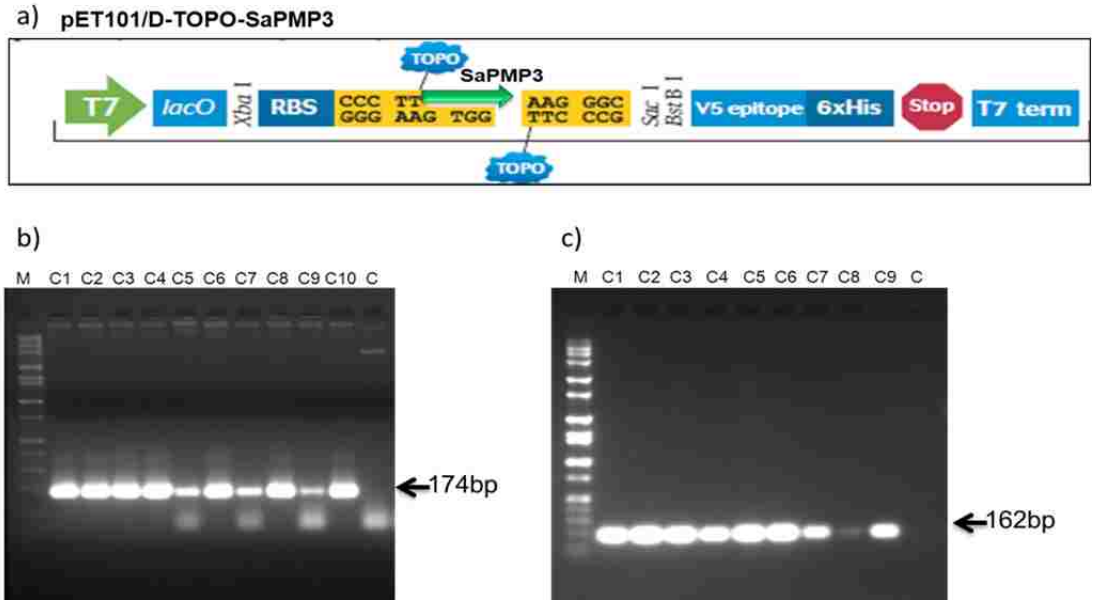

Figure 4.10 Cloning of *SaPMP-2* and *SaPMP3-1* into *E. coli* expression vector pET101/D-TOPO® (a) Schematic representation pET101/D-TOPO vector with cloning site. (b) Agarose gel showing the amplification of *SaPMP3-2* from recombinant plasmid DNA isolated from transformed BL21 (DE3) cells, C1-C10: Independent clones carrying recombinant plasmid *SaPMP3-2* (c) *SaPMP3-1* gene confirmation from transformed BL21 (DE3) cells, C1-C9: Independent clones carrying recombinant plasmid *SaPMP3-1*, M: 1 Kb DNA ladder and C: Control plasmid without the gene of interest. The arrow indicates the position of *SaPMP-2* gene (174bp) and *SaPMP-1*gene (162bp).

4.2.2 Overexpression of *SaPMP-2* and *SaPMP3-1* in *E. coli*

To investigate the function of the *SaPMP3-2* and *SaPMP3-1* in bacterial cells, growth of transformed *Escherichia coli* cells was compared under different concentrations of NaCl. *E. coli* strain BL21 (DE3), transformed with recombinant plasmids pET-SaPMP3-2 and pET-SaPMP3-1 as well as negative control plasmid pET-C from log phase were streaked on selection media plates with 200, 400, 600, 800 mM NaCl and incubated at 37 ^{o}C (Figure 4.11). Normal bacterial colony growth was observed under control, 200, and 400 mM NaCl concentrations and there was no significant difference between the growths of transformed and control *E. coli* cells.

Interestingly, more colony growth was observed only with transformed *E. coli* cells at 600 mM NaCl as compared to control *E. coli* cells (Figure 4.11). The growth of transformed and control cells ceased at NaCl concentrations beyond 600 mM.

Figure 4.11 Differences in the growth of *E. coli* cells grown under control and different NaCl concentrations. (a) Expression control: pET-C (b) pET-SaPMP3-2, and (c) pET-SaPMP3-1. The experiment was conducted in two replications, R-I and R-II.

Quantitative growth difference at 600 mM NaCl was measured using liquid growth curve assay to validate the visible observation. The growth of bacterial cells in terms of optical density (OD 600nm) was plotted at different time intervals to examine the relationship between protein expression and salt tolerance. An interesting pattern was noticed under control and salt stress conditions. Even under control condition, significant difference in the growth of *E. coli* cells transformed with expression vector control and cells transformed with *SaPMP3-1* was observed (Figure 4.12a). Moreover, the optical density (OD) of the cells transformed with *SaPMP3-1* was more than the expression control and the magnitude of this effect increased with the incubation time. This observation indicated that SaPMP3-1 protein has little influence on the growth of *E. coli* cells under control condition.

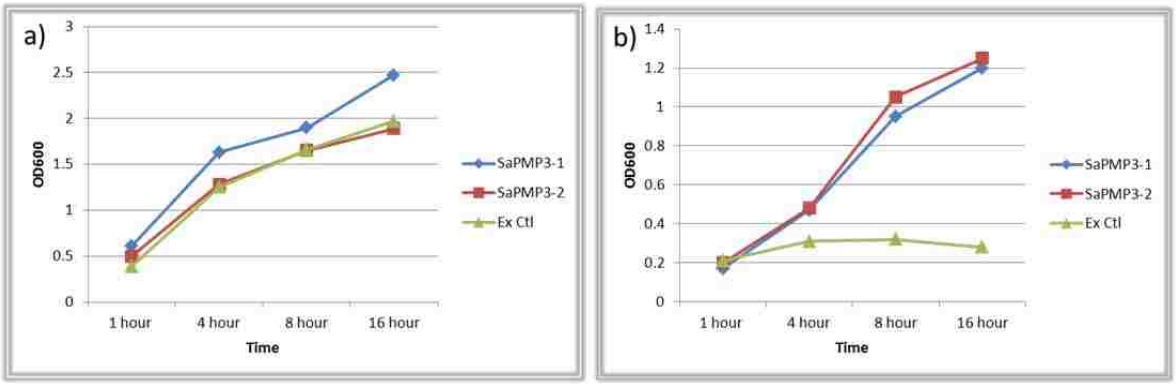

Figure 4.12 Differences in the growth response curve of *E. coli* cells grown under control and salinity conditions. (a) *E. coli* cells grown in the absence of NaCl in liquid LB culture. (b) Growth of *E. coli* cells after adding 600 mM NaCI to liquid LB media. SaPMP3-2: pET-SaPMP3-2, SaPMP3-1: pET-SaPMP3-1, ExCt1: pET-C. The mean of three independent measurements (OD_{600}) are indicated at different time intervals.

Interestingly, when these cells were grown under 600 mM NaCl and their OD600 was compared at several time points, there was clear difference in the growth of *E. coli* cells transformed with expression control vector and cells transformed with *SaPMP3-1* and *SaPMP3-2* (Figure 4.12b). Moreover, cells transformed with transgenes showed normal growth curve pattern at 600 mM NaCl whereas *E. coli* cells transformed with expression control vector did not grow normally. Faster growth of transformed *E. coli* cells was immediately observed after approximately 1 hour of salt exposure. Based on the *E. coli* expression results, it can be inferred that SaPMP3-2 and SaPMP3-1 played important role in the adaptation of transformed *E. coli* cells under salt stress.

4.3 Overexpression of *SaPMP3-2* in yeast

4.3.1 Cloning of *SaPMP-2* into yeast expression vector

The purpose of this experiment was to evaluate the effect of *SaPMP3-2* gene in yeast at different levels of salt concentrations. For expression of *SaPMP3-2* gene into yeast, *SaPMP3-2* was cloned into a yeast expression vector pYES2 between *Hind*III and *Bam*HI restriction sites

(Figure 4.13a). The recombinant plasmid was transformed into two yeast strains, BY4741 (wild type) for overexpression of *SaPMP3-2* and Y03635 (mutant YDR276c::kanMX4) for genetic complementation test. Plasmid DNA was isolated from transformed yeast colonies of both overexpression and mutant complementation strains growing on uracil drop out selection media. Transformed yeast colonies were confirmed by the PCR amplification of *SaPMP3-2* gene from both experiments (Figure 4.13b).

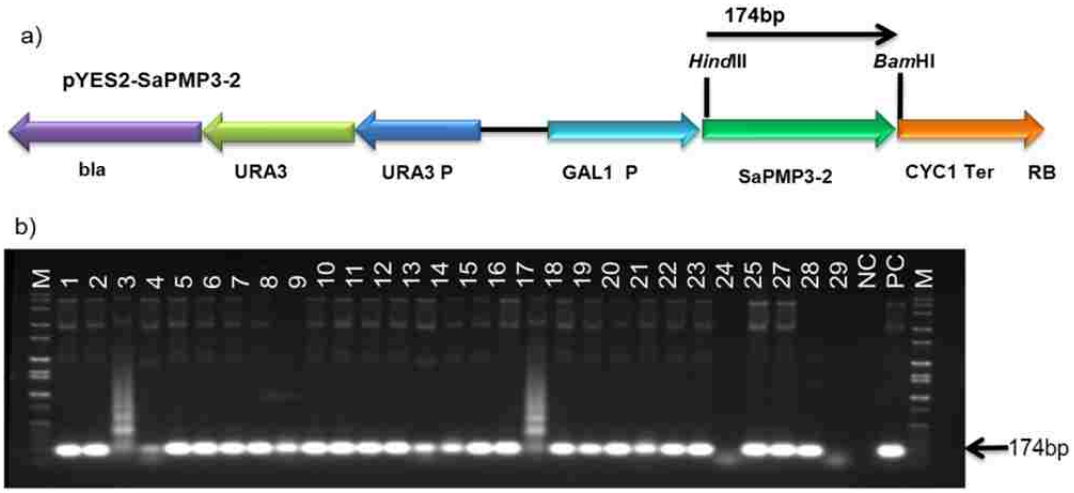

Figure 4.13 Cloning of *SaPMP3-2* into yeast expression vector pYES2. (a) Schematic representation pYES2 vector with cloning sites, GAL1 promoter, uracil selection gene, bla: ampicillin resistance gene, N and C: N-terminal to C-terminal direction of the amino acid sequences of *SaPMP3-2*. (b) Agarose gel image confirming the presence of *SaPMP3-2* from recombinant plasmid DNA isolated from transformed yeast colonies. M: 1 Kb DNA ladder, 1-14: Independent clones carrying recombinant plasmid SaPMP3-2 from transformed WT BY4741, 15-29: Independent clones carrying recombinant plasmid *SaPMP3-2* from transformed mutant strain Y03635, NC: Negative control without template, and PC: Positive control plasmid. The arrow indicates the position of *SaPMP-2* gene (174bp).

4.3.2 Screening of yeast cells for NaCl tolerance

Results from serial dilution assay in Figure 4.14 clearly indicated the genetic complementation of mutant strain at 1000 mM NaCl after 3 days of incubation. There was no difference in the visible growth of mutant and transformed strains at lower than 1000 mM NaCl. Similarly, at higher salt concentrations, mutant and transformed mutant ceased to grow due to

salt toxicity. The results indicated that *SaPMP3-2* rescued the mutant strainY03635, which lacks native *ScPMP3* gene, at 1000 mM NaCl stress.

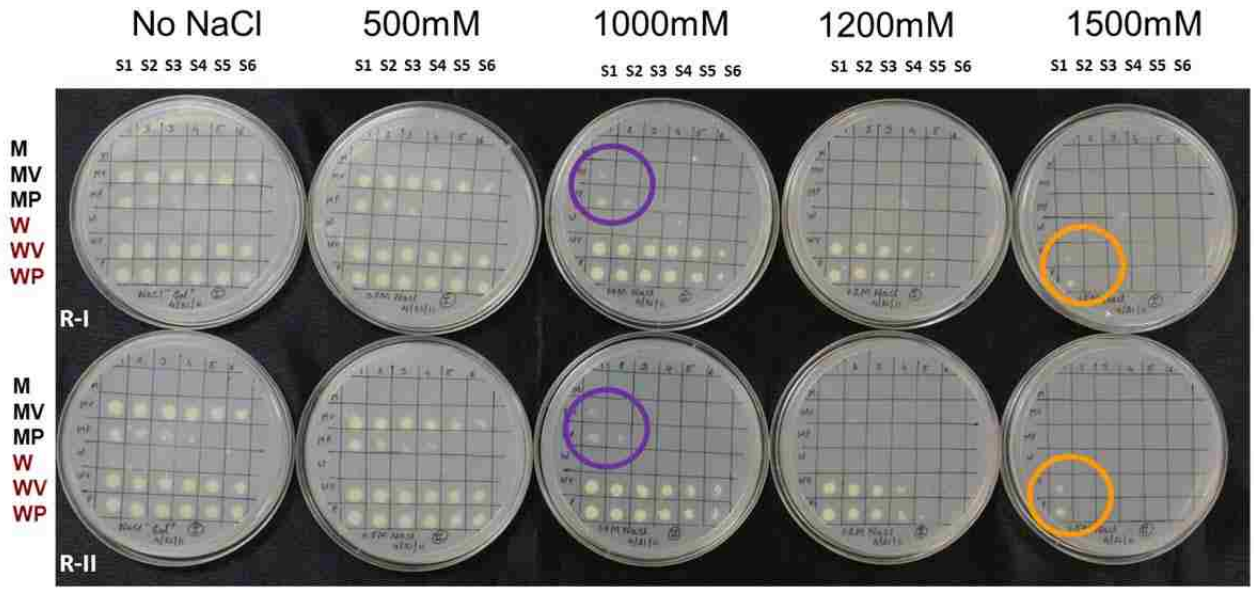

Figure 4.14 Serial dilution assay of yeast showing the functional complementation in mutant strain Y03635 and overexpression in WT strain BY4741 by *SaPMP3-2*. M: Mutant, MV: Mutant + Vector, MP: Mutant + *SaPMP3-2*, W: Wild Type, WV: Wild Type + Vector control, WP: Wild Type +*SaPMP3-2*. Salt Treatments: 0, 500, 1000, 1200 and 1500 mM NaCl. Replications: R-I and R-II. Fivefold serial dilution: S1, S2, S3, S4, S5, and S6. Purple circle highlights mutant complementation and orange circle highlights overexpression in WT.

Likewise, serial dilution assay results showed enhancement of salt tolerance in wild type strain BY4741. There was a clear difference in the growth of WT, WT transformed with vector control, and WT transformed with *SaPMP3-2* only at 1500 mM NaCl. Yeast strains transformed with *SaPMP3-2* showed more intense growth than the control strains (Figure 4.14). However, this difference in the visual growth was not detectable at salt concentrations less than 1500 mM NaCl. It was concluded that overexpression of *SaPMP3-2* successfully enhanced salt tolerance of WT yeast.

Since the growth difference was difficult to see visually, liquid growth assay was conducted to account for quantitative difference among different yeast strains. The growth of

yeast cells in terms of optical density (OD600nm) was plotted at different time intervals to examine the relationship between protein expression and salt tolerance (Figure 4.15). There was significant difference between wild type yeast transformed with vector control and *SaPMP3- 2* under 600 mM NaCl. During initial incubation time up to 10 hours, no significant difference in the growth was noticed between two strains under comparison. Similarly, there was no growth difference between the yeast strains under control condition. However, after 10 to 46 hrs of incubation at 600 mM NaCl, there was significant increase in the growth of *SaPMP3-2* transformed WT yeast strain than vector control. The genetic complementation in mutant and overexpression experiment in WT yeast suggested that enhanced salt tolerance could be due to *SaPMP3-2* gene expression.

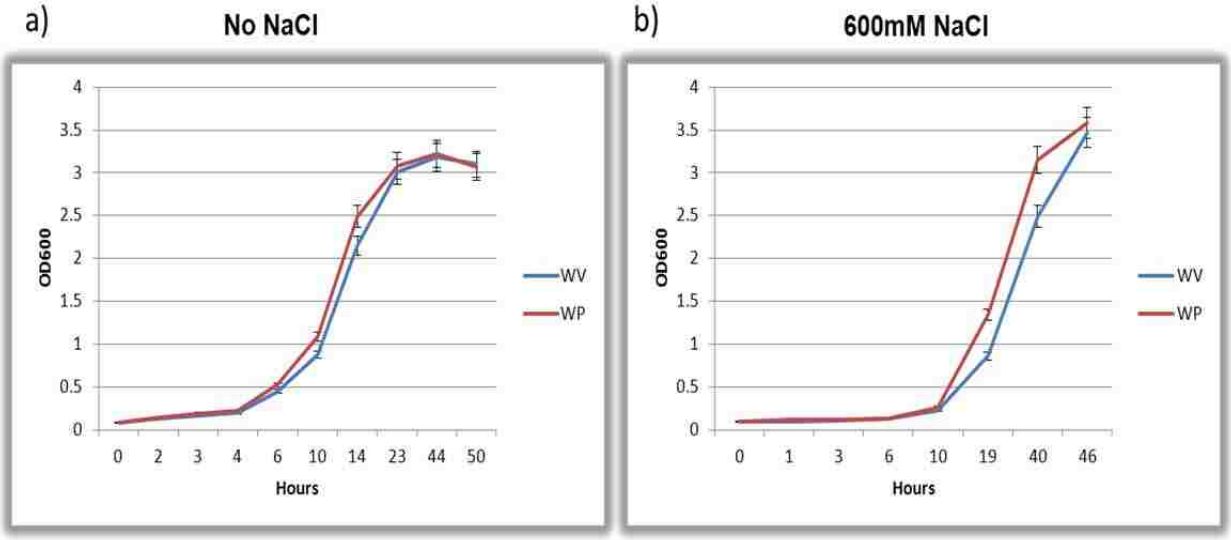

Figure 4.15 Overexpression of *SaPMP3-2* showing the differences in the growth response curves of yeast cells grown under control and saline conditions. (a) Yeast cells grown in the absence of NaCl in liquid YP culture with Gal. (b) Growth of yeast cells after adding 600 mM NaCI to liquid YP media with Gal. WV: Wild Type + Vector control, WP: Wild Type +*SaPMP3-2*. The mean of three independent measurements (OD_{600}) are indicated at different time intervals.

4.4 Overexpression *SaPMP3-2* and *SaPMP3-1* in rice

4.4.1 Cloning of *SaPMP3-2* and *SaPMP3-1* in plant expression vector

To evaluate the effect of *SaPMP3-2* and *SaPMP3-1* genes on salt tolerance in higher plants, rice plants were transformed with binary vectors cloned with the above genes. The *SaPMP3-2* gene was cloned at *Bgl*II and *Pml*I restriction sites of the pCambia1305.2 binary vector (Figure 4.16 and 4.17a) as described in the materials and methods. The schematic diagram of the plant transformation binary vector pCAMBIA 1305.2 vector is shown in Figure 4.16. The recombinant binary vector was transformed into competent dH5α *E. coli* cells. Plasmid DNA was isolated from selected colonies growing on selection media for further transformation of competent *Agrobacterium tumefaciens* strain EHA105. Before *Agrobacterium* transformation, the plant expression vector was confirmed by colony PCR and sequencing (Figure 4.17b and Appendix 3).

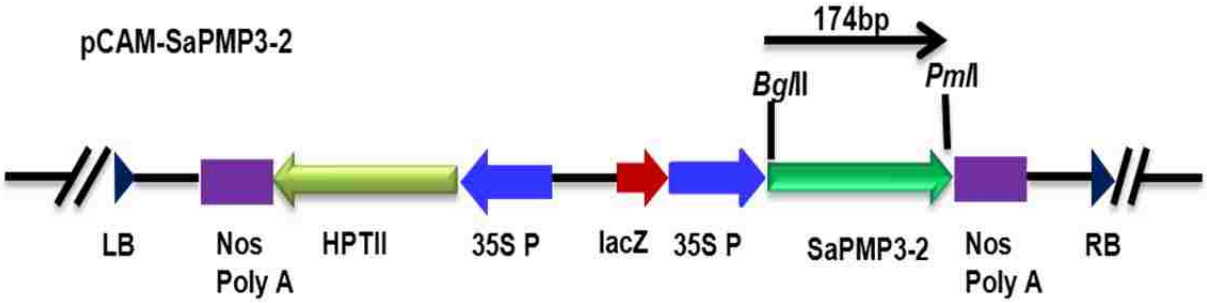

Figure 4.16 The schematic diagram of the partially linear plasmid vector pCAMBIA 1305.2 showing the position of insertion of the *SaPMP3-2* gene. The diagram depicts the right and left borders, 35S CaMV promoter, plant selection marker, and termination sites.

Similarly, *SaPMP3-1* was cloned into binary vector pCAMBIA 1305.2 at *Bgl*II and *BstE*II restriction sites following similar approach as described for *SaPMP3-2* (Figure 4.18).The partial linear map of the resulting recombinant binary vector, pC1305.2-SaPMP3-1, was shown in Figure 4.19.

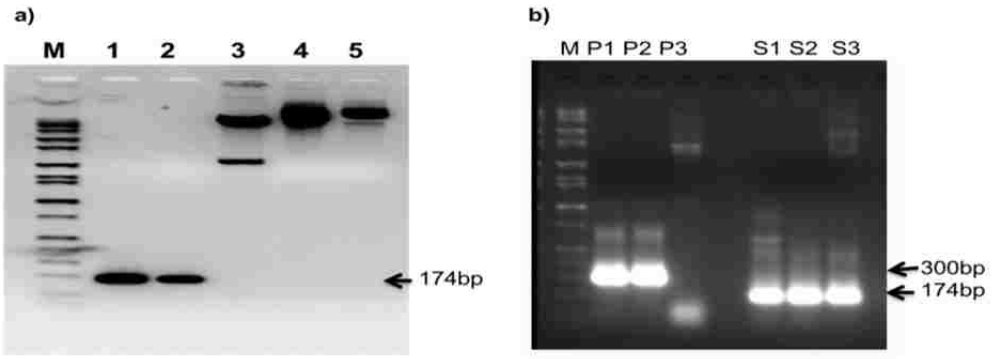

Figure 4.17 Cloning of *SaPMP3-2* into plant expression binary vector pCAMBIA1305.2. (a) Restriction digestion of *SaPMP3-2* amplified product and plasmid DNA for cloning and transforming *E. coli* and *Agrobacterium*. M: 1 Kb DNA ladder, 1: EH277593, 2:EH277593, 3: Double cut pCAMBIA 1305.2 (*Bgl*II and *Pml*I), 4: Single cut plasmid pCAMBIA 1305.2 (*Bgl*II), 5: Single cut plasmid pCAMBIA1305.2 (*Pml*I). (b) PCR confirmation of recombinant vector pC-SaPMP3-2 by the amplification of *SaPMP3-2* specific primers, M: 1 Kb DNA ladder, P1-P3: PCR amplification by 35S forward and *SaPMP3-2* reverse primers, P1 and P2: Recombinant pC-SaPMP3-2 DNA, P3: Plasmid DNA from EH277593 and S1-S2: PCR amplification by SaPMP3-2 specific primers from the recombinant pC-SaPMP3-2, S3: PCR amplification of SaPMP3-2 from the cDNA clone (GenBank Acc. No.EH277593).

The recombinant binary vector pC1305.2-SaPMP3-2 1 was confirmed by colony PCR (Figure 4.20) and transformed into *Agrobacterium tumefaciens* strain EHA105 for plant transformation. In addition to this, the DNA isolated from positive colonies was sequenced before transforming into an *Agrobacterium tumefaciens* (Appendix 4).

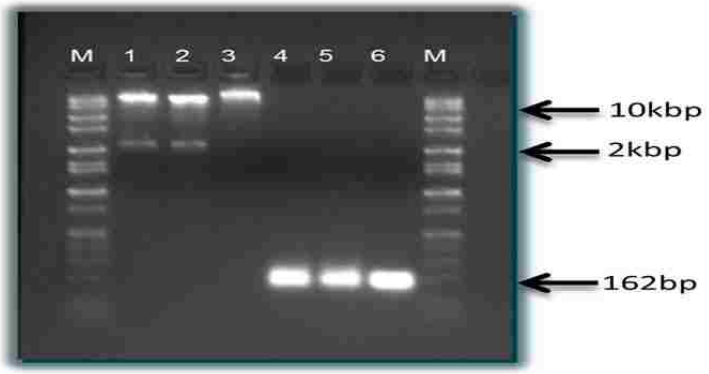

Figure 4.18 Restriction digestion of *SaPMP3-1* amplified product and plasmid DNA for cloning and transforming *E. coli* and *Agrobacterium*. M: 1 Kb DNA ladder, 1 and 2: Linearized plasmid after digestion of pCAMBIA1305.2 with *Bgl*II and *Bst*EII enzymes, 3: Uncut plasmid, 4 and 5: Double digested PCR product of SaPMP3-1 by *Bgl*II and *Bst*EII, 6: Uncut SaPMP3-1.

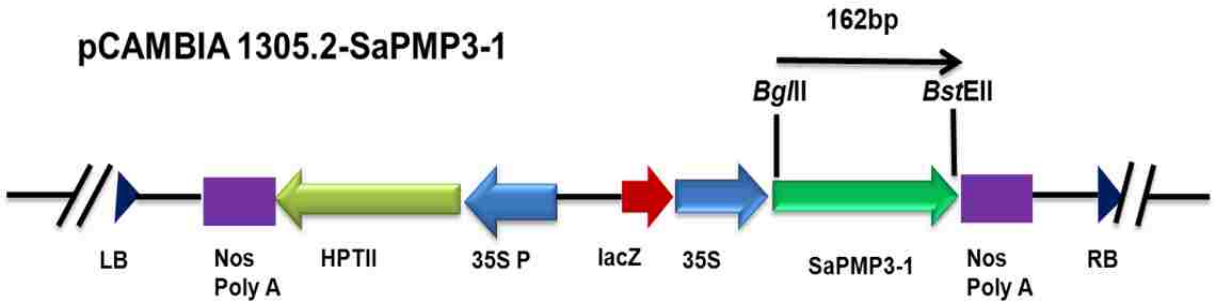

Figure 4.19 The schematic diagram of the partial linear plasmid vector and position of the gene. The diagram depicts the details of the pCAMBIA1305.2 vector containing right and left borders, *SaPMP3-1* gene between *Bgl*II and *Bst*EII sites, 35S CaMV promoter, plant selection marker, multiple cloning sites, and termination sites.

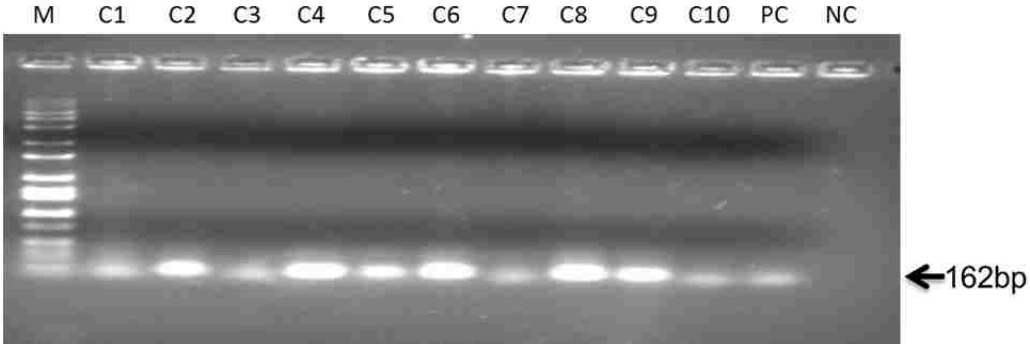

Figure 4.20 PCR confirmation of recombinant vector pC-SaPMP3-1 by the amplification of *SaPMP3-1* specific primers. M: 1 Kb DNA ladder, C1 to C10: Plasmid DNA isolated from transformed dH5α cells, PC: Positive control plasmid and NC: Negative control without any DNA.

4.4.2 *Agrobacterium* mediated transformation of rice

Two months old light yellowish and compact embryogenic calli of Cocodrie variety were transformed using *Agrobacterium* mediated transformation as described in the materials and methods. Healthy growing antibiotic (Hygromycin) resistant calli were selected during three cycles of selection on antibiotic media. After third cycle of selection, these calli were regenerated into seedlings and subsequently transferred to hydroponic media for initial hardening. Figure 4.21 shows the different stages of transformation and regeneration of transgenic rice plants, Cocodrie-*SaPMP3-2* and Cocodrie-*SaPMP3-1*.

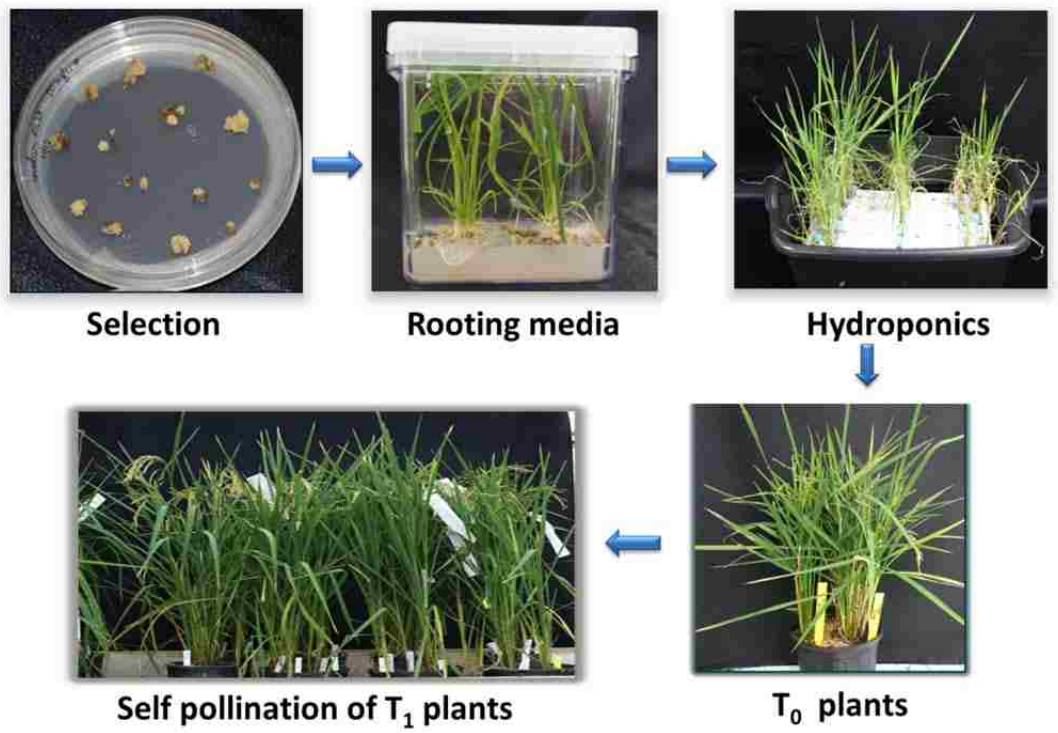

Figure 4.21 Transformation and regeneration of Cocodrie-*SaPMP3-2* and Cocodrie-*SaPMP3-1* transgenic rice plants. Different stages of selection, regeneration, and self-pollination of transgenic plants after *Agrobacterium* mediated transformation.

4.4.3 Confirmation of transgene integration in rice plants

At early seedling stage, DNA from each T_0 transgenic plant was isolated to confirm the integration of transgene by PCR analysis. PCR amplification of both *SaPMP3-2* (174bp) and hygromycin B phosphotransferase gene (780 bp) confirmed the successful integration of target gene into Cocodrie rice genome (Figures 4.22a and 4.22b). Five T_0 transgenic plants were confirmed for the presence of *SaPMP3-2* gene but only three plants showed the presence of the hygromycin B phosphotransferase gene. The positive seedlings were grown in a greenhouse and self-pollinated to make them homozygous. The growth of T_0 transgenic plants was comparable to the wild type Cocodrie for most of the phenotypic characters, but seed sterility (> 50 %) was observed in most of the primary transgenic plants.

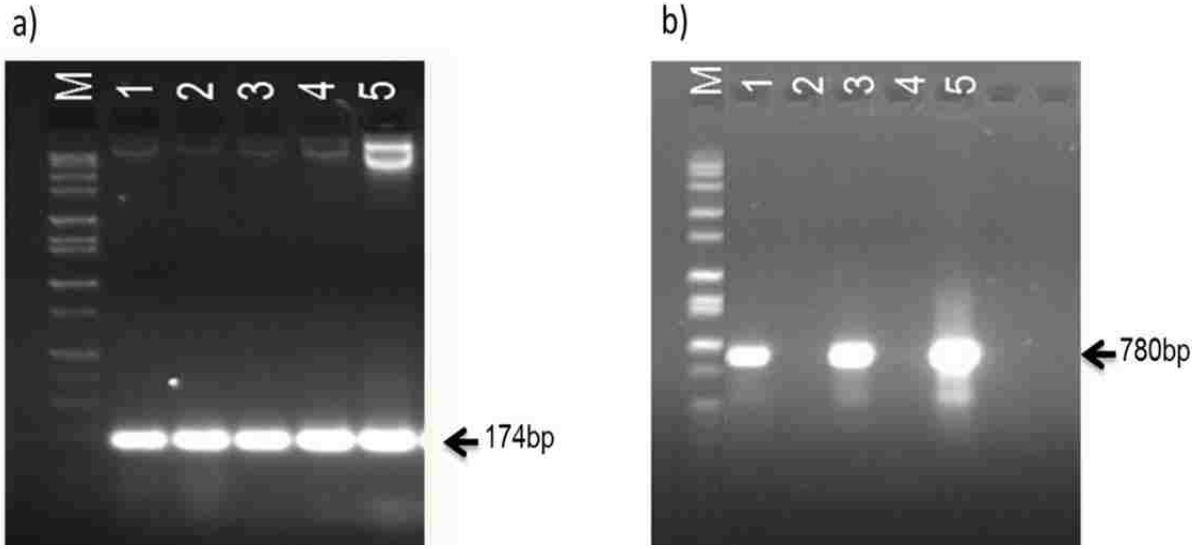

Figure 4.22 PCR confirmation of *SaPMP3-2* containing T_0 rice transgenic plants. (a) PCR analysis of *SaPMP3-2* transgenic plants rice plants by gene specific primers (b) PCR confirmation of T_0 plants using hygromycin specific primers. M: 1 Kb DNA ladder, 1 to 5: DNA isolated from individual T_0 transgenic plants.

Similarly, the integration of *SaPMP3-1* gene into genome of Cocodrie was confirmed by PCR amplification using the gene specific primers (Figure 4.23a and Appendix 1). The transgenic plants were further confirmed by amplification of the hygromycin B phosphotransferase gene (Figure 4.23b). Eleven independent plants were identified in T_0 generation out of which seven plants were confirmed to have hygromycin B phosphotransferase gene.

The positive T_0 seedlings showing the integration of *SaPMP3-1* gene were self-pollinated to generate the homozygous transgenic plants. The transgenic plants carrying *SaPMP3-1* gene were crossed to homozygous transgenic lines containing a vacuolar ATPase subunit c1 (*SaVHAc1*) gene from *Spartina alterniflora* L (Baisakh et al. 2012) for pyramiding both genes. Reciprocal crosses were also made by emasculating and then pollinating both transgenic plants (Figure 4.24).

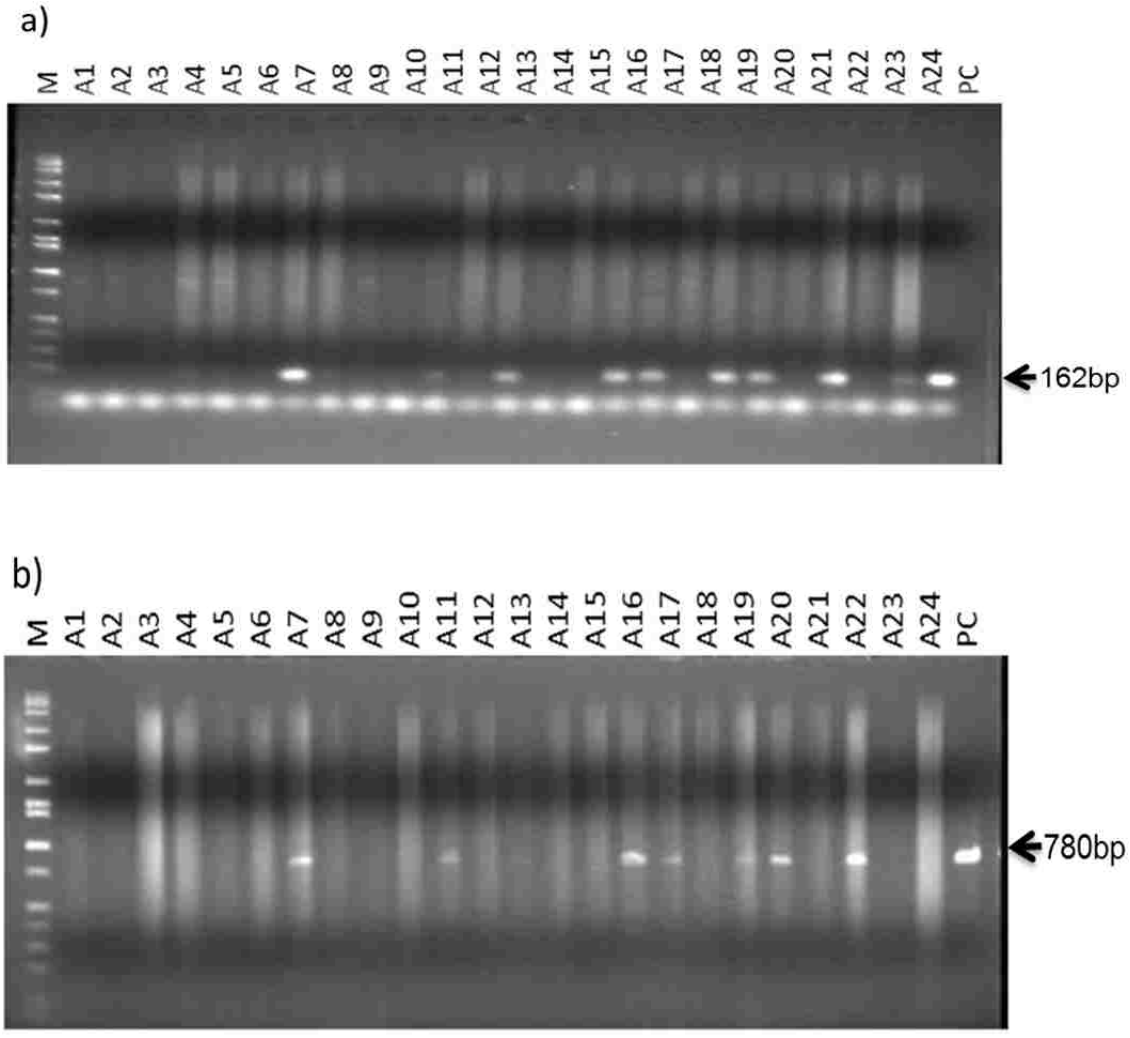

Figure 4.23 Confirmation of *SaPMP3*-1 containing T_0 rice transgenic plants by PCR. (a) PCR confirmation using *SaPMP3-1* specific primers (b) PCR confirmation of T_0 plants using hygromycin specific primers. M: 1 Kb DNA ladder, A1 to A24: DNA isolated from individual T_0 transgenic plants, PC: Positive control.

Pyramided progenies were identified in the following generation by PCR amplification of transgenes, *SaPMP3-1* and *SaVHAc1*. Five independent plants with both *SaPMP3-1* and *SaVHAc1* genes were confirmed by PCR analysis (Figure 4.25). Seeds from the PCR positive plants were further self-pollinated for one more generation to generate homozygous pyramided lines.

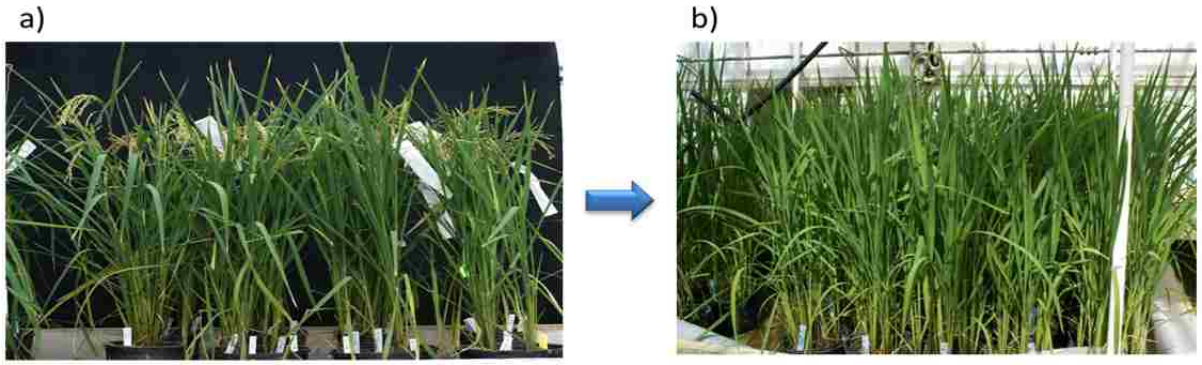

Figure 4.24 Transgenic plants grown in green house. (a) Pyramiding of *SaPMP3-1* (T_1) and *SaVHAc1* (T_2) genes by crossing homozygous plants during flowering stage, (b) Growth of pyramided progenies with both *SaPMP3-1* and *SaVHAc1*.

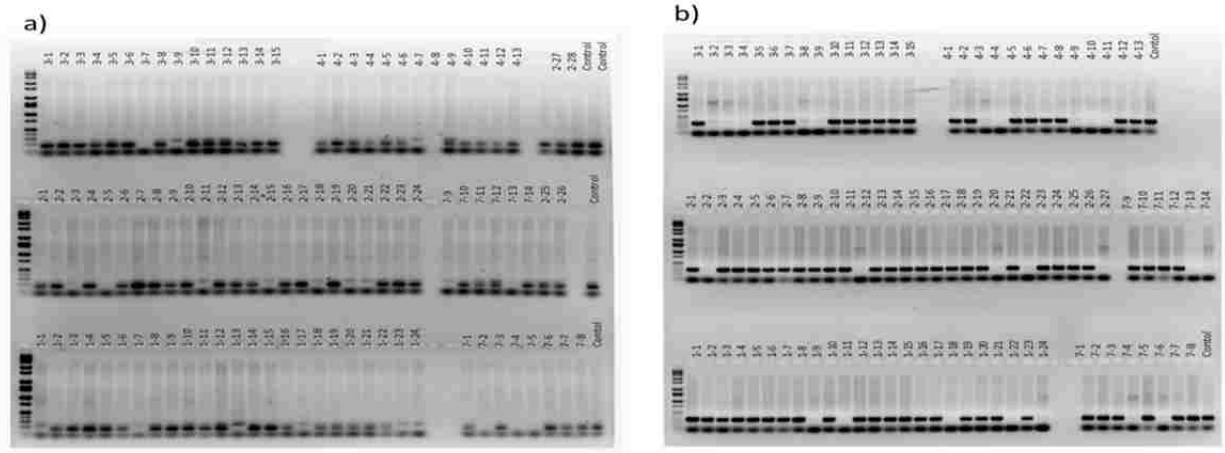

Figure 4.25 PCR analysis to confirm the integration of *SaPMP3-1* and *SaVHAc1* genes in pyramided progenies. (a) PCR amplification showing the presence of *SaPMP3-1* gene, (b) Pyramided transgenic rice plants showing the amplification of *SaVHAc1* gene using gene specific primers.

Transgenic plants with *SaPMP3-2* and *SaPMP3-1* genes were further checked for the integration of transgenes into the genome of Cocodrie rice. Southern hybridization revealed the presence of *SaPMP3-2* and *SaPMP3-1* specific bands in the individual transgenic plants. The Cocodrie plant had two copies of native PMP3 genes, which are noticeable in both *SaPMP3-2* and *SaPMP3-1* transgenic plants (Figure 4.26a and b). The results also indicated the integration of single copy of *SaPMP3-2* in all T_1 transgenic plants. Similarly, *SaPMP3-1* transgenic plants had one (plant# S2, S4, and S5), two (plant #S1) and three (plant #S6) copies of the transgene.

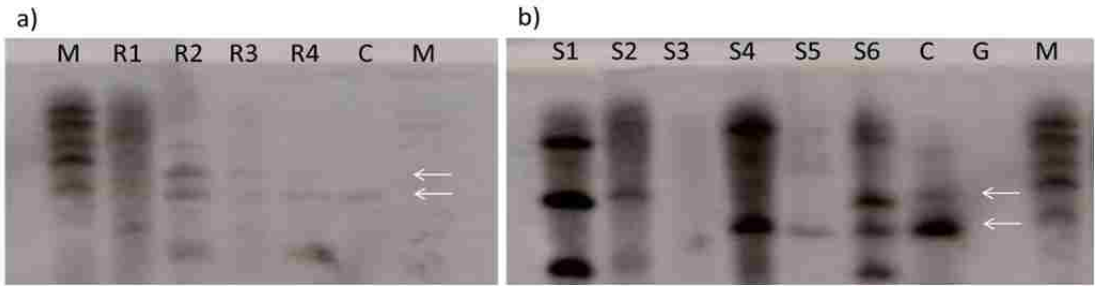

Figure 4.26 Confirmation of the transgene by southern hybridization. (a) Southern hybridization of *SaPMP3-2* T_1 plants using labeled gene fragment as a probe, (b) T_1 *SaPMP3-1* plants confirmation using labeled gene fragment as a probe. M: λ phage *Hind*III digested standard, R1 to R4: Genomic DNA from individual *SaPMP3-2* T_1 plants, S1-S6: Genomic DNA from individual *SaPMP3-1* T_1 plants and arrows indicate the position of native *PMP3* members from Cocodrie.

Figure 4.27 shows the cDNA amplified products obtained from the RT-PCR performed with RNA extracted from leaf samples collected from seedlings grown in greenhouse. The RT-PCR results confirmed the detection of *SaPMP3-1* mRNA during seedling stage. In contrast, *SaPMP3-1* mRNA was not detected in WT Cocodrie plants. The detection of elongation factor 1-α (ef1α) expression, a housekeeping internal control mRNA, in all the samples confirmed that the *SaPMP3-1* mRNA detection was unbiased.

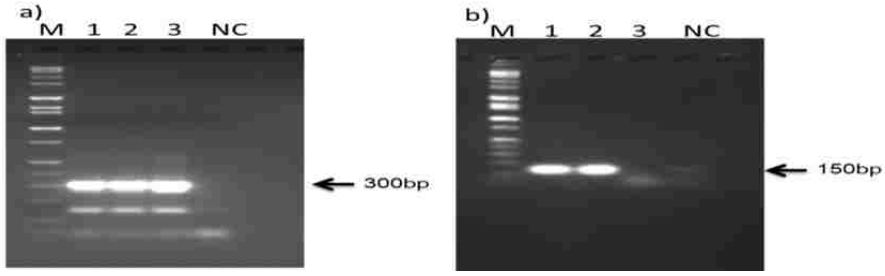

Figure 4.27 Detection of *SaPMP3-1* mRNA by conventional RT-PCR analysis of transgenic seedlings grown in greenhouse condition. (a) Internal expression control of elongation factor 1-α (ef1α) detection in transgenic and WT plants, (b) A representative RT-PCR experiment for detection of *SaPMP3-1* mRNA expression in seedling stage leaf samples. M: 1 Kb ladder, 1 and 2: transgenic plants with *SaPMP3-1* gene, 3: Cocodrie, and NC: Negative control without any sample. The expected PCR product is indicated by an arrow.

4.5 Physiological analysis of *SaPMP3-2* and *SaPMP3-1* transgenic rice

4.5.1 Salt screening at vegetative stage

Leaf samples were collected from 45 days old plants during vegetative stage and various physiological analyses were made. Healthy growing leaf, one below the flag leaf, was used for the physiological assays.

4.5.1.1 Leaf disc salt assay

The leaf disc assay is very rapid for preliminary identification of salt tolerant plants under stress condition. Floated leaf discs on an equal volume of distilled water (control), were visually compared to assess the difference in their chlorophyll retention after four days. Based on visual observation, the retention of chlorophyll was more in transgenic plants as compared to WT (Cocodrie) under two salt stress conditions (Fig 4.28). We also noticed that there was no visible difference under control condition.

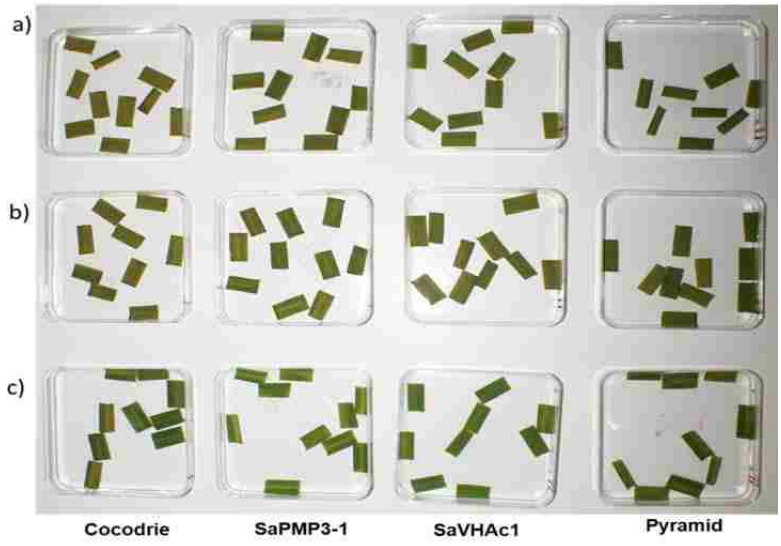

Figure 4.28 Leaf disc assay showing salt tolerance in transgenic plants. Equal size leaf discs were floated on 1/4th Yoshida media (Yoshida et al 1976) with different salt concentrations for 4 days: (a) no NaCl, (b) 100 mM, and (c) 150 mM NaCl.

4.5.1.2 Estimation of leaf chlorophyll content

To further examine the quantitative difference in the net chlorophyll content among the transgenic plants, changes in chlorophyll retention was measured from the leaf discs. Transgenic plants retained more chlorophyll than WT (Cocodrie) both under control and salt stress condition. Under salt stress, transgenic plants having *SaPMP3-1*, *SaVHAc1*, and pyramided plants maintained significantly higher chlorophyll content. In contrast, the *SaPMP-2* transgenic plant showed less chlorophyll, which was comparable to WT. The chlorophyll retention was quite similar at both 100 and 150 mM NaCl stress (Figure 4.29).

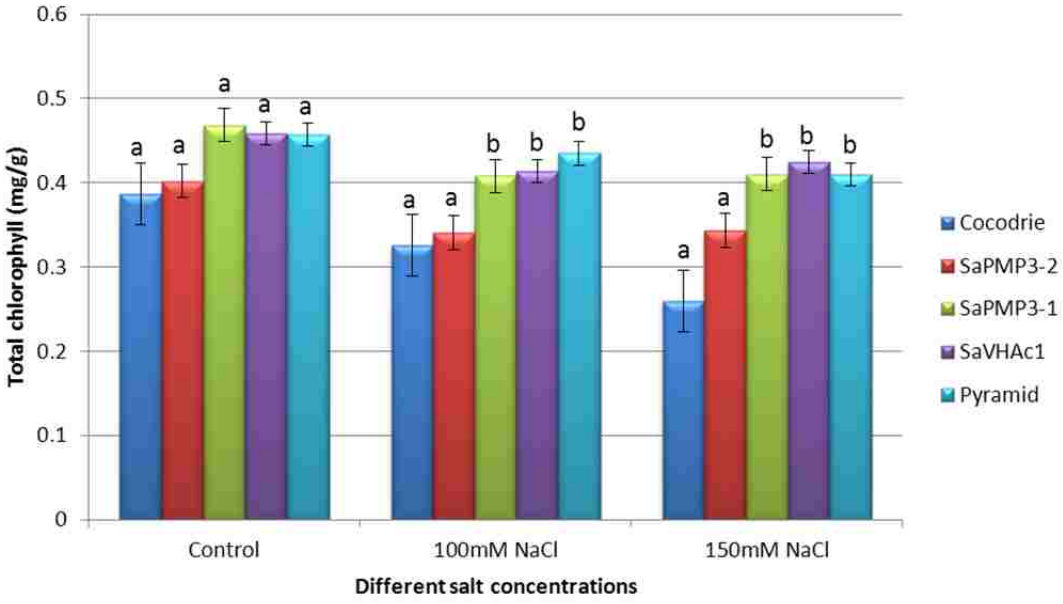

Figure 4.29 Chlorophyll content in leaf samples from different transgenic and WT plants. Leaf chlorophyll content was measured after 4 d of salt stress. Cocodrie (WT), *SaPMP3-2*, *SaPMP3-2*, *SaVHAc1*, and pyramided transgenic plants. Values represent means ± SD of five independent replicates. Different letters on bar diagram indicates the statistical significance at 5% level based on Dunnett's test.

4.5.1.3 Relative water content (RWC) in transgenic plants

The mean values of RWC measured using five replicates from control, 60 mM, and 80 mM NaCl are presented in Figure 4.30. Transgenic plants maintained higher RWC as compared

to the WT in response to 60 mM salt stress for one week. Plants with *SaPMP3-1*, *SaVHAc1* and pyramided plants maintained nearly 95 percent of RWC and transgenic plant with *SaPMP3-2* had little less RWC (90 percent). The WT Cocodrie plant maintained 86.9 percent of RWC. Similarly, when salt stress was further increased to 80 mM, all transgenic plants maintained significantly higher RWC compared to WT. However, under control condition, there was no difference in RWC between transgenic and WT plants.

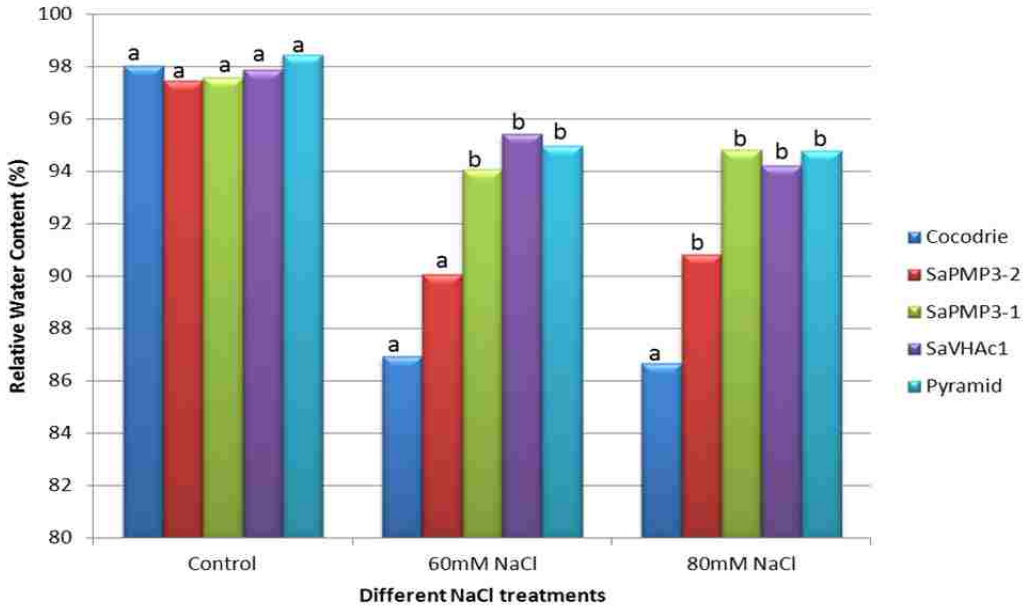

Figure 4.30 Comparison of Relative Water Content (RWC) between WT, individual transgenic lines, and pyramided lines under control condition and salt stress. Values represent means ± SD of five independent replicates. Different letters on bar diagram indicates statistical significance at 5% level based on Dunnett's test.

4.5.1.4 Estimation of free proline content in plant tissue

As proline is known to accumulate under various abiotic stresses, proline content was estimated after 10 days of NaCl stress using ninhydrin method. All plants, transgenic and WT, showed increased accumulation of proline content after exposure to 60 and 80 mM NaCl stress. All plants showed 5 times increase in proline accumulation at 60 mM NaCl and approximately 35 times increase in leaf proline content at 80 mM NaCl compared to control condition (Figure

4.31). Although there was slight increase in proline content in the transgenic plant, it was not significantly different from WT under salt stress.

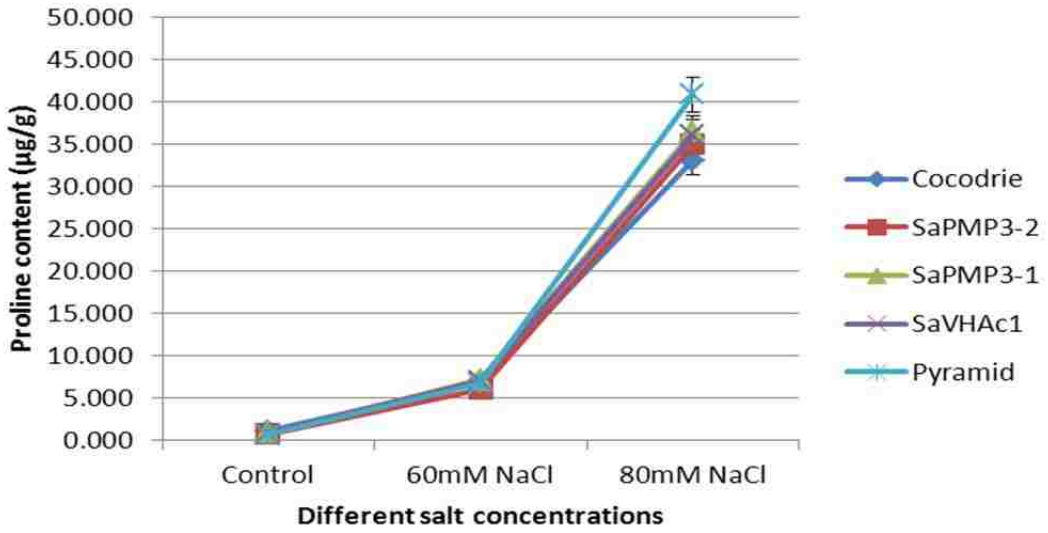

Figure 4.31 Proline accumulation in transgenic and WT plants under salt stress. Proline content was estimated from control and salt stressed leaf samples after 10 d of salt stress. Values represent means ± SD of five independent replicates.

4.5.2 Seedling stage screening under salt stress condition

4.5.2.1 Comparison of shoot and root length

To compare the variation in seedling growth, 5 day old uniform size seedlings were transferred to hydroponic culture and after 7 days of growth seedlings were transferred to different salt treatment. Seedlings shoot length and root length were compared after 12 days of salt stress. Notable changes in plant height were observed at 100 mM NaCl compared to the control condition. All transgenic plants showed better shoot growth when compared to WT under salt stress condition (Figures 4.32a, 4.33b, 4.34 and Table 4.5). However, the growth of transgenic and WT seedlings was normal under control condition and all plants had similar shoot length. Statistical analysis using paired t-test also confirmed the increase in shoot and root length of transgenic plants compared to the WT under salt stress (Table 4.5 and 4.6). Under salt stress condition, root length in all transgenic and pyramided plants was significantly higher than the

WT (Figure 4.33b and Table 4.6). Interestingly, pyramided plants showed higher root growth than the single gene transgenic plants and Cocodrie at 100 mM NaCl concentration. In contrast, roots of Cocodrie plants ceased to grow at 100 mM NaCl (Table 4.6).

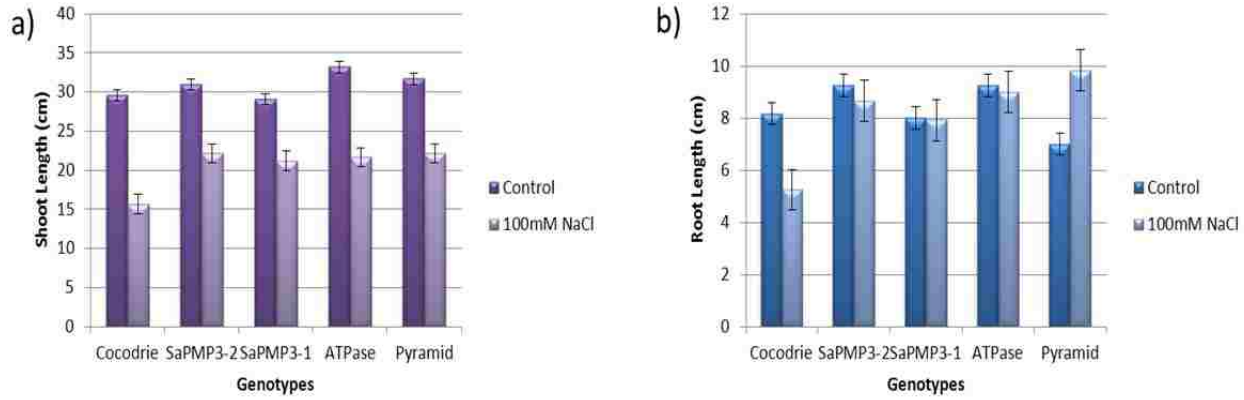

Figure 4.32 Comparison of shoot and root length under control and 100 mM NaCl stress at seedling stage. (a) Shoot length and (b) Root length.

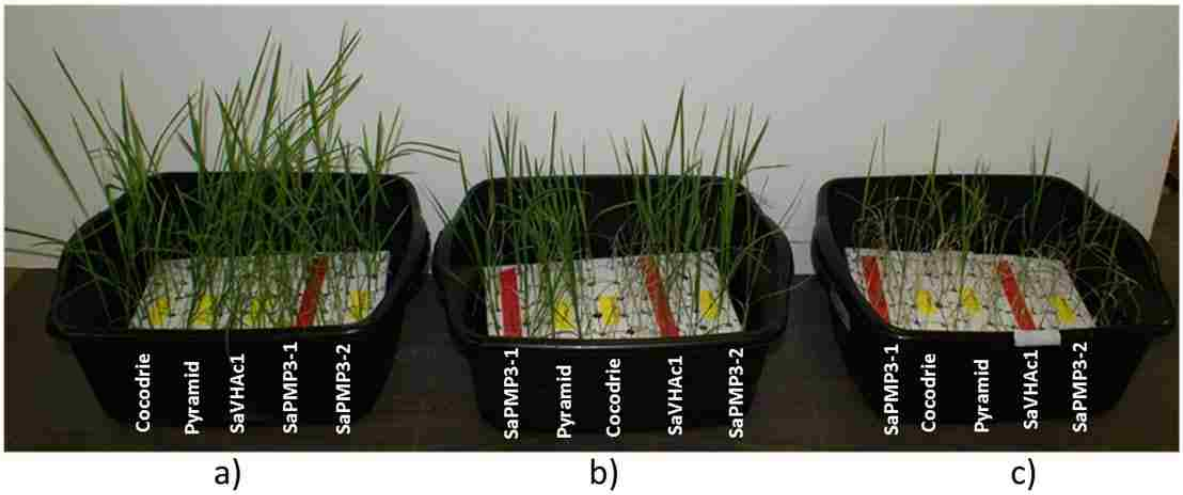

Figure 4.33 Seedling screening for salt tolerance at different levels of salt stress under hydroponic growing condition. (a) No NaCl, (b) 100 mM NaCl, and (c) 150 mM NaCl. Plants: Cocodrie (WT), *SaPMP3-2*, *SaPMP3-1*, *SaVHAc1*, and Pyramided lines. Wilting symptoms are visible in Cocodrie (WT) at 100 mM NaCl stress.

Table 4.5 Paired t-test results showing the differences in shoot length under control and 100 mM NaCl conditions after 12 days of stress.

Genotypes	Shoot length	DF	t Value	Pr > |t|
Cocodrie	diff	5	7.03	0.0009**
SaPMP3-2	diff	5	5.5	0.0027**
SaPMP3-1	diff	5	5.35	0.0031**
SaVHAc1	diff	5	11.31	<.0001**
Pyramid	diff	5	14.72	<.0001**

*: Significance at 5% level and **: significance at 1% level, diff: mean difference of genotype before and after stress

Table 4.6 Paired t-test results showing the differences in root length under control and 100 mM NaCl after 12 days of stress.

Genotypes	Root length	DF	t Value	Pr > |t|
Cocodrie	diff	5	2.96	0.0016*
SaPMP3-2	diff	5	0.65	0.5447
SaPMP3-1	diff	5	0.1	0.9249
SaVHAc1	diff	5	0.28	0.7926
Pyramid	diff	5	-3.08	0.0274*

*: Significance at 5% level, diff: mean difference of genotype before and after stress

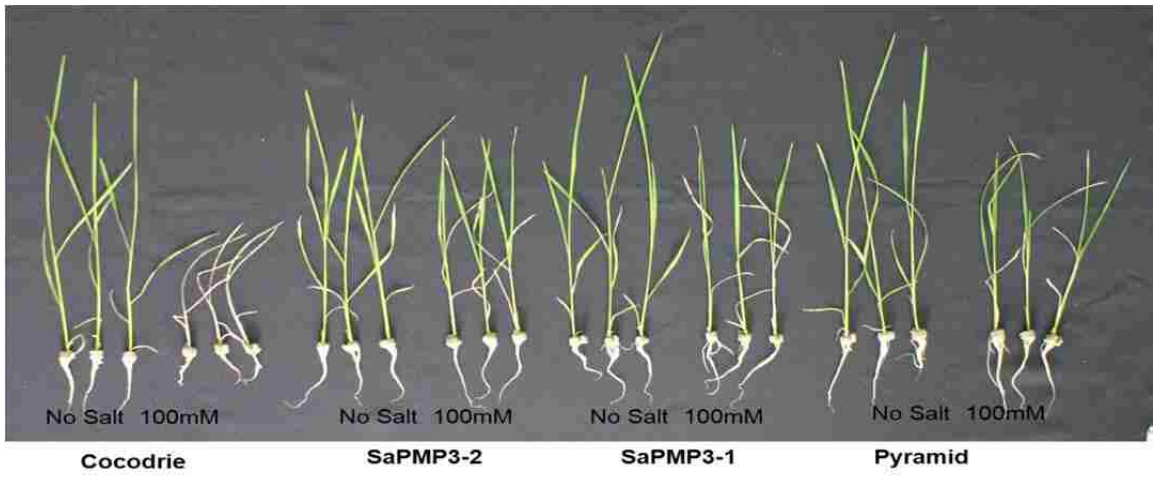

Figure 4.34 Responses of transgenic and wild type rice plants to 100 mM NaCl stress during seedling stage. Transgenic plants with single gene and pyramided transgenic plants showed better growth than Cocodrie (WT) under salt stress.

4.5.2.2 Estimation of Na^+ and K^+

To further verify the differential uptake and accumulation of Na^+ and K^+ by transgenic plants, Na^+ and K^+ content in shoot and root were estimated by flame photometer. Leaf samples from the seedling stage screening were used for the analysis. The results from Dunnett's t-test (Figure 4.35) indicated that *SaPMP3* overexpressing transgenic plants had significantly higher K^+/Na^+ ratio than non-transformed plants under salt stress condition. The shoot tissue had more available K^+ ion in all transgenic plants compared to WT. In contrast, leaf K^+ and Na^+ ratio under control condition did not show significant difference between transgenic lines and WT.

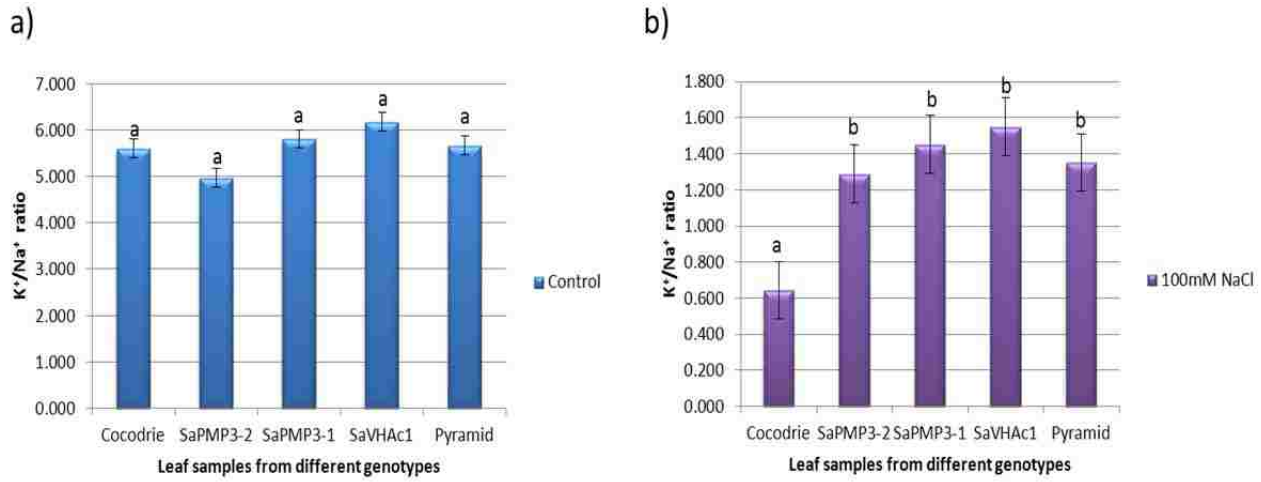

Figure 4.35 Estimation of K^+/Na^+ ratio in transgenic plants after 12 days of 100 mM salt stress. (a) Control condition and (b) 100 mM NaCl. Values represent means ± SD of five independent replications. Different letters on bar diagram indicates the statistical significance at 5% level based on Dunnett's test.

4.5.7 Analysis of yield parameters in transgenic plants

` After observing tolerance of transgenic plants at the seedling stage, the performance of transgenic plants was evaluated under salt stress condition at reproductive stage. Matured plants were harvested and yield related traits were analyzed. Under control conditions, there was no difference in grain yield per plant between transgenic and control plants (Figure 4.36a).

However, at 60mM NaCl concentration during reproductive stage, significant higher grain yield per plant was observed in pyramided plants, while other transgenic plants did not show any yield advantage over the WT (Figure 4.36b). Further, we measured the test weight by weighing one hundred seeds to check any difference among the genotypes. Like grain yield, there was no significant difference in the test weight between transgenic lines and Cocodrie under control condition (Figure 4.37a). However, the pyramided lines and *SaVHAc1* transgenic plants had higher test weight than *SaPMP3-1* and *SaPMP3-2* transgenic rice plants and Cocodrie at 60mM salt stress condition (Figure 4.37b).

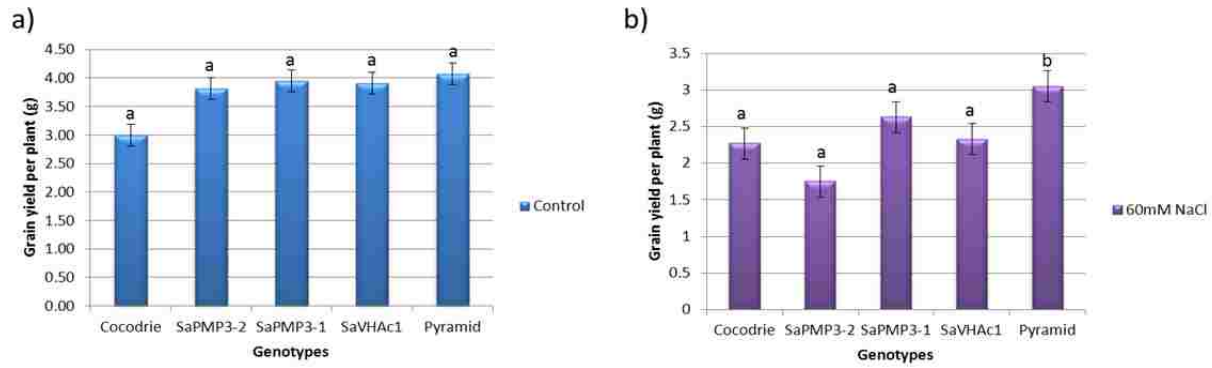

Figure 4.36 Comparison of grain yield per plant in wild type and transgenic plants under salt stress during reproductive stage in greenhouse. (a) Control condition and (b) 60 mM NaCl. Different letters on bar diagram indicate the statistical significance at 5% level based on Dunnett's test.

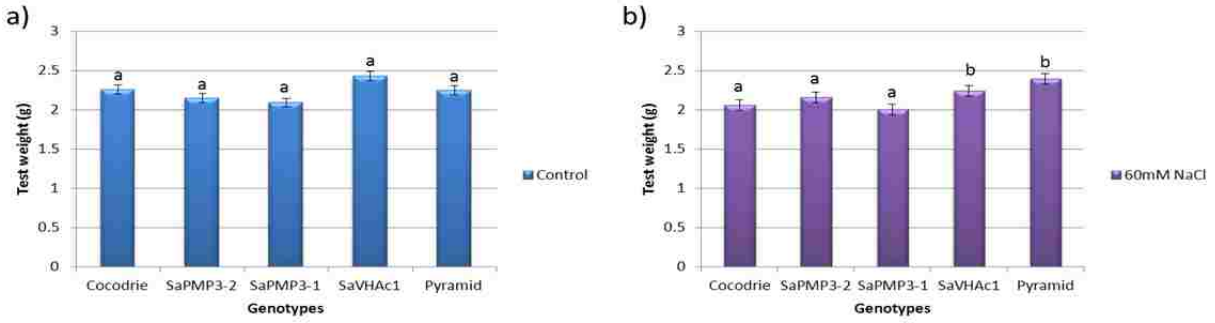

Figure 4.37 Variation in test weight due to salt stress during reproductive stage. (a) Control condition and (b) 60 mM NaCl. Different letters on bar diagram indicate the statistical significance at 5% level based on Dunnett's test.

The number of sterile seeds for each genotype was recorded and the percent seed sterility was calculated. Without NaCl addition during flowering stage, there was no detectable difference inseed sterility among the genotypes (Figure 4.38a). In contrast, the seed sterility was significantly lower in all transgenic lines as well as pyramided lines than Cocodrie at 60 mM salt stress condition (Figure 4.38b). These results suggest that salt stress during reproductive stage has more negative effect on seed setting and grain filling, which was significantly improved in transgenic and pyramided plants.

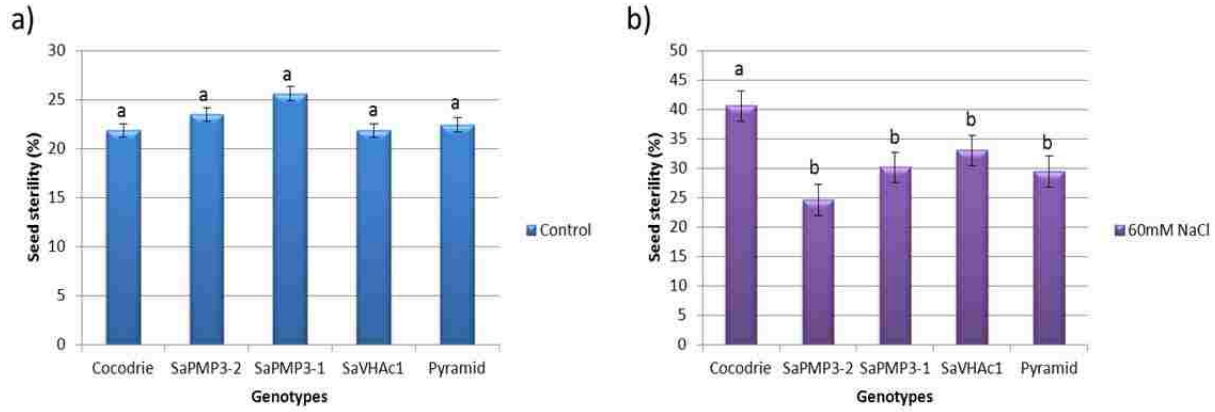

Figure 4.38 Impact of salt stress at the reproductive stage on grain filling and seed sterility in transgenic rice plants. (a) Control condition and (b) 60 mM NaCl. Different letters on bar diagram indicate the statistical significance at 5% level based on Dunnett's test.

CHAPTER 5. DISCUSSION

5.1 Sequence homology at DNA and amino acid level

The *SaPMP3-2* and *SaPMP3-1* genes isolated from *Spartina alterniflora* L. cDNA library (Baisakh et al. 2008) shared high homology to *PMP3* genes from different species. Ortholologs of *PMP3* genes from yeast (Navarre and Goffeau 2000), *Arabidopsis* (Medina et al. 2007; Mitsuya et al. 2005; Nylander et al. 2001), major cereals (Fu et al. 2012; Morsy et al. 2005), and halophytes (Inada et al. 2005) have been investigated to assess their role in salt tolerance. The BLASTN results showed high similarity of *SaPMP3-2* and *SaPMP3-1* genes to homologs from different vascular plants. Sequence homology of *SaPMP3-2* was highest with *Cleistogenes songorica* (96*%)* followed by barley (94%), rice (93%), *Leymus chinensis* (89%), and sorghum (89%) (Table 4.1). Similarly, SaPMP3-1 showed high similarity with different species of halophytes, major cereals, dicots, and yeast (Table 4.2). Amino acid sequences of SaPMP3-1 showed 13.2% similarity with SaPMP3-2 (Figure 4.6). The presence of high level of similarity at nucleotide and amino acid level demonstrated that *PMP3* sequences are highly conserved during the evolution of higher plants.

The results from BLASTN search in The Rice Annotation Project Database (RAP-DB) revealed presence of *SaPMP3-2* and *SaPMP3-1* related sequences on six different rice chromosomes whereas Fu et al. (2012) reported eight members of *ZmPMP3* present on different chromosomes of maize with similarity of 42.17% to 95.38% among them. The *ZmPMP3s* were expressed in response to salt stress, ABA, and PEG.

Phylogenetic analysis of amino acid sequences from different organisms showed the presence of two highly conserved regions in SaPMP3-2 like other organisms. Multiple sequence alignment and phylogenetic tree indicated high similarity of SaPMP3-2 with *Cleistogenes*

songorica (93%) followed by cereals, dicots, and yeast (Figures 4.3, 4.4, and 4.5). These two conserved regions corresponded to the predicted two transmebrane domains, TMD1 and TMD2. Further the secondary structure analysis showed that these two transmembranes were made up of two α-helix structures. Similar evolutionary relationship of PMP3 proteins was observed with the PMP3 homolog in *Arabidopsis* (Medina et al. 2007; Nylander et al. 2001), *Aneurolepidium chinense* (Inada et al. 2005), and maize (Fu et al. 2012). The high amino acid sequence similarity observed in SaPMP3 with its homologs of yeast and vascular plants indicated that PMP3 proteins have diverged from a common ancestor.

Further, the presence of high sequence homology was used as the basis for the prediction of three-dimensional (3D) structures. The predicted 3D protein of SaPMP3-2 showed that TMD1 forms the transport protein and TMD2 can either form a single transmembrane helix or CH domain-like protein (Figure 4.8). Hopf et al. (2012) reported that 3D structure prediction is a reliable tool to predict the folding of transmembrane proteins. They have used amino acid sequences and precisely predicted 11 previously unknown transmembrane proteins based on 3D structure prediction tool. The presence of highly conserved TMDs and their 3D structures indicated the presence of functional homologs in different organisms and their conserved ability to tolerate the salt stress by preventing the accumulation of excess Na^+.

5.2 *SaPMP3-2* and *SaPMP3-1* enhances salt tolerance in *E. coli*

For preliminary functional characterization, salt tolerance screening was performed in *E. coli* system. There was a hint of differences between the growth of *E. coli* cells transformed with *SaPMP3-1* and *SaPMP3-2* and expression control on LB plates at 600mM NaCl (Figure 4.11). In liquid growth assay, the degree of salt tolerance was further quantitatively elucidated. This study revealed an interesting association between overexpression of *SaPMP3-1* and *SaPMP3-2* in *E.*

coli and their salt tolerance at 600 mM NaCl (Figure 4.12). Transformed cells maintained normal growth pattern, while *E. coli* cells transformed with expression control vector did not grow significantly under 600 mM NaCl (Figures 4.11 and 4.12). Growth advantage was noticed immediately after 1 hour of salt exposure and the differences were clearly evident through the log phase. Apart from this, growth was better in *SaPMP3-1* transformed cells than expression control and *SaPMP3-2* under normal condition. These observations provided evidence in support for the enhanced salt tolerance of *E. coli* cells due to overexpression of *SaPMP3-2* and *SaPMP3-1* genes.

Functional characterization of genes in microbial model organisms such as *Escherichia coli* and yeast have been widely used for screening salt-sensitive mutations (Yamada et al. 2003) as overexpression results implicate the existence of a stress resistance system in higher plants. Yamada et al. (2002) conducted a similar *E. coli* overexpression study to test the salt tolerance of mangrove Allene Oxide Cyclase (*AOC*) and characterize the salt tolerance role of *AOC* in *Escherichia coli*, yeast, and tobacco cells. Apart from overexpression, another advantage of *E. coli* system is the feasibility of protein purification and subsequent enzymatic characterization. Liu et al. (2011) overexpressed Ascorbate Peroxidase (*APX*) gene from *Brassica napus* in *Escherichia coli* BL21 (DE3) and demonstrated that enzymatic assay conducted from the purified protein increased activity of APX, which decompose H_2O_2 produced during salt stress.

Therefore, the significant growth observed in *SaPMP3-2* and *SaPMP3-1* overexpressing *E. coli* at 600 mM implied that their cellular activity is crucial for survival under salt stress. This is the first line of evidence indicating crucial role of *PMP3* genes from *Spartina alterniflora* L. in salt tolerance mechanism in our study.

5.3 Genetic complementation of yeast mutant with *SaPMP3-2* gene

Functional characterization of any gene can be easily tested by genetic complementation; however the availability of mutant strain for a particular gene is the basic requirement.
In an earlier study, Navarre and Goffeau (2000) demonstrated that mutant cells lacking *PMP3*, *pmp3Δ,* showed accumulation of excess Na^+ in their intracellular space and hypersensitive reaction to 1M NaCl compared to the wild-type strain.

A complementation study was undertaken to test the function of *SaPMP3-2* using the yeast mutant strain lacking *ScPMP3* gene. The results from serial dilution assay indicated that *SaPMP3-2* rescued the suppression of growth in yeast mutant strain Y03635 at 1M NaCl (Figure 4.14). There was noticeable increase in the growth of transformed mutant cells compared to mutant transformed with vector control, which is a clear indication of genetic complementation. This observation was consistent with the result of salt stress screening in yeast by Navarre and Goffeau (2000). In addition to complementation study, the overexpression of *SaPMP3-2* in WT strain BY4741 also showed increased growth of transformed yeast cells at 1.5M NaCl (Figure 4.14 and 4.15).

Complementation involving the *PMP3* gene from different sources of origin demonstrated restoration of the plasma membrane potential and enhanced salt tolerance in yeast mutants (Medina et al. 2001; Nylander et al. 2001; Inada et al. 2005; Medina et al. 2007; Chang et al. 2008). Inada et al. (2005) have shown that *AcPMP3-2* from *Aneurolepidium chinense* complemented the Δnha1, Δpmr2, and Δpmp3 yeast mutants, which lacks the major Na^+ efflux systems (Na^+/H^+ antiporter and Na^+-ATPase). Transformed cells indicated the prevention of excess accumulation of Na^+ and K^+ than the mutant. Similarly, the orthologous genes *PutPMP3-1* and *PutPMP3-2* from alkali grass *Puccinellia tenuiflora* (Chang et al. 2008) and *AtRCI2A* from

Arabidopsis (Nylander et al. 2001 and Medina et al. 2007) complemented the functions of *PMP3* in mutant strains.

Further functional characterization results from mutant complementation studies in yeast showed that *PMP3* is not associated with the functioning of plasma membrane Na^+-ATPases, Na^+/H^+ antiporter activity, and K^+ transporters (Navarre and Goffeau 2000). Therefore, the observed genetic complementation in mutant strain by *SaPMP3-2* and its overexpression in wild type strain could result in prevention of increased uptake and accumulation of Na^+ under salt stress.

5.4 Overexpression of *SaPMP3-2* and *SaPMP3-1* enhances salt tolerance in rice

To investigate the possible role of *SaPMP3-2 and SaPMP3-1* in improving the salt tolerance of rice, transgenic plants with single genes were screened at different NaCl concentrations. Additionally, to complement the effect of single transgene *SaPMP3-1*, pyramided lines were developed by crossing *SaVHAc1* transgenic plants (Baisakh et al. 2012) with *SaPMP3-1* transgenic plants and evaluated for different physiological parameters.

5.4.1 Salt tolerance screening at vegetative stage

Transgenic plants with single gene as well as pyramided plants showed improved salt tolerance at different NaCl concentrations during vegetative stage. Leaf disc assay results revealed retention of more chlorophyll in single gene transgenic plants (*SaPMP3-1, SaPMP3-2,* and *SaVHAc1)* and pyramided plant (*SaPMP3-1* and *SaVHAc1)* than WT (Figure 4.28). These visual differences observed in leaf disc assay was further supported by the chlorophyll content from the leaf samples. Statistically, significant retention of chlorophyll was evident 4 d after exposure to 100 and 150 mM NaCl stress in *SaPMP3-1, SaVHAc1,* and pyramided plants (Figure 4.29). In contrast, *SaPMP3-2* and Cocodrie plants showed bleaching of chlorophyll from their

leaves. These results indicated that photosynthetic apparatus was least affected at higher salt stress in transgenic plants compared to WT. This is an indication that these transgenes maintain the cellular ion homeostasis without affecting photosynthesis. Higher chlorophyll content was reported in transgenic rice overexpressing the *SaVHAc1* (Baisakh et al. 2012) as well as in transgenic *Arabidopsis* plants overexpressing *ZmPMP3-1* (Fu et al. 2012).

Significantly higher relative water content (RWC) was observed by *SaPMP3-1, SaVHAc,* and pyramided transgenic plants at 60 mM and by all transgenic plants at 80 mM NaCl than in the Cocodrie plants (Figure 4.30). This result suggested that *SaPMP3-1* and *SaPMP3-2* genes may be vital for improving salt stress during vegetative stage.

Based on the findings in yeast (Navarre and Goffeau 2000) and *Arabidopsis* (Mitsuya et al. 2005), the decrease in total chlorophyll content and low RWC in Cocodrie can be attributed to the over accumulation of excess Na^+ by WT plants. Higher chlorophyll and RWC observed in transgenic plants with *SaPMP3-1* and *SaPMP3-2* could be due to prevention of excess Na^+ accumulation in root and shoot, which in turn maintained ion homeostasis and normal cellular functions. The evidences from *Arabidopsis* transgenic plants expressing *ZmPMP3* revealed a direct evidence of chlorophyll fluorescence (Fu et al. 2012). The wild type plants in their study showed reduction in chlorophyll content, leaf senescence, and the photo-oxidative stress, whereas maximum fluorescence ratios (Fv/Fm) indicated higher chlorophyll content in transgenic lines. Their findings also indicated the differential response of various *ZmPMP3* genes to exogenous ABA treatment but with different patterns. In addition to the findings in maize (Fu et al. 2012), *PMP3* homologs from rice, *OsLti6a* and *OsLti6b,* were expressed by exogenous application of ABA due to the presence of ABA-Responsive Element (ABRE) in the

promoter of *OsLti6a* (Morsy et al. 2005). The *OsLti6a* and *OsLti6b* were highly expressed under low temperature during seedling emergence.

Interestingly, there was significantly higher chlorophyll retention and RWC observed in pyramided plants (Figure 4.29 and 4.30). Perhaps it could be due to the prevention of salt accumulation by *SaPMP3-1* as well by complementary effect of vacuolar proton pump gene *SaVHAc1*. Baisakh et al. (2012) have already shown the retention of more chlorophyll and high RWC under salt stress condition in *SaVHAc1* transgenic rice plants than WT.

Accumulation of proline content was observed as a characteristic behavior under salt stress in both transgenic and WT plants. Although proline content was not statistically different between transgenic and Cocodrie plants, it was increased 5-fold at 60 mM NaCl and nearly 35 times when salt stress was raised to 80 mM (Figure 4.31). The paradox of independence in proline accumulation noticed in transgenic plants of *SaPMP3-1, SaPMP3-2, SaVHAc1,* and pyramided plants may be ascribed to their separate role in salt tolerance without influencing the proline accumulation. Proline accumulation in response to the imposed environmental stresses such as drought, high salinity, and low temperature has been reported in plants (Handa et al. 1986; Nanjo et al. 1999). It is reported to act as a free radical scavenger under salt and drought stress conditions (Miller et al. 2010; Yonamine et al. 2004). The increased proline accumulation by both transgenic and Cocodrie plants suggested the possibility of other salt tolerance genes that acted independently from *SaPMP3* in Cocodrie . It is quite possible to have such an independent determinant of salt tolerance as yeast mutant complementation study by Navarre and Goffeau (2000) indicated the unique mechanism of salt tolerance by *ScPMP3*, which is independent from the proven candidates for salt tolerance such as plasma membrane Na^+-ATPases, Na^+/H^+ antiporter activity, and K^+ transporters.

5.4.2 Seedling stage evaluation in transgenic rice

As seedling stage of rice is most sensitive to salinity, the relative growth of shoot and root under control and 100 mM NaCl was compared. Transgenic plants clearly showed improved salt tolerance by maintaining normal growth up to 12 days of salt stress There was significant increase in shoot and root length of all transgenic plants when compared to WT under salt stress (Tables 4.5, 4.6 and Figures 4.32 and 4.33). Fu et al. (2012) reported similar results in transgenic *Arabidopsis* plants overexpressing PMP3 orthologs from maize. Seedling growth and photosynthetic rate were enhanced in transgenic plants compared to WT. Similarly, higher fresh weight of shoot and root was reported by Mitsuya et al. (2005) in *Arabidopsis* plants containing *RCI2A* gene compared to mutant plants.

Navarre and Goffeau (2000) proposed that the primary mechanism of *ScPMP3* is the prevention of excess accumulation of salts in yeast system. Similarly, prevention of excess accumulation of Na^+ ions has been demonstrated due to the expression of PMP3 orthologs from *Aneurolepidium chinense* (Inada et al. 2005), *Arabidopsis* (Medina et al. 2007; Mitsuya et al. 2005; Nylander et al. 2001), maize (Fu et al. 2012), and *Puccinellia tenuiflora* (Chang et al. 2008) in mutant yeast strains. Accumulation of Na^+ was significantly reduced in transgenic plants compared to WT at 100 mM NaCl during seedling screening stage (Figure 4.35). Therefore, the plausible explanation for the observed better growth of transgenic rice plants with *SaPMP3-1* and *SaPMP3-2* gene could be due to prevention of the excess accumulation of NaCl.

Improvement in seedling growth and high K^+/Na^+ ratio has been demonstrated in transgenic rice plants overexpressing *SaVHAc1* gene (Baisakh et al. 2012) and in transgenic *Arabidopsis* plants overexpressing of *SaSce9 (*Karan and Subudhi 2012). In addition, better vegetative growth and low K^+/Na^+ ratio were reported in transgenic rice plants overexpressing the Na^+/H^+ antiporter gene *OsNHX1* (Fukuda et al. 1999; Fukuda et al. 2004). These findings

suggest that *SaPMP3-1* and *SaPMP3-2* may play an important role in seedling establishment and survival under salt stress by preventing excess accumulation of Na^+.

5.4.3 Salt tolerance screening at reproductive stage

Since salt stress during reproductive stage results in increased seed sterility and reduced yield, we examined the effect of overexpression of *SaPMP3-1* and *SaPMP3-2* genes on yield related traits in rice under salt stress. Grain yield per plant, test weight, and seed sterility were affected at 60 mM NaCl salt stress imposed from booting stage to maturity. There was a significant increase in grain yield per plant only in pyramided plants and test weight in *SaVHAc1* and pyramided plants compared to Cocodrie. However, single transgenic plants of *SaPMP3-1* and *SaPMP3-2* did not show any yield and test weight advantage.

The effect of salt stress on seed sterility was distinguishable between transgenic and Cocodrie plants. Cocodrie plants showed 40 percent seed sterility, which was significantly higher compared to all transgenic plants under salt stress (Figure 4.38b). In contrast, seed sterility was not affected either Cocodrie or transgenic plants under control condition (Figure 4.38a**)**. The lower sterility in *SaPMP3-1* and *SaPMP3-2* expressing rice plants implied that these genes either have some role in grain filling or they maintain the ion homeostasis by preventing the over accumulation of salt in the reproductive structures. Since there was no correlation between salt tolerance at vegetative and reproductive stage in rice (Lutts et al. 1995), the observed non significance for grain yield in *SaPMP3-1* and *SaPMP3-2* transgenics suggested that these genes may be important for improving tolerance to salt stress in seedling stage stress tolerance rather than reproductive stage.

The superior performance of *SaPMP3-1* and *SaPMP3-2* transgenic plants provided evidences in support for its role in salt tolerance. Navarre and Goffeau (2000) have shown its

role in preventing increased uptake and accumulation of Na^+ under salt stress. Further support for this mechanism was confirmed by *RCI2A* in *Arabidopsis* (Mitsuya et al. 2005), which is a homolog of *PMP3*. In addition to salt stress, expression of *AcPMP3* genes in response to exposure to cold, drought, abscisic acid, H_2O_2, and salicylic was reported in sheep grass (*Aneurolepidium chinense*), a monocotyledonous wild halophyte plant (Inada et al. 2005). Similar gene expression in response to salt, drought, and cold was noticed in *Arabidopsis* (Medina et al. 2001). Increased transcription of three ion transporter and four antioxidant genes as well as enhanced growth was observed in transgenic *Arabidopsis* plants overexpressing *ZmPMP3-1* (Fu et al. 2012).

The observed increase in grain yield and test weight of pyramided plants indicated complementary effect of *SaPMP3-1* and the *SaVHAc1* genes. The improvement in salt tolerance at the early stage of salt stress in rice transgenics with *SaVHAc1* gene could be due to preparatory physiological adjustments (Baisakh et al. 2012). The SaVHAc1-expressing plants showed increase in the transcription of genes involved in cation transport and ABA signaling. Physiological observations indicated its role in maintaining higher RWC, K^+/Na^+ ratio, root and leaf growth, and yield in SaVHAc1 transgenic rice. Therefore, the complementary effects of both genes might be playing important role for overall better performance and yield of pyramided plants over Cocodrie and single transgenic plants. However, more yield evaluations will be necessary to further validate the role of *SaPMP3-1* and *SaPMP3-2* genes during reproductive stage salt stress.

CHAPTER 6. SUMMARY AND FUTURE DIRECTIONS

6.1 Summary

The results from this dissertation demonstrated enhancement of salt tolerance by *SaPMP3-2 and SaPMP3-1* in *E. coli*, yeast, and rice system. The bioinformatics analysis indicated that the *PMP3* gene is highly conserved in different organisms from unicellular yeast to *Arabidopsis,* major cereals, and grass halophytes. Amino acid sequence analysis also confirmed conservation of PMP3 orthologs in different organisms. Two transmembrane domains corresponding to the two α helices of SaPMP3-2 were predicted like other organisms. One of the α-helix forms the single transport protein and the second α-helix can either form a single transmembrane helix or CH domain-like protein. In SaPMP3-1, only one TMD was predicted.

Functional characterization results from overexpression of *SaPMP3-2* and *SaPMP3-1* in *E. coli* revealed enhanced salt tolerance of transformed cells. The expression of *SaPMP3-2* in yeast mutant rescued the growth of mutant at 1M NaCl. The overexpression of *SaPMP3-2* in WT yeast strain showed improvement in salt tolerance at 1.5M NaCl. These results indicated the conserved functional role of SaPMP3 in improving salt tolerance from yeast to halophyte.

Transgenic rice plants overexpressing *SaPMP3-2* and *SaPMP3-1* showed enhanced tolerance to salinity stress at seedling and vegetative stage. Chlorophyll content and relative water content (RWC) were significantly less affected in transgenic plants with single gene as well as in pyramided transgenic lines than Cocodrie. All the transgenic plants showed significantly increased growth of shoot and root than WT under salt stress condition at the seedling stage. Interestingly, these plants also showed higher K^+/Na^+ ratio under salt stress.

Salt stress evaluation during reproductive stage indicated that transgenic plants overexpressing *SaPMP3-2* and *SaPMP3-1* had significantly better grain filling than WT under

60 mM NaCl stress. Further analysis of other yield related data revealed better grain yield per plant and more test weight in pyramided plants than Cocodrie and plants with single transgenes.

The salt tolerance of *SaPMP3-2* and *SaPMP3-1* transgenic plants was mainly attributed to the prevention of excess salt accumulation, which in turn helped to maintain the ion homoeostasis and normal functioning of cell. Tolerance to salt stress was further enhanced in pyramided transgenic lines suggesting the synergism between *SaVHAc1* and *SaPMP3-1* genes.

6.2 Future directions

Subcellular localization of *SaPMP3-2* and *SaPMP3-1* is needed to understand their cellular function within the plant cell and the nature of proteins with which it may interact in imparting salt tolerance. Comparison of gene expression patterns of abiotic stress responsive genes in transgenic plants may help to understand the influence of *SaPMP3-2* and *SaPMP3-1* on stress responsive pathways as well as their involvement in imparting tolerance to other abiotic stresses. Detailed analysis of members of *PMP3* in *Spartina alterniflora* L. and rice is necessary to unravel the functions of PMP3 homologs as their exons are present in varying size and number on rice chromosomes. Detailed analysis of unique regulatory sequences associated with *SaPMP3* members from smooth cordgrass will accelerate exploitation of these genes to improve salt tolerance in crop plants through genetic engineering.

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APPENDIX 1. OLIGONUCLEOTIDE PRIMERS USED IN THE STUDY

Primer Name	Sequence (5'- 3')	Purpose
PMP31BglIIF	GGAAGATCTATGCGGGCACGAG	Cloning *SaPMP3-1* in plant expression vector
PMP31BstEIIR	GGGTWACCTCACCACCGGGCA	Cloning *SaPMP3-1* in plant expression vector
PMP32BglIIF	GGAAGATCTATGGCGGACGAAGGGAC	Cloning *SaPMP3-2* in plant expression vector
PMP32PmlIR	CGCATGTGTTATTTGGTGATGGCGTAGACGGC	Cloning *SaPMP3-2* in plant expression vector
HPHF	TACTTCTACACAGCCATC	Hygromycin B phospho-transferase gene
HPHR	TATGTCCTGCGGGTAAAT	Hygromycin B phospho-transferase gene
PMP31EFUSF	CACCATGCGGGCACGAGTTCTGGAT	Cloning *SaPMP3-1* in *E .coli* expression vector
PMP31EFUSR	CCACCGGGCACCTGCAGCTGC	Cloning *SaPMP3-1* in *E .coli* expression vector
PMP32ECF	GGAAGATCTATGGCGGACGAAGGGAC	Cloning *SaPMP3-2* in *E .coli* expression vector
PMP32ECR	TTATTTGGTGATGGCGTAGACGGC	Cloning *SaPMP3-2* in *E .coli* expression vector
PMP32HindIIIF	CCCAAGCTTATGGCGGACGAAGGGACG	Cloning *SaPMP3-2* in yeast expression vector
PMP32BamHIR	CGGGATCCTTATTTGGTGATGGCGTAGAC	Cloning *SaPMP3-2* in yeast expression vector
PMP31BglIIF	GGAAGATCTATGCGGGCACGAG	GFP localization of *SaPMP3-1*
PMP31GFPR	GGACTAGTTCACCACCGGGCACCT	GFP localization of *SaPMP3-1*
PMP32BglIIF	GGAAGATCTATGGCGGACGAAGGGAC	GFP localization of *SaPMP3-2*
PMP32GFPR	GGACTAGTTTATTTGGTGATGGCGTAGACGGC	GFP localization of *SaPMP3-2*
PMP31RTF	GGAAGATCTATGCGGGCACGAG	Reverse transcription PCR of *SaPMP3-1*
PMP31RTR	GGGTWACCTCACCACCGGGCA	Reverse transcription PCR of *SaPMP3-1*
PMP32RTF	ATGGCGGACGAAGGGA	Reverse transcription PCR of *SaPMP3-2*
PMP32RTR	AAAGTGAGCAGCAGGCAGAT	Reverse transcription PCR of *SaPMP3-2*
PCAM35SF	GGAGAGAACACGGGGGACTCTTG	35S CaMV promoter

APPENDIX 2. COMPOSITION OF REGENERATION AND ROOTING MEDIA USED IN THIS STUDY

Components	Regeneration medium	Rooting medium
MS basal medium	4.4 g/L	4.4 g/L
NAA	0.5 mg/L	0.1 mg/L
Kinetin	0.2 mg/L	No
BAP	2.0 mg/L	No
Sucrose	30.0 g/L	30.0 g/L
Agar	8.0 g/L	8.0 g/L

MS basal medium (Sigma, St. Louis) as described by Murashige and Skoog 1962

APPENDIX 3. SEQUENCE CONFIRMATION OF *SAPMP3-2* IN RECOMBINANT VECTOR PC-SAPMP3-2.

```
  1  NNNNNNNNN  NNNNTTGNC TCTTGCCNTC CTTGTCCTCCT TAGCATTGGT
 51  ANGACCACCA GTGCAAGAAC CCTCCTAGAT CTATGGCGGA CGAAGGGACG
101  GCCAACTGTA TCGACATCAT CATCGCCATC CTCCTCCCGC CGCTCGGCGT
151  CTTCCTCAAG TTCGGATGCG GGCACGAGTT CTGGATCTGC CTGCTGCTCA
201  CTTTCCTCGG CTACATCCCC GGGATCATCT ACGCCGTCTA CGCCATCACC
251  AAATAACACG TGCGGTGTGA ATTGGTGACC AGCTCGAATT TCCCCGATCG
301  TTCAAACATT TGGCAATAAA GTTTCTTAAG ATTGAATCCT GTTGCCGGTC
351  TTGCGATGAT TATCATATAA TTTCTGTTGA ATTACGTTAA GCATGTAATA
401  ATTAACATGT AATGCATGAC GTTATTTATG AGATGGGTTT TTATGATTAG
451  AGTCCCGCAA TTATACATTT AATACGCGAT AGAAAACAAA ATATAGCGCG
501  CAAACTAGGA TAAATTATCG CGCGCGGTGT CATCTATGTT ACTAGATCGG
551  GAATTAAACT ATCAGTGTTT GACAGGATAT ATTGGCGGGT AAACCTAAGA
601  GAAAAGAGCG TTTATTAGAA TAATCGGATA TTTAAAAGGG CGTGAAAAGG
651  TTTATCCGTT CGTCCATTTG TATGTGCATG CCAACCACAG GGTTCCCCTC
701  GGGATCAAAG TACTTTGATC CNACCCCTCC GCTGCTATAG TGCAGTCGGC
751  TTCTGACGTT CAGNGNAGCC GTCTTCTGAA AACGACNTGT CGCACAAGTC
801  CTAAGTTACG CGACAGGCTN CCGCCCTGCC CTTTTCCTGG NNTTTTCTTG
851  NCGCGNGTTT TAGTCGCATA AAGTANAATA CTNGCGACTA NAANNGGANA
901  NATTACGCCA TNANNANGNN CNNNCNCTNG NNNGNTGGNT ATNNNNCGTC
                          //
```

APPENDIX 4. SEQUENCE CONFIRMATION OF *SAPMP3-1* IN RECOMBINANT VECTOR PC- SAPMP3-1.

```
  1  NNNNCNNNNN NNNNNNNGNN CNNNNNCNNN NTNGNNNNNN NNTTGAAGAT
 51  GCCTCTGCCG ACAGTGGTCC CAAAGATGGA CCCCCACCCA CGAGGAGCAT
101  CGTGGAAAAA GAAGACGTTC CAACCACGTC TTCAAAGCAA GTGGATTGAT
151  GTGATATCTC CACTGACGTA AGGGATGACG CACAATCCCA CTATCCTTCG
201  CAAGACCCTT CCTCTATATA AGGAAGTTCA TTTCATTTGG AGAGAACACG
251  GGGGACTCTT GACCATGGCT ACTACTAAGC ATTTGGCTCT TGCCATCCTT
301  GTCCTCCTTA GCATTGGTAT GACCACCAGT GCAAGAACCC TCCTAGATCT
351  ATGCGGGCAC GAGTTCTGGA TCTGCCTGCT GCTCACTTTC CTCGGCTACA
401  TCCCCGGCAT CATCTACGCC GTCTACGCCA TCACCAAATA AGAGATAGCA
451  GCAGCTTCAC AAAGCTGTGT TCGTACTCAA GGATACGTGC AGCTGCAGGT
501  GCCCGGTGGT GAGGTTACCC GGTGTGAATT GGTGACCAGC TCGAATTTCC
551  CCGATCGTTC AAACATTTGG CAATAAAGTT TCTTAAGATT GAATCCTGTT
601  GCCGGTCTTG CGATGATTAT CATATAATTT CTGTTGAATT ACGTTAAGCA
651  TGTAATAATT AACATGTAAT GCATGACGTT ATTTATGAGA TGGGTTTTTA
701  TGATTAGAGT CCCGCAATTA TACATTTAAT ACGCGATAGA AAACAAAATA
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VITA

Hanamareddy Biradar was born in Talikoti, Karnataka state, India. He completed high school education at Jawahar Navodaya Vidyalaya, Almatti, India. Reddy graduated from the University of Agricultural Sciences, Dharwad, in 1998 with a Bachelor of Science in agriculture. In 2001, he received his Master of Science in genetics and plant breeding with minor in molecular biology from G.B. Pant University of Agriculture and Technology, Pantnagar, India. After his graduation, he worked at University of Agricultural Sciences, Bangalore as a research associate in rice breeding program for five years. In August 2007, he moved to Baton Rouge to pursue a Ph.D. in rice genetics in the School of Plant, Environmental, and Soil sciences at Louisiana State University. Hanamareddy Biradar will graduate in May 2013.