Drought and salt tolerance characteristics of rice plants transformed with the *SNAC*1 transcription factor

Ph.D. Thesis

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By

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Certificate

This is to certify that Rumana Sultana Tammi has conducted her thesis work entitled, 'Drought and salt tolerance characteristics of rice plants transformed with the *SNAC*1 transcription factor' under my supervision for the fulfillment of the degree of 'Doctor of Philosophy in Biochemistry and Molecular Biology' from the University of Dhaka.
The work or any part of the thesis has not been submitted anywhere for any other degree.

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Dedication

To my beloved family
for unconditional love and never-ending support

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Abbreviation

A

A	Amino acid
ABA	Abscisic acid
ABRE	ABA responsive element
ABF	ABRE binding factor
AP2	*Arabidopsis thaliana* protein 2
AP2/ERF	APETALA2/ethylene-responsive element binding factor
ARF	Auxin response factor
ATAF1/2	*Arabidopsis thaliana* activating factor 1/2

B

BA	Binnatoa
bp	Base pair
BRRI	Bangladesh Rice Research Institute
bZIP	Basic leucine zipper
bHLH	Basic helix-loop-helix

C

C	Celsius
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary DNA
CPN20	20-kDa Chaperonin
CTAB	Cetyl trimethylammonium bromide
CUC2	Cup-shaped cotyledon 2
cv.	Cultivar
CYP72A5	Cytochrome 72A5

D

ddH2O	Distilled deionized water
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic acid

DRE	Dehydration-responsive element
DRE/CRT	Dehydration -responsive element/C-repeat
dS/m	Decisiemens per meter

E

e.g.	For example
et al.	And others
EDTA	Ethylene diamine tetra acetic acid
ERF	Ethylene responsive element binding protein
EtBr	Ethidium bromide
etc	Etcetera

F

FeSOD	Iron superoxide dismutase

G

g	Gram
GUS	β -Glucuronidase

H

Ha	Hectare
hr	Hour
HD-ZIP	Homeodomain-Leucine Zipper

I

IAA	Isoamyl alcohol
ICGEB	International Centre for Genetic Engineering and Biotechnology
IRRI	International Rice Research Institute

J

JA	Jasmonic acid

K

KAT1	Potassium channel in *Arabidopsis* 1
kb	kilo base
kDa	kilodalton

L

L	liter
LEA	Late embryogenesis abundant proteins

M

M	Molar
MDA	Malondialdehyde
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MS medium	Murashige and Skoog media
MYB	Myeloblastosis
MYC	Myelocytomatosis

N

NAM	No apical meristem
NAC	NAM, ATAF1/2, and CUC2
NACBS	NAC binding site
NACRS	NAC recognition sequence
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
NLS	Nuclear localization signal
No./no.	Number

O

OST 1	Open stomata 1

P

PCR	Polymerase Chain Reaction
pH	Negative logarithm of hydrogen ion concentration
Pok	Pokkli
PP2C	Protein phosphatase 2C
PYL	Pyrabactin like
PYR	Pyrabactin resistance

Q

qRT-PCR	Reverse transcription-quantitative polymerase chain reaction

R

RCAR	Regulatory Component of ABA receptor
RCD	Radical-induced cell death
RD26	RESPONSIVE TO DEHYDRATION 26
RD29A	RESPONSIVE TO DEHYDRATION 29A
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Rotation per minute
RT	Room temperature

S

SLAC1	Slow anion channel associated 1
SNAC1	Stress responsive NAC1
SnRK2	Sucrose Non-fermenting Related Kinase2
SRO1c	Similar to RCD one c

T

TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

TE	Tris EDTA
TRIZOL	Reagent contains guanidinium thiocyanate and phenol

U

µg	Microgram
µl	Microliter
UV	Ultraviolet

V

vol	Volume

W

WRKY	Transcription factors containing conserved core WRKYGQK motif

Abstract

Abiotic stresses are one of the major problems for restricting food production around the world. Plant survival and crop production has been hampered due to environmental stresses such as salinity and drought. Some plants may however show tolerance to abiotic stress through activating different genes including those for transcription factors. In the current study, stress tolerance was shown to be increased in rice which was transformed with the transcription factor *SNAC*1 (stress responsive *NAC*1).

My purpose was to increase the stress tolerance in existing commercial and farmer popular high yielding rice varieties, which directly influences the lives and livelihood of farmers. The *SNAC*1 transcription factor was isolated and cloned from the salt tolerant rice landrace, Pokkali, under the constitutive promoter, *CaMV*35S. It was at first overexpressed and characterized in the tissue culture responsive *indica* rice cultivar Binnatoa, at the two developmental stages at seedling and maturity for both the stresses salinity and drought. Transgenic lines were confirmed by PCR and Southern blot hybridization indicating stable transgene integration into the genome. Assays for leaf senescence and chlorophyll content at 100mM and 200mM salt and survival rates at 200 mM salt and drought condition showed that transgenic lines were significantly increased in their stress tolerance compared to wildtype at seedling stage. At reproductive stages, the transgenic lines showed significantly enhanced spikelet fertility, yield and 1000 grain weight compared to wildtype under both salinity and drought stresses. Thus, the transgenic rice overexpressing *SNAC*1 showed significantly improved tolerance to salinity and drought stresses at the vegetative as well as reproductive stages. After characterization in *indica* rice, the *SNAC*1 gene was transformed in three high yielding rice varieties (BRRI Dhan-55, BRRI Dhan-56 and BRRI Dhan-49) for stress inducible expression under rd29A promoter. All high yielding varieties were transformed through tissue culture independent *Agrobacterium*-mediated *in planta* transformation method which bypasses the problems associated with tissue culture-based *indica* rice transformation methods. Transgenic lines showed correct sized bands in PCR and integration of the *SNAC*1 gene into the genome were confirmed by Southern blot hybridization. Significantly higher transgene expression (*SNAC*1) was found by real time (qRT) PCR. Transgenic lines showed 3:1 segregation ratio at T_2 generation following the Mendelian law of inheritance. Transgenic lines also showed significantly higher level of

stress tolerance in salinity and drought condition compared to their respective wildtype plants.

A comparative assay was done in BRRI Dhan-45 (salt sensitive) and BRRI Dhan-56 (moderately drought tolerant) transgenic lines showing that the level of tolerance differ in different genotypes. Transgenic lines of BRRI Dhan-56 showed more stress tolerance than BRRI Dhan-49 in seedling and reproductive stages and both varieties showed significantly higher stress tolerance compared to their respective wildtype plants. To understand the promoter effects on *SNAC*1 gene expression comparative assay was done in BRRI Dhan-55 transgenic lines between *SNAC*1 expressed under stress inducible promoter and constitutively *SNAC*1 overexpression lines. It was found that at both seedling and reproductive stages under both salinity and drought stresses, induced expression of *SNAC*1 provided significantly more tolerance in plants. In this study, six up-regulated genes were selected from reported microarray data and tested for expression in BRRI Dhan-55 *SNAC*1 transgenic lines under inducible promoter. It was found that the expression of all 6 genes was induced under salt stress condition. The increased expression of these *SNAC*1 downstream genes likely helped in providing a higher level of stress tolerance in abiotic stresses. The underlying pathway and possible interactions of the selected genes were also predicted by literature and network analysis. This work provided a promising approach for the genetic improvement in the salinity and drought tolerance of commercial, high yielding *indica* rice cultivars through transformation with the *SNAC*1 transcription factor under stress inducible promoter.

1. Introduction

Agriculture has been feeding the total population for more than 20,000 years. Food security of a country only be ensured by a stable agricultural industry. Worldwide many crops are grown to feed people. Among them rice is the most widely consumed staple food for a large part of the world's population. Increased frequency of extreme weather conditions as well as simultaneous occurrence of abiotic stresses such as drought, high salinity, and high temperature cause extensive losses to agricultural production worldwide (Mittler and Blumwald, 2010). According to PRB's World Population Data, in the year 2020 the total population of the world is 7.8 billion and by 2050 the population will reach 9.9 billion, which is an increase of more than 25% from the current population. Growing population will have increasing demand for food. By 2050, the world may not be able to feed its population if the productivity not increased. Food production will need to be nearly doubled in developing countries. So, countries need to focus on agriculture and the growing of food by more viable methods. For this combination of practices is needed, including technologies that are designed for farmers in their areas.

Rice is one of the most important cereal crops in the world which provide one-third of the total carbohydrate source for us. Three billion people consider rice as their stable food which accounts for 50–80% of their daily calorie intake. However, rice is a salt sensitive monocot (Singh and Sengar, 2014). And rice productivity has decreased due to different environmental stresses such as salinity and drought. Due to the current global climate change and increasing levels of salinity in coastal areas, it is the present necessity to increase the stress tolerance in high-yielding commercial rice varieties. Transgenic rice engineered for higher level of expression of regulatory genes which can influence multiple downstream genes to help plants in adaptation under different abiotic stress conditions is therefore highly desirable. These plants will likely produce higher yields under stressed conditions and have the potential of providing a significantly positive impact on world food production.

1.1.Rice and Bangladesh:

Rice *(Oryza sativa)* is the staple food of about 135 million people of Bangladesh. Rice provides about one-half of the total protein intakes, about two-third of total calorie supply of an average person in the country. Nearly 48% of rural employment is depends on rice related work. In Bangladesh, one-sixth of the national income and one-half of the agricultural GDP provide by rice sector contribution. Rice is grown by almost 13 million

farm families in the country. About 80% of the total irrigated area and over 75% of the total cropped area are involved in rice production. Thus, rice plays a vital role in the livelihood of the people of Bangladesh. The volume of Rice production in FY2018-19 stood at 363.91 lakh MT, of which Aus accounted for 27.75 lakh MT, Aman 140.55 lakh MT, Boro 195.61 lakh MT and for the FY2019-20 it was targeted 389.50 lakh MT (Economic Review 2020).

Table 1.1: Rice grains production status during FY2012-13 to FY2019-2020.

Rice	2012-13	2013-14	2014-15	2015-16	2016-17	2017-18	2018-19	2019-20*
Aus	21.58	23.26	23.28	22.89	21.34	27.09	27.75	30.12
Amon	128.97	130.23	131.90	134.83	136.56	139.94	140.55	155.02
Boro	187.78	190.07	191.92	189.38	180.16	195.76	195.61	204.36
Total	338.33	343.56	347.10	347.10	338.06	362.79	363.91	389.50

Source: *Bangladesh Bureau of Statistics (BBS), Ministry of Agriculture. Amount in Lakh MT (Metric ton)* target. (Economic Review 2020).*

Despite the success in rice production, with time, as the due to the increased population, the gap between rice production and food requirement becomes expanded with times. This will create a major threat to food security of the millions of people in our country, particularly those that are living in different districts, faces environmental stress almost each year. In this context, it is the challenges for us to help maintain an increase in food production and ensure food security for the growing population with limited natural resources, declining arable land, and adverse climatic conditions.

1.2.Salinity and drought- two major abiotic stresses:

Salinity is one of major problems for rice production. It covers about 400 million hectares lands in the world including 54 million hectares found in south and south East Asia. Hence, production of rice is under pressure due to saline condition because salinity may cause plant demise, growth and development and reduced yield up to 50% (Nozulaidi et al., 2015).

Abiotic stress includes high salinity, water deprivation, high temperatures, excessive light, pollutants such as ozone and herbicides, excessive UV radiation, high concentrations of heavy metals, and so on (Petrov et al., 2015). Among the abiotic stresses, high salinity and drought are severe environmental stresses which impair plants growth and crop production. About 1125 million hectares of lands are salt affected worldwide that is at least 20% of irrigated land worldwide (Hossain, 2019). High salinity affects almost all aspects of plant physiology and metabolism by causing both hyper ionic and hyperosmotic stresses, which lead to plant death. High salt deposition in soil leads to a declaration of a low water potential

zone in the soil which makes it difficult for the plant to uptake water as well as nutrients. External Na^+ can negatively impact intracellular K^+ influx. For plant growth potassium ions are one of the essential elements, alterations in K^+ ions can change the osmotic balance as well as the function of stomata, and also functions of many essential enzymes. Salinity increases Na^+ and Cl^- concentrations in the cytosol which is detrimental to the cell. Higher concentrations of sodium ions (above 100 mM) are toxic to cell metabolism and regular functions by inhibiting the activity of many essential enzymes. Cell faces interruption in cell division and expansion, membrane disorganization, and osmotic imbalance, which leads to growth inhibition of plants (Tuteja, 2007).

In Bangladesh, salinity is one of the major abiotic stress that hinders crop production. About 20% area in Bangladesh are in coastal region and 53% of these areas are affected by different degrees of salinity (Hossain et al., 2012). Above a soil conductivity of 3 dS/m, rice plant shows sensitivity. Greater than 1 million ha of coastal areas in Bangladesh are affected by varying degrees of soil salinity. Depending the proximity to the sea or season, the soil conductivities vary from 4-20 dS/m in coastal areas. Farmers can grow crops in the coastal areas during the monsoon season, when the soil salinity levels go down, rest of the year the areas left fallow. It will be beneficial for small and marginal farmers to produce two crops per year.

Drought is another abiotic stress, took great concern. Bangladesh also have 2.68 million hectares drought prone areas. Bangladesh has experienced drought at regular intervals; on average at least ones in every 2.5 years. In Bangladesh, the northern and southwestern districts of the north-west region are more drought prone (Kamruzzaman et al., 2019). During the pre-monsoon period the drought impact is becomes more severe in Bangladesh. It has great impact on Boro rice, high yielding variety, which is cultivated in 88% of the total available areas of the country, grows during this time. A deficit of rainfall during this period causes huge damage to agriculture and to the economy of the country (Shahid, 2008). There is no alternative to producing more salinity and drought tolerant varieties, or increasing the level of tolerance of the existing varieties, particularly the staple food rice (*Oryza sativa* L).

1.3.Transcription factors are the best means to combat stress:

High salinity and dehydration adversely affect the plant growth and productivity of crops, including rice (*Oryza sativa* L.). Plants have changed a range of physiological, biochemical, and molecular responses to confer tolerance to different biotic and abiotic stresses. Plant adaptation to environmental stresses such as salinity is reliant on the activation of cascades of molecular networks involved in stress perception, signal transduction and the expression of specific stress related genes and metabolites.

Salinity tolerance is a multigenic trait. It is difficult to obtain salt tolerance by using single gene. Many genes are now reported to confer salt tolerance in plants (Tuteja, 2007). Transcription factors are the best possible way to confer response to different stresses altogether. The gene expression is enhanced or repressed by binding of the transcription factors (TFs) that are DNA-binding proteins in sequence specific manner. Some of these TFs are master regulators of signaling and regulatory pathways of stress adaptation. Genetic engineering of TFs to overexpressed or induced expression in plants may be sufficient to enhance stress tolerance. These TFs becomes attractive targets for engineering. It is reported that by genetic engineering over-expression of some of the TFs like bZIP, NAC, ERF, WRKY, CBF/DREB, MYB, AP2/ERF gives different type of stress tolerance (Lata et al., 2011).

The NAC family of transcription factors, a plant-specific transcription factors with a variety of biological functions, containing highly conserved and specific NAC domains in the N-terminal of proteins. NAC plays an important role in plant stress tolerance to abiotic stresses by directly or by regulating the expression of different stress-related genes involved in stress response. NAC proteins are involved in different functions in plant such as involved in cell division, development of shoot apical meristem, formation of secondary wall, lateral root formation, and leaf senescence (Ge et al., 2014; Nikovics et al., 2006; Mitsuda et al., 2005; Zhong et al., 2010; Xie et al., 2000; Guo and Gan, 2006; Yang et al., 2011), and response to many biotic and abiotic stresses (Xia et al., 2010; Puranik et al., 2012; Nakashima et al., 2012).

1.4.*SNAC*1 (Stress Responsive NAC1):

*SNAC*1, stress responsive NAC1, is one of the members of plant specific NAC transcription factor protein family. Transcription factor SNAC1 was first reported to overexpressed in *Japonica* rice *cv*. Nipponbare, showed salt tolerance and drought resistance at the

vegetative stage and also showed higher seed setting in field condition compared to wildtype under drought condition (Hu et al., 2006). Thereafter, Os*SNAC*1 was overexpressed into wheat, cotton, ramie to enhance drought and salt tolerance (Saad et al., 2013; Liu et al., 2014; An et al., 2015). Overexpression of *SNAC*1 homologous gene Ta*SNAC*1 in wheat, Hv*SNAC*1 in barley, Musa *SNAC*1 in banana also showed stress tolerance by ROS homeostasis in transgenics (Liwei et al., 2012; Abdallat et al., 2014; Negi et al., 2018; Negi et al., 2021). Other NAC proteins in rice SNAC2, SNAC3, OsNAC5, OsNAC6, OsNAC9, OsNAC10 were also overexpressed in rice and found similar results (Hu et al., 2008; Fang et al., 2015; Takasaki et al.,2010; Nakashima et al., 2007; Redillas et al., 2012; Jeong et al., 2010).

In stress response *SNAC*1 follows the ABA-independent pathways, containing two DRE sequence in its promoter region, expressed under DREB binding proteins. It was found that protein phosphatase PP2C (OsPP2C18) was a downstream target gene of *SNAC*1, which is ABA insensitive, regulates the stress response pathway by ROS homeostasis (You et al., 2014).

1.5.Effects of constitutive and inducible promoters on gene expression:

Regulated expression of transgenes is an important aspect of transgenic technology. The promoter helps to increase the level of expression of the gene, also tissue specificity and correct timing of transgene expression are an important consideration. Thus, for tailoring plant response to stresses, the strength of the promoter as well as the possibility of using stress-inducible, or developmental stage, or tissue-specific promoters have need to be large consideration.

For the production of abiotic stress-tolerant plants, the promoters that have been most commonly used so far include the *CaMV*35S, ubiquitin, and actin promoters (Fang et al., 2015). Being constitutive in nature, these promoters express the downstream transgenes in all organs and at all the developmental stages largely. These promoters have been successfully applied to overexpress the transgene in plants and thus, the development of stress-tolerant transgenic crops. However, the strong constitutive expression of functional genes or transcription factors often shows undesirable phenotypes on transgenic plants. For example, in stress-tolerant transgenic *Arabidopsis* overexpressing transcription factor DREB1A under *CaMV*35S promoter displayed growth retardation and a severe reduction in seed production (Kasuga et al., 1999). Similarly, transgenic rice plants overexpressing

OsNAC6 under the maize ubiquitin promoter became stunted (Takasaki et al., 2010). In such cases, the use of stress-inducible promoter may be more desirable. In plants, a large number of promoters are induced by various types of abiotic stresses. Certainly, the use of a stress-inducible promoter is the viable alternative strategy for the elimination of abnormal phenotypes induced by constitutive transgene expression.

In *Arabidopsis* rd29A is a stress-responsive downstream gene in ABA signaling pathway, induced under abiotic stress conditions. rd29A promoter includes both DRE and ABRE elements, as a results high salinity, dehydration, high and low temperatures induce the gene. It was reported that the expression of DREB1A driven by the stress-inducible rd29A promoter has minimal effects on plant growth, as compared with the *CaMV*35S promoter, although conferring a greater tolerance to stress conditions (Kasuga et al.,1999). Use of rd29A promoter in rice also showed enhanced yield potential of transgenic plants (Datta et al., 2012). In this study rd29A promoter is used as stress inducible promoter for transgene expression.

1.6.Significance of this study:

During osmotic stresses, due to salinity or drought, plants will undergo both ABA-dependent and ABA-independent pathways of stress response (Yoshida et al., 2014; Du et al., 2018). AREB/ABFs are main transcription factors involved in ABA-dependent pathway and DREBs are predominant transcription factors involved in gene expression in ABA-independent pathway. Both pathways regulate different stress-responsive genes and other downstream transcription factors to switch on different processes for stress response or tolerance.

*SNAC*1 works under ABA-independent pathway in osmotic stress and dehydration (Puranik et al., 2012). *SNAC*1 promoter region contains two DREs (dehydration-responsive elements), stress-related cis-acting element (Nakashima et al., 2012). In this study, *SNAC*1 is expressed under rd29A promoter, which contains two DREs, one DRE/CRT core and one ABRE (ABA-responsive element) cis-acting elements (Narusaka et al., 2003). As a result, *SNAC*1 now serve as the intermediate for both ABA-dependent and ABA independent signaling pathways. Enhancement of whole cascade of stress responsive mechanisms will be activate in transgenic plants. This may give synergistic effect in transgenic plants for better tolerance in osmotic stress due to salinity and drought condition.

1.7.Objectives:

Salinity and Drought are two major environmental stresses that affect in plant growth and productivity. Our main concern is to develop plants that can minimize the adverse stress effect, particularly with respect to our staple food, rice. Rice plants is fully dependent on high amounts of fresh water throughout its life cycle. Its vegetative growth and yield production are therefore highly dependent on the soil water and mineral condition.

The purpose of this work is to enhance drought and salinity tolerance in existing commercial high yielding rice varieties by transformation with *SNAC*1 (Stress Responsive NAC1) transcription factor under stress inducible promoter rd29A.

I divided the work into four parts with specific objectives-

Part One: Characterization of transcription factor *SNAC*1 in *indica* rice variety Binnatoa for drought and salinity tolerance.

For characterization in *indica* rice, *SNAC*1 transcription factor was overexpressed in a tissue culture responsive rice cultivar Binnatoa. Transformed plants were confirmed by molecular analysis (PCR, Southern blot hybridization, semi quantitative RT-PCR) as well as phenotypic screening such as leaf disk senescence assay, survival rate, etc. Drought and salinity tolerance among transgenic lines were tested at both seedling and reproductive stages.

Part Two: Transformation of high yielding BRRI rice varieties with transcription factor *SNAC*1 under stress-inducible promoter for conferring both salinity and drought tolerance.

Three high yielding rice varieties BRRI Dhan-55, BRRI Dhan-56 and BRRI Dhan-49 were transformed with *SNAC*1 transcription factor under stress inducible promoter rd29A. These rice varieties were transformed by tissue culture independent *in planta* transformation method. Thus rd29A_*SNAC*1_BR-55, rd29A_*SNAC*1_BR-56 and rd29A_*SNAC*1_BR-49 transgenic lines were formed. For confirmation of transgenic lines molecular analysis and seedling level screening were done. Transgene expression was analyzed under stress and without stress. According to the best level of tolerance at both drought and salinity stress condition two lines were selected for further physiological characterization.

Part Three: Comparative assay in different rice genetic background on the ability of *SNAC*1 to confer stress tolerance.

Comparative assay was done in between two genetic background, among rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines. BRRI Dhan-56 is a moderate drought tolerant variety and BRRI Dhan-49 is a salt sensitive variety. This work was done to understand how far these transgenic lines give stress tolerance in both salinity and drought condition. Another comparison was done in BRRI Dhan-55 rice variety. *SNAC*1 overexpressed transgenic lines *CaMV*35S_*SNAC*1_BR-55 (previously developed in our lab) and rd29A_*SNAC*1_BR-55 were compared to understand the comparative effect of *SNAC*1 under constitutive and inducible promoter.

Part Four: Evaluation of the effect of *SNAC*1 expression on downstream genes.

The expression analysis of six stress-related genes known to be downstream of *SNAC*1 was done. At salt stress, the level of gene expression was analyzed in *SNAC*1 transgenic plants (under stress inducible promoter) and their integrated functions were explored by using bioinformatic tools and literature mining.

2. Review of Literature

2.1.Abiotic stresses:

Abiotic stresses are a major constraint to crop production. Plant growth and productivity are adversely affected by such environmental stresses. Stress condition triggers a series of physiological and biochemical changes in plants. Drought, high temperature and high level of saline in soil are the most common abiotic stresses that plants are facing throughout its life. Abiotic stress causes more than 50% of yield reduction throughout the world. More than 50% of grain yield must be increased in major crops like rice, wheat or maize to maintain the food supply requirement for world's population by 2050 (Godfray et al., 2010). Therefore, improving abiotic stress-tolerance in plants has its important significance in order to increase the cereal production for feeding the world in the near future.

2.2.Salinity and Drought- The two major concerns for crop production:

In Bangladesh, drought and salinity are of great concern due to poor irrigation facilities and lack of infrastructure to store water in the monsoon season. Moreover, a huge area, in the southern part of Bangladesh suffers from high salinity, particularly in the dry winter season. The northern and southwestern districts of the north-west region of Bangladesh are more drought prone (Kamruzzaman et al., 2019). So, salinity and drought are well-documented as a limiting factor in crop productivity.

2.2.1.Salt stress:

High salinity impedes plant growth and development by introducing physiological drought conditions and ion toxicity (Zhu 2002). In saline condition, ion specific stress is increased in plants which alters the cellular K^+/Na^+ ratio. Salinity leads to the accumulation of Na^+ and Cl^- ions in the cytosol, which is detrimental to the cell. High concentration of sodium ions are toxic to cell metabolism and causes inhibition of the activity of many essential enzymes and proteins involved in cell division and expansion. Finally, growth is inhibited by osmotic imbalance due to higher Na^+ concentration. Production of reactive oxygen species are the common consequences of higher concentrations of sodium ions (Chaves et al., 2009). This also reduces the photosynthesis level in plants. Also, the alterations in K^+ ions due to the influence of high salinity stress directly affects some K^+-dependent enzymes and disturbs the osmotic balance in plants. As a result, the function of stomata and many essential enzymes are repressed. Rice is sensitive to salinity at the seedling stage with consequent poor growth and development. It is even more sensitive during reproduction

where salinity can severely affect flowering, grain formation and yield (Moradi et al., 2007).

2.2.2.Drought stress:

Excess water or water deficit can both cause water stress in plants. During flood condition, excess water results in reduced oxygen supply to the roots, resulting in failure of critical root functions including less nutrient uptake and respiration. The major water stress is the water deficit stress, also known as the drought stress (Mahajan et al., 2005). Plant's growth, mainly at the stage of flowering and seed development, is very susceptible to drought. Drought stress disrupts the normal bilayer structure of membrane, resulting in loss of membrane integrity, selectivity and loss of enzyme activity. Also, cellular metabolism is disrupted due to the dehydration of the protoplasm (Mahajan et al., 2005).

2.3.Plant response to stress:

Plants respond to abiotic stresses such as salinity and drought through abscisic acid (ABA)-dependent and/or independent signaling pathways, which ultimately lead to the expression of many stress related genes that causes physiological and biochemical changes for adaptation to the stress conditions (Xiong et al., 2002). During stress, diverse regulatory proteins are activated through signal transduction and different stress-responsive gene expression. Drought and salt stress also cause osmotic stress and the change in osmotic pressure triggers many signaling proteins, such as transcription factors, protein kinases and phosphatases (Figure 2.1). Different proteins like chaperones, late embryogenesis abundant (LEA) proteins are macromolecules and involved in cell protection and development. Different proteases, detoxification enzymes, ion and water channels and transporters, osmolyte biosynthesis enzymes among others which are involved in the stress tolerance response are called functional proteins. Different regulatory proteins are also involved in stress responses like transcription factors, protein kinases and phosphatases. Enzymes in phospholipid metabolism and Abscisic acid (ABA) biosynthesis are also includes in regulatory proteins (Shinozaki and Yamaguchi-Shinozaki, 2007).

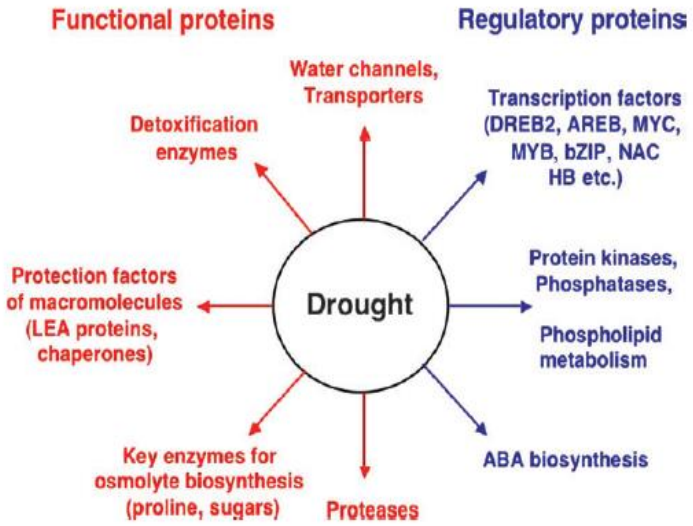


Figure 2.1: Drought stress-inducible genes in stress tolerance and response which are different functional proteins and regulatory proteins (Shinozaki and Yamaguchi-Shinozaki, 2007)

2.3.1.ABA pathway in stress tolerance:

Under abiotic stress condition, Abscisic acid (ABA) is produced and acts through downstream signaling pathways to control the stress response and tolerance of plants. ABA signaling pathway was identified in *Arabidopsis*, where it was reported that the PYR/PYL/RCAR ligand acts as a receptor which binds with ABA and inactivates the PP2C phosphatases in the presence of ABA, resulting in the activation of SnRK2 kinases. SnRK2 kinases in turn regulate downstream stress-responsive gene expression by reversible phosphorylation (Nakashima et al., 2009). The rice homolog was found for all these proteins (Zong et al., 2016) which are functionally similar. This pathway functions through stomatal closure mediated by the activation of different ion transporters or different transcription factors by direct phosphorylation. Activated transcription factors further regulate the expression of other stress responsive genes.

2.3.2.Role of osmolytes in salinity and drought stress:

Plants defend against abiotic stress involved to the association with different metabolites. Glycine betaine (N,N,N-trimethylglycine betaine), proline, raffinose are solutes, which are able to stabilize proteins and cellular structure and help in osmotic adjustment by maintaining cell turgor (Krasensky and Jonak, 2012). In plants overexpression of glycine betaine leads plants to tolerate stresses, including salinity stress (Chen and Murata, 2011). Accumulation of glycine betaine by overexpressing choline oxidase, which is the enzyme

for GB biosynthesis from glycine and choline, enhances drought tolerance in plants (You et al., 2019).

In plants proline acts as an osmolyte, a ROS scavenger and stabilize the structure of proteins, resulting in stress tolerance (Moukhtari et al., 2020). In higher plants, pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) are biosynthesis proline from glutamic acid (Delauney et al., 1993). It was found that overexpression of the P5CS gene resulting increased production of proline and higher level of salinity and drought tolerance in transgenic tobacco (Kishor et al., 1995). The exogenous application of proline also acts as osmo-protectant and helped the growth of plants under saline condition. During oxidative stresses, proline protect cell membranes by upregulating the activities of various antioxidants (Wang et al., 2017).

2.3.3.Reactive Oxygen Species (ROS) in early stress response in plants:

Accumulation of ROS (reactive oxygen species) to toxic levels, cause cellular damage under stress condition such as salinity and drought. In plant ROS are generated as the by-product of photoreaction and cellular oxidation. They are highly reactive molecules such as peroxides, superoxide, hydroxyl radical and singlet oxygen. Plant detoxify ROS and always maintained to low level. During stress condition high levels of ROS are produced in plants, that must be maintained for plant survival. Among different ROS, two most common nitric oxide (NO) and hydrogen peroxide (H_2O_2) have important signaling roles in plant stress (Jewell et al., 2010). Different antioxidant metabolites such as ascorbate, glutathione, tocopherols are scavenged ROS by ROS detoxifying enzymes such as superoxide dismutase, ascorbate peroxidase, and catalase (Mittler, 2002; Neill et al., 2002). Different transcription factors such as bHLH92 and WRKY33 helps in enhanced level of ROS detoxification in plants under stress condition (Jiang et al., 2009). It was also found that the transcription factors are linked with ROS scavenging pathways by increasing the level of expression of peroxidases and glutathione-S-transferases under osmotic and oxidative stresses (Miller et al., 2008).

2.3.4.Mitogen-Activated Protein Kinases in abiotic stress tolerance:

The mitogen-activated protein kinase (MAPK) mediated signaling pathway have plays an important role in the integration of physiological and cellular responses to abiotic stresses in plants. MAPK signaling pathway includes MAP3Ks (MAP2K kinase) serine/threonine

kinases, MAP2K (MAPK kinase) dual-specificity kinases and MAPK serine/threonine kinases (Colcombet and Hirt, 2008). This pathway involved in drought and salt stress response in rice and *Arabidopsis* (Kiegerl et al., 2000; Ning et al., 2010). MAPKs are enzymes that catalyse reversible phosphorylation, by sequential phosphorylation of a kinase by its upstream kinase via cascades (Xiong et al., 2006). MAPK cascade involved in osmotic stress responses during the accumulation of ROS. Oxidative stress induced ROS accumulation was controlled by MPK6 through the MAPK pathway (Kim et al., 2012). The function of MAP kinases in salinity stress response in plants also mentioned by Mishra et al., 2006.

2.4.Transcription Factors:

Transcription factors are proteins that binds to specific DNA sequences and controls gene expression. A large groups of transcription factor families in plants have been identified as AP2/ERF, bZIP, MYB and MYC, zinc-finger proteins, HD-ZIP, NAC, and etc. Among them many transcription factors play important roles in stress tolerance and response, by regulating the expression of many downstream genes under different stress conditions (Hirayama and Shinozaki, 2010; Song et al., 2016; Samad et al., 2017). They involved in feedback regulation of the upstream stress related genes and also interacting with different regulatory proteins to form a complex network for stress response. They are the most important regulatory proteins involved in abiotic stress responses. Abiotic stress tolerance is enhanced in plants characterized by different members of the DREB, MYB, bZIP, zinc finger, and NAC transcription factor families (Wang et al., 2008; Xiang et al., 2008; Huang et al., 2009; Su et al., 2010; Hu et al., 2006).

In *Arabidopsis thaliana* more than about 1500 transcription factors are found which are involved in stress tolerance and response through stress related gene expression (Riechmann et al., 2000). They function in both ABA dependent and independent manner in response to abiotic stresses. During abiotic stresses different stress responsive pathways are simultaneously activated by the influences of different transcription factors (Figure 2.2). Among them MYB (myeloblastosis), MYC (myelocytomatosis), AREB/ABF (ABA-responsive element-binding protein/ABA-binding factor), NAC (RD26) act through ABA dependent pathway and The NAC (NAM, ATAF1/2 and CUC2), HD-ZIP (Leucine zipper

homeodomain), DREB2 (AP2/ERF) involved in ABA independent pathway. The involvement of both pathways can provide good tolerance during stress condition.

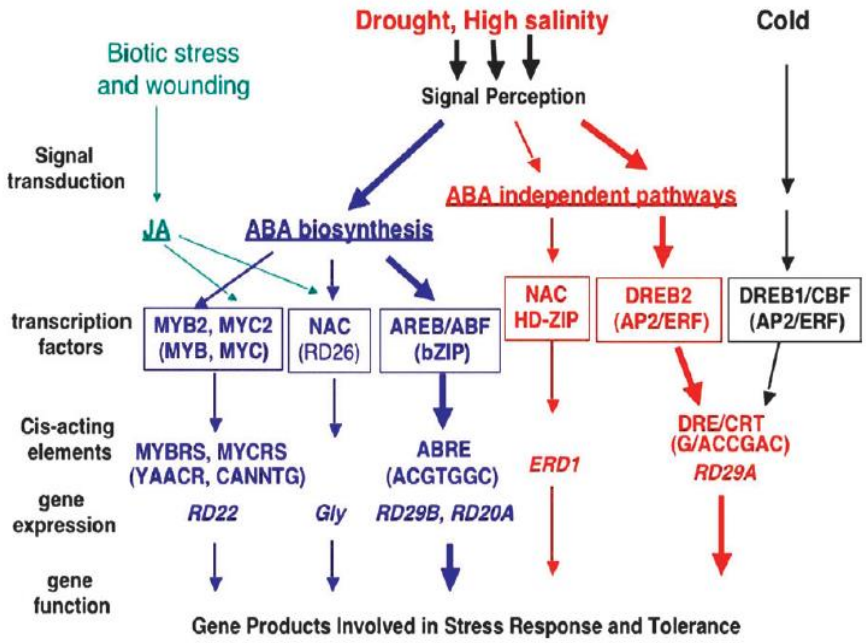


Figure 2.2: A schematic representation of transcriptional regulatory networks of cis-acting elements and transcription factors involved in abiotic stress responses (Shinozaki and Yamaguchi-Shinozaki, 2007).

2.5.NAC-transcription factors involved in abiotic stresses:

NAC (NAM, ATAF1/2, and CUC2) transcription factors are one of the largest members in transcription factor families. They are plant specific transcription factors (Hu et al., 2006). NAC transcription factors are involved in stress responses as well as different developmental processes. There are 151 NAC proteins present in rice (Nuruzzaman et al., 2010). Different plants have different number of NAC proteins. It was reported that 117 NAC genes are present in *Arabidopsis*, 79 in grape, 163 in poplar and 152 each in soybean and tobacco (Puranik et al., 2012). In stress response, NAC proteins are involved in both ABA dependent and ABA independent pathway (Nakashima et al., 2012).

2.5.1.NAC-recognition sites:

The transcription factors bind with the promoter of downstream genes and regulates their expression. They require the specific recognition sites in promoter region of these genes. For NAC transcription factors, recognition site is called NACRS which is CGT(G/A). in *Arabidopsis*, the promoter region of EARLY RESPONSIVE TO DEHYDRATION 1

(ERD1) gene contains core-DNA binding motif CACG for NAC transcription factors (Tran et al., 2004). This recognition sequence is conserved in plants (Bhattacharjee et al., 2017). NACRS also found in several pathogenesis-related (PR) gene promoters, responsive to biotic stress (Seo et al., 2010).

2.5.2.NAC-promoter contains different cis-elements:

Different cis-elements are identified in the promoter of many stress-responsive NAC genes such as, ABRE (ABA responsive element), DRE (dehydration responsive element), salicylic acid responsive element, and jasmonic acid responsive etc. (Nakashima et al., 2012). Under stress condition, these stress-related cis-elements are involved in the NAC genes regulation.

In rice several NAC gene promoters were studied for the presence of different abiotic stress-related cis-acting elements. The OsNAC5 and OsNAC6 promoters contained three ABA-responsive elements (ABREs; ACGTG G/T C) each, which are involved with abscisic acid (ABA)-responsiveness (Nakashima et al., 2007). One dehydration response element (DRE; G/A CCGAC) was found in the OsNAC3 promoter and two DREs were found in the *SNAC*1 promoter (Nakashima et al., 2012). DREs are the binding site for DREB (dehydration responsive element binding) proteins. Thus, although NAC genes are induced by similar abiotic stresses (salinity, drought, temperature, cold etc.), their level of expression may be regulated by different mechanisms.

2.6.*SNAC*1 (stress responsive NAC1) transcription factor in salt and drought tolerance:

*SNAC*1 (Stress-Responsive NAC1) transcription factor is one of the members of rice NAC protein family, which play a vital role in plant growth, transcription of downstream genes and stress responses. At vegetative stage, *SNAC*1 overexpressing rice plants showed better tolerance under drought and salinity stress conditions, and at reproductive stage showed greater seed setting under drought stress (Hu et al., 2006). Four haplotypes were found in genotyping of the *SNAC*1 promoter in rice germplasm. In field drought condition, the C1 haplotype confers stronger gene induction and showed better drought resistance than others haplotype (Songyikhangsuthor et al., 2014). It was localized in nucleus with transcriptional activation property (Hu et al., 2006). The *SNAC*1 promoter region contains two DREs sequences. *SNAC*1 protein is 314 aa long and NAC domain in sequence 1-176 bp in N-

terminal end (Figure 2.3). The C-terminal region diverse in length, called transcription regulatory (TR) region. The C-terminal regions of Os*NAC* proteins are highly diverse called transcription regulatory (TR) region and do not form any known protein domains. These different amino acid sequences in the C terminus provide diversification in the biological functions of OsNAC proteins (Takasaki et al., 2010). *SNAC*1 gene expression is regulated by DREB1/CBF-type transcription factors, which play an important role in tolerance to low temperatures, drought, and high-salt stress, by binding with CRT/DRE elements in promoter regions.

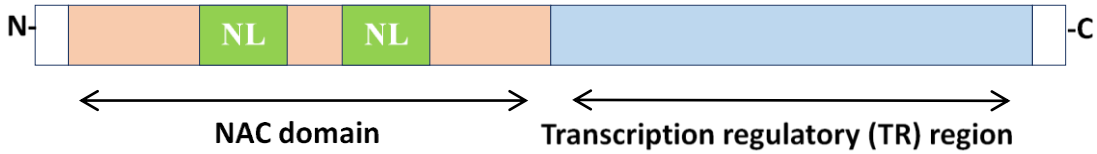


Figure 2.3: *SNAC*1 gene structure. It contains two Nuclear localization signal (NLS) sequence in NAC domain (Hu et al., 2006).

The crystal structure of *SNAC*1 NAC domain (Chen et al., 2011) showed the similar fold pattern with transcription factor ANAC NAC domain (Ernst et al., 2004), NAC domain in *Arabidopsis*. This folding provides conserved interaction which facilitates dimerization of the NAC proteins. The dimerization is conserved in NAC family (Figure 2.4).

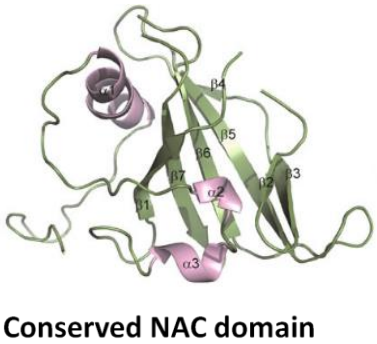


Figure 2.4: The crystal structure of *SNAC*1 NAC domain (Chen et al., 2011).

2.7.*SNAC*1 homolog and other NAC genes involved in stress tolerance:

Other than rice, many crops overexpressed their own *SNAC*1 gene and get better tolerance against salinity and drought stresses. Ta*SNAC*1 cloned and characterized in wheat showing stress tolerance in abiotic stresses (Liwei et al., 2012). Hv*SNAC*1 overexpressed in barley and Musa*SNAC*1 overexpressed in banana showed similar results (Abdallat et al., 2014;

Negi et al., 2018). *SNAC*2, a homolog of *SNAC*1, is responsive to various stresses and rice plants overexpressing *SNAC*2 results in increased cold tolerance and ABA sensitivity (Hu et al., 2008). *SNAC*3 gene also responsive to diverse stresses, confers increased resistance to both heat and drought stress in overexpressing rice plant. *SNAC*3 functions through regulating genes for detoxification of reactive oxygen species (ROS) in rice (Fang et al., 2015). OsNAC10 and OsNAC5 are also stress tolerance genes, conferred increased drought resistance and produced more grains when overexpressed in rice plants (Jeong et al., 2010, 2013; Takasaki et al., 2010). Overexpression of ONAC022 in rice (Hong et al., 2016) and T*sNAC*1 (Liu et al., 2018) also showed increased tolerance and resistance in abiotic stresses in plants.

Overexpression of OsNAC genes in another species may also improve drought tolerance. For example, overexpression of *SNAC*1 in wheat, cotton and ramie resulted in increased drought tolerance (Saad et al., 2013; Liu et al., 2014; An et al., 2015). In *Arabidopsis*, a rose NAC gene RhNAC3, induced by dehydration and ABA, confers drought tolerance through osmotic adjustment regulation (Jiang et al., 2014). It has been reported that in *Arabidopsis* Pg NAC21, ZmNAC55, Zm*SNAC*1, TaNAC2, Sb*SNAC*1 was characterize and confirms stress tolerances (Shinde et al., 2019; Mao et al.,2016; Lu et al., 2012; Mao et al., 2012; Lu et al., 2013). EcNAC1 from finger millet, overexpressed in tobacco confer abiotic stress tolerance (Ramegowda et al., 2012). Overexpression of MusaNAC68 in banana also enhance drought tolerance (Negi et al., 2016).

2.8.*SNAC*1 targeted downstream genes:

*SNAC*1 transcription factor binds to the cis-acting elements of their downstream gene promoters, regulating their expression and facilitate plant adaptation to different abiotic stresses (Leng and Zhao, 2020). It was found that two genes, OsSRO1c and OsPP2C18, which showed increased expression in *SNAC*1 overexpressing plants (microarray data, Hu et al., 2006), were directly regulated by *SNAC*1 transcription factor (You et al., 2013, 2014). OsSRO1c is predominantly expressed in guard cells under drought stress. (You et al., 2013). Overexpression of OsSRO1c (similar to RCD one, radical-induced cell death 1 in *Arabidopsis*) increased plants stress tolerance through reduced water loss by stomatal closure and oxidative stress tolerance by regulating H_2O_2 homeostasis in rice. It was found that OsPP2C18 suppressed rice plants, through artificial microRNA, were very hypersensitive to drought stress and overexpression of OsPP2C18 in rice led to enhanced

osmotic and oxidative stress tolerance. Different reactive oxygen species (ROS) scavenging enzymes were downregulated in the ospp2c18 mutant, indicating that this protein works on ROS pathway. OsPP2C18 expression was induced by drought stress but not induced by abscisic acid (ABA), indicating that this protein phosphatase enzyme did not work through the SNF1-RELATED PROTEIN KINASE2 (SAPK2, a component of ABA signal transduction in rice identified by Kim et al., 2012) protein kinases, which function in ABA signaling pathway. *SNAC*1 directly regulate OsPP2C18 resulting enhanced drought and oxidative stress tolerance by regulating ROS homeostasis through ABA-independent pathways.

*SNAC*1 targeted or downstream genes were identified in genome-wide scale (Li et al., 2019) by Chromatin immunoprecipitation sequencing (ChIP-Seq) and RNA-Seq, in *SNAC*1 overexpressed rice and wildtype (WT) under normal and drought stress conditions. 93 *SNAC*1-targeted genes were identified related to drought resistance. Most of these genes are involved in transcriptional regulation, response to water loss, and other stress processes. Among these genes it was found that *SNAC*1 can bind to the OsbZIP23 promoter, a key ABA signaling regulator (Zong et al., 2016). It was reported that Os*MFT*2 is involved in the regulation of ABA signaling-mediated seed germination through interacting with OsbZIP23 in rice (Song et al., 2020). It was found that *OsMFT2* was negatively regulates seed germination in rice. Pre harvest sprouting was found in *OsMFT2* knock-out lines, whereas delayed germination was observed in *OsMFT2* overexpressed lines. Drought responsive genes were regulated by transcription factor OsbZIP23 and histone modification occur synergistically (Zong et al., 2020). Os*NAC*5, a transcriptional activator, also localized to the nucleus. Pull-down assays revealed that Os*NAC*5 interacts with *SNAC*1 (Takasaki et al., 2010). The *SNAC*1-targeted genes provide light insights into the molecular mechanism of drought response and abiotic stress tolerance pathways.

2.9.rd29A-Stress inducible promoter:

Growth retardation was found by constitutively expressing the transcription factors under unstressed conditions (James et al., 2008; Kasuga et al.,1999). However, recent evidence indicates that inducible promoters can be used to drive transgenes expression in an ideal temporal and spatial fashion. In comparison with constitutive promoter, this type of promoter only switches on genes transcription when the internal or external stimuli appear,

this strategy thus can provide better potential for the improvement of abiotic stress tolerance in plants.

To date, the research on the promoter of LEA-protein-like *rd29A* gene in *Arabidopsis* shows important advances. They discovered that the expression of DREB1A/CBF3 with the inducible rd29A promoter reflects no visible effects on *Arabidopsis* and wheat plants growth while providing an even greater tolerance to several stress conditions than do overexpression of DREB1A/CBF3 with *CaMV*35S promoter (Kasuga et al., 1999; Pellegrineschi et al., 2004). Similarly, another report also showed no deleterious effects on the transgenic Bahiagrass plants transformed with the inducible-promoter HVA1-*DREB1A/CBF3* fusion (James et al., 2008).

The promoter regulates gene expression by those regulatory elements either enhancing (enhancers) or repressing (repressors) the transcription efficiency. Many such kinds of *cis*-acting elements were discovered in the response to one specific or several environmental signals, such as ABA-responsive element (ABRE) responsive to ABA treatment and drought-responsive element/C-repeat (DRE/CRT) in response to high salinity, dehydration, and low temperature. rd29A promoter contains two DRE sequences, one DRE/CRT core and one ABRE element (Figure 2.5), shows that it can induced in both ABA responsive and other stress induced pathways.

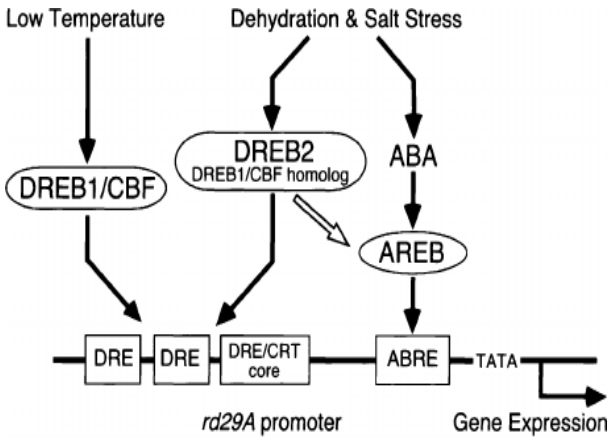


Figure 2.5: Stress inducible promoter rd29A (Kasuga et al.,1999)

There are some promoters for use with stress-inducible gene expression in plants, and in particular for monocotyledonous crops such as rd29B, Wsi18, Lea3, Uge1, Dip1, R1G1B

that were induced by drought stress in rice microarray experiments (Ingram and Bartels, 1996; Msanne et al., 2011).

As we don't need the large number of genes, related with stress resistance or tolerance will be expressed continuously, this will negatively affect plant growth and crop yield. The genes will only be expressed in response to stresses is desirable. Use of rd29A stress inducible promoter along with target gene can provide the advantage.

2.10.Focus on high yielding rice varieties:

High yielding rice varieties are superior cultivars, with high crop yield per area (hectare). They were created as a consequence of the green revolution to increase global food production. They are dwarf in size, with early maturation. These however require higher level of fertilizer and controlled water supply. Bangladesh Rice Research Institute (BRRI) developed a group of high-yielding rice varieties BRRI Dhan for different seasons with their own different level of tolerance to abiotic stresses.

BRRI Dhan-55 -This is an Aus and Boro season variety, released by Bangladesh Rice Research Institute (BRRI) in 2011. Plant height remains 100 cm with white slender grain. This variety considered as slightly saline and drought stress tolerant. At the Aus season, it has an early maturation of about 105 days and possesses yields of about 5.0 ton/hector. At the Boro season, its life span is 145 days with high yield of about 7.0 ton/hector.

BRRI Dhan-56 -This is an Aman variety, released by Bangladesh Rice Research Institute (BRRI) in 2011. This is a slightly drought tolerant variety. It possesses tall and erect leaves. It contains long and bold white seed. The plant height is 115 cm and yield level is about 4.5-5.0 ton/hectors. With a short life span of about 105-110 days, it is suitable for growing in drought-prone areas. Strong plants, medium height and high yield make this variety farmer popular.

BRRI Dhan-49 -This is an Aman variety, released by Bangladesh Rice Research Institute (BRRI) in 2007. Plant height remain 100 cm with upright leaf and strong stem. It contains small white seeds and yield level is about 5.0 ton/hectors. Its needs 135 days for maturation. This is a salt sensitive variety.

3. Methods and Materials

Transcription factors are playing their role in plant growth, development and stress tolerance. The NAC transcription factor family is involved in drought and salinity stress directly (Fang et al., 2008). *SNAC*1 (a member of NAC family) was reported that, over expression of *SNAC*1 in *japonica* rice cultivar Nipponbare showed enhanced salt and drought tolerance in transgenics and increase in grain yield under field stress condition compared to control (Hu et al., 2006).

The objectives of this study were to characterize *SNAC*1 transcription factor in *indica* rice Binnatoa (BA) under constitutive promoter *CaMV*35S. Previously *SNAC*1 was cloned from tolerant cultivar Pokkali and inserted into destination vector pH7WG2 to produce pH7WG2_*CaMV*35S_*SNAC*1 construct (Abdullah-Al-Emran et al., 2010). Binnaroa (BA) was transformed with this construct by tissue culture method (Abdullah-Al-Emran et al., 2010). Here, in this present work *CaMV*35S_*SNAC*1_BA lines were characterized in both seedling and reproductive stages. They were subjected to both salinity and drought stresses. To analyze the effect of *SNAC*1 under stress inducible promoter, rd29A promoter was clone from *Arabidopsis* and pH7WG2_rd29A_*SNAC*1 construct were prepared. Different high yielding *indica* rice cultivars were transformed with pH7WG2_rd29A_*SNAC*1 construct to understand different genomic background effect. For these slightly drought tolerant BRRI Dhan-56 and salt sensitive BRRI Dhan-49 were transformed with *SNAC*1 under stress inducible promoter (pH7WG2_rd29A_*SNAC*1 construct). Farmer popular moderately drought and salt tolerant variety BRRI Dhan-55 was transformed with *SNAC*1 under both constitutive promoter and stress inducible promoter (both pH7WG2_rd29A_*SNAC*1and pH7WG2_*CaMV*35S_*SNAC*1 constructs). All high yielding rice varieties were transformed by tissue culture independent *in planta* transformation.

3.1.Rice varieties used for transformation:

In this study one local landrace Binnatoa (BA) and three high yielding rice varieties BRRIdhan-55, BRRIdhan-56 and BRRIdhan-49 were used. All these are *indica* rice varieties. High yielding rice varieties were developed by BRRI (Bangladesh Rice Research Institute) and approved by National Seed Board. These are farmer popular high yielding varieties with their own specialties.

Binnatoa is an *indica* landrace, grown in the coastal regions of Bangladesh. Binnatoa has salt-tolerance equivalent to about 60% of Pokkali, the benchmark for salt tolerance in rice.

It also shows about 96% regeneration potency from callus derived from mature embryo. (Seraj et al., 1997).

Table 3.1: Characteristics of high yielding rice varieties used in this study.

Characteristics	BRRI Dhan-55	BRRI Dhan-56	BRRI Dhan-49
Yield	high yield	high yield	high yield
Season	Aus and Boro variety	Aman variety	Aman variety
Grain	White, Long and slender	White and medium slender	White and small
Tolerance level	Moderate salinity, drought and cold stress tolerant	Slightly drought tolerant	Salt sensitive
Height (cm)	100	115	100
Life span (days)	105/145	105-110	135
Sowing time	April/ November	July	June-July
Harvesting time	July/March	October-November	October
Yield (ton/hectare)	5.0-7.0	4.5-5.0	5.0

Source: *Bangladesh Rice knowledge Bank*

3.2.Construct preparation for transformation:

The stress inducible promoter rd29A (597bp) was isolated from *Arabidopsis* and cloned by restriction digestion directional cloning into pH7WG2.0 binary vector by removing the constitutive promoter *CaMV*35S from the vector and inserting the rd29A promoter in place of *CaMV*35S (Muntasir, unpublished data). This replacement created a new rd29A_pH7WG2.0 binary vector where the gene cloned through LR reaction will be expressed under stress inducible promoter rd29A.

*SNAC*1 cDNA (1051 bp) was isolated from salt tolerant rice variety Pokkali, and successfully cloned into the pENTR vector and subsequently mobilized into the *Agrobacterium* compatible destination vector pH7WG2 (Figure 3.1) for rice transformation (Abdullah-Al-Emran et al., 2010). Here *SNAC*1 was expressed under *CaMV*35S constitutive promoter (*CaMV*35S_*SNAC*1 construct). *SNAC*1 cDNA was also mobilized into rd29A_pH7WG2.0 vector to produce rd29A_*SNAC*1 construct where *SNAC*1 was expressed under rd29A stress inducible promoter (Figure 3.2). Two constructs were used in this study namely pH7WG2_*CaMV*35S_*SNAC*1 construct and pH7WG2_rd29A_*SNAC*1 construct.

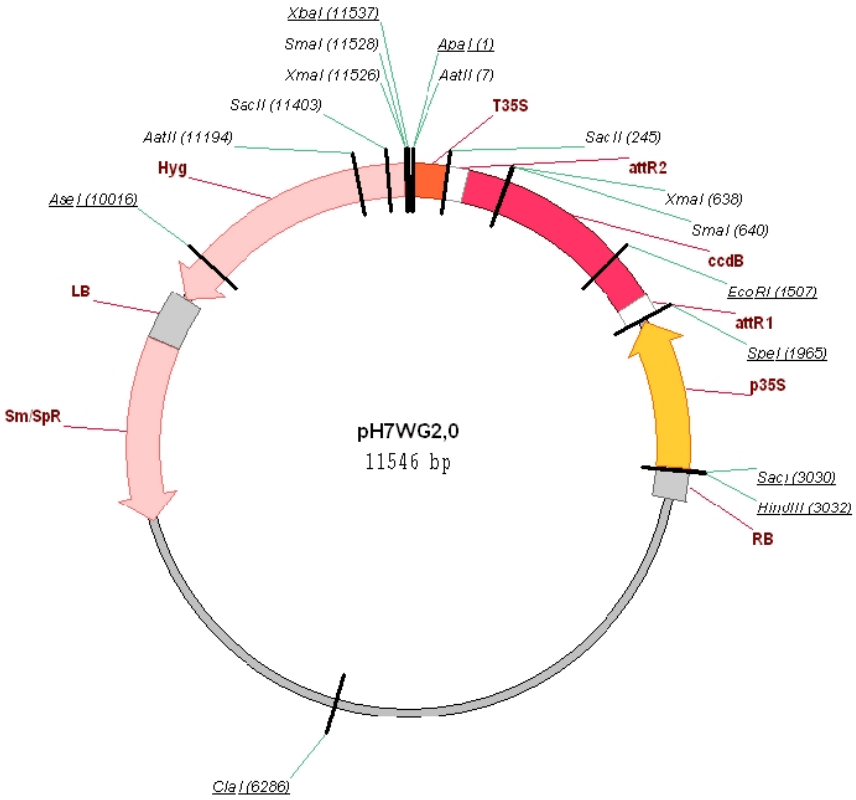


Figure 3.1: Map of binary vector pH7WG2.0.

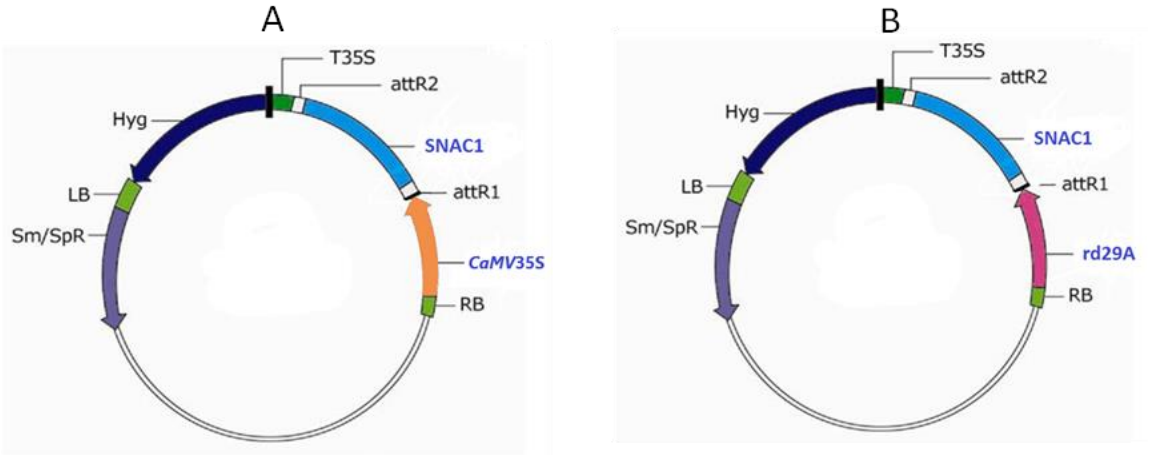


Figure 3.2: Modified pH7WG2.0 construct. **A)** *CaMV* 35S_*SNAC*1 construct. **B)** rd29A_*SNAC*1 construct where *CaMV*35S promoter is replaced by rd29A promoter. The ccdb region is replaced by *SNAC* I gene after LR recombination in both cases.

3.3.Plant Transformation Methods:

Local land race Binnatoa was transformed with *CaMV* 35S_*SNAC*1 construct by tissue culture technique (Abdullah-Al-Emran et al., 2010). But High yielding rice varieties are not tissue culture responsive. So tissue culture independent *in planta* transformation

method (Lin et al., 2009) was used to transform BRRIdhan-55, BRRIdhan-56 and BRRIdhan-49 with rd29A_*SNAC*1 construct.

3.3.1.*In planta* transformation:

a) Sterilization and germination of seed:

Mature seeds of individual variety were washed in 99% ethanol for 3 minutes. The ethanol was then poured off and washed in 30% chlorox with 1 drop of tween20 for 3 minutes. Then chlorox was poured off and the seeds were washed with ddH_2O for five times. On a filter paper the seeds were placed and soaked with ddH_2O and incubated at 37˚C for 2 days. It would take 2 days the embryo region turned white (it depends on individual varieties).

b) Preparation of bacterial solution:

Agrobacterium tumefaciens strain LBA4404 containing pH7WG2_promoter_gene construct was cultured on solid YM [Appendix 04, Table1.1] agar media containing appropriate antibiotics and incubated at 28˚C for 2 days for colony formation. Next *Agrobacterium* single colony was picked up from petri-dish, inoculated into the liquid YM medium and were *incubated* at 28˚C for overnight. Next morning *Agrobacterium* culture mediums were precipitated by centrifugation (8000 rpm, 15-20 min) and the pellet were re-suspended in Bacterial Re-suspension Media [Appendix 04, Table 1.2]. After Re-suspending, centrifugation was repeated (8000 rpm, 15-20 min) for removing the trace of antibiotic. Then *Agrobacterium* culture pellets were re-suspended again in Bacterial Re-suspension Media. Finally, at OD_{600} bacterial density was measured and the final absorbance was made to 0.6. This bacterial cell suspension was used as the *Agrobacterium* inoculums.

c) Infection of seeds by *Agrobacterium* inoculum:

Agrobacterium tumefaciens was inoculated into the embryonic apical meristem of the soaked seed by pierced to a depth of 1-1.5 mm with a needle that dipped in the bacterial solution. (to avoid seriously damage of the embryo the needle should pierce the side of the plumule).

d) Vacuum infiltration:

The pierced seeds were then placed in a reagent bottle and soaked in the *Agrobacterium* inoculums. The reagent bottle with the seeds was placed into a bell jar. A vacuum pump

was used to drawn out the air in the bell jar at a pressure of 80kpa for 15min. After 2 min the vacuum was released and pumped again for 3min.

e) Antibiotic treatment and transfer in hydroponic solution:

The inoculated seeds were transferred onto wet filter paper containing petri-dishes. Petri-dishes were kept in the dark for 6-7 days at 28˚C temperature. After 7 days of incubation the inoculated seeds began to germinate. The seedlings were treated with 250mg/L carbenicillin solution for 1h to remove the remainder *Agrobacterium*. Seedlings were washed well with ddH_20 and transferred to new petri dishes containing wet filter papers. Seedlings were then kept in light for 16 hours and in dark for 6 hours. When the seedlings turn into green, they were transferred to hydroponic solution [Appendix 04, Table 1.3]. After 7-10 days later, the seedlings were mature enough to transfer to soil.

3.4.Confirmation of the transformation

Each putative transformed plant was tested to find the transgenic lines. Phenotypic and physiological screening were done as well as molecular analysis. The tests were done mentioned below-

- **Phenotypic and physiological screening-**
  a) Leaf disk senescence (LDS) assay
  b) Measurement of chlorophyll content
  c) Hygromycin resistance assay
  d) Measurement of relative electrolyte leakage
  e) Malondialdehyde (MDA) test
  f) Measurement of hydrogen peroxide (H_2O_2) level
  g) Relative water loss
  h) Survival assay in salt condition
  i) Survival assay in drought condition
- **Molecular analysis of the transformants-**
  a) Isolation of DNA
  b) Polymerase chain reaction (PCR) analysis of transformants
  c) Southern blot hybridization
  d) Isolation of RNA
  e) Expression analysis by semi-quantitative RT-PCR
  f) Expression analysis real time PCR (qRT-PCR)
- **Data analysis**
  a) Calculation of the transformation efficiency
  b) Segregation analysis of the transformed plants
  c) Statistical analysis

3.4.1.Phenotypic and physiological screening:

a) Leaf disk senescence (LDS) assay:

After *in planta* transformation, the seedlings were transferred in hydroponic solution and finally in soil. They were allowed to grow. Leaf disk senescence test was done when the flag leaf emerged. A small length of flag leaf was cut. The piece of leaf was cut into approximately same size (~1.0 cm). Leaf disks were taken from both transgenic lines and wildtype plants. The disks were floated in a petri-dish with 20ml solution of NaCl with 100mM or 200mM salt concentration or water (for control) for 5-7 days and the temperature were maintained at 25˚C. Three independent experiments were done with three biological replicates.

b) Measurement of the chlorophyll content of transformants:

Leaf disk of both wildtype and transgenic lines from used in leaf disk senescence (LDS) assay were weighed and kept in a bottle containing 12.5 ml of 80% acetone. After 48 hrs absorbance of leaf tissues extract was measured by spectrophotometer at wavelength 663 and 645 nm for chlorophyll a and chlorophyll b. The total amount of chlorophyll was calculated following the protocol by Yoshida et al. (1976) and Chutia and Borah (2012). The chlorophyll content was calculated in the diluted sample using the following equation.

$A = ECd$

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

Here,

$A = observed\ absorbance$

$E = a\ proportionality\ constant (extinction\ co\ efficient)\ \left({}^{36mL}/_{cm}\right)$

$C = chlorophyll\ concentration\ \left({}^{mg}/_{mL}\right)$

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

Reduction of chlorophyll content was determined using following formula:

$$Chlorophyll\ reduction = {}^{(Control - Stress)}/_{Control} \times 100$$

The spectrophotometer was prepared to read the absorbance of the diluted chlorophyll extract. The wavelength was adjusted to read 645nm and 663nm. The spectrophotometer was set to blank with the reagent blank (80% acetone) to read 0 absorbance (right-hand

knob). Some of the diluted chlorophyll extract was transferred to a cuvette and the absorbance was read. Finally, the absorbance was recorded.

c) Hygromycin resistance assay:

The T_2 seeds are germinated normally at 37 ˚C temperature. The germinated plants when began to be green, hygromycin solution (20 mg/L) with ½ strength MS media added to the plants in plates. They kept in solution until the wildtype plants died. The transformed plants remain green and healthier than the untransformed and wildtype plants. Then the selected plants were transferred to hydroponic solution (Parvin et al., 2015).

d) Measurement of relative electrolyte leakage:

Measurement of relative electrolyte leakage was done by using the protocol of Cao et al., 2007, Yasmin et al., 2015. The leaf segments from the seedlings of transgenic and wildtype plants were taken into a bottle containing deionized water and kept in shaker for 2 hours. Then the conductivities (C1) of the solutions were measured. Then the leaf segments in deionized water were autoclaved. After cooling in room temperature, the conductivities (C2) of the solutions were measured. The values of C1 to C2 (C1/C2) were calculated and used to estimate the relative electrolyte leakage. Results represent average from five replicates.

e) Estimation of Malondialdehyde (MDA) content:

Malondialdehyde (MDA) is one of the end products of oxidative alteration of lipids and a good indicator of cell membrane damage due to lipid peroxidation under stress conditions. To measure MDA content, protocol of Negi et al., (2015) was followed. 100mg of leaves was homogenized in 10ml of 10% TCA (Trichloro Acetic acid). Then the homogenate was transferred to screw capped tube and centrifuged at 4000 rpm for 20 minutes. After centrifugation, 2 ml of supernatant was added to 2 ml of 0.6% TBA (Thiobarbituric acid, made in 10% TCA) and the mixture boiled at 95º-100ºC for 20 minutes. After boiling the tubes were placed quickly on ice to stop the reaction. Then the absorbance of the supernatant was measured at 532 nm and adjusted for nonspecific absorbance to 600 nm. five replicates were measured for each line.

The amount of MDA was calculated using the following equation:

$$MDA\ content = 155 \times (OD_{532nm} - OD_{600nm})\ mmol\ g^{-1}FW$$

$$155 = Extinction\ coefficient$$

$$OD_{532\ nm} = For\ MDA - TBA\ adduct$$

$$OD_{600mm} = For\ non - specific\ turbidity$$

f) Measurement of hydrogen peroxide (H_2O_2) level:

0.3 g of plant leaf were taken from the seedlings of both wildtype and transgenics. In presence of liquid nitrogen, they were ground to a fine powder and 5 ml of 0.1% (w/v) TCA was added. At 12,000 rpm, the homogenate was centrifuged for 15 min at room temperature. In a fresh screw capped tube 3 ml of supernatant was collected. Then 1 ml of potassium iodide (1 M) and 0.5 ml of 1 M potassium phosphate buffer (pH 7.0) were added in the tube. Then the absorbance of the mixture was taken at 390 nm. Here, 1 ml of 0.1% (w/v) TCA and 1 ml of potassium iodide was used as the blank (Negi et al., 2015).

The amount of H_2O_2 was calculated using the equation:

$$H_2O_2(\mu mol.\ g^{-1}FW) = 1 + (227.8 \times OD_{390})$$

Percentage increase in hydrogen peroxide level was determined using following formula:

$$Increase\ in\ H_2O_2\ level\ = \left({}^{(Stress\ -\ Control)}/_{Control}\right) \times 100\%$$

g) Relative water loss:

Relative water loss was measured under dehydration conditions. Fully grown leaf was detached from plants and exposed to air at room temperature (25˚C). The leaves were weighed at 0, 15 min, 30 min, 45 min, 1hr, 2hr, 3hr, 4hr and 24 hr after being cut off. Zero (0) hr weight were count as fresh weight (FW). The leaves were finally dried in oven at 80˚C for 48 hrs to a constant dry weight (DW) (Zhang et al., 2011).

The water loss rates were calculated by the formula:

$$water\ loss\ rate\ (\%) = {}^{(FW - desiccated\ weight)}/_{FW} \times 100$$

h) Survival assay in salt condition:

The germinated seeds of transgenic lines and their respective wildtype parents were grown in netted Styrofoam floater in PVC tray containing 10L Yoshida solution (Yoshida et al., 1976). At four-leaf stage (14–18 days from germination) of seedlings, NaCl stress was applied gradually starting from 6 dS/m to 20 dS/m at 24 h increments of 2 dS/m. After 7-10 days, when 90% of wildtype plants about to die, the stress was stopped, and the floater were kept in Yoshida solution without salt for recovery. After one week of recovery, the number of survived plants were counted. The experiment was repeated three times.

i) Survival assay in drought condition:

Both transgenic and their respective wildtype plants were grown in the same pot. The pot was filled with same weight of thoroughly mixed soil. 12 plants of each type were grown for two weeks (contain four leaves). Drought stress was applied by withholding water for 12 days, wildtype plants were about to die. The plants were re-watered for 14 days. After recovery, survived wildtype and transgenic plants were counted (Hu et al., 2006). Experiment was repeated three times.

3.4.2.Molecular Analysis:

a) Plant DNA isolation (CTAB method):

Genomic DNA was isolated from the upper leaves of wildtypes and transformants. In liquid nitrogen the leaves were crushed to powder and DNA was isolated using CTAB method and mini preparation. The CTAB method provides a less expensive procedure and is characterized by high yields of DNA from a small amount of tissue (Doyle and Doyle, 1987). The main drawbacks of this procedure are less pure DNA will obtain, time consuming and laborious.

[DNA Isolation, quantification and concentration determination are described in Appendix 05. DNA isolation of T_0 plants by Short Method (IRRI): This procedure of DNA isolation explained in Appendix 06].

b) Polymerase Chain Reaction (PCR) analysis of transformants:

The pH7WG2_*CaMV*35S_*SNAC*1 construct and pH7WG2_rd29A_*SNAC*1 construct contain *CaMV*35S and rd29A promoter region respectively and *SNAC*1 gene both. Primers for these genes were used for DNA amplification by PCR to confirm the transformation.

The primers were diluted in TE buffer. PCR program was carried out as follows: initial denaturation at 95˚C for 5 min, followed by 35 cycles of 1 min at 95˚C, 1.30 min at respective annealing temperature and 1.30 min at 72˚C, then a final extension of 10 min at 72˚C.

Table 3.2: The primers used in Polymerase Chain Reaction (PCR) analysis of T_0 and T_1 transformants.

Primer	Sequence	Tm	Product size
*SNAC*1_F	5' AGAAGCAAGCAAGAAGCGAT 3'	57^0C	1051bp
*SNAC*1_R	5' CCGAGCCATCTCTTGAC 3'		
*CaMV*7wg2 _F	5' GTTTGTTGTTTGTTTTGTTGTGG 3'	62.5^0C	1000bp
*CaMV*7wg2 _R	5' GGTCGACTAGAGCCAAGCTG 3'		
rd29A_F	5' CACCTGAGGAATATTCTCTAGTAAGATA 3'	63^0C	597 bp
rd29A_R	5' GTAATCAAACCCTTTATTCCTGATGATTG 3'		

c) Southern blot hybridization:

20 µg of genomic DNA from both wild-type and transgenic plants of T_3 generation of *CaMV*35S_*SNAC*1_BA and rd29A_*SNAC*1_56 was digested with N*he*I restriction enzyme. The digested products were electrophoresed and blotted onto a positively charged nylon membrane (Hybond N+ membrane, Amersham, UK) and probed using DIG-labelled PCR-amplified product (intron spanning 500 bp, only binds with transgene not internal own gene) from *SNAC*1 gene following DIG Luminescent Detection Kit standard protocol (Roche Diagnostics Inc., Mannheim, Germany).

The genomic DNA from transformants of the *CaMV*35S_*SNAC*1_55, rd29A_*SNAC*1_55, and rd29A_*SNAC*1_49 was digested by B*amH*I restriction enzyme, with same probe and same protocol. This work was done at ICGEB (International Centre for Genetic Engineering and Biotechnology), New Delhi, India, under the India Science and Research Fellowship (ISRF) Programme, 2015.

Table 3.3: *SNAC*1 primers (Internal) for Southern blot hybridization.

Primers	Sequence	Annealing temperature
*SNAC*1_P_F	5' GCCGAGGTGGATCTCTACAA 3'	63.3 ˚C
*SNAC*1_P_R	5' GTTGTCCACGATCTCCGACT 3'	62 ˚C

d) RNA extraction and semi quantitative RT-PCR analysis:

Total RNA was extracted from the transgenic lines of *CaMV35S_SNAC*1_BA and wildtype BA for semi-quantitative RT-PCR analysis to find transgene expression. The Trizol method was used for RNA isolation [Appendix 07] and followed the manufacturer's protocol. From 1.5 µg of total RNA, first-strand cDNA was synthesized using the Invitrogen Superscript III reverse transcription RT-PCR as described by the manufacturer's protocol (Invitrogen, USA). Elongation Factor-α (EF- α) was used as the normalization control.

Table 3.4: The primers for elongation factor-1α.

Name	Sequence	Length (bp)	Tm
eEF_1α_F	5' TTTCACTCTTGGTGTGAAGCAGAT 3'	24	62˚C
eEF_1α_R	5' GACTTCCTTCACGATTTCATCGTAA 3'	25	63.4˚C

e) Quantitative real time PCR for *SNAC*1 gene expression in high yielding transgenic lines:

Total RNA was extracted from plant leaf of 20-day-old seedlings of T_3 generation of transgenic lines and wildtype rice varieties according to the TRIzol_reagent (Invitrogen) manufacturer's instructions. First-strand cDNA was synthesized from 2.0 µg of total RNA using the Thermo Scientific Revert Aid H Minus First Stand cDNA synthesis kit following the manufacturer's protocol. Quantitative Real-time PCR was performed in a 15 µl reaction using Power SYBR® Green PCR Master Mix of Applied biosystems with *SNAC*1 internal primers in Applied Biosystems 7500 Fast Real-Time PCR System**.** Elongation Factor-α (EF-α) was used as the normalization control. Relative transcript abundance was calculated using the comparative cycle threshold method described by Chen et al (2014). This work was done at ICGEB (International Centre for Genetic Engineering and Biotechnology), New Delhi, India, under the India Science and Research Fellowship (ISRF) Programme, 2015.

Table 3.5: The Primers used for real time PCR (qRT-PCR).

Primer	Sequence	Length (bp)	Tm
Forward primer	5' GTGGGCGCACGCTTGGGATC 3'	20	63.3^0C
Reverse primer	5' GTTGTCCACGATCTCCGACT 3'	20	62^0C

3.4.3.Data Analysis:

a) Calculation of the transformation efficiency:

Transformation efficiency was calculated based on the result of the leaf disk senescence (LDS) assay. Following formula was used-

$$Transformation\ efficiency(TE) = \frac{\substack{No.of\ leaf\ disk\ positive\ \ plants \\ tested\ on\ flag\ leave\ (b)}}{\substack{No.of\ the\ germinated\ seedlings \\ after\ infection\ (a)}} \times 100\%$$

b) Segregation analysis of the transgenes for T_1 transgenic lines:

Segregation analysis was done based on the result of the leaf disk senescence assay/ positive PCR test of the T_1 seedlings. Chi-square is a statistical test performed to determine the difference between the observed and the expected data under the specific hypothesis.

$$Chi - square\ test\ or\ X^2test = \frac{(observed - expected)^2}{(expected)}$$

The **p** value is the probability that the deviation of the observed from that expected is due to chance alone (no other forces acting)

$$P\ value = \left[\frac{0.5^{df/2}}{T\left(df/2\right)}\right] \times (\chi^2)^{\left(df/2\right)-1} \times e^{-\chi^2/2}$$

Chi-square test (χ^2) was done to analyze the inheritance pattern of T_1 transformants of transgenic lines.

c) Statistical analysis (ANOVA):

Data Analysis ToolPak of Microsoft Office Excel 2007 and CropStat was used for statistical analysis. Analysis of variance (ANOVA) was done, and results were prepared assuming equal variance or unequal variance as applicable, to compare significant differences between the transgenic and the wildtype plants at $P<0.05$ level. The F test was performed to verify equal variance of the independent set of samples and based on that results the Student's t test was done (*, **, *** at $P<0.05$, $P<0.01$, $P<0.001$). Duncan's Multiple Range Test (DMRT) was used to differentiate between the treatments and lines in comparative assay.

3.5.Comparative assay in between high yielding rice varieties:

3.5.1. Salinity stress tolerance at seedling stage:

The phenotypic screening for the salinity tolerance at seedling stage was done on the T_3 population of transgenic lines and respective wildtypes. Pokkali and IR 29 rice varieties were used as salt tolerant and salt sensitive control in screening. Germinated seeds were sown in netted Styrofoam, floated in PVC trays containing 10L Yoshida solution (Yoshida et al., 1976). Each tray contains six lines of transgenics (9 for each in 6 rows) arranged in a completely randomized way and wildtype, salt tolerant and salt sensitive control in other different three rows. At four-leaf stage (14–18 days from germination) of seedlings, NaCl stress was applied gradually starting from 6 dS/m to 12 dS/m at 24 h increments of 2 dS/m. Two trays were remained as control tray without salt stress. By using a conductivity meter (Lutron CD4301, Taiwan), the EC (Electrical Conductivity) of the solution was maintained at 12 dS/m until the end of the experiment. After 8–10 days, when 90% of IR29 (sensitive control) were about to dead in stress condition, tolerance-related traits (Standard Evaluation System (SES) score, root length, shoot length, shoot weight) were measured from all stressed and controlled plants. The level of salinity tolerance was calculated based on the percentage of leaf damage and score accordingly (Gregorio et al., 1997). The chlorophyll content and electrolyte leakage of the stressed and control transgenic plants as well as WT were measured at this stage. Also, Na^+ /K^+ ratio in shoot and root were measured at this stage (Amin et al., 2012).

3.5.2.Drought tolerance at seedling stage:

For seedling stage drought screening, all the transgenic lines were grown in the same pot with their respective wildtypes. 10 of each types of plants were grown in the same pot until four leaf of stage (about two weeks of age) (Fukao et al., 2011). Drought stress was applied by the total withheld of water for 8 days and then re-watering for 14 days. Fresh weight, shoot length, root length and chlorophyll content of both wildtype and transgenic lines were measured after recovery (Hu et al., 2006).

3.5.3.Salinity tolerance at the reproductive stage:

Germinated seeds of transgenic lines and wildtype were grown in hydroponic system in Yoshida solution. Here IR29 and Pokkali were used as salt tolerant and salt sensitive control respectively. Two weeks old seedlings were transferred into soil filled perforated pots. Each

pot contained single plant. The temperature of the net house was varied from 30-32 °C in the day and 24-25°C during the night and humidity were 72%. The pots were placed in bowls of water, with six pots in each bowl. Tolerant and sensitive control, wildtype and three transgenic lines were placed in each bowl. At 4weeks of age, near booting stage, pots were transferred in bowls filled with 8 dS/m NaCl in Yoshida solution. Some bowl with same transgenic and control plant were kept aside with water, served as plants without stress. Seven biological replicates of each lines were maintained here. The salinity was kept at the same level throughout the experiment until completion of the life cycle of the plants. When 80% grain mature, some physiological parameters were recorded such as flowering date, plant height (from the base of each plant up to the tip of the panicle), number of total tiller, effective tiller per plant, panicle length, flag leaf length. At the end of reproductive stage screening, seeds were collected from transgenic and wildtype plants as well as tolerant and sensitive control plants. Other yield-related traits such as spikelet per panicle, spikelet fertility, yield (g/plant), 1000 grain weight were also measured.

3.5.4.Reproductive stage drought screening:

At T_4 generation all transgenic lines and respective wildtype plants were germinated and grown in floater in hydroponic solution with Yoshida solution. After two weeks they were transferred into individual soil filled pot. Same sized pot was used, contained 8 kg of thoroughly mixed soil with fertilizer. Plants were there grown for 30 days and fully irrigated by watering every day until the drought treatment. At booting stage (panicle initiation stage) drought stress were individually applied (Yue et al., 2006) to each plant. At that stage makes three holes at the bottom of the pot and makes the water drain out. During drought stress the leaves were rolled during day but open at night. When all leaves of a stressed plant became fully rolled, and never open at night, a point corresponding to the relative water content in the range of 72–75%, watering was applied to the full capacity of the pot. And applying water were continued to allow recovery at the flowering and seed maturation stage (Xiao et al., 2009). All the phenotypic parameters and yield related traits were recorded after seed harvest.

3.6.Expression analysis of the *SNAC*1 downstream genes:

3.6.1.RNA extraction and Real-time PCR analysis:

RNA was isolated from wildtype and two lines P7 and P8 from rd29A_*SNAC*1_BR-55 transgenic plants. Two weeks old seedlings were kept under both 0mM and 100mM NaCl stress for 24 hours. Quantitative Real-time PCR was performed using SYBR Green (Bio-Rad, USA) with individual gene specific primers in CFX96 TM Real-Time PCR detection system (Bio-Rad, USA). Relative transcript abundance was calculated using the comparative cycle threshold method described by Chen et al (2014). Elongation Factor- α (EF- α) was used as the normalization control.

Table 3.6: Primers used in downstream specific genes.

Name	Primers	Annealing temperature
NAM family protein	F: 5' GCCGAGGTGGATCTCTACAA 3' R: 5' GTTGTCCACGATCTCCGACT 3'	63˚C
sodium/decarboxylate cotransporter	F: 5' GGCTTTTGCAGAGAAGATGG 3' R: 5' GTCGTGGTTGAGTCGTCAGA 3'	62˚C
protein phosphatase 2C	F: 5' ATCGCTTGCTGGTTGCTAAT 3' R: 5' AGCAGGCTCATTGTTTGCTT 3'	62˚C
20-kDa chaperonin	F: 5' CAAGCCCTTGGCTGATAGAG 3' R: 5' CGCTAAGAGGCTTCATGTCC 3'	62˚C
cytochrome p450	F: 5' ATGAAGGAGAACAGCCGAGA 3' R: 5' CCTGAGCACCTCGTGCAGTA 3'	62˚C
Myb family transcription factor	F: 5' ACAAATGAGCTGCATGAACG 3' R: 5' ATCAGGGAACTGATCGCTTG 3'	62˚C

3.7.Searching the underlying pathway of the *SNAC*1 downstream genes and deciphering their interaction

Expression of various genes depend upon *SNAC*1 transcription factor under different abiotic stresses. From microarray data (Hu et al., 2006) of *SNAC*1 transgenic plant it was clear that *SNAC*1 transcription factor is the master regulator of many stress related transcription factors and genes. The objectives of this section were to decipher the molecular function of the genes which are upregulated by *SNAC*1 transcription factor in *SNAC*1-transgenic plants. For this reason, six genes were selected, and their mechanism of

action were determined by literature mining. Analysis of the functional pathway of individual genes was done by pubMed (NCBI) and google scholar, sequences were aligned by NCBI blastn Nucleotide database, Locus ID gathered by RAP-DB (Rice Genome Annotation Project Database) and finally interaction was observed in STRING 9.05.

4. Results

4.1.Part ONE: Characterization of transcription factor *SNAC*1 in *indica* rice variety Binnatoa for drought and salinity tolerance.

Transcription factors are involved in plant stress responses. Transgenic plants overexpressing transcription factors can enhance their tolerance to various stresses (Jaglo-Ottosen et al., 1998; Lu et al., 2009). Transcription factors play essential roles in stress responses by regulating their target genes through binding to the cognate cis-acting elements (Tran et al., 2004). The NAC family is one of the largest plant transcription factor families which are characterized by the NAC domain in the N-terminal region, which is a highly conserved DNA binding domain. The C-terminal region of NAC proteins, is highly differentiated both in length and sequence, usually contain the transcriptional activation domain (Fang et al., 2008). Previously one of the NAC genes, *SNAC*1 (Stress Responsive NAC1) characterized in *japonica* rice (Hu et al., 2006) was shown to have induced expression in guard cells under drought stress conditions. Overexpression of this gene in rice resulted in significantly increased stomatal closure and drought resistance under field conditions. There is no report found about the characterization of *SNAC*1 in *indica* rice.

Previously in our lab *SNAC*1 gene was cloned from *O. sativa* cultivar Pokkali and was transformed into a traditional *indica* rice variety Binnatoa through tissue culture method (Abdullah-Al-Emran et al., 2010). In my present work the transformed transgenic lines were characterized in both seedling and reproductive stages. Transgenic lines and their wildtype parent Binnatoa were subjected to both drought and salinity stress at both seedling and reproductive stages. The transgenic rice overexpressing *SNAC*1 showed significantly improved tolerance to salinity and drought stresses at both the vegetative stage and reproductive stage. These results suggested that *SNAC*1 is a potential candidate for enhancing drought and salt tolerance in rice.

4.1.1.Plant materials and vectors:

O. sativa cultivar Binnatoa is an *indica* landrace, grown in the coastal regions of Bangladesh. Binnatoa has salt-tolerance equivalent to about 60% of *O. sativa* cultivar Pokkali, the benchmark for salt tolerance in rice. It also shows high regeneration ability (~96%) from mature embryo-derived callus (Seraj et al., 1997) It was chosen for its high responsiveness in tissue culture.

The pH7WG2 is a binary destination vector of GATEWAY^{TM} system for easy insertion of genes in plants through transformation. This vector is widely used for plant transformation. In this system the gene of interest integrates downstream of the Cauliflower Mosaic Virus Promoter *CaMV*35S by recombination. *SNAC*1 gene was isolated from tolerant variety *O. sativa* cultivar Pokkali and was first cloned into pENTR vector. After LR recombination *SNAC*1 gene was transferred to pH7WG2 downstream of *CaMV*35S promoter (Figure 4.1). This pH7WG2_*CaMV*35S_*SNAC*1 construct was used to transform Binnatoa (Abdullah-Al-Emran et al., 2010). *SNAC*1 transformed plants were named *CaMV*35S_*SNAC*1_ BA. *CaMV*35S_*SNAC*1_ BA transgenic lines were characterized in this section of the present work.

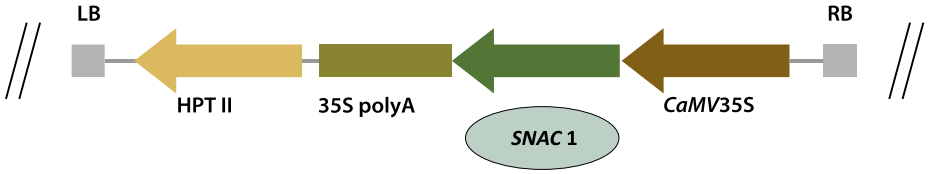


Figure 4.1: T-DNA border of pH7WG2_*CaMV*35S_*SNAC*1 construct

4.1.2.Workflow chart:

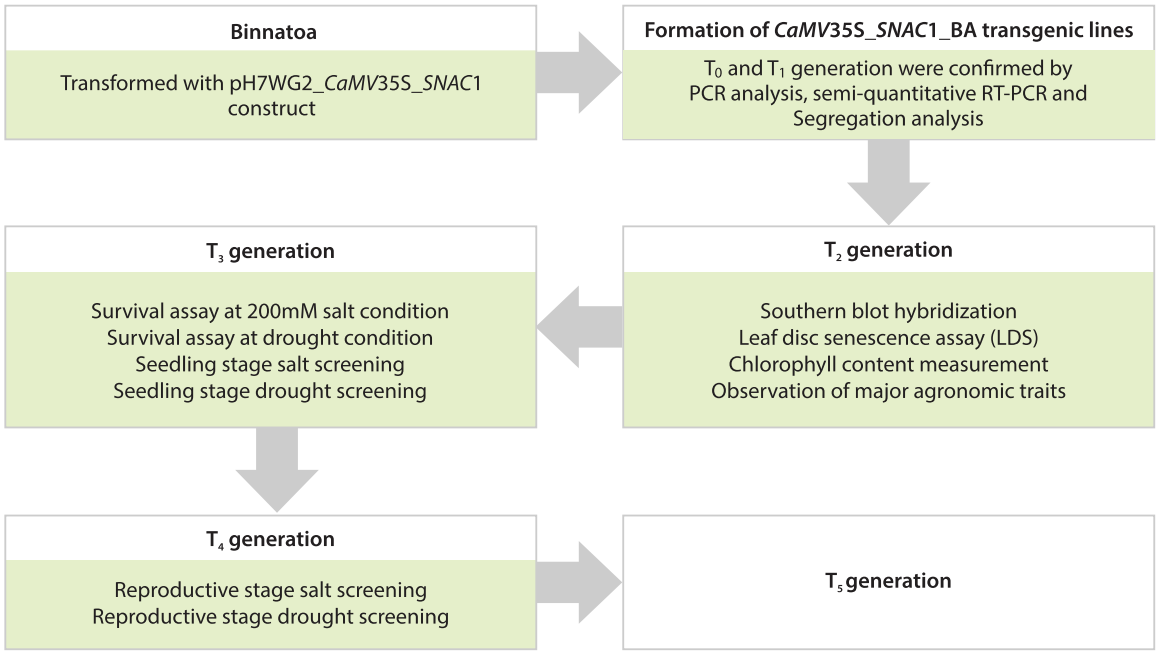


Figure 4.2: workflow chart about molecular and physiological characterization of *CaMV*35S_*SNAC*1_BA lines

4.1.3.Molecular Analysis:

a) PCR analysis:

After transformation by tissue culture method, transformed putative transgenic lines were confirmed by PCR analysis. 50 ng of DNA was amplified with *SNAC*1 gene specific primers. Here wildtype Binnatoa (BA) was used as parents, water sample and pH7WG2_*CaMV35S_SNAC*1 construct were used as negative and positive sample respectively. All the transgenic lines showed correct sized bands (1051bp) and no band was found in wildtype Binnatoa (BA) (Figure 4.3).

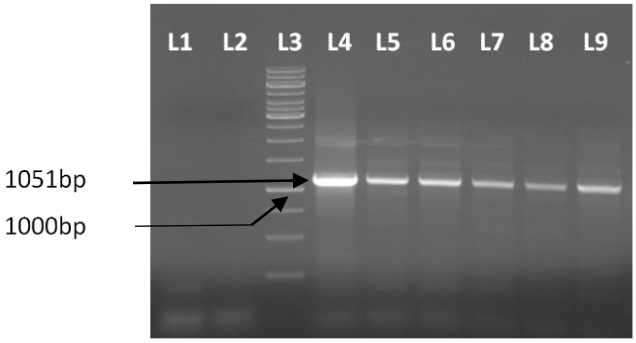


Figure 4.3: PCR analysis at T_1 plants of pH7WG2_*CaMV*35S_*SNAC*1 transgenic lines. L1: wildtype BA; L2: Water control; L3: 1 kb DNA ladder; L4: positive control (plasmid); L5-L9: transformed lines (P2, P3, P5, P7, P8). The transformed lines showed the correct sized band.

b) Semi quantitative RT-PCR:

PCR positive transformed lines were analyzed for Transgene *SNAC*1 expression. Total RNA was isolated from six transgenic lines at T_1 generation and wildtype BA. Semi quantitative RT (reverse transcriptase) PCR was performed with *SNAC*1 specific primers. cDNA was optimized for all plants using eEF- α (eukaryotic elongation factor- 1α) gene specific primers. As a housekeeping gene, the level of expression of eEF- α were found same for all transgenic lines and wildtype.

Semi-quantitatitve RT (reverse transcriptase) PCR was done at 28 cycles with *SNAC*1 specific primers. Desired sized band (1051bp) was found for all lines. The transgene expression level is much higher in overexpressed transgenic lines than wildtype plants (Figure 4.4). Four lines (P2, P5, P7, P8) out of five showed higher *SNAC*1 expression, were selected and advanced to next generation. Line P2 showed highest expression level.

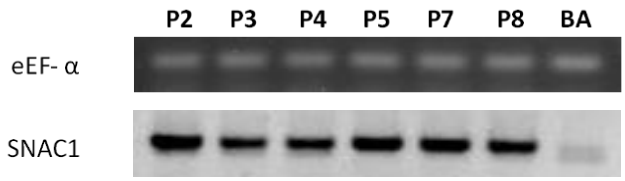


Figure 4.4: Expression pattern analysis of T_1 transgenic lines and wildtype BA by semi-quantitative RT (reverse transcriptase) PCR. All the transgenic lines showed higher *SNAC*1 expression than wildtype Binnatoa. Line P2 showed higher expression than other lines.

c) Southern blot hybridization:

Stable integration of *SNAC*1 in transgenic lines was confirmed at T_2 generation. Total DNA was isolated from four transgenic lines (P2, P5, P7, P8) and wildtype BA. Through Southern blot analysis, two copies of *SNAC*1 gene was shown in transgenic line P2 whereas Line P5 and Line P8 showed single copy of insertion and Line P7 showed no band. Wildtype BA showed no band and positive control (pH7WG2_*CaMV*35S_*SNAC*1 construct) showed the correct sized band of 6kb (Figure 4.5).

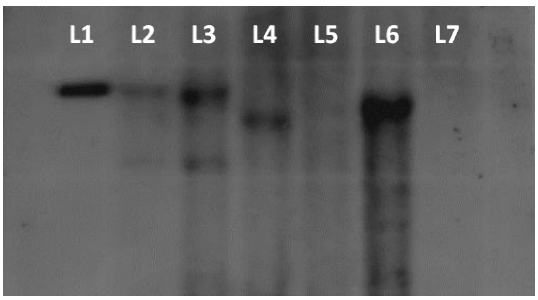


Figure 4.5: Southern blot hybridization for T_2 transgenic lines. L1: positive control, L2-L3: line P2, L4: Line P5, L5: Line P7 (no band), L6: Line P8 and L7: wildtype BA. Transgenic lines except Line P7 showed transgene insertion.

4.1.4.Segregation analysis at T_1 generation:

The three transgenic lines (P2, P5, P8) were selected for generation advancement. Segregation analysis was done at T_1 generation. Random 15 plants were chosen from three lines and tested for PCR analysis. PCR positive plants were selected as transgenic (resistant) and PCR negative plants were selected as non-transgenic (susceptible)(Table 4.1). Plants from all three lines maintained the Mendelian law of inheritance (3:1).

Table 4.1: Segregation analysis of transgenic (resistant) and non-transgenic (susceptible) seedlings in the T_1 plants.

Name of plants	Number of resistant seedlings	Number of susceptible seedlings	Chi-square test or χ^2 test = (observed-expected) ^{2}/ (expected)	P-value
P2	15	4	0.157895	0.69119*
P5	15	6	0.142857	0.705513*
P8	15	6	0.142857	0.705513*

* Followed the Mendelian law of inheritance (3:1)

4.1.5.Selection of transgenic lines and generation advancement:

Among all transgenic lines, three lines (P2, P5 and P8) were selected through molecular analysis (PCR, gene expression and Southern blot hybridization) and segregation analysis. Generation advancement was done with these three lines. Phenotypic and physiological screening was done with these three lines. All the screening were done at seedling stage as well as reproductive stage, both in salinity and drought condition.

4.1.6.Leaf disk senescence assay:

Leaf disk senescence assay was performed among three transgenic lines (P2, P5, P8) along with their wildtype BA. Leaf disk were excised from healthy and fully expanded leaves of same aged plants. Assay was performed at 0mM, 100mM and 200mM NaCl for 7 days following the protocol described in section 3.4.1.a. After 7 days of stress transformed plant's disk were remains healthier and greener than wildtype plants. Line P2 showed better tolerance among transgenic lines (Figure 4.6 A).

a) Measurement of chlorophyll content from LDS assay:

After leaf disk senescence assay, leaf disks were further used for the measurement of chlorophyll content (section 3.4.1.b). The total amount of chlorophyll was measured from both transgenic and wildtype plants from the leaf disks. In each level of salt stress (100mM and 200 mM NaCl) chlorophyll content of transgenic leaf disks were more than wildtype. Percent reduction of chlorophyll content in transgenic lines were significantly lower than wildtype ($p<0.01$ for 100mM salt and $p<0.001$ for 200mM salt) (Figure 4.6 B).

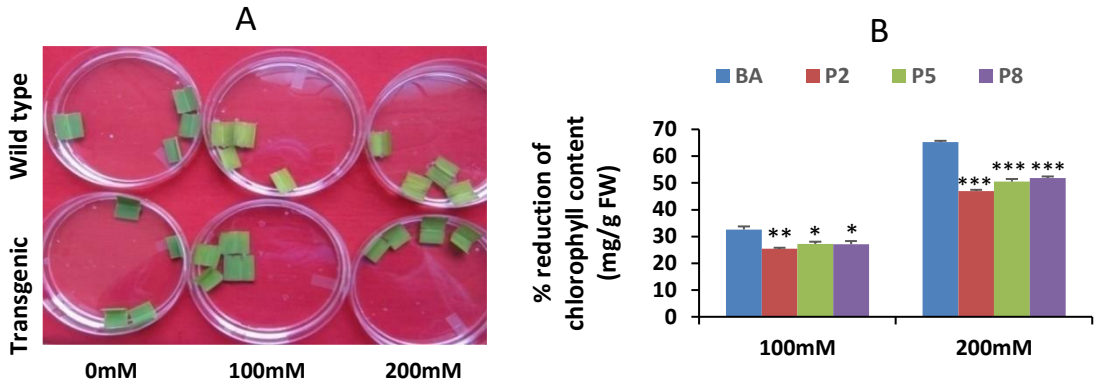


Figure 4.6: Leaf disk senescence (LDS) assay and chlorophyll content measurement. Leaf disks of transgenic line's remains much healthier and greener compared to wildtype. Transgenic lines also contained significantly more chlorophyll at 100mM and 200mM salt condition. Student's *t* test (*, **, *** P<0.05, P<0.01, P<0.001)

4.1.7.Agronomic trait of transgenic lines:

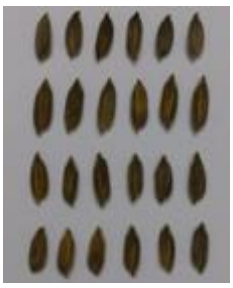
Selected transgenic lines (P2, P5, P8) were advanced to T_3 generation in net house in soil filled pot. During seed harvest the agronomic traits were observed and counted. Seeds were collected only from PCR positive plants for next generation advancement. Any phenotypic changes were observed as well as yield related traits. It was observed that Line P2 remained significantly taller than wildtype with more effective tillers. Line P2 showed the best results with highest seed setting, spikelet fertility and yield per plant (significantly differed from wildtype). Line P5 had a similar height compared to wildtype (WT) Binnatoa but with more tillers. So, all yield related parameters, showed statistically better results than WT. Line P8 was also about the same height as WT but showed higher values in yield related parameters (Table 4.2). In seed length, seed width and 1000 grain weight, line P2 also showed better results (Table 4.3).

Table 4.2: Comparison of major agronomic traits between *SNAC*1 overexpressing transgenic lines and wildtype under normal condition

Name of plants	Plant height	No of panicle	Panicle length	% of Spikelet fertility	yield
BA	115 ± 1.13	12.3 ± 1.4	17.5 ± 0.4	48.1 ± 1.1	3.2 ± 0.4
P2	120 ± 2.6*	14.6 ± 1.3*	21.3 ± 0.2***	66.1 ± 2.6***	7.3 ± 1.3**
P5	116 ± 0.5	13.6 ± 0.6	18.7 ± 0.4	65.0 ± 1.4**	6.2 ± 0.4**
P8	115.6 ± 1.4	13.3 ± 0.8	18.6 ± 0.8	54.8 ± 0.5**	4.6 ± 0.48

Table 4.3: Seed length, Seed width and 1000 grain weight of transgenic lines and wildtype.

	Length (mm)	Width (mm)	1000 grain weight(g)
Wildtype	6.8 ± 0.16	2.4 ± 0.09	17.1 ± 0.9
P2	8.5 ± 0.06**	2.7 ± 0.04**	18.1 ± 0.2*
P5	7.7 ± 0.10***	2.6 ± 0.04*	17.9 ± 0.4
P8	7.5 ± 0.09***	2.4 ± 0.01	17.9 ± 0.5



WT
P2
P5
P8

4.1.8.Survival assay at saline condition:

T_3 transgenic plants were subjected to survival assay at 200mM salt condition. At four leaf (two weeks old) stage, after 10 days of salt stress and one week of recovery, survival rates were counted. Survival rates of the transgenic lines in 200mM salt varied from 14% to

80% whereas in the wildtype BA it was from 6% to 19% (Figure 4.7), which is significantly higher than wildtype plants (*t* test, P<0.001, P<0.01, P<0.05).

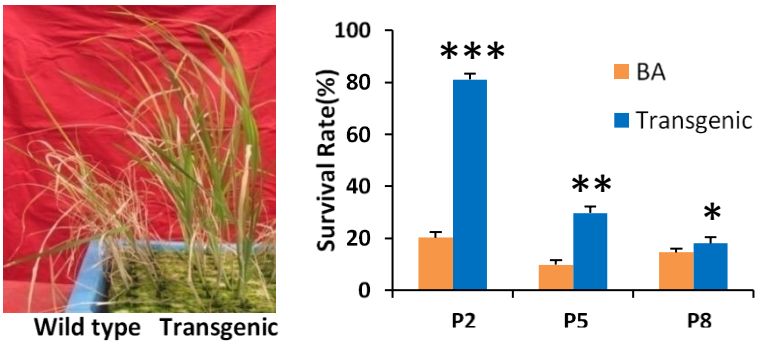


Figure 4.7: Survival rate at 200mM salinity stress. Line P2 showed 80% survival rate whereas wildtype 19%. Student's *t* test (*, **, *** P<0.05, P<0.01, P<0.001).

4.1.9.Survival assay at drought condition:

Transgenic lines P2, P5 and P8 were grown with wildtype Binnatoa in soil in the same pot. At two weeks of age (four leaf stage) drought stress was applied for 12 days by total withholding of water followed by 14 days of recovery (Hu et al., 2006). Recovered plants were then counted. Transgenic lines showed significantly higher survival rates of 43% to 50% compared to the 14%-30% of the WT parent Binnatoa (Figure 4.8). Among the three lines P2 showed best survival rate.

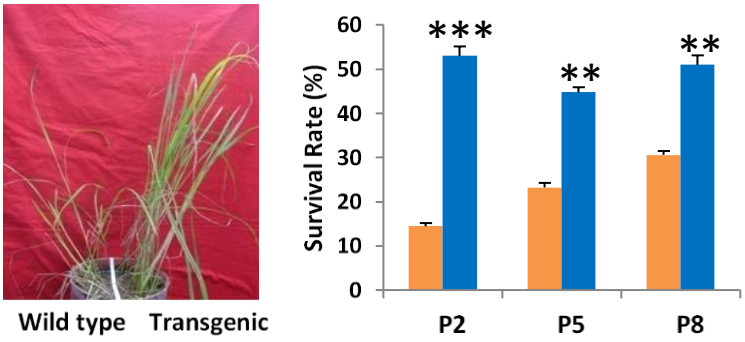


Figure 4.8: Survival rate at drought stress. Line P2 showed 50% survival rate compared to wildtype 14%. Student's *t* test (**, *** P<0.01, P<0.001).

4.1.10.Seedling stage salt screening:

T_3 plants were selected for seedling stage salinity screening. In hydroponic system 18 days old seedlings were kept at 12 dS/m salt (NaCl) for 10 days. After the salt stress, leaf drying

scores were recorded for the seedlings. Under stressed condition, transgenic lines showed significantly lower SES value than their parent (Figure 4.9).

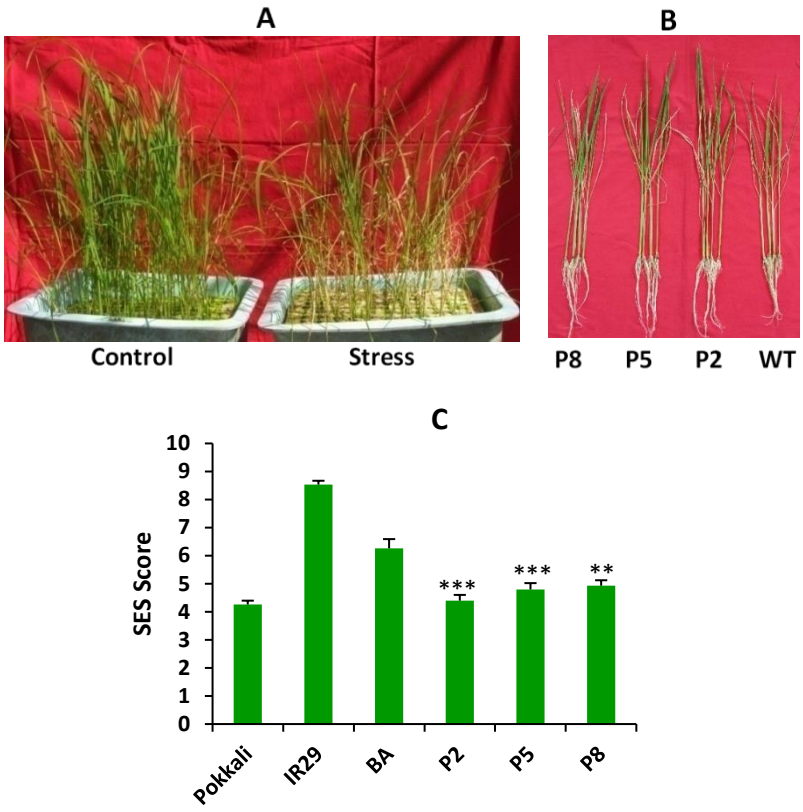


Figure 4.9: Seedling stage salinity screening at T_3 generation. **A)** control and stressed plants after 10 days of 120 mM salt. **B)** Phenotype of transgenic lines and wildtype plants after stress. **C)** Standard Evaluation System (SES) Score for wild type and transgenic lines. Pokkali and IR29 represent as salt tolerant and salt sensitive controls respectively. Transgenic lines showed significantly lower SES score than wildtype. Student's *t* test (**, *** P<0.01, P<0.001).

The stability of cell membrane under salt stress was also measured by relative electrolyte leakage. More chlorophyll content indicates plants remai ned greener in stressed condition. Lower reduction in shoot length and differences in root length were also important parameters indicating stress tolerance of the plants. Transgenic lines showed significantly lower percent reduction of chlorophyll content and shoot length than wildtype (Figure 4.10 A and C). Wildtype plant showed higher percent increase of electrolyte leakage in salt stress condition. At stress the wildtype plants showed reduced root length, but transgenic lines showing increased root length, tolerant characteristics of plant in stress condition (Liu et al., 2014). In non-stress condition there were no significant differences in all these parameters between wildtype and transgenic lines. Among the transgenic lines P2 performed well in all parameters (Figure 4.10).

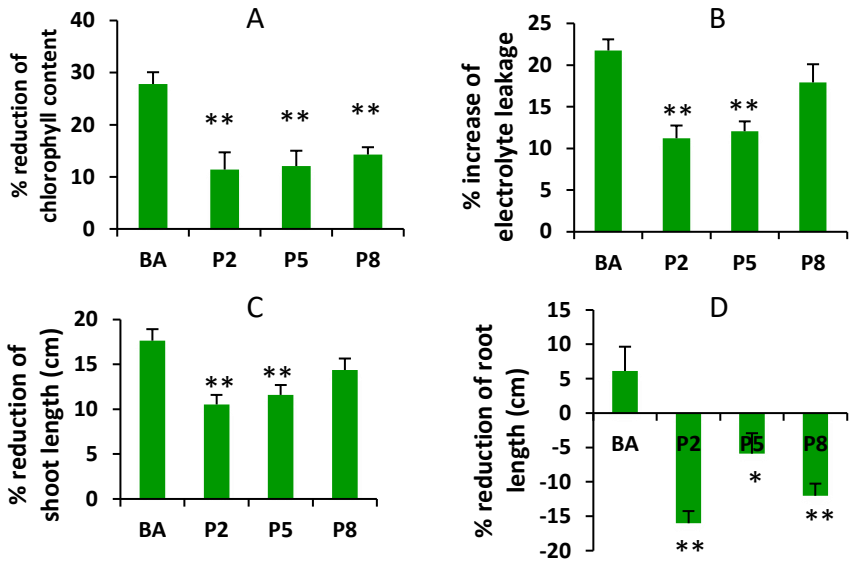


Figure 4.11: Chlorophyll content, electrolyte leakage, shoot length and root length measurement. **A)** Percent reduction of chlorophyll content is significantly higher in wildtype plants than all transgenic lines.**B)** Percent increase of electrolyte leakage is much higher in wildtype than transgenics, showed much damage in wildtype plants. **C)** Shoot length is significantly reduce in wildtype than transgenics and **D)** root length is reduced in wildtype plants but increased in all transgenics. Error bar represents the mean ± SE (n = 5). Significance was analyzed by student's *t* test (*, ** P<0.05, P<0.01).

During salinity stress, Na^+ concentration is increased in the shoot and root region. Na^+ is present in higher concentration in root region than in shoot. Higher Na^+/ K^+ ratio indicates more salt accumulation. Transgenic lines showed significantly lower Na^+/ K^+ ratio in both shoot and root region compared to the wildtype BA. In non- stress condition there was no significant differences in Na^+/ K^+ ratio in shoot and root region in transgenics and wildtype (Figure 4.11).

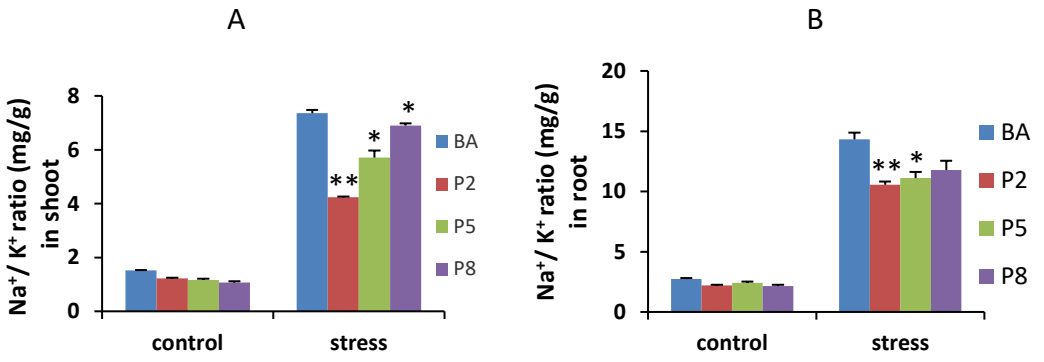


Figure 4.10: Na^+/K^+ ratio of shoot and root in transgenic lines and wild type Binnatoa. . **A)** Na^+/K^+ ratio in shoot and **B)** in root were measured in both non-stress and 12dS/m salt stress plants. Transgenic plants showed significantly lower Na^+/K^+ ratio in both shoot and root region. *, ** Significant differences between wildtype and transgenic lines at P<0.05, P<0.01 respectively.

4.1.11.Seedling stage drought screening:

The seedlings of three transgenic lines and their wildtype plants were subjected to drought screening at the T_3 generation. Two weeks old seedlings were subjected to withdrawal of water for 12 days (section 3.5.2). During stress wildtype plant leaves were rolled and almost about to die but transgenic plants showed less damage. After one week of recovery by watering of the plants, most of the transgenic lines recovered with green leaves compared to wildtype BA. After recovery chlorophyll content, shoot length and root length were measured. Drought stress recovered transgenic plants contained 4.3- 3.6 mg/g chlorophyll content whereas the wildtype had 3.1 mg/g chlorophyll content. Among transgenic lines P2 showed significantly higher shoot and root length than wildtype Binnatoa (Figure 4.12).

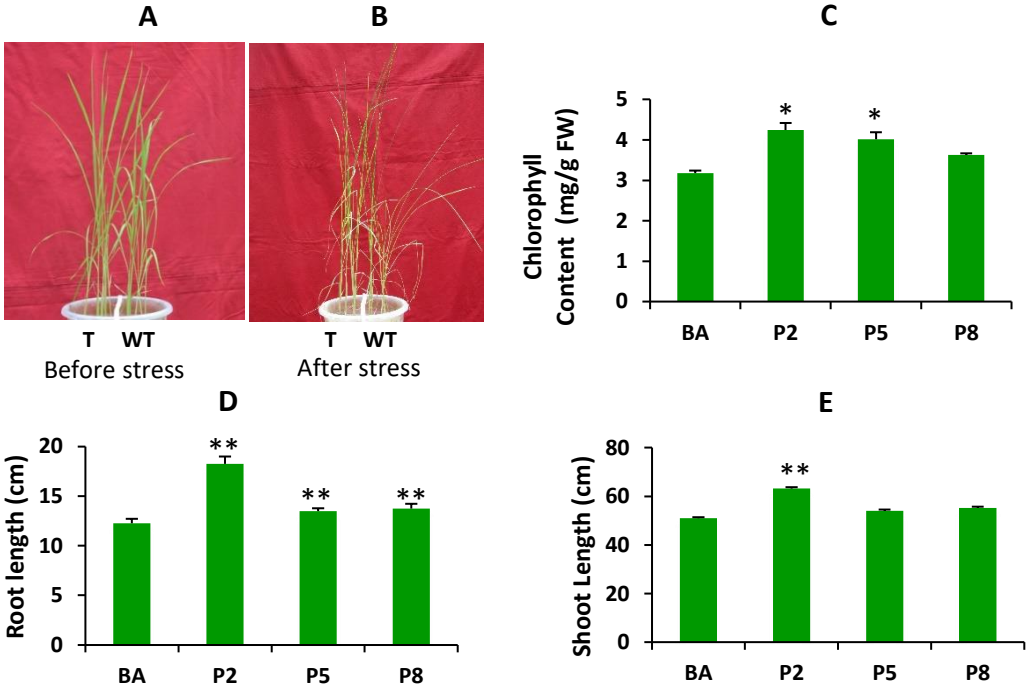


Figure 4.12: Seedling stage drought stress at T_3 *CaMV*35S_*SNAC*1_BA transgenic lines. **A)** and **B)** Phenotype of the wildtype BA and transgenic lines before and after drought stress. **C)** Chlorophyll content, measurement of **D)** Shoot length and **E)** Root length measurement of transgenic lines and wildtype after drought stress recovery. Transgenic lines showed significantly better stress tolerance than wildtype BA. Student's *t* test (*, **, *** P<0.05, P<0.01, P<0.001).

4.1.12.Reproductive stage screening at salinity stress:

Reproductive stage salinity screening was done at the T_4 generation. Three transgenic lines (P2, P5, P8) and wildtype BA were subjected to 8 dS/m salt (NaCl) at booting stage until harvest. Tillering, panicle formation, flowering and seed setting were done under the influence of salt. During harvest all phenotypic and yield related parameters were recorded (Figure 4.13).

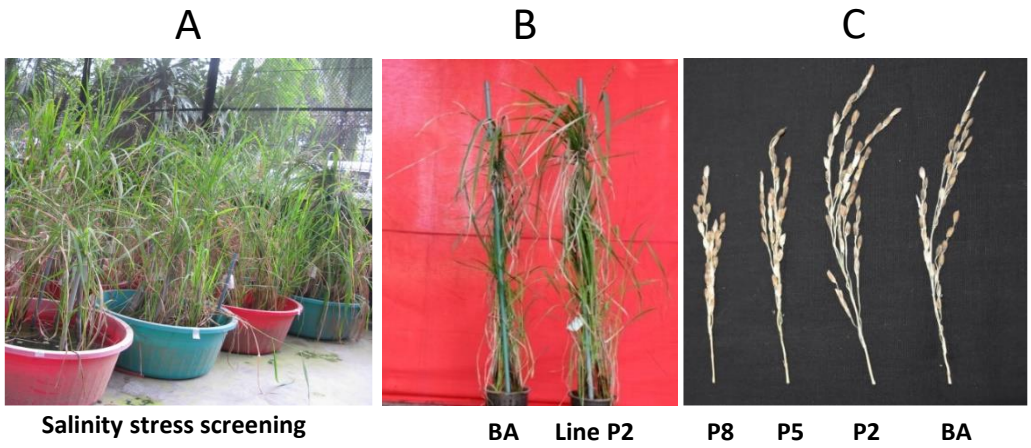


Figure 4.13: Salinity stress at reproductive stage of transgenics and wildtype plants. **A)** Plants under 8 dS/m salt condition. **B)** Phenotype of transgenic line P2 and wildtype BA under stress condition. **C)** The panicles of transgenic lines and wildtype BA after salinity stress.

Total number of tiller and number of panicles were measured from both transgenic lines and wildtype. Line P2 showed significantly higher tiller number and panicle number than wildtype. P5 and P8 produced more panicle in stress condition. It was observed that at salt stress condition, line P5 and line P8 had smaller panicle length than wildtype but higher number of panicles produce more seeds in plants (Table 4.4).

Table 4.4: Yield data of transgenic lines and wildtype Binnatoa under 8 dS/m NaCl stress.

	Name of plants	Total number of tiller	Panicle number per plant	Panicle length (cm)	Number of total grain
Stress	BA	7.8 ± 0.73	6.6 ± 0.50	11.2 ± 0.32	172.8 ± 10.94
	P2	12 ± 0.54**	11.2 ± 0.58***	12.5 ± 0.20*	257.4 ± 9.42***
	P5	8.8 ± 0.37	8.2 ± 0.37*	10.0 ± 0.12	196 ± 6.14
	P8	8.2 ± 0.48	7.4 ± 0.50	10.0 ± 0.05	182.6 ± 9.88
Control	BA	11 ± 0.57	10.3 ± 0.33	11.2 ± 0.56	220.3 ± 5.78
	P2	15 ± 0.57	13.3 ± 0.33	12.0 ± 0.50	294.3 ± 5.45
	P5	11.6 ± 0.33	11 ± 0.57	11.0 ± 1.2	252.6 ± 3.48
	P8	12.3 ± 0.33	11 ± 0.57	9.0 ± 0.28	255.3 ± 6.38

Yield related traits were measured for both wildtype and transgenics after salt stress and in non-stressed plants. Wildtype and transgenic plants did not differ in these parameters in non-stressed condition. Percent reduction of these parameters were calculated under stress. 11.5% reduction of plant height were measured for wildtype whereas 8-10% for the

transgenics. Wildtype plant showed 30% reduction of spikelet fertility and 45% reduction of yield whereas transgenics showed 22-28% reduction of spikelet fertility and 28-44% of yield reduction. Wildtype and transgenics showed 12.5% and 8-10% of 1000 grain weight reduction respectively. Among transgenic lines, P2 showed significantly higher tolerance in saline condition (Figure 4.14).

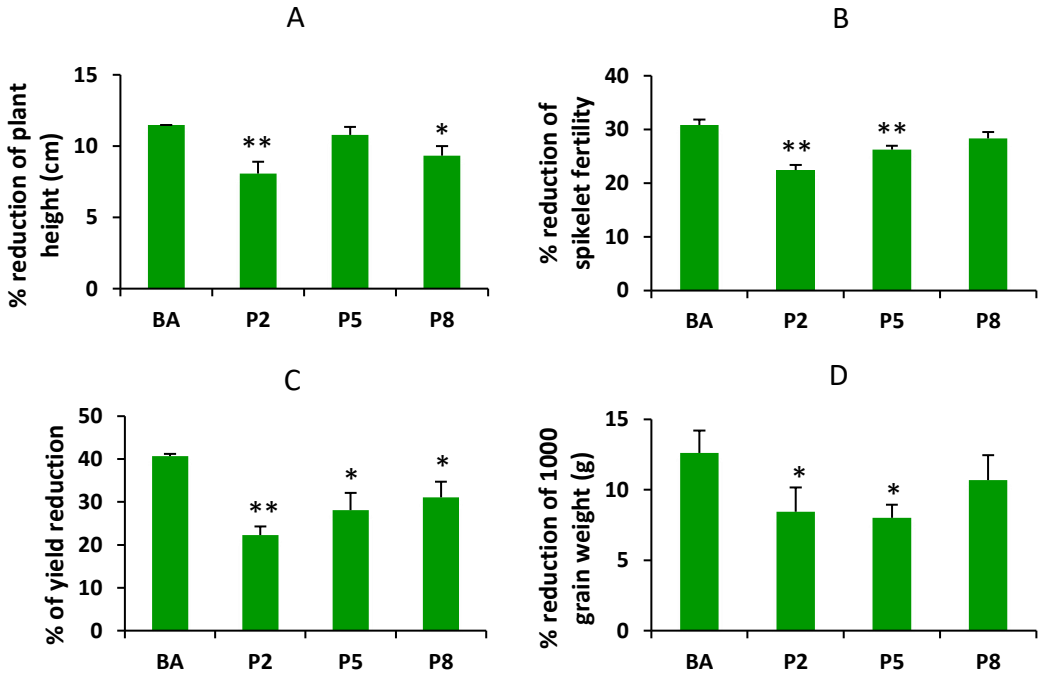


Figure 4.14: Yield related parameters at reproductive stage salinity screening. **A)** % reduction of plant height, **B)** % reduction of spikelet fertility, **C)** % of yield reduction and D) % reduction of 1000 grain weight. All transgenic lines showed significantly good results in above parameters than wildtype. Student's *t* test (*, ** P<0.05, P<0.01).

4.1.13.Reproductive stage screening under drought stress:

Yield performance at reproductive stage was assessed under drought stress. Both wildtype and transgenic plants were kept in drought stress by withdrawing water at panicle initiation stage. Plants were stressed until total leaves were rolled, indicating 70-72% of water content (section 3.5.4). After stress all plants were kept in water until seed maturation. This stress level provides a proper comparison in yield related parameters between wildtype and transgenic lines (Figure 4.15).

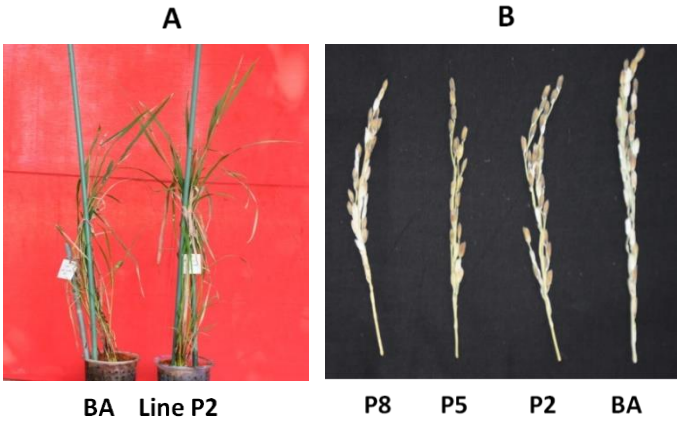


Figure 4.15: Drought stress screening at reproductive stage of transgenics and wildtype. **A)** Phenotype of transgenic line P2 and wildtype BA under stress condition. **B)** The panicles of transgenic lines and wildtype BA plants collected after drought stress.

Yield related traits such as, tiller number, panicle number, panicle length, total grain and filled grain were measured from both drought stressed and non-stressed plants. Transgenic lines contained higher number of tillers and panicles than wildtype. Also, significantly higher amount of total grain and filled grain per plant were found in transgenic lines than wildtype (Table 4.5).

Table 4.5: Yield data of transgenic lines and wildtype Binnatoa under drought stress.

	Name of plants	Total number of tiller	Panicle number per plant	Panicle length (cm)	Number of total grain
Stress	BA	6.6 ± 0.24	4.8 ± 0.37	7.4 ± 0.22	87.4 ± 3.64
	P2	10.2 ± 0.37***	9.4 ± 0.24***	8.7 ± 0.15**	150.8 ± 9.43***
	P5	8.2 ± 0.37**	6.4 ± 0.40*	8.1 ± 0.33	117.6 ± 5.53**
	P8	9.2 ± 0.37***	7.4 ± 0.24***	8.2 ± 0.12*	125.8 ± 6.27**
Control	BA	9.3 ± 0.33	8.6 ± 0.33	11.2 ± 0.56	172 ± 5.85
	P2	12.3 ± 0.88	11.0 ± 0.57	11.8 ± 0.13	217.6 ± 6.35
	P5	9.6 ± 0.33	8.3 ± 0.33	11.4 ± 0.30	183 ± 8.88
	P8	11.0 ± 0.57	9.6 ± 0.33	10.0 ± 0.22	195.6 ± 8.68

After drought stress percent reduction of plant height, spikelet fertility, yield per plant and 1000 grain weight were calculated. Transgenic lines showed 6-14% reduction in plant height whereas wildtype reduced 27%. About 35% of spikelet fertility was reduced for wildtype whereas 8-14 % for transgenic line plants (Figure 4.16).

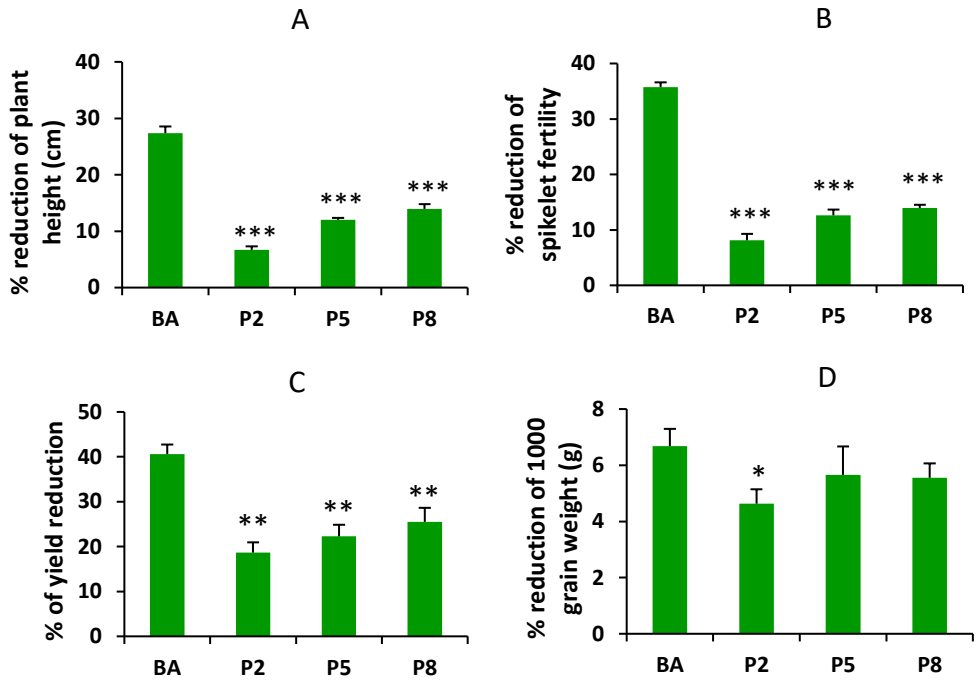


Figure 4.16: Yield related traits at reproductive level drought stress. **A)** % reduction of plant height, **B)** % reduction of spikelet fertility, **C)** % of yield reduction and **D)** % reduction of 1000 grain weight. All transgenic lines showed significantly good results in above parameters than wildtype. Student's *t* test (*, **,*** P<0.05, P<0.01, P<0.001).

In summary, land race Binnatoa was successfully transformed with *SNAC*1 transcription factor. *SNAC*1 was stably inserted in the ge nome and highly expressed in transgenic plants. Transgenic lines maintain 3:1 Mendelian law of segregation through several generations. At seedling stage salt and drought screening transgenic plants showed better salt tolerance and drought resistance. Also, in reproductive stage salt and drought screening transgenic plants showed better performance in yield related parameters. Over expression of *SNAC*1 gene provided salt and drought tolerance in both seeding and reproductive stages. Therefore, overexpression of *SNAC*1 provides enhanced drought and salt tolerance in rice.

4.2.Part Two: Transformation of high yielding BRRI rice varieties with transcription factor *SNAC*1 under stress-inducible promoter for conferring both salinity and drought tolerance.

Drought and salinity are two major abiotic stresses which limit on crop productivity. (Wang et al., 2003). In Bangladesh, salinity is one of the major environmental hazards impeding crop production in the coast. The Southern coastal area, which is 20% total land area in Bangladesh. Among these area about 53% are affected by different degrees of salinity (Hossain et al., 2012). Also, our North-Western regions are drought prone. Both salinity and drought bring adverse effect on crop production, including rice. This situation lowers rice production each year and threaten our food security. Plant adaptation to these environmental stresses depends on the activation of cascades of molecular networks including stress perception, signal transduction and the expression of specific stress related genes and metabolites (Oh et al., 2009; Tang et al., 2019). Plants show tolerance to abiotic stress through activating different genes like transcription factors. *SNAC*1 (Stress responsive NAC1), is one of the members of a large plant transcription factor family NAC protein. The NAC transcription factors are widely distributed in plants such as *Arabidopsis*, wheat, soya bean, cotton and rice (Puranik et al., 2012). These proteins are well characterized for their roles in plant growth, development, and stress tolerance (Nakashima et al., 2012). *SNAC*1 has already been reported as an enhancer of salt and drought tolerance in rice plants under field stress conditions compared to control in japonica rice (Hu et al. 2006). In My present work, *SNAC*1 under constitutive promoter *CaMV*35S was characterized in *indica* rice variety Binnatoa (results: part one). *SNAC*1 overexpressed plants showed higher tolerance in salinity and drought stresses both in seedling and reproductive stages.

In this part two, three farmer popular high yielding varieties were selected for transformation with *SNAC*1 gene. Farmers will only be benefitted if popular commercial rice varieties are also stress tolerant. In this study, the high yielding rice varieties BRRI Dhan 55, BRRI Dhan 56 and BRRI Dhan 49 were transformed with *SNAC*1 under the stress inducible (*rd29A*) promoter (Kasuga et al., 1999). Transformations were done by *Agrobacterium* mediated *in planta* transformation. For each transformation event, best transgenic lines were selected by molecular analysis as well as seedling stage physiological screening. Transgenic lines were confirmed by PCR and Southern blot hybridization proving stable integration of the transgene in the genome. Transgene expression was

analyzed by quantitative real time PCR. Transgenic lines showed higher survival rate at 200mM salt condition and at drought condition. At leaf disk senescence assay (0, 100 and 200mM salt condition) transgenic lines contained more chlorophyll compared to their respective wildtypes. All transgenic lines showed better tolerance than respective wildtypes. It was found that *SNAC*1 performed better under the inducible promoter. This work provided a promising approach to improve stress tolerance of high yielding rice cultivars through the *SNAC*1 transcription factor.

4.2.1.Vector used for transformation:

The *CaMV*35S promoter is widely used for transgene over expression. It was reported that constitutive overexpression of some stress-responsive transcription factor genes, such as DREBs, under *CaMV*35S promoter frequently caused unwanted phenotypes, such as reduced plant growth, which caused significant yield reduction (Shen et al., 2003). Use of the stress inducible rd29A promoter instead of the constitutive *CaMV*35S promoter for the overexpression of *DREB1A* minimized the negative effects on plant growth in transgenic *Arabidopsis* (Kasuga et al., 1999). Previously in our lab rd29A was cloned from *Arabidopsis* and promoter_GUS constructs was prepared to characterize the promoter after plant transformation. It was found that rd29A promoter shows higher GUS gene expression compared to the *CaMV* 35S promoter in shoot and root (Sarker et al., 2016).

In the present work *CaMV*35S promoter was removed from pH7WG2 destination vector and rd29A promoter was ligated to produce an acceptor vector where genes were expressed under stress inducible promoter. Primers with restriction enzyme cutting sites were designed (rd29A_*Sac*I_F and rd29A_*Spe*I_R) to amplify the rd29A promoter. The amplified product was gel extracted and digested by the restriction enzymes (*Sac*I and *Spe*I). *CaMV*35S promoter from pH7WG2 vector was removed by digestion with *Sac*I and *Spe*I restriction enzymes. pH7WG2 vector without promoter region was eluted from gel extraction. Both restriction enzyme digested PCR amplified rd29A promoter and pH7WG2 vector fragment were ligated to form pH7WG2_rd29A acceptor vector. *SNAC*1 gene was further inserted downstream of rd29A promoter by LR recombination. Thus, the prepared pH7WG2_rd29A_*SNAC*1 vector was used to transform high yielding rice. T-DNA region of pH7WG2_rd29A_*SNAC*1 vector are shown in figure 4.17.

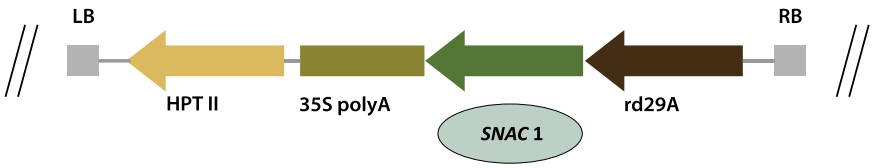


Figure 4.17: T-DNA border of pH7WG2_rd29A_*SNAC*1 construct

4.2.2.High yielding rice varieties:

High yielding rice varieties are superior cultivars, with high crop yield per area (hectare). In this study three high yielding rice varieties were selected namely BRRI dhan-55 (BR-55), BRRI Dhan-56 (BR-56) and BRRI Dhan-49 (BR-49) to be transformed with the *SNAC*1 transcription factor under the stress inducible promoter rd29A.

BRRI Dhan-55 -This is an Aus and Boro season variety. It has an early maturation of about 105 days and possesses yields of about 5.0 ton/hector. At the Boro season, its life span is 145 days with high yield of about 7.0 ton/hector.

BRRI Dhan-56 -This is an Aman variety, with life span of 105-110 days. This is a slightly drought tolerant variety. Strong plants, medium height and high yield make this variety farmer popular. This variety is selected to find how much further *SNAC*1 can increase its tolerance.

BRRI Dhan-49 -This is an Aman variety, with life span of 135 days. This is a salt sensitive variety. This variety is selected to understand how far *SNAC*1 makes it salt tolerant.

4.2.3.Workflow chart:

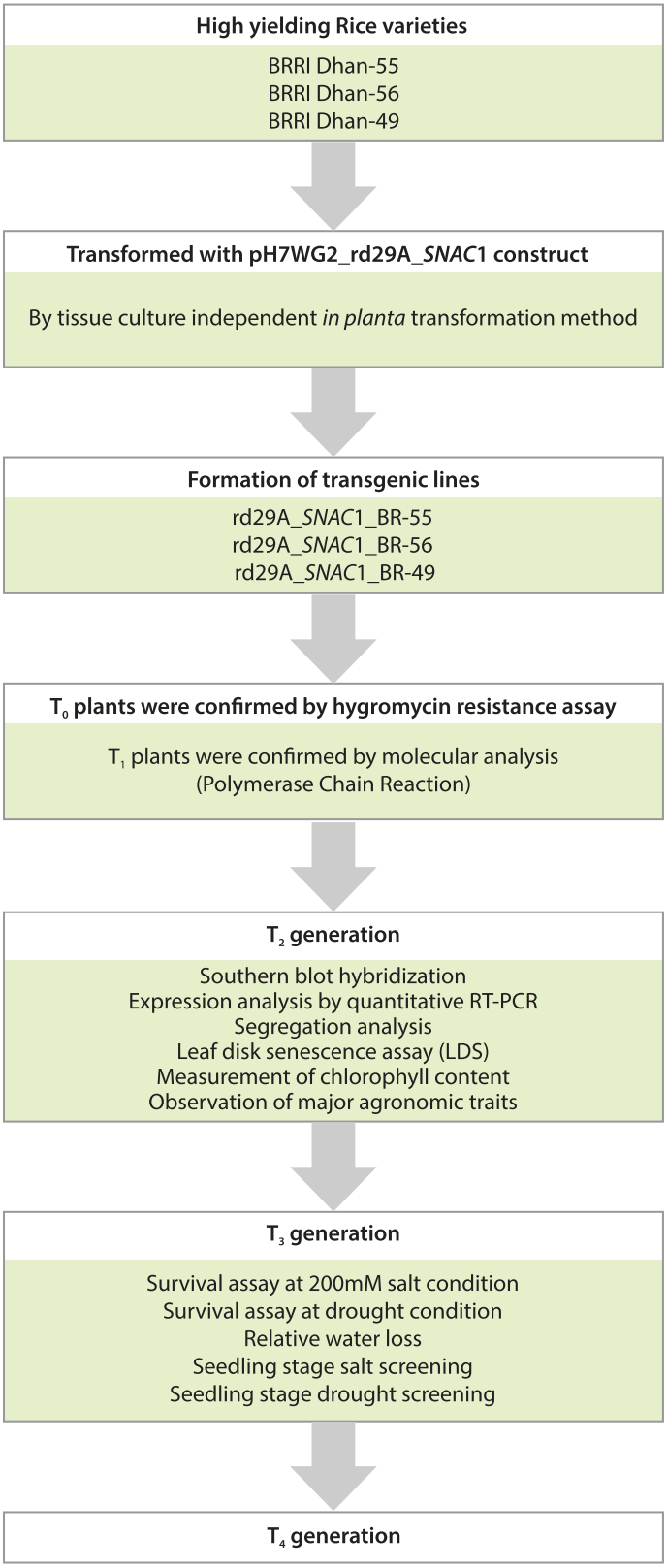


Figure 4.18: Workflow chart for transformation of high yielding rice varieties.

4.2.4.*In planta* Transformation:

For plant transformation tissue culture is a commonly practiced technique. But not all plants response well in tissue culture. High yielding rice varieties showed lower regeneration potency in tissue culture in normal condition. After infection they become recalcitrant and did not regenerate at all. So as an alternative we used *in planta* transformation technique (Supartana et al., 2005; Lin et al., 2009), a simple and efficient transformation method for any rice variety.

During transformation by *in planta* method (section 3.3.1), the mature embryos of soaked rice seeds were pierced by a needle, and then soaked in the *Agrobacterium* strain LBA4404 harboring pH7WG2_rd29A_*SNAC*1 construct as inoculum under vacuum infiltration. The inoculated seeds were germinated. Germinated seedlings were transferred to soil and grew to maturation (Figure 4.19).

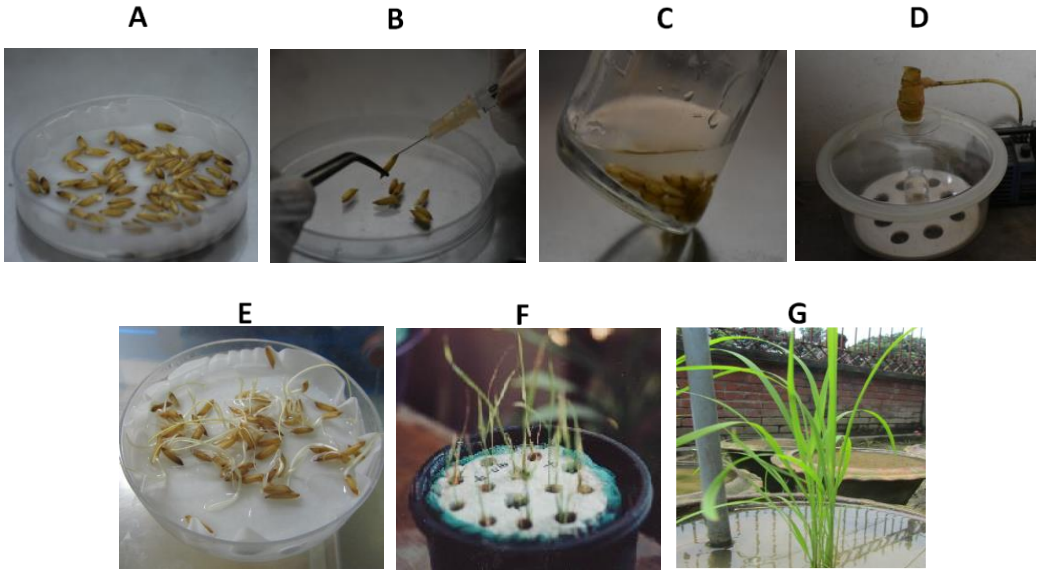


Figure 4.19: transformation of high yielding rice varieties through in planta transformation method. **A)** Seeds soaked in water. **B)** Inoculation of a seed with a needle. **C)** The pierced seeds soaked in the *Agrobacterium* inoculum. **D)** drawn vacuum for infiltration. **E)** Incubation of infected seeds. **F)** Transfer to hydroponic solution. **G)** Transformed plant in soil.

Confirmation of transformation was carried out both at T_0 and T_1 generation. The plants from inoculated seeds (T_0) might be chimeras. Hygromycin resistance assay (section 3.4.1.c.) was done with flag leaf samples when the plants were mature, and grain were set. After confirmation, seeds (T_1) from confirmed flag leaf panicle were collected. Transformation efficiency were also calculated for rd29A_*SNAC*1_BR-55, rd29A_*SNAC*1_BR-56 and rd29A_*SNAC*1_BR-49 lines.

4.2.5.Hygromycin resistance assay of T_0 transformants:

The pH7WG2_rd29A_*SNAC*1 construct contains hygromycin phosphotransferase (HPT) gene under *CaMV*35S promoter in the T-DNA region. This gene was used as marker gene which ensure the transfer of transgene into transformed plants. Hygromycin phosphotransferase (HPT) gene helps transformed plants remain healthy even in presence of the antibiotic hygromycin.

The flag leaf of T_0 plants (from all transformed lines) was used in the hygromycin resistance assay. This assay was used for the confirmation of successful transformation. During hygromycin resistance analysis, the leaf pieces from both wildtype and T_0 plants for all three rice varieties were placed in the hygromycin solution. Leaf disk from wildtype plants and non-transformed flag leaf began to show necrosis and dark-brown spots after 7 days (Figure 4.20). Flag leaves which were remain green and healthy (hygromycin resistant) were primarily selected as putative transformed plants and T_1 seeds were collected from those flag leaf panicles.

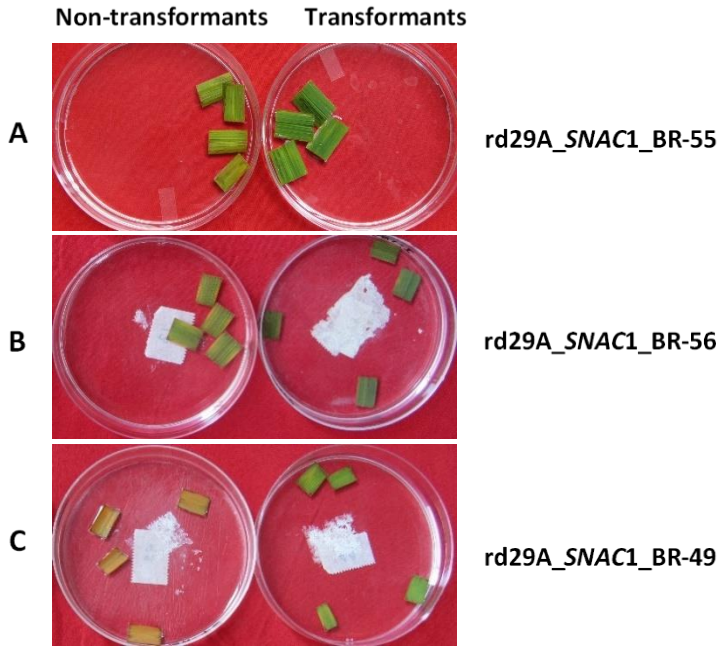


Figure 4.20: Hygromycin resistance assay of flag leaves of T_0 transformants. **A)**, **B)** and **C)** shows results from rd29A_*SNAC*1_BR-55, rd29A_*SNAC*1_BR-56 and rd29A_*SNAC*1_BR-49 lines. Transformants remained healthy and green (right side) than the non-transformants which showed necrosis and dark-brown strips (left side) at 50 mg/L hygromycin solution.

4.2.6.Transformation efficiency of the *in planta* method:

The transformation efficiency of *in planta* method for *indica* rice varieties was reported only about 6.0 % (Lin et al., 2009). With some modifications of this method transformation efficiency for high yielding *indica* rice varieties, were enhanced from 16 to 20 % (Table 4.6). Addition of acetosyringone in the bacterial inoculum plays a vital role in increase in transformation efficiency. Furthermore, instead of the ½ strength MS media, here the bacterial solution was centrifuged and re-suspended in bacterial re-suspension media. It was ensured the removal of trace amount of *Agrobacterium* selection antibiotic by double wash in centrifugation.

After hygromycin assay 6, 4, 4 out of 30, 22, 23 germinated plants of BRRI Dhan-55, BRRI Dhan-56, BRRI Dhan-49 respectively were found tolerant in hygromycin solution. The transformation efficiency was measured 20%, 18% and 17% for BRRI Dhan-55, BRRI Dhan-56, BRRI Dhan-49 respectively (Table 4.6). The plants that showed hygromycin resistance phenotype were regarded as putative transformed plants.

Table 4.6: Transformation efficiency of *in planta* transformation at T_0 generation (based on hygromycin resistance assay)

Construct name	Variety	No. of the germinated seedlings after infection (a)	No. of hygromycin positive plants tested on flag leave (b)	Transformation efficiency (b/a*100)
pH7WG2_rd29A_*SNAC*1	BRRI Dhan-55	30	6	20%
	BRRI Dhan-56	22	4	18.18%
	BRRI Dhan-49	23	4	17.39 %

4.2.7.Molecular analysis of the transformants by PCR analysis:

For confirmation of the T_1 transformants at molecular level, total DNA was isolated from all transgenics as well as their respective wildtypes. PCR analysis was performed with rd29A promoter specific primers. Only transformed plants showed 597 bp band and selected for generation advancement. No band was found in wildtype plants and in water control (Figure 4.21).

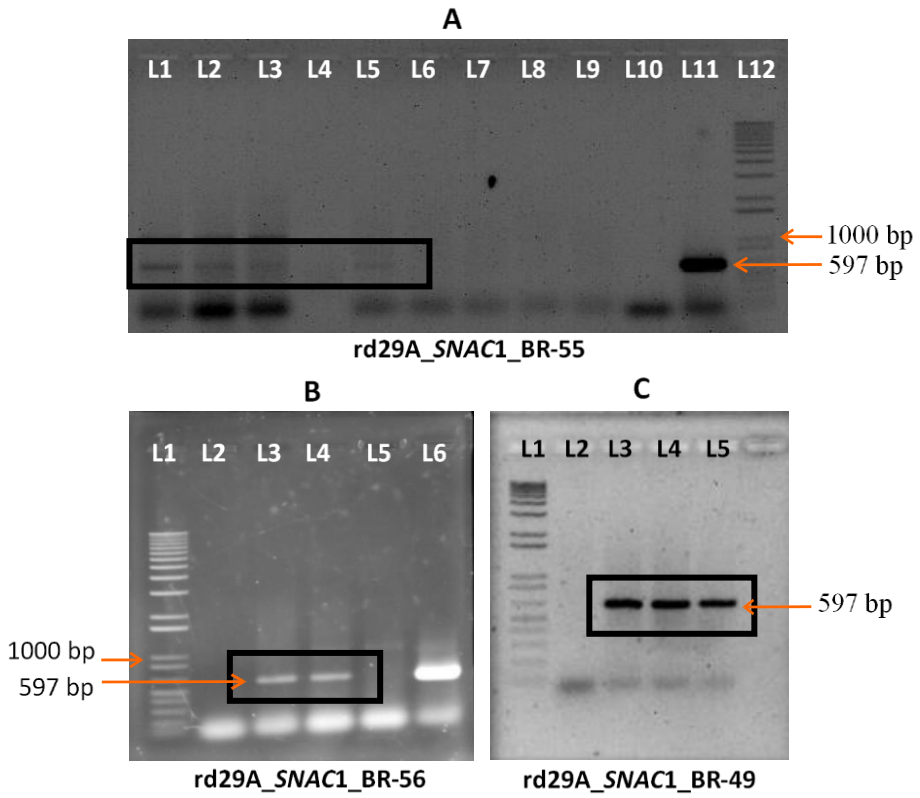


Figure 4.21: PCR amplification of rd29A promoter in T_1 transgenic lines. **A)** L1-L8: Transgenics; L9: wildtype BRRI Dhan-55; L10: water control; L11: positive control; L12: 1kb$^+$ ladder. **B)** L1:1Kb$^+$ ladder; L2: water control; L3-L4: transgenics; L5: wildtype BRRI Dhan-56; L6: positive control. **C)** L1: 1kb$^+$ ladder; L2: wildtype BRRI Dhan-49; L3-L5: transgenics.

4.2.8.Selection of the best transformed plants and generation advancement:

Generation advancement is necessary to obtain gene stability. As the generation is advanced gene becomes stabilized and homozygosity is obtained. T_0 plants were chosen on their performance in hygromycin resistance assay, T_1 seeds were collected from hygromycin resistant flag leaf panicle. T_1 plants were tested by PCR analysis with promoter rd29A specific primers. PCR positive plants were selected for each variety. Among all positive plants three lines for each variety were selected for further T_2 generation advancement. In the case of rd29A_*SNAC*1_BR-55, line P4, P7, P8 and in the case of rd29A_*SNAC*1_BR-56, line P2, P3, P6 and in the case of rd29A_*SNAC*1_BR-49, line P1, P6, P8 were selected. At T_2 generation, selection processes were done within these three transformed lines for each variety.

4.2.9.Southern blot hybridization:

Genomic DNA from all transgenic lines at T_2 generation and their respective wildtypes were digested with restriction endonuclease. The digested DNAs were electrophoresed, transferred to nylon membrane and hybridized to DIG-labeled probe (Figure 4.22). *SNAC*1

gene specific PCR amplified product was used as probe. Three lines of rd29A_*SNAC*1_BR-55 were found positive, whereas two lines for rd29A_*SNAC*1_BR-56 and three lines were found positive for rd29A_*SNAC*1_BR-49. The pH7WG2_rd29A_*SNAC*1 construct was used as positive control. No band was found in wildtypes. Plasmid pH7WG2_rd29A_*SNAC*1was used as positive control.

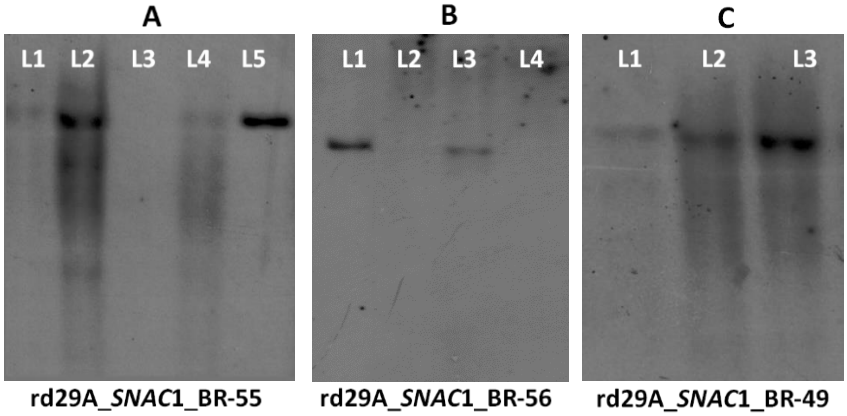


Figure 4.22: Southern blot analysis of transgenic lines with *SNAC*1 gene specific probe. **A)** Southern blot of rd29A_*SNAC*1_BR-55. L1-L4: transgenic lines, L5: plasmid pH7WG2_rd29A_*SNAC*1. **B)** Southern blot of rd29A_*SNAC*1_BR-56. L1-L3: transgenic lines, L4: wildtype. **C)** Southern blot of rd29A_*SNAC*1_BR-49. L1-L3: transgenic lines.

4.2.10.Expression analysis:

After stable integration analysis it is necessary to quantify the expression of the *SNAC*1 transcription factor in different transgenic lines. Quantitative real-time PCR was done with the transgenic lines as well as the wildtype varieties. Transgenic plants of rd29A_*SNAC*1_BR-55 lines showed significantly higher *SNAC*1 gene expression in quantitative RT-PCR compared to wildtype BR-55. *SNAC*1 transcription Factor was significantly more expressed in transgenic plants of rd29A_*SNAC*1_BR-56 line than wildtype BR-56. In rd29A_*SNAC*1_BR-49 lines transgenic plants showed higher gene expression for line P6 and line P1 showed lower gene expression than wildtype BR-49 (Figure 4.23).

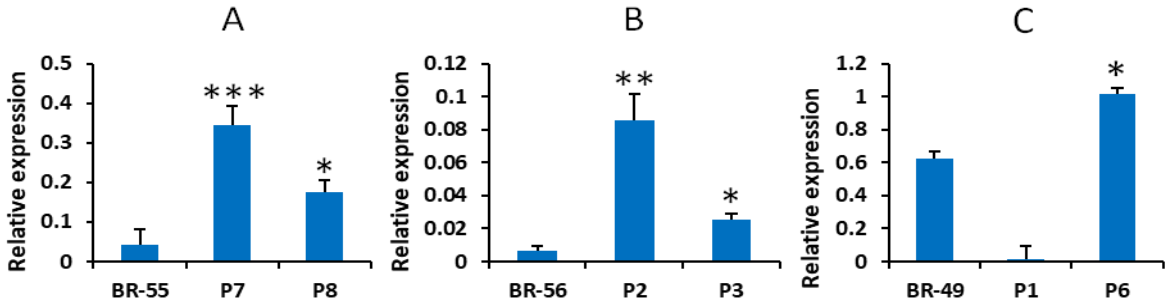


Figure 4.23: Relative expression analysis of *SNAC*1 gene. **A)** qRT- PCR in rd29A_*SNAC*1_BR-55 transgenic lines and wildtype BR-55. **B)** qRT- PCR in rd29A_*SNAC*1_BR-56 transgenic lines and wild-type BR56. **C)** qRT- PCR in rd29A_*SNAC*1_BR-49 transgenic lines and wild-type BR-49.

4.2.11.Leaf disk senescence assay:

T_2 seeds were collected from PCR positive T_1 plants. Germinated T_2 seeds were transferred in soil and leaf disk senescence assay was done in mature plants to confirm the inheritance of genes at T_2 generation. Leaf disks from non-transformed wildtype plants and T_2 transformed plants were floated separately on 0 (H_2O only), 100 or 200 mM NaCl for 7 days (section 3.4.1.a). The damage caused by salinity stress was reflected in the degree of bleaching observed in the leaf tissue after 7 days. The leaves of the control plants bleached, whereas the leaf disks of transformed plants were remained healthy and retained more chlorophyll (Figure 4.24).

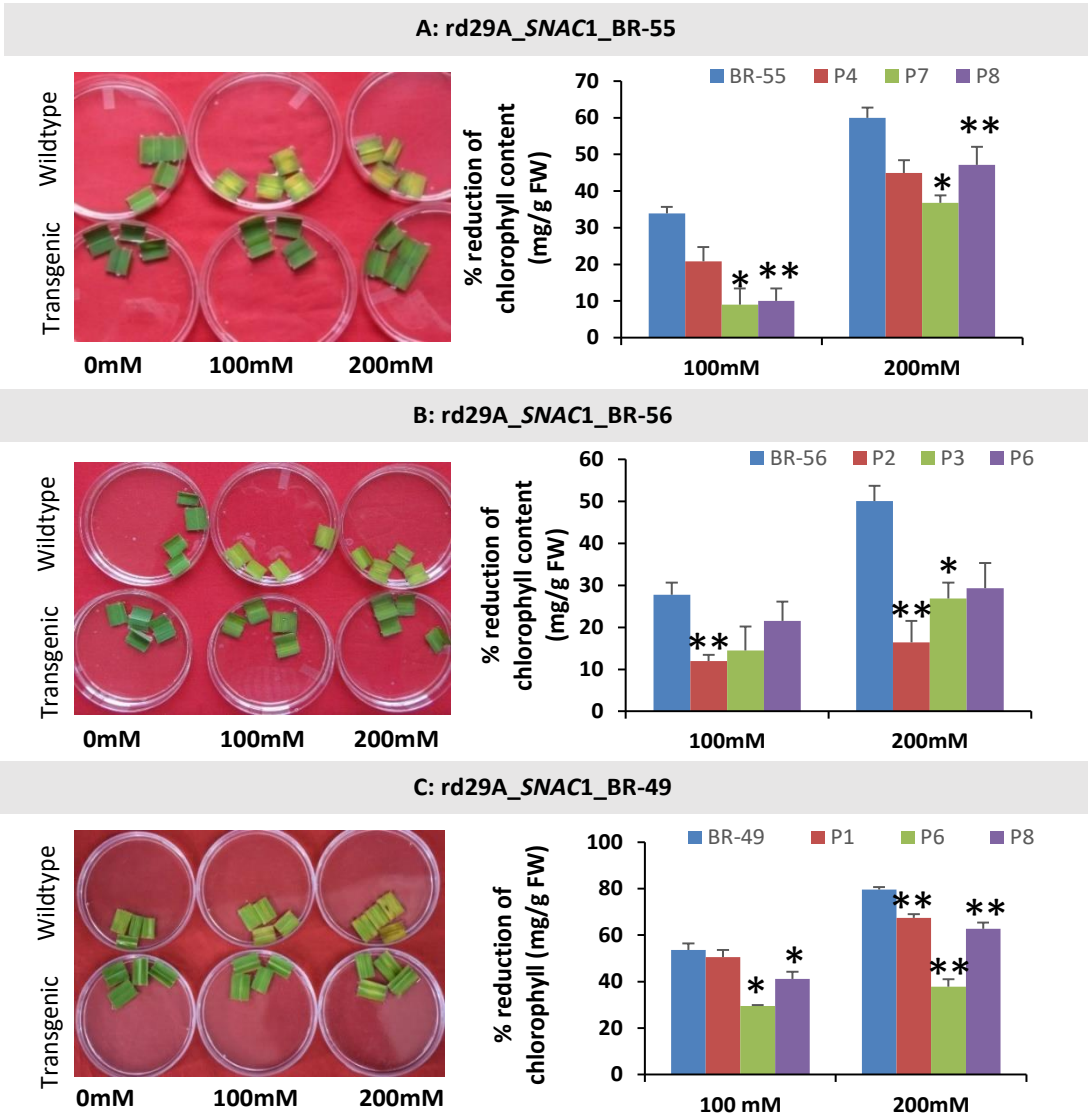


Figure 4.24: Leaf disk senescence assay and measurement of chlorophyll content of T_2 plants. **A)** rd29A_*SNAC*1_BR-55 **B)** rd29A_*SNAC*1_BR-56 and **C)** rd29A_*SNAC*1_BR-49 transgenic lines. Chlorophyll content reduced significantly in wildtype plants than transformed plant at 100 mM and 200 mM NaCl salt solution. Student's *t* test (*, ** P<0.05, P<0.01).

Plants having the transgenes showed resistance in salt water and remained greener than the non-transformed plants (wildtype). Plants which remained healthier comparative to the wildtype were selected for the chlorophyll content measurement. Chlorophyll content was measured (section 3.4.1.b) and found that without salt condition chlorophyll content for both wildtype and transgenic were almost same. But under salt stress, the percent reduction of chlorophyll content was much higher in wildtype than the transgenic plants. Percent reduction of chlorophyll content at 200mM salt condition was much higher than 100 mM salt condition (Figure 4.24).

4.2.12.Segregation ratio at T_2 generation:

Segregation analysis of transgenes was done based on the result of leaf disk senescence assay of the T_2 seedlings. In tissue culture independent transformation, Mendelian inheritance (3:1) is usually followed by the positive transformants. At T_2 generation they also showed (3:1) segregation ratio. Transgenic lines which were following Mendelian inheritance (3:1), advanced for next generation. Previously selected all three lines for each high yielding variety followed Mendelian inheritance of *SNAC*1 gene (Table 4.7).

Table 4.7: Segregation analysis of transformed (resistant) and non-transformed (susceptible) seedlings in the T_2 plants

Transgenic lines	Name of plants	Number of resistant seedlings (T_2)	Number of susceptible seedlings (T_2)	*chi-square test* or χ^2 test = (observed-expected)^2/ (expected)	P-value
rd29A_*SNAC*1_BR-55	P4	4	1	0.066667	0.796353*
	P7	5	2	0.047619	0.827293*
	P8	6	3	0.333333	0.563722*
	P9	6	1	0.428571	0.512726*
rd29A_*SNAC*1_BR-56	P2	5	1	0.222222	0.637369*
	P3	6	3	0.333333	0.563722*
	P4	7	2	0.037037	0.847465*
	P6	10	4	0.095238	0.757668*
rd29A_*SNAC*1_BR-49	P1	6	1	0.428571	0.512726*
	P2	8	3	0.030303	0.861811*
	P6	9	4	0.230769	0.631005*
	P8	8	4	0.444444	0.505006*

4.2.13.Agronomic trait at T_2 generation:

Positively selected transformants from three high yielding varieties and their respective wildtypes were grown in soil filled pot. Three plants were grown in each pot (Figure 4.25). Primary selection was done based on molecular analysis, leaf disk senescence assay and measurement of chlorophyll content. It was observed that selected transgenic lines were significantly phenotypically taller, producing more effective tiller with longer panicle.

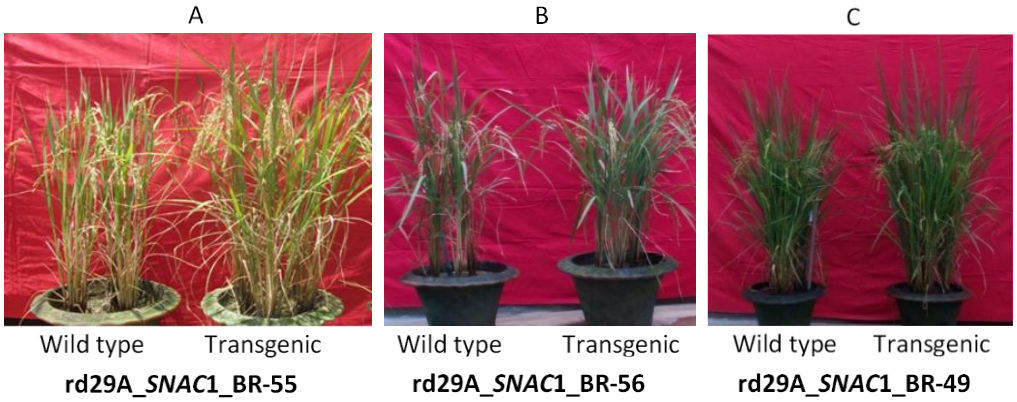


Figure 4.25: Phenotype of transgenic plants and respective wildtypes. **A)** rd29A_*SNAC*1_BR-55, **B)** rd29A_*SNAC*1_BR-56 and **C)** rd29A_*SNAC*1_BR-49 transgenic lines and wild type BRRI Dhan-55, BRRI Dhan-56 and BRRI Dhan-49 respectively.

In percentage of spikelet fertility and yield related traits they performed significantly better than their respective wildtypes (Table 4.8). The seed length, seed width and 1000 grain weight of the transgenic lines were significantly higher compared to their respective wildtypes. Among these three transgenic lines better two lines were selected. Seed length, seed width and 1000 grain weight of these two lines were mentioned here in Table 4.9.

Table 4.8: Comparison of major agronomic traits between rd29A_*SNAC*1_BR-55, rd29A_*SNAC*1_BR-56 and rd29A_*SNAC*1_BR-49 transgenic lines and wildtypes.

Name of plants	Plant height	No of panicle	Panicle length	% of Spikelet fertility	Yield
BR-55	78.3 ± 0.5	11.0 ± 0.5	21.0 ± 0.4	71.7 ± 4.9	9.6 ± 0.2
P4	82 ± 1.6	10.6 ± 0.3	22.0 ± 0.4	77.6 ± 0.6	11.1 ± 0.7
P7	86.3 ± 0.5***	11.6 ± 0.3	23.6 ± 0.2**	81.1 ± 2.9*	12.9 ± 1.1*
P8	81.6 ± 0.6*	10.6 ± 0.3	22.6 ± 0.2*	80.5 ± 2.2	10.9 ± 0.3*
BR-56	82 ± 0.4	9.60 ± 0.3	22.5 ± 0.2	66.4 ± 4.4	10.1 ± 0.4
P2	86.6 ± 0.9**	10.3 ± 0.3	23.6 ± 0.2*	82.0 ± 2.6**	12.6 ± 1.0*
P3	85.3 ± 0.2*	10.0 ± 0.5	23.3 ± 0.2	74.4 ± 0.2	12.6 ± 0.7
P6	81.3 ± 0.6	9.4 ± 0.5	23.0 ± 0.4	73.4 ± 0.6	10.6 ± 0.1

Name of plants	Plant height	No of panicle	Panicle length	% of Spikelet fertility	Yield
BR-49	77.6 ± 1.2	13.3 ± 0.3	21.6 ± 0.2	68.8 ± 4.1	7.9 ± 0.4
P1	77.3 ± 1.2	13.0 ± 0.5	22.6 ± 0.2	76.8 ± 4.7	8.4 ± 0.9
P6	78 ± 1.3	14.0 ± 0.5	22.3 ± 0.2	81.3 ± 3.5**	9.1 ± 0.8*
P8	77 ± 1.0	12.3 ± 0.6	21.0 ± 0.4	74.4 ± 6.9	8.1 ± 1.0

Table 4.9: Seed length, seed width and 1000 grain weight of rd29A_*SNAC*1_BR-55, rd29A_*SNAC*1_BR-56 and rd29A_*SNAC*1_BR-49 transgenic lines and wildtypes.

Name of variety	Seed length (mm)	Seed width (mm)	1000 grain weight (g)
BR-55	9.93 ± 0.11	2.37 ± 0.02	21.95 ± 0.23
P7	10.67 ± 0.17**	2.73 ± 0.02***	25.2 ± 0.81*
P8	10.13 ± 0.09	2.69 ± 0.03***	24.0 ± 0.22
BR-56	8.79 ± 0.05	2.63 ± 0.04	20.45 ± 0.39
P2	9.15 ± 0.05**	2.72 ± 0.06	22.55 ± 0.20*
P3	8.86 ± 0.03	2.76 ± 0.02*	21.25 ± 1.10*
BR-49	7.24 ± 0.09	2.27 ± 0.03	15.2 ± 0.18
P1	7.72 ± 0.14*	2.37 ± 0.01	16.58 ± 0.55
P6	7.82 ± 0.06**	2.39 ± 0.02*	17.3 ± 0.23*

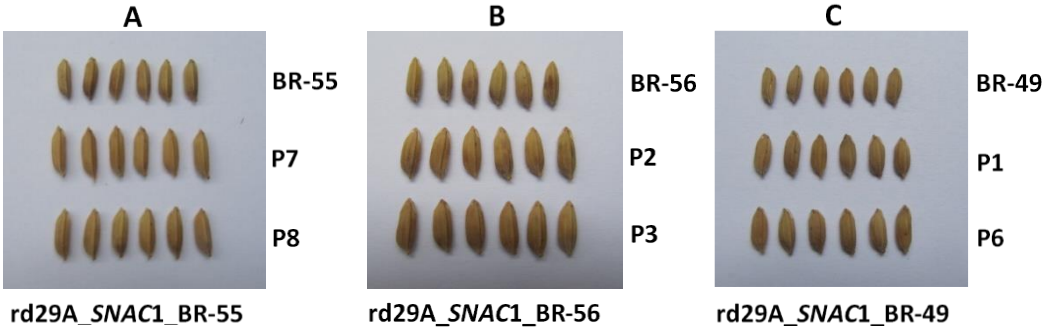


Figure 4.26: Seeds of transgenic lines and respective wildtypes. **A)** rd29A_*SNAC*1_BR-55, **B)** rd29A_*SNAC*1_BR-56 and **C)** rd29A_*SNAC*1_BR-49 transgenic lines and wild type BRRI Dhan-55, BRRI Dhan-56 and BRRI Dhan-49 respectively.

4.2.14.Survival assay after salinity stress in T_3 plants:

Among the three lines, two best lines were selected as they performed consistently well compared to wildtype plants. Survival assay in 200 mM salt condition was done with these two best lines along with their respective wildtype. Transgenic lines and wildtype plants were grown in same Styrofoam floater in PVC tray containing Yoshida solution. At four

leaf stage (two weeks old) of seedling 200 mM salt was administered gradually. After 10 days of stress when 90% of wildtype plants were about to die, the stress was stopped and after one-week recovery the survival rate was measured. For rd29A_*SNAC*1_BR-55, survival rate for wildtype was 60% whereas for transgenics 64-68%. About 47% BR-56 plants survived compared to 73-74% for transgenics. For rd29A_*SNAC*1_BR-49 lines, transgenic plants survived 37-55% whereas 25% plants survived for wildtype BR-49 (Figure 4.27). In all three varieties survival rate of transgenic lines were significantly higher than wildtypes.

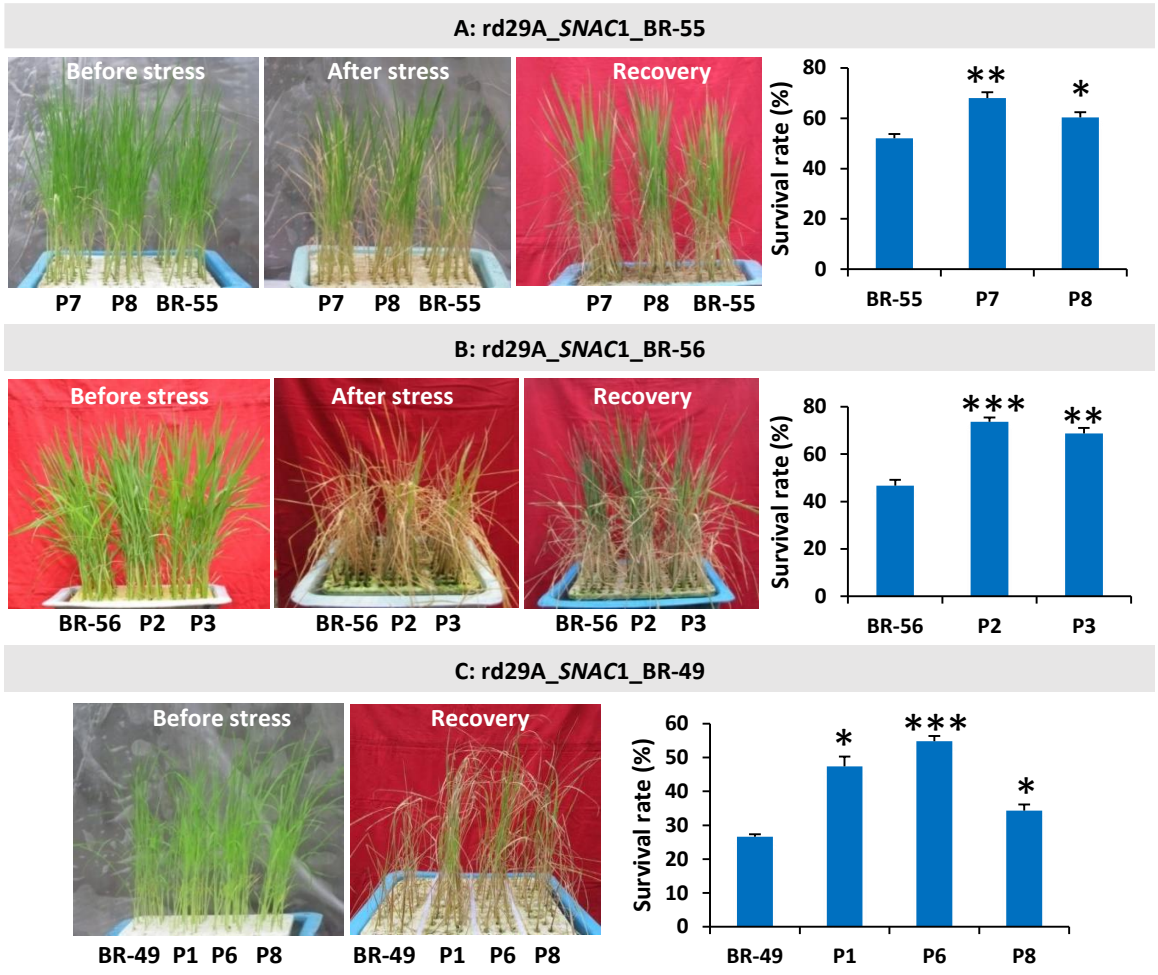


Figure 4.27: Survival assay at 200 mM salinity stress. **A)** rd29A_*SNAC*1_BR-55, **B)** rd29A_*SNAC*1_BR-56 and **C)** rd29A_*SNAC*1_BR-49, transgenic lines showed better survival rate compared to their wild type BRRI Dhan-55, BRRI Dhan-56 and BRRI Dhan-49 respectively. Student's *t* test (*, **, *** P<0.05, P<0.01, P<0.001).

4.2.15.Survival assay at drought condition:

Two weeks old seedling of transgenic lines and wildtype plants were subjected in drought stress by withholding of water for 12 days, or until the wildtype plants were nearly dead. After 14 days of recovery in water, the condition of the survived plants were analyzed. Drought survival rate of transgenic lines for each high yielding varieties, were significantly higher than their respective wildtypes (Figure 2.28).

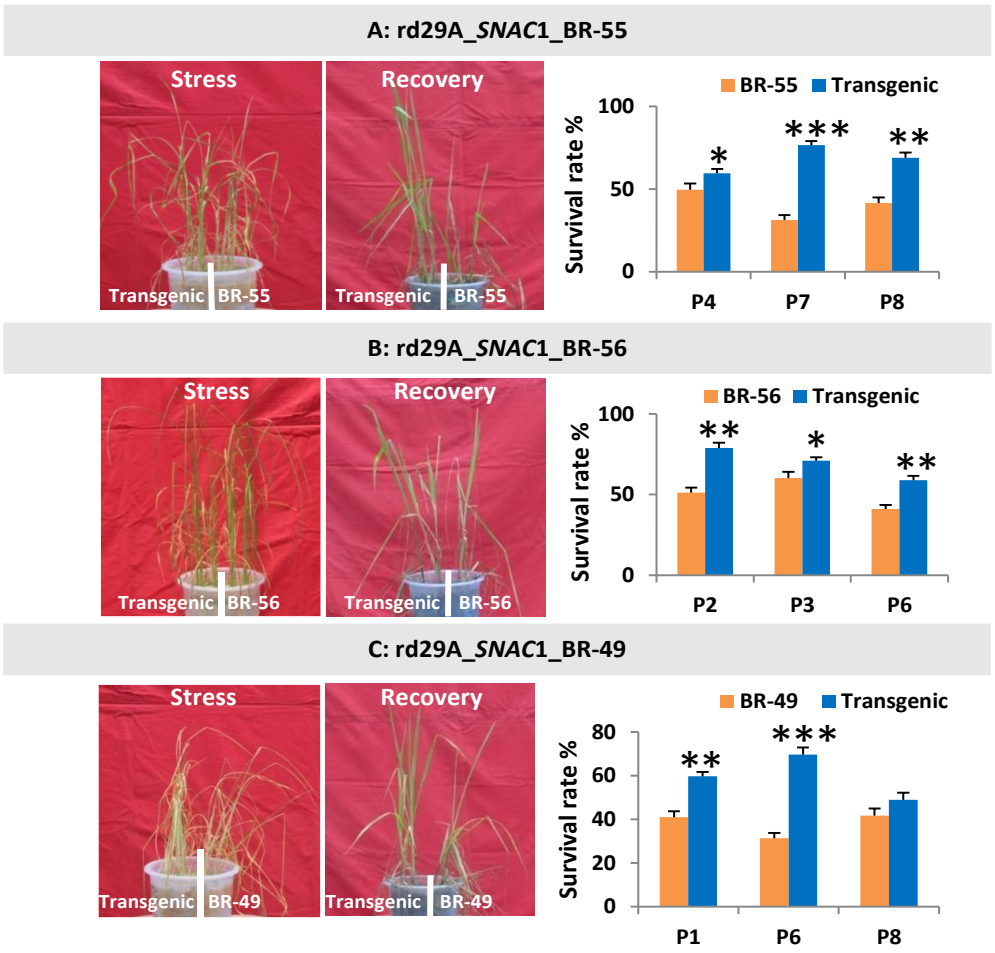


Figure 4.28: Survival assay at drought stress. **A)** rd29A_*SNAC*1_BR-55, **B)** rd29A_*SNAC*1_BR-56 and **C)** rd29A_*SNAC*1_BR-49. Transgenic lines showed better survival rate at drought condition than wildtype plants. Student's *t* test (*, **, *** P<0.05, P<0.01, P<0.001).

4.2.16.Relative water loss of excised leaves:

Leaves from three high yielding rice varieties and their *SNAC*1 transformed lines were collected and the relative water loss from detached leaves were measured at 0 min, 15 min, 30min, 45min, 1hr, 2hr and 3hr time points (Figure 4.29). Final readings were calculated up to at 24hr (not shown). Transgenic lines showed lower relative water loss than their

respective wildtypes. Among the three variety transgenic lines of BRRI Dhan-55 showed better results.

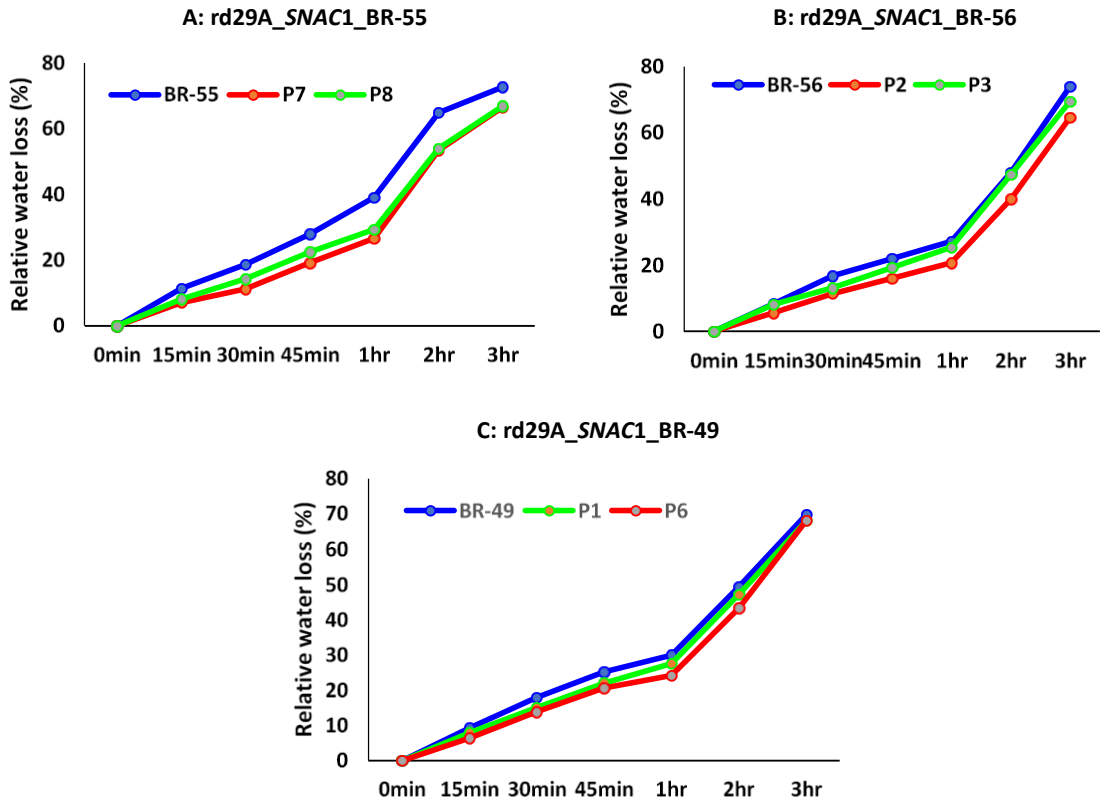


Figure 4.29: Relative water loss from excised leaves. **A)** rd29A_*SNAC*1_BR-55, **B)** rd29A_*SNAC*1_BR-56 and **C)** rd29A_*SNAC*1_BR-49 lines. Transgenic lines showed lower rate of water loss compared to their wildtypes.

4.2.17.Expression analysis of *SNAC*1 gene under salinity stress:

*SNAC*1 gene expression was analyzed under 150mM salt conditions in both wildtype and their respective transgenic lines for all three rice varieties by real time PCR. Two weeks old seedling of rd29A_*SNAC*1_BR-55, rd29A_*SNAC*1_BR-56, rd29A_*SNAC*1_BR-49 transgenic lines and their respective wildtype were stressed at 150 mM salt for 24 hours. After 24 hours, RNA was isolated from both control (without stress) and stress (150mM NaCl) condition. Real time PCR was performed using *SNAC*1 internal primers (Table 3.5, chapter 3). Elongation Factor-α (EF-α) was used as the normalization control (Table 3.4, chapter 3).

Relative expression of *SNAC*1 gene in transgenic lines were calculated along with the gene expression in wildtype plants under salt stress. At 150mM salt stress for 24 hours, transgene expression was significantly increased in transgenic lines than wildtype plants under stress

condition. Among the three variety, transgene expression is much higher in rd29A_*SNAC*1_BR-55 transgenic lines (Figure 4.30).

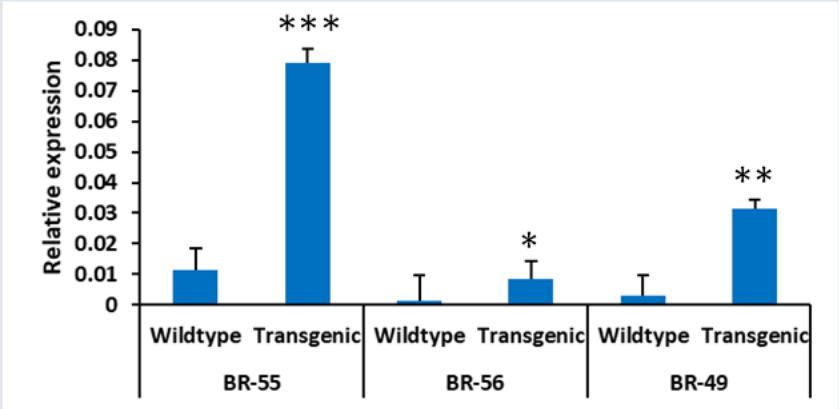


Figure 4.30: Relative *SNAC*1 gene expression under 150mM salt condition by real time PCR. After 24hours of 150mM salt stress, transgenic lines rd29A_*SNAC*1_BR-55, and rd29A_*SNAC*1_BR-56 and rd29A_*SNAC*1_BR-49 showed significantly higher gene expression compared to their wildtype plants. Student's *t* test (*, **, *** P<0.05, P<0.01, P<0.001).

4.2.18.Seedling stage salinity screening:

Seedling stage salinity screening was done at T_3 generation. Selected three transgenic lines and their respective wildtypes were subjected to 12 dS/m salt (NaCl) condition for 10 days. No obvious phenotype differences were found between the wild-type and transgenic plants before salt treatment. After salt stress different parameters were measured to find the best transgenics. Pokkali and IR29 were used as salt tolerant and salt sensitive controls respectively.

For rd29A_*SNAC*1_BR-55 lines, transgenic lines showed significantly lower SES value than BRRI Dhan-55. Transgenic lines showed 8-13% reduction of chlorophyll content whereas this was 23% for wildtype. Transgenic lines showed better membrane stability since their electrolyte leakage was 19-24%, whereas it was 42% for wildtype. Percent reduction of shoot length and fresh weight for wildtype plant was 29% and 46% respectively, whereas it was 13-16% and 28-31% respectively for transgenics. Transgenic lines did significantly better in all these parameters than wildtype plants. Under stressed conditions, the root length was reduced in wildtype plants, but increased in transgenic lines (Figure 4.31).

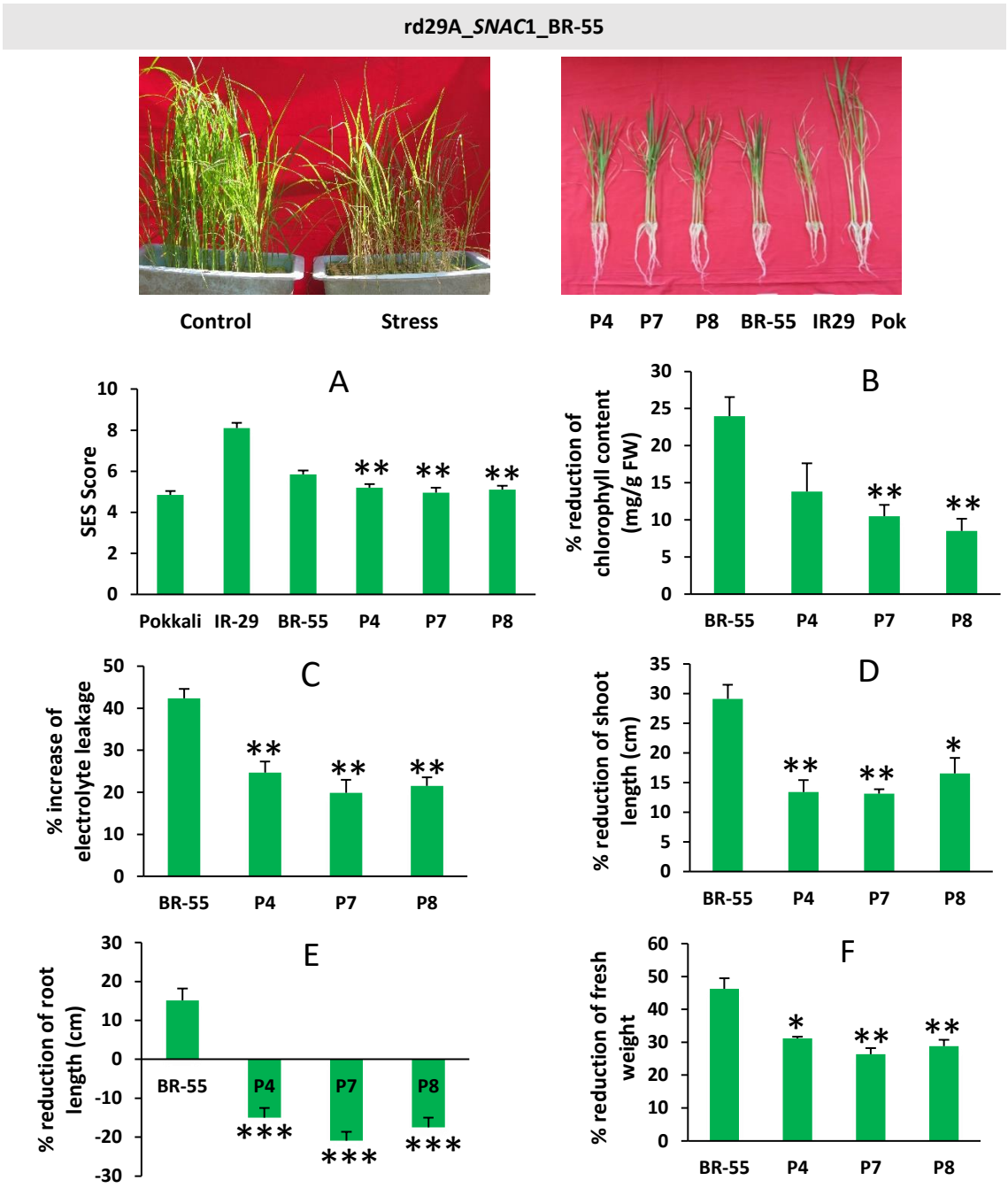


Figure 4.31: Seedling stage salinity screening at T_3 plants of rd29A_*SNAC*1_BR-55 lines. (Upper left) Control and stressed plants. (Upper right) Phenotype of transgenic lines and wildtype plants after stress. **A)** Standard Evaluation System (SES) Score. **B)** Percent reduction of chlorophyll content. **C)** percent increase of electrolyte leakage. Percent reduction of **D)** shoot length **E)** root length and **F)** fresh weight. In all parameters transgenic plants showed better salt tolerance than wildtypes BRRI Dhan-55. Each bar represents the mean ± SE (n = 5). Student's t test (*, **, *** P<0.05, P<0.01, P<0.001).

BRRI Dhan-56 is reportedly slightly drought tolerant, but it also did significantly well in salt stress screening. Wildtype plants showed 25% reduction of chlorophyll content whereas this was 14-18% for the corresponding transgenic plants. Transgenic plants showed 9-11% increase of electrolyte leakage compared to 27% in wildtype. Transgenic lines showed significantly lower percent reduction in shoot length and fresh weight than wildtype BRRI Dhan-56 (Figure 4.32).

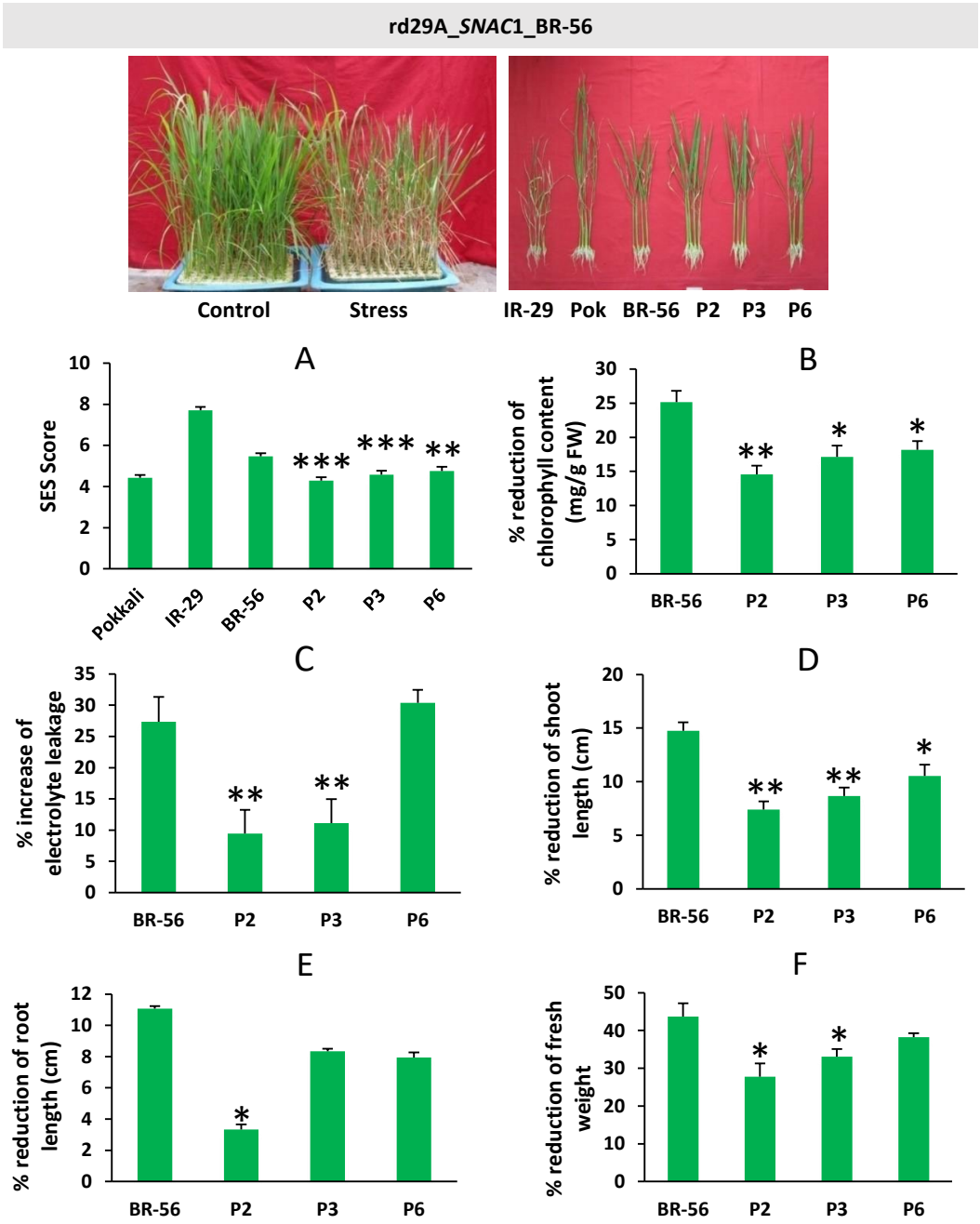


Figure 4.32: Seedling stage salinity screening at T_3 plants of rd29A_*SNAC*1_BR-56 lines. (Upper left) Control and stressed plants. (Upper right) Phenotype of transgenic lines and wildtype plants after stress. **A)** Standard Evaluation System (SES) Score. **B)** Percent reduction of chlorophyll content. **C)** percent increase of electrolyte leakage. Percent reduction of **D)** shoot length **E)** root length and **F)** fresh weight. In all parameters transgenic plants showed better salt tolerance than wildtypes BRRI Dhan-56. Each bar represents the mean ± SE (n = 5). Student's *t* test (*, **, *** P<0.05, P<0.01, P<0.001).

BRRI Dhan-49 transgenic lines showed significantly lower reduction of 22-37% of chlorophyll content compared to 53% in wildtype. Percent increase in electrolyte leakage was measured as 64% for wildtype plants whereas this was 24-46% for transgenic lines. Transgenic lines showed significantly lower percent reduction in fresh weight and shoot length than wildtype BRRI Dhan-49. Root length was also reduced in both wildtype and transgenics (Figure 4.33).

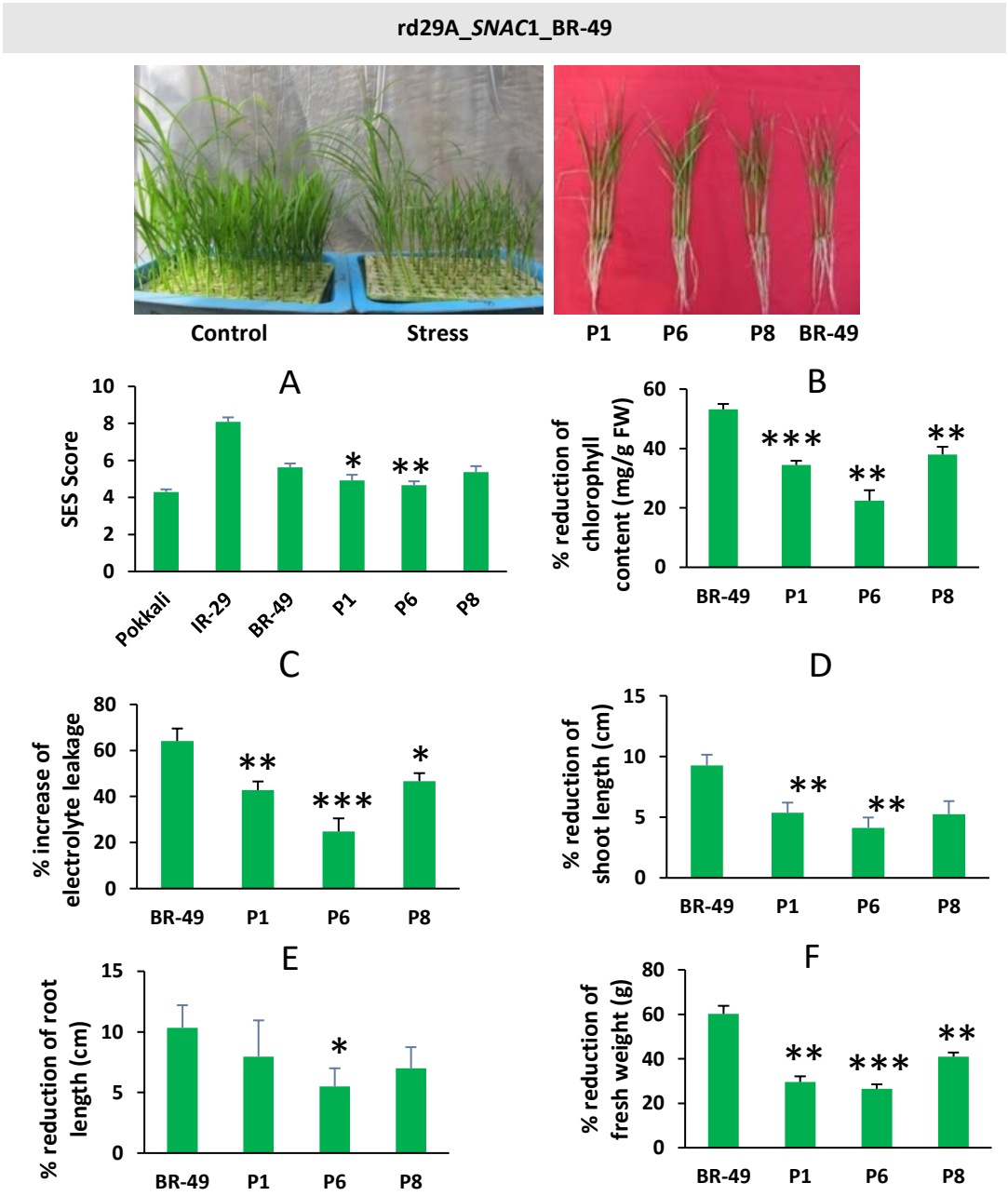


Figure 4.33: Seedling stage salinity screening at T_3 plants of rd29A_*SNAC*1_BR-49 lines. (Upper left) Control and stressed plants. (Upper right) Phenotype of transgenic lines and wildtype plants after stress. **A)** Standard Evaluation System (SES) Score. **B)** Percent reduction of chlorophyll content. **C)** percent increase of electrolyte leakage. Percent reduction of **D)** shoot length **E)** root length and **F)** fresh weight. In all parameters transgenic plants showed better salt tolerance than wildtypes BRRI Dhan-49. Each bar represents the mean ± SE (n = 5). Student's *t* test (*, **, *** P<0.05, P<0.01, P<0.001).

4.2.19.Seedling stage drought screening:

Two transgenic lines and their respective wildtype plants were subjected in drought stress at T_3 generation. Two weeks old seedlings of transgenic and wildtype plants in the same pot were kept under water withhold for 8 days. During stress transgenic lines showed better tolerance than wildtype. After 8 days of stress, plants were re-watered for 14 days for recovery. Chlorophyll content and shoot length were measured after recovery. Transgenic lines contained significantly more chlorophyll and longer in shoot length compared to their wildtypes (Figure 4.34).

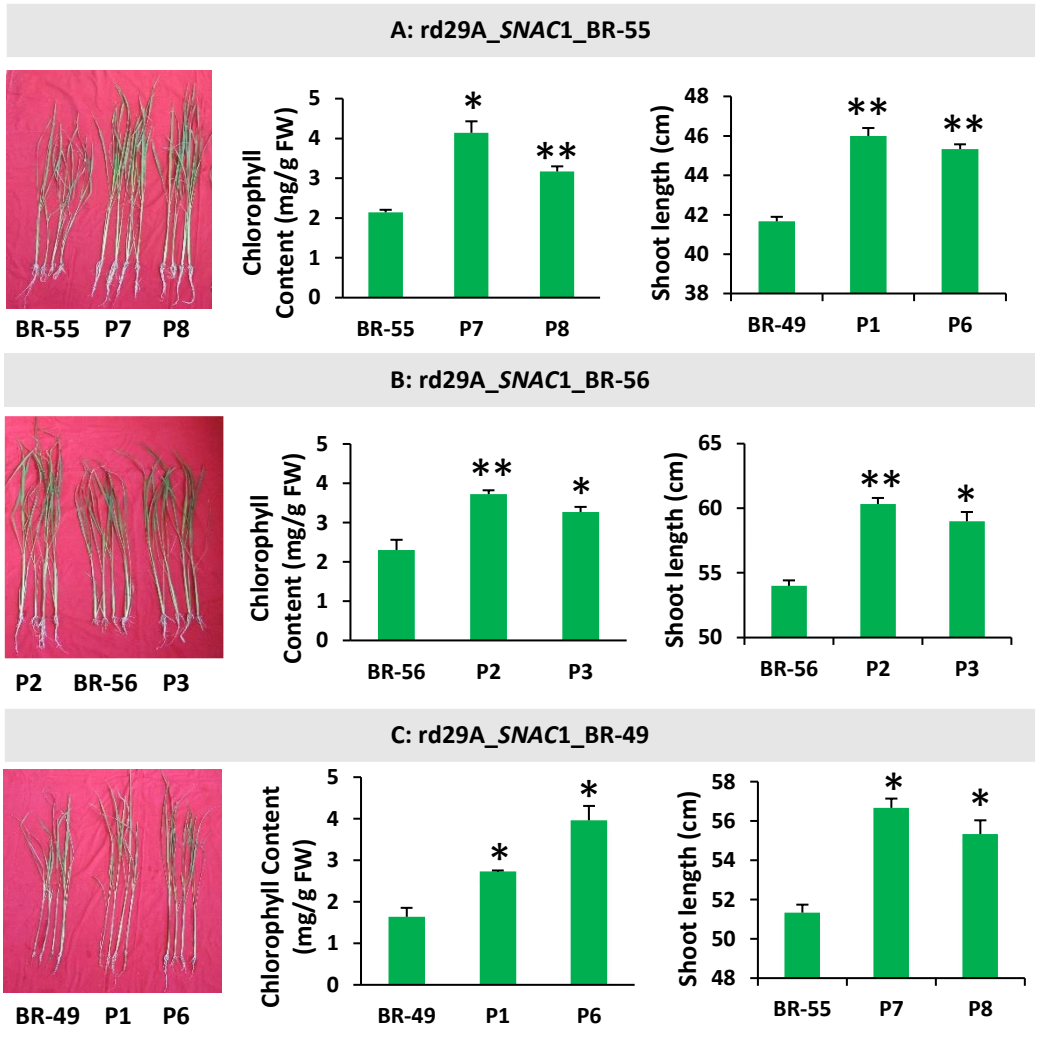


Figure 4.34: Seedling stage drought screening at T_3 generation. **A)** rd29A_*SNAC*1_BR-55, **B)** rd29A_*SNAC*1_BR-56 and **C)** rd29A_*SNAC*1_BR-49 showed phenotype, chlorophyll content and shoot length after drought stress recovery. Transgenic lines showed significantly better chlorophyll content and more shoot length compared to their wildtypes. *, ** significant differences between wildtype and transgenic lines at $P<0.05$, $P<0.01$ respectively.

In this study, three high yielding rice varieties were successfully transformed with *SNAC*1 transcription factor by *in planta* transformation method. Transgenic plants showed normal morphology and growth. Best transgenic lines were selected through molecular analysis and seedling level screening. Stable *SNAC*1 insertion was identified by Southern blot hybridization and real time PCR showed transgene expression pattern. Transgenic plants showed higher survival rate in both salt and drought condition. Selected transgenic lines for each high yielding varieties performed better in stress tolerance tests for salt and drought conditions than respective wildtypes. Finally, two best transgenic lines were selected for each rice variety comparison purposes assay amongst the rice varieties. Therefore, the tolerance level achieved due to *SNAC*1 integration suggests that this transcription factor shows great promise for the genetic improvement of stress tolerance in commercial *indica* varieties of rice.

4.3.Part Three: Comparative assay in different rice genetic background on the ability of *SNAC*1 to confer stress tolerance.

Salinity and drought cause the most adverse effect on crop production in the world. Due to global climate changes, abiotic stress now occurs more frequently. Plant survival and yield are often significantly reduced by abiotic stresses, such as drought and high level of salt (Nakashima et al., 2012). Crop yield became reduced up to 70 % by environmental factors (Agarwal et al., 2006). Therefore, understanding plant responses to abiotic stresses at the physiological and morphological levels provides an essential foundation for future farming. During abiotic stresses plants activate cascades of molecular networks involved in the expression of specific stress related genes and metabolites. *SNAC*1 (a member from NAC transcription factor family) is one of the versatile and established genes that has been previously mentioned to attain stress tolerance as an enhancer of salt and drought tolerance in rice plants (Hu et al.,2006).

Here in this present work, *SNAC*1 transcription factor under *CaMV*35S constitutive promoter was characterized in *indica* rice landrace cultivar Binnatoa (results: part one) and three high yielding rice varieties BRRI Dhan-49, BRRI Dhan-56 and BRRI Dhan-55 were transformed with *SNAC*1 transcription factor under stress inducible rd29A promoter (results: part two), thus three rd29A_*SNAC*1_BR-49, rd29A_*SNAC*1_BR-56 and rd29A_*SNAC*1_BR-55 transgenic lines were produced. Previously, another *SNAC*1 over-expressed transgenic line *CaMV*35S_*SNAC*1_BR-55 was prepared in our lab by transformation of BRRI Dhan-55 with pH7WG2_*CaMV*35S_*SNAC*1 construct. *CaMV*35S_*SNAC*1_BR-55 transgenic lines also showed better stress tolerance in salinity and drought condition at seedling stage (Parvin et al., 2015). These transgenic lines were further analysed at reproductive stages and finally two lines P4 and P5 were selected according to their stress tolerance at both seedling and reproductive stages.

In this results part three, comparative assay was done between rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 lines along with their wildtype BRRI Dhan-49 and BRRI Dhan-56, under salinity and drought condition at both seedling and reproductive stages to understand how far *SNAC*1 transformed rice varieties with different genetic background showing their stress tolerance with salinity and drought condition. Comparative assay was also done between rd29A_*SNAC*1_BR-55 and *CaMV*35S_*SNAC*1_BR-55 transgenic lines to find out

the effect of *SNAC*1 transcription factor under constitutive and stress inducible promoter in the same rice variety BRRI Dhan-55.

4.3.1.Workflow chart:

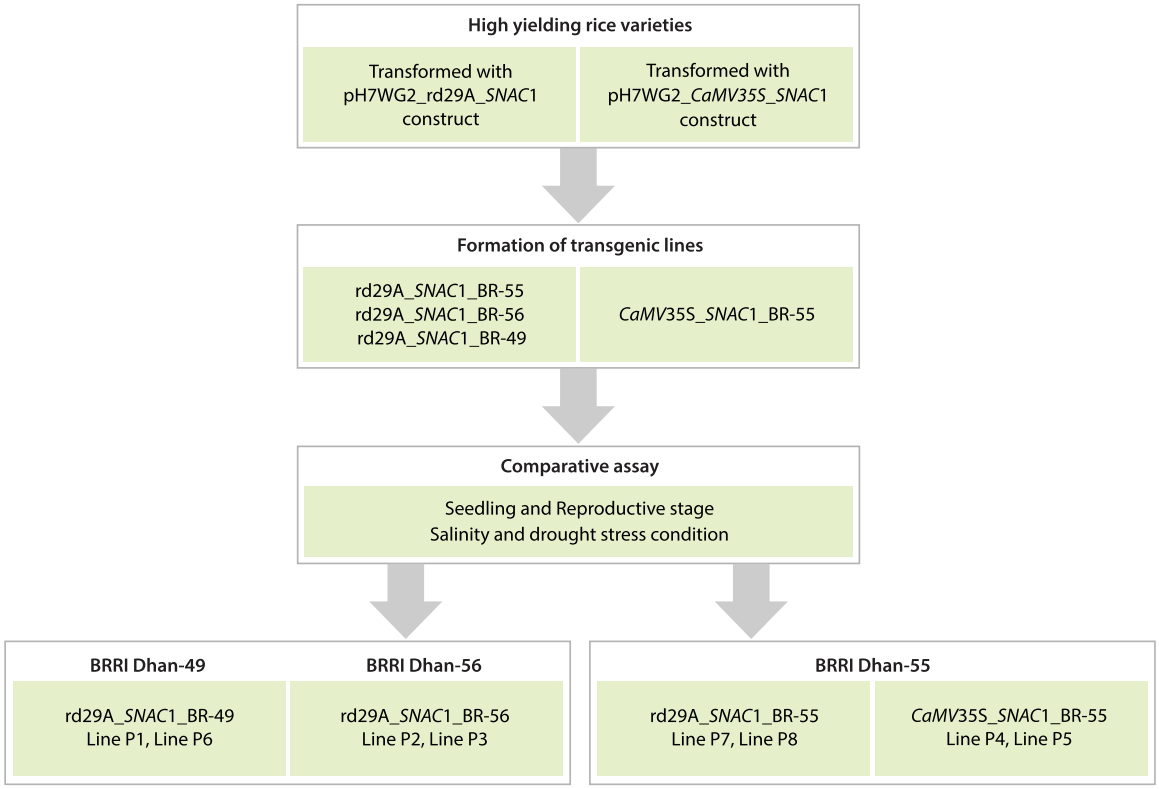


Figure 4.35: Workflow chart for comparative assay.

4.3.2.Comparative assay under salinity and drought condition:

Rice plants are tolerant to salinity during germination but are more sensitive during the young seedling stage (2-3 leaf stage). Salinity applied at the seedling stage frequently induces premature senescence of leaves, also influences shoot and root growth. It is easy to compare stress effect upon varieties during the seedling stage. Salinity and drought both affect plant height, shoot weight, numbers of tillers and effective tillers per plant and overall yield related traits. Stress condition affects panicle initiation, spikelet formation, germination of pollen grains and fertilization of florets and hence increases the number of sterile florets. So, comparative assay was conducted two-three leaves stage for seedling level and booting stage for reproductive level.

4.3.3.Comparative assay in different genetic background:

High yielding varieties BRRI Dhan-56 and BRRI Dhan-49 were transformed with *SNAC*1 transcription factor under stress inducible promoter rd29A. As moderately drought tolerant (BRRI Dhan-56) and salt sensitive (BRRI Dhan-49) rice varieties, comparative analysis showed the effect of *SNAC*1 transcription factor in two different genetic rice background. Comparative analysis was done at both seedling stage and reproductive stages under salinity stress and drought stress both. Two best lines, line P1 and P6 for rd29A_*SNAC*1_BR-49 transgenic plants and line P2 and P3 for rd29A_*SNAC*1_BR-56 transgenic plants were selected for comparative assay.

a) Comparative analysis at seedling stage:

For comparative assay two selected lines of both transgenic plants and their respective wildtype were subjected to 120 mM salt stress for 7 days. The rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines varied significantly in stress related parameters and in their stress tolerance. Under salinity stress rd29A_*SNAC*1_BR-56 lines showed better tolerance than rd29A_*SNAC*1_BR-49 transgenic lines. Transgenic plants from both varieties showed better phenotype than their wildtype (Figure 4.36). Both wildtype plants showed lower tolerance than transgenics.

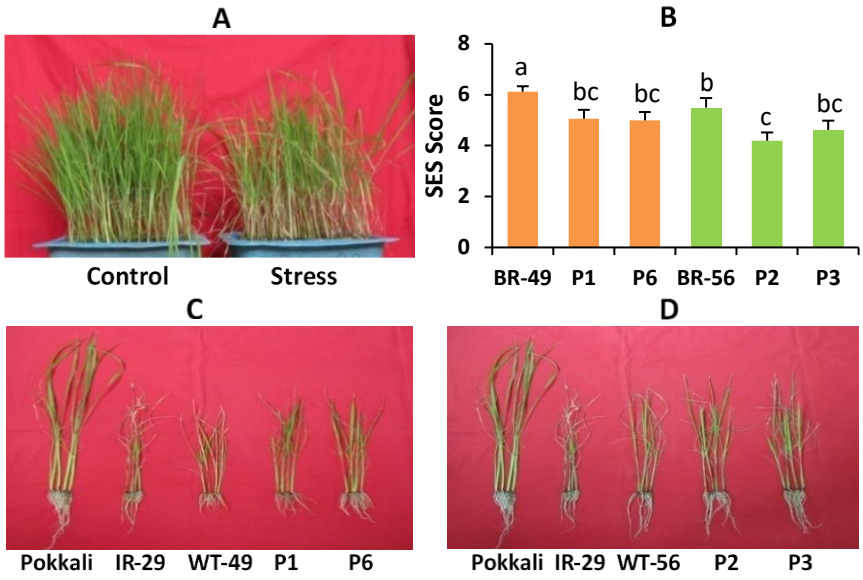


Figure 4.36: Seedling stage salinity stress. **A)** control and stressed plants after 10 days of 120 mM salt condition. **B)** Standard Evaluation System (SES) Score for rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines with their respective wildtypes. **C)** and **D)** Phenotype of rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines and wildtype plants after stress. Pokkali and IR29 represent as salt tolerant and salt sensitive controls respectively.

At seeding stage, drought stress was applied by withdrawal of water for 10 days. After application of the stress, leaf damage scores were measured. Plants from rd29A_*SNAC*1_BR-56 transgenic line showed better tolerance than plants from rd29A_*SNAC*1_BR-49 transgenic lines (Figure 4.37). Both wildtype plants showed significantly lower stress tolerance than the transgenic plants.

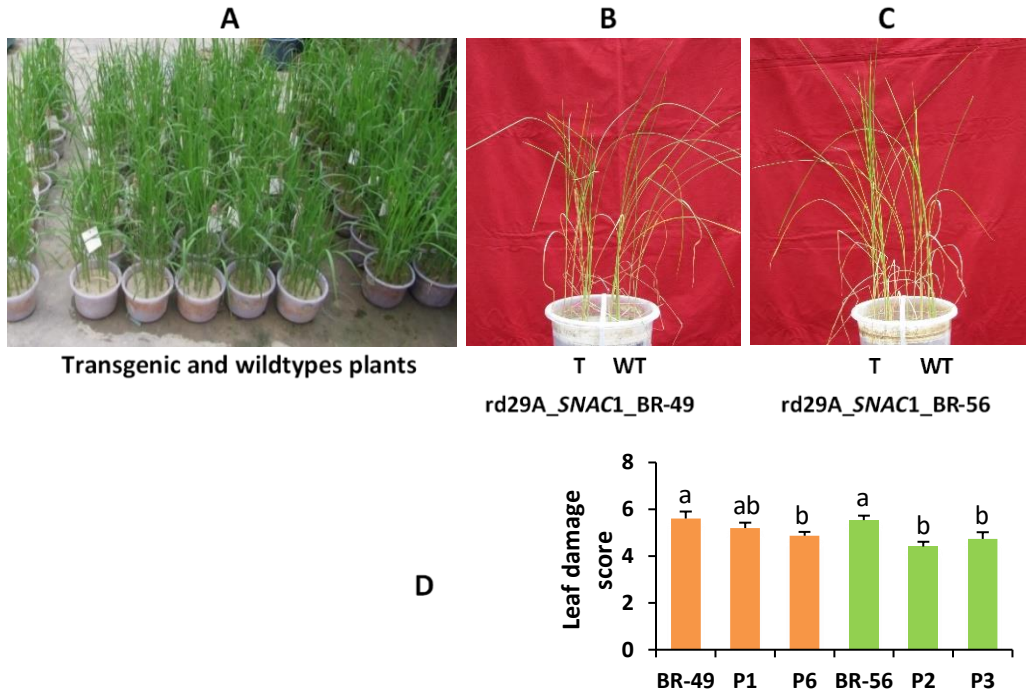


Figure 4.37: Seedling stage drought stress. **A)** rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines and wild type plants before drought stress. **B)** and **C)** rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines and wild type plants after drought stress respectively. **D)** Leaf damage score of rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines after drought stress.

The percent reduction of chlorophyll content was measured after stress application for both varieties. After application of both salinity and drought stress conditions, salt sensitive rd29A_*SNAC*1_BR-49 transgenic plants were more affected than rd29A_*SNAC*1_BR-56 transgenic lines. rd29A *SNAC*1_BR-49 transgenic plants showed 17-23 % and 20-21 % reduction of chlorophyll content in salinity and drought condition whereas rd29A_*SNAC*1_BR-56 transgenic lines showed 7-18 % and 8-10 % reduction respectively. Wildtype BRRI Dhan-49 showed 33% and 31% chlorophyll reduction compared to 30% and 22% reduction in wildtype BRRI Dhan-56 in salinity and drought condition (Figure 4.38). The differences between wilt type and transgenic plants were statistically significant.

The percent increase of electrolyte leakage in both rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic plants was 44-63% and 33-50% in salinity stress and 31-44% and 36-56% in drought stress. About 93% and 83% of electrolyte leakage was recorded for wildtype BRRI Dhan-49 compared to 69% and 72% for wildtype BRRI Dhan-56 in salinity and drought stresses (Figure 4.38).

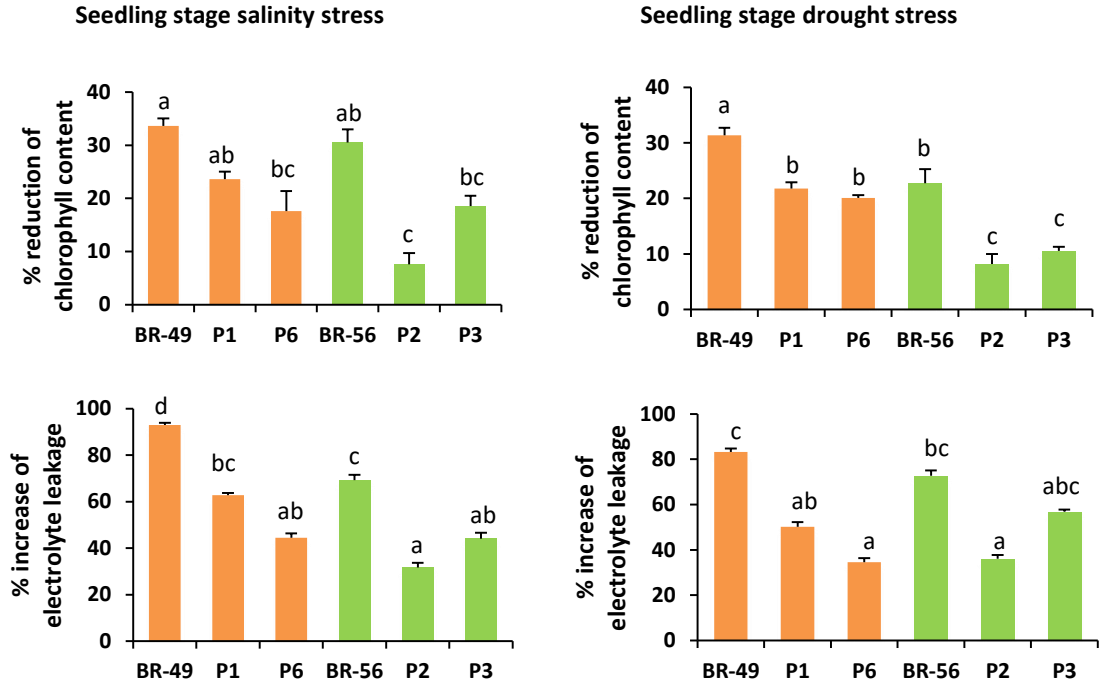


Figure 4.38: Comparative assay at seedling stage. (Left) percent reduction of chlorophyll content and percent increase of electrolyte leakage after salinity stress and (right) percent reduction of chlorophyll content and percent increase of electrolyte leakage after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–d) indicate significant differences (P<0.05, ANOVA and Duncan test)

Lipid peroxidation assay was done to measure the oxidative stress by measuring the amount of degradation product, Malondialdehyde (MDA), for both rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines. Both lines showed more oxidative damage in salinity stress than drought stress. Under stress condition, the MDA concentration is increased in all transgenic and wildtype plants. But the percentage of increase in MDA content was significantly lower in the transgenic plants than the wildtype (Figure 4.39).

Higher level of H_2O_2 indicates more tolerance by increasing stomatal closure that plays crucial role in preventing water loss and retention of water content in plants. Transgenic plants showed significantly higher H_2O_2 level than respective wildtypes at both salinity and

drought stress. But H_2O_2 level is much higher in drought condition than in salt stress for both transgenic lines (Figure 4.39).

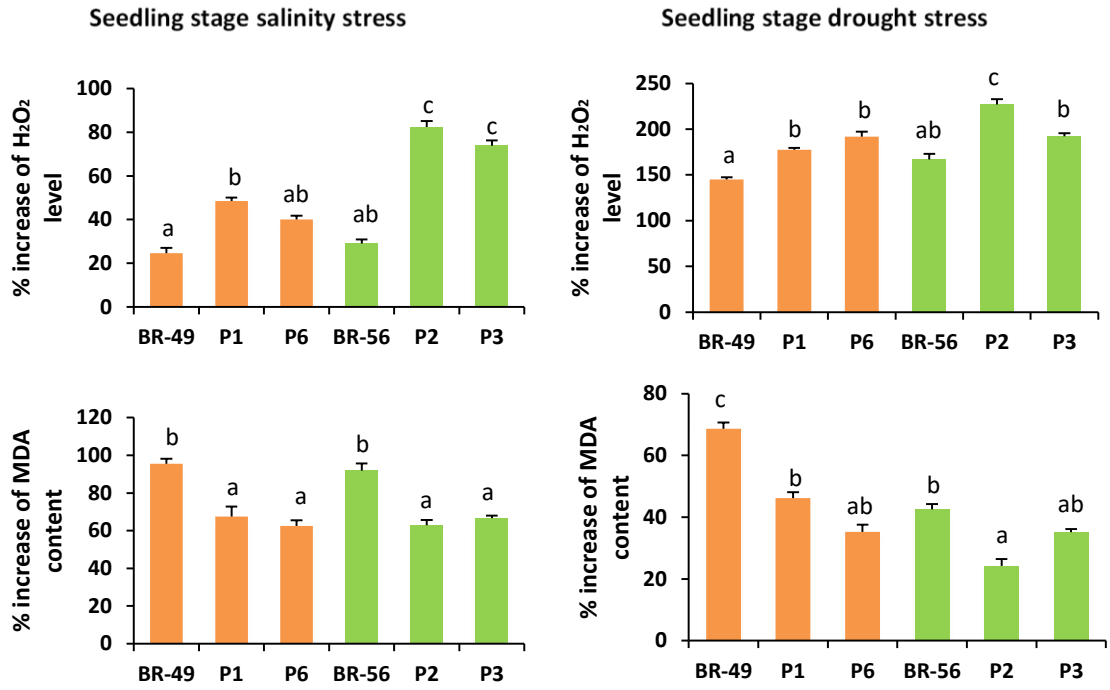


Figure 4.39: Comparative assay at seedling stage. (Left) percent increase of hydrogen peroxide (H_2O_2) level and percent increase of Malondialdehyde (MDA) content after salinity stress and (right) percent increase of hydrogen peroxide (H_2O_2) level and percent increase of Malondialdehyde (MDA) content after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–c) indicate significant differences ($P<0.05$, ANOVA and Duncan test).

Percent reduction of shoot length and shoot weight were measured after stress for both varieties. Here, in terms of damage, drought affects more than salinity stress. At both stress condition, Salt sensitive rd29A_*SNAC*1_BR-49 transgenic plants were more affected than rd29A_*SNAC*1_BR-56 transgenic lines. Transgenic lines from both rice varieties showed significantly better tolerance than their wildtypes plants at both stress condition (Figure 4.40).

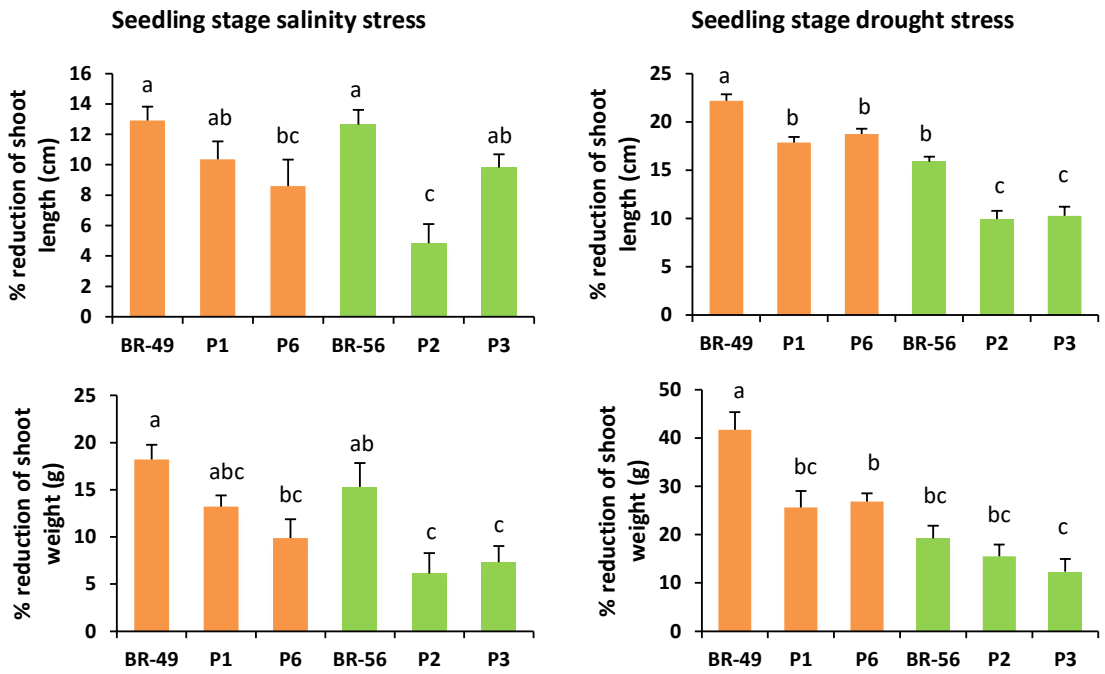


Figure 4.40: Comparative assay at seedling stage. (Left) percent reduction of shoot length (cm) and percent reduction of shoot weight (g) after salinity stress and (right) percent reduction of shoot length (cm) and percent reduction of shoot weight (g) after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–c) indicate significant differences (P<0.05, ANOVA and Duncan test)

b) Comparative analysis at reproductive stage:

One-week old seedling of rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines and respective wildtypes were transferred into individual pot. For salinity stress, perforated pots were used, and six pots were arranged into one bowl. At booting stage, the bowl was filled with 8 dS/m NaCl in Yoshida solution. Five replicates were set for each line. Plants were present under saline condition until harvest and yield related data were measured (Figure 4.41).

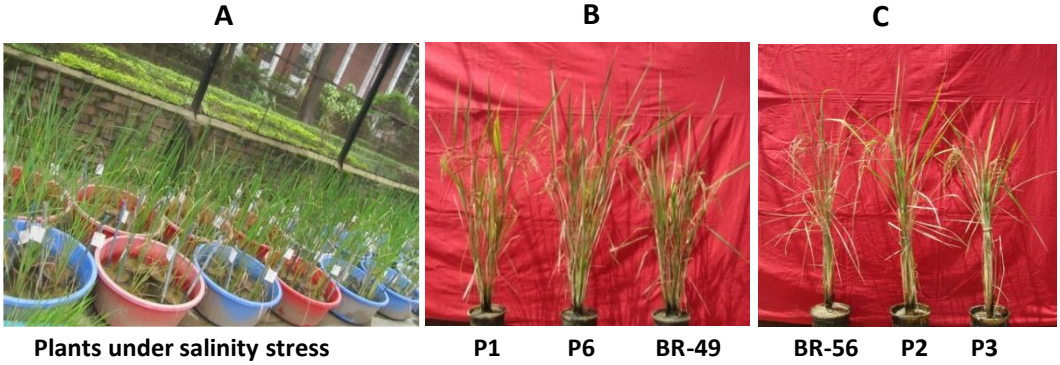


Figure 4.41: Reproductive stage salinity stress. **A)** transgenic and wildtype plants under 8 dS/m salt condition. **B)** and **C)** Phenotypes of transgenic rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines and wildtype plants under stress condition at maturity.

Drought stress was applied at panicle initiation stage. At that stage, water was stopped, and three holes were made at the bottom of the pot to drain out water. Stress was applied until leaves were fully rolled, considering 70-72% water content (section 3.5.4). After drought stress the plants were rewatered and remained in water until harvest. Transgenic lines visibly showed better stress tolerance than wildtype plants (Figure 4.42).

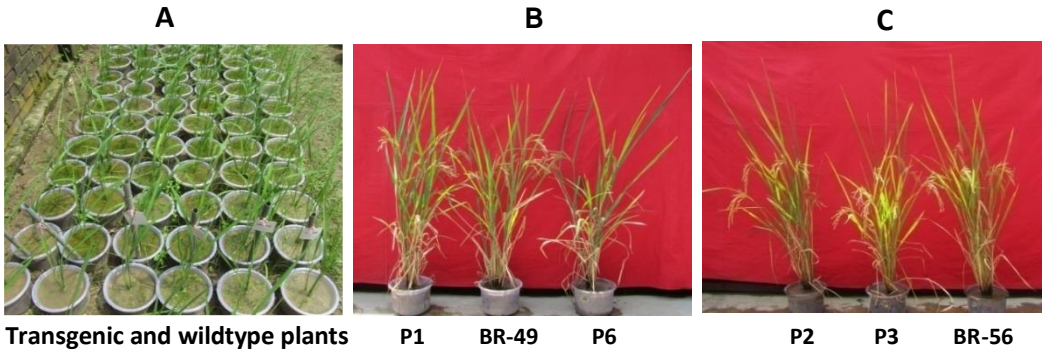


Figure 4.42: Reproductive stage drought stress. **A)** Transgenic and wildtype plants before drought stress. **B)** and **C)** Phenotypes of rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines and wildtype plants respectively after stress condition at maturity.

Salinity and drought stress significantly inhibited all yield attributing characteristics. Salt sensitive BRRI Dhan-49 variety differed in their yield related data from drought tolerant BRRI Dhan-56 variety. BRRI Dhan-49 variety showed less stress tolerance at both salinity and drought condition than BRRI Dhan-56 lines. Furthermore, both BRRI Dhan-49 and BRRI Dhan-56 transgenic lines were affected more in salt stress than drought stress. These two varieties showed significant variation in percent reduction of spikelet fertility, also in percent reduction of yield compared to wildtype plants.

Under salt stress about 15-25% spikelet fertility was reduced in rd29A_*SNAC*1_BR-49 transgenic lines whereas 8-12% for rd29A_*SNAC*1_BR-56 transgenic lines and under drought stress it was found 12-14% for rd29A_*SNAC*1_BR-49 transgenic lines and 9-12% for rd29A_*SNAC*1_BR-56 transgenic lines. For both varieties, there were no physiological or morphological difference between transgenic lines and their wildtype without any stress (data not shown). For rd29A_*SNAC*1_BR-49 transgenic lines, 23-34% yield was reduced in salinity condition but 12-21% yield reduced in drought condition. In salinity condition, rd29A_*SNAC*1_BR-56 transgenic lines showed 27-30% yield reduction and in drought condition, 17-20% yield was reduced (Figure 4.43).

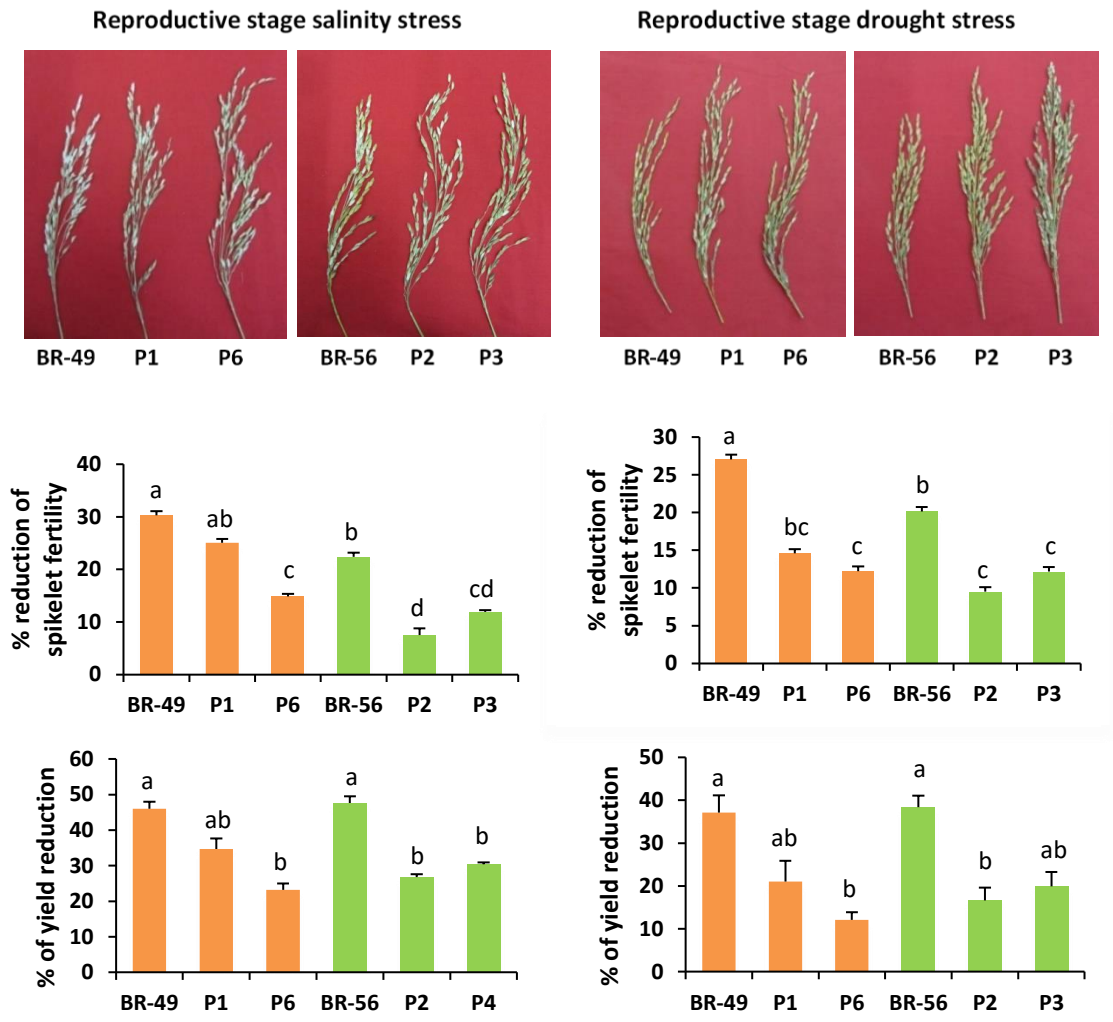


Figure 4.43: Comparative assay at reproductive stage. (left) the panicles of transgenic lines and wildtype plants, percent reduction of spikelet fertility and percent reduction of yield after salinity stress and (right) the panicles of transgenic lines and wildtype plants, percent reduction of spikelet fertility and percent reduction of yield after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–d) indicate significant differences (P<0.05, ANOVA and Duncan test)

Salinity stress showed more effect on plant height and 1000 grain weight than drought stress in both rice varieties. Wildtype BR-49 plants showed 11% and 10% plant height reduction in salinity and drought condition respectively whereas wildtype BR-56 plants showed 6% and 10% plant height reduction. About 6.5-8.5% and 4-6.5% plant height reduced for rd29A_*SNAC*1_BR-49 transgenic lines compare to 3.5-4.5% and 3.8-4.6% for rd29A_*SNAC*1_BR-56 transgenic lines in salinity and drought condition (Figure 4.44). Transgenic lines from both varieties showed significantly lower percent reduction of 1000 grain weight than respective wildtype plants in both stress condition, indicating better tolerance (Figure 4.44).

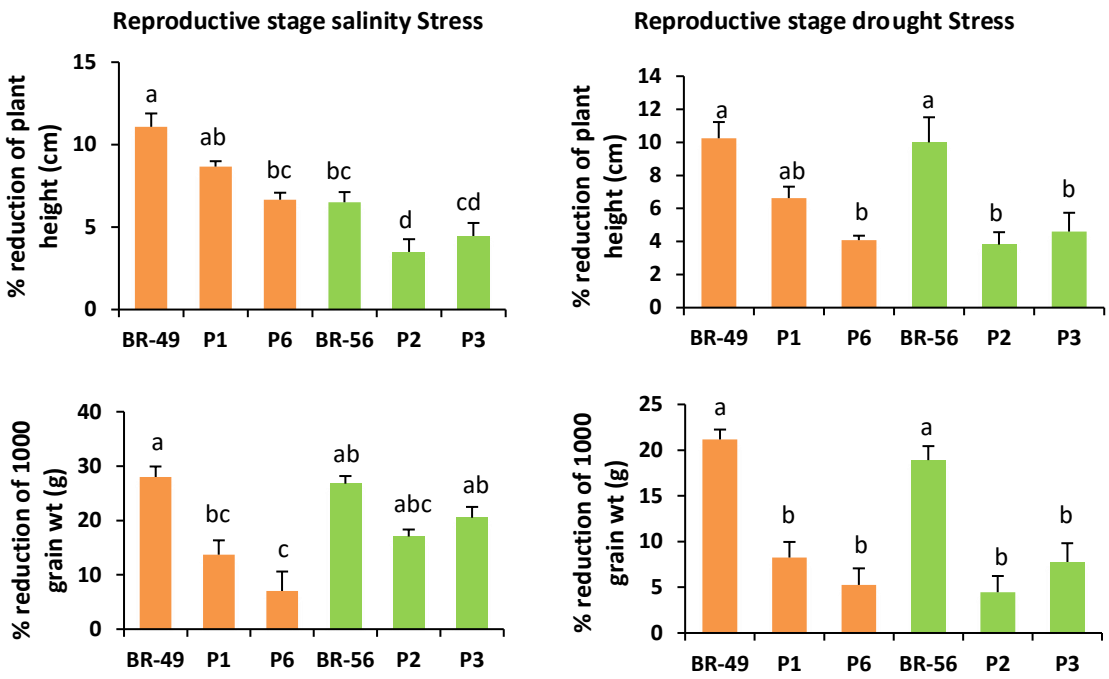


Figure 4.44: Comparative assay at reproductive stage. (left) percent reduction of plant height and percent reduction of 1000 grain weight after salinity stress and (right) percent reduction of plant height and percent reduction of 1000 grain weight after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–d) indicate significant differences (P<0.05, ANOVA and Duncan test)

For rd29A_*SNAC*1_BR-49 transgenic lines, percent reduction of panicle length was higher in salt stress than rd29A_*SNAC*1_BR-56 lines but in drought stress it was lower compare to rd29A_*SNAC*1_BR-56 transgenic lines. And in both salinity and drought stress rd29A_*SNAC*1_BR-49 transgenic lines showed lower % reduction of flag leaf length than rd29A_*SNAC*1_BR-56 transgenic lines (Figure 4.45). In both, parameters wildtype plants had higher % reduction than transgenic plants.

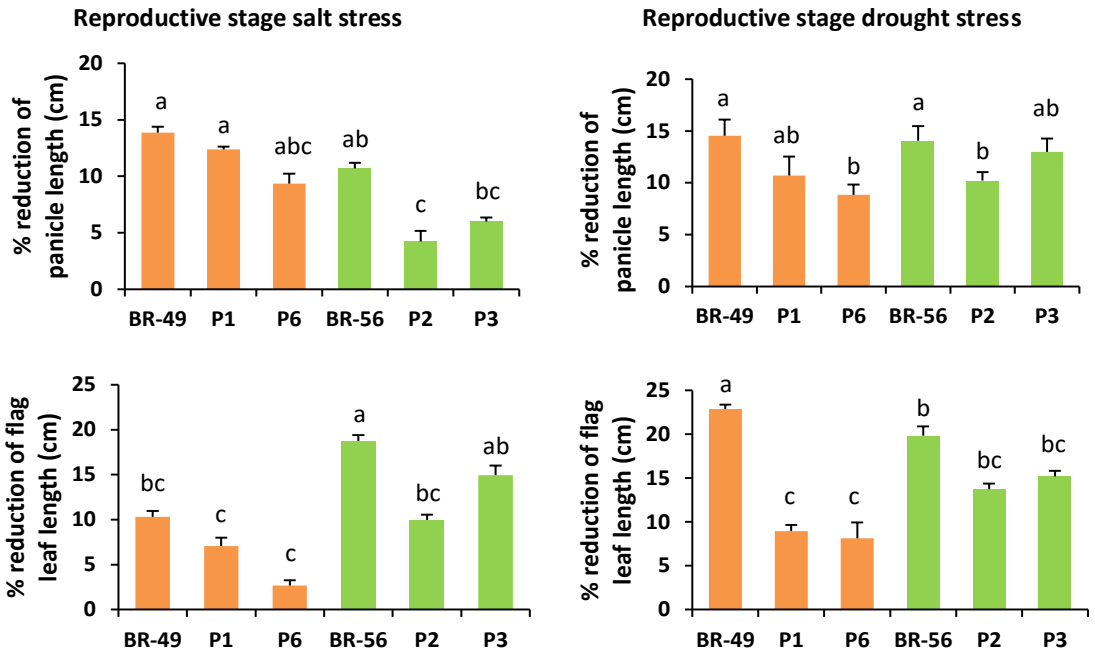


Figure 4.45: Comparative assay at reproductive stage. (left) percent reduction of panicle length (cm) and percent reduction of flag leaf length (cm) after salinity stress and (right) percent reduction of panicle length (cm) and percent reduction of flag leaf length (cm) after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–c) indicate significant differences (P<0.05, ANOVA and Duncan test)

Both varieties showed significantly higher total grain number in transgenic lines than wildtype plants under both salt and drought stresses. Compared to salinity stress, higher number of grains were found in drought condition. But less number of effective tillers were found in drought condition than salinity stress (Table 4.10).

Table 4.10: Number of total tillers, effective tiller and total grain of rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56 transgenic lines at salinity and drought stresses.

	Plants	Total no. of tiller		No. of effective tiller		No. of total grain	
		salt	*Drought*	*salt*	*Drought*	*salt*	*Drought*
Stress	BR-49	9.75 ± 0.39 ^{d}	9 ± 0.33 ^{d}	8.25 ± 0.20 ^{d}	6.25 ± 0.20 ^{d}	386.25 ± 3.6 ^{g}	487.5 ± 2.7 ^{d}
	P1	10.75 ± 0.20 ^{c}	9.5 ± 0.23 ^{c}	9.25 ± 0.20 ^{cd}	7 ± 0.33 ^{c}	491 ± 4.5 ^{f}	610.75 ± 9.6 ^{c}
	P6	10.5 ± 0.23 ^{c}	9.75 ± 0.39 ^{c}	9.5 ± 0.23 ^{c}	7.5 ± 0.23 ^{c}	520.75 ± 5.5 ^{e}	644.75 ± 4.5 ^{b}
	BR-56	8.5 ± 0.23 ^{e}	8.5 ± 0.23 ^{d}	6.5 ± 0.23 ^{f}	5.5 ± 0.23 ^{e}	395.5 ± 7.6 ^{g}	458.75 ± 6.2 ^{e}
	P2	8.25 ± 0.39 ^{e}	9.25 ± 0.20 ^{cd}	7.5 ± 0.23 ^{e}	6.5 ± 0.23 ^{d}	484.75 ± 6.1 ^{f}	622.75 ± 4.2 ^{bc}
	P3	8.5 ± 0.23 ^{e}	9.5 ± 0.23 ^{c}	7.25 ± 0.39 ^{e}	6.25 ± 0.20 ^{d}	468 ± 7.1 ^{f}	606 ± 10.7 ^{c}

	Plants	Total no. of tiller		No. of effective tiller		No. of total grain	
		salt	*Drought*	*salt*	*Drought*	*salt*	*Drought*
Control	BR-49	15.75 ± 0.48 ^{a}	11.25 ± 0.25 ^{a}	13 ± 0.70 ^{a}	9.25 ± 0.20 ^{a}	780 ± 8.1 ^{d}	749 ± 5.8 ^{b}
	P1	15.75 ± 0.47 ^{a}	11.75 ± 0.47 ^{a}	13 ± 0.40 ^{a}	9.5 ± 0.23 ^{a}	858.75 ± 12.8 ^{c}	776 ± 5.8 ^{a}
	P6	15 ± 0.40 ^{b}	12 ± 0.40 ^{a}	13.5 ± 0.28 ^{a}	9.5 ± 0.23 ^{a}	895.5 ± 11.4 ^{c}	778.25 ± 8.5 ^{a}
	BR-56	10.25 ± 0.25 ^{cd}	9.5 ± 0.28 ^{c}	9.75 ± 0.25 ^{c}	8.5 ± 0.23 ^{b}	950.5 ± 14.6 ^{b}	761 ± 11.7 ^{b}
	P2	10.75 ± 0.47 ^{c}	10.25 ± 0.25 ^{b}	10.25 ± 0.25 ^{b}	8.5 ± 0.23 ^{b}	1076.25 ± 15.3 ^{a}	776.75 ± 6.6 ^{a}
	P3	10.5 ± 0.50 ^{c}	10.25 ± 0.25 ^{b}	9.75 ± 0.25 ^{c}	8.75 ± 0.20 ^{b}	978 ± 4.1 ^{b}	774.5 ± 5.0 ^{a}

4.3.4.Comparative assay in same genetic background under constitutive and inducible promoter:

Plants under inducible promoter (rd29A_*SNAC*1_BR-55) and overexpressed (*CaMV*35S_*SNAC*1_BR-55) transgenic lines were analyzed under salinity and drought stress at both seedling and reproductive stages. After stress, different parameters were measured, which help to compare the constitutive and inducible expression of *SNAC*1 transcription factor in BRRI Dhan-55 under salinity and drought both abiotic stresses.

a) Comparative analysis at seedling stage:

Two selected lines (line P7, P8 for rd29A_*SNAC*1_BR-55 and line P4, P5 for *CaMV*35S_*SNAC*1_BR-55) from transgenic plants and wildtype BRRI Dhan-55 plants were grown in hydroponic system for approximately 14 days and then 120 mM salt stress was applied. Seven days after salt stress, when IR-29 (used as sensitive control) showed

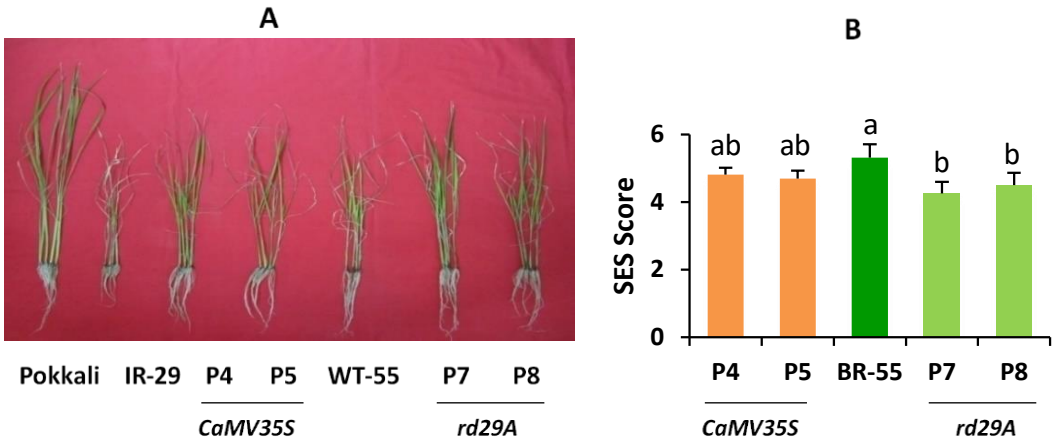


Figure 4.46: Seedling stage salinity stress. **A)** Phenotype of *CaMV*35S_*SNAC*1_BR-55 and rd29A_*SNAC*1_BR-55 transgenic lines and wildtype plants after10 days of 120 mM salt stress. **B)** Standard Evaluation System (SES) Score for *CaMV*35S_*SNAC*1_BR-55 and rd29A_*SNAC*1_BR-55 transgenic lines and wildtype plants. Pokkali and IR29 represent as salt tolerant and salt sensitive controls respectively.

visible signs of damage, stress related parameters were measured to compare between the rd29A_*SNAC*1_BR-55 and *CaMV*35S_*SNAC*1_BR-55 transgenic lines. In this comparative assay Pokkali was used as tolerant control.

At salinity stress transgenic lines were significantly differed from wildtype plants. Among transgenic lines, plants with stress inducible promoter showed significantly lower SES score than *SNAC*1 overexpressed lines (Figure 4.46). After 10 days of water withdrawal, both wildtype and transgenic lines showed visible changes, leaves were rolled, and old leaves were almost brown in colour. But transgenic plants were remained greener than the wildtype plants (Figure 4.47).

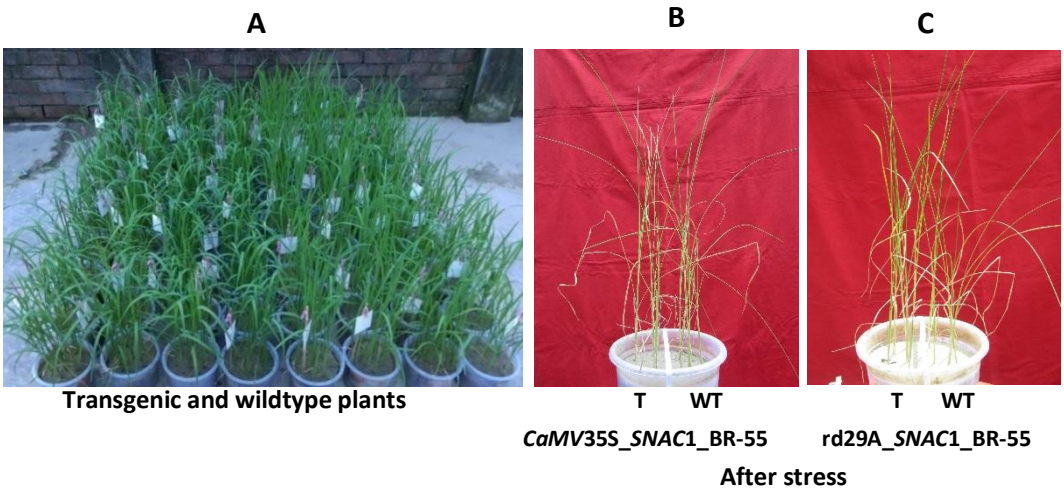


Figure 4.47: Seedling stage drought stress. **A)** *CaMV35S_SNAC*1_BR-55 and rd29A_*SNAC*1_BR-55 transgenic lines and wild type plants before drought stress. **B)** and **C)** *CaMV35S_SNAC*1_BR-55 and rd29A_*SNAC*1_BR-55 transgenic lines and wild type plants after drought stress respectively.

All transgenic lines had been performing consistently better than wildtype plants in terms of percent reduction of chlorophyll content and percent increase of electrolyte leakage. In both salinity and drought stress condition transgenic lines with rd29A inducible promoter showed significantly better tolerance than *CaMV*35S_*SNAC*1_BR-55 transgenic lines (Figure 4.48).

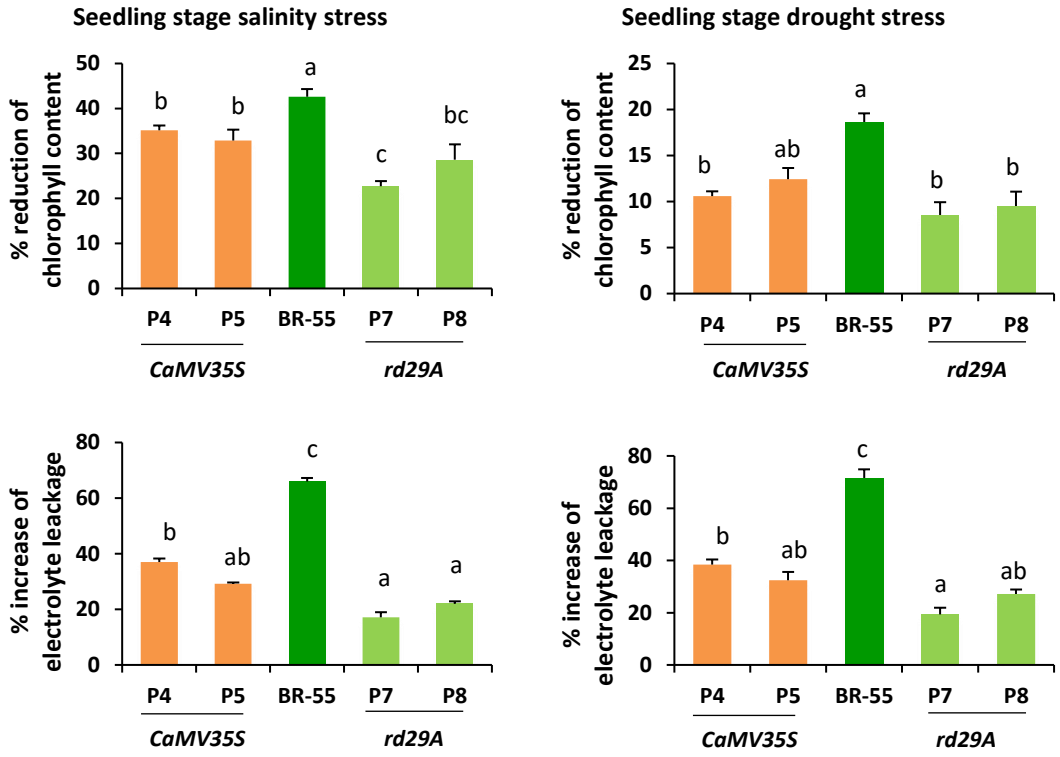


Figure 4.48: Comparative assay at seedling stage. (Left) percent reduction of chlorophyll content and percent increase of electrolyte leakage after salinity stress and (right) percent reduction of chlorophyll content and percent increase of electrolyte leakage after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–c) indicate significant differences (P<0.05, ANOVA and Duncan test).

The analysis variance of data revealed that the transgenic lines under constitutive promoter and inducible promoter varied significantly in their stress related parameters such as percent increase of malondialdehyde (MDA) content or percent increase of hydrogen peroxide (H_2O_2) level. Under stress condition, the MDA concentration is increased in all transformed and wildtype plants. But the percent of increase in MDA was significantly lower in the transgenic plants than the wildtype. rd29A_*SNAC*1_BR-55 showed better tolerance. More MDA content was found under salt stress than in drought stress (Figure 4.49).

Higher percent increase of H_2O_2 level was found under drought stress condition than salt stress in transgenic and wildtype plants. Under salinity stress rd29_*SNAC*1_BR-55 transgenic lines showed significantly better tolerance than *CaMV35S_SNAC*1_BR-55 transgenic lines. Under drought stress no significant difference was found in two transgenic plants (Figure 4.49).

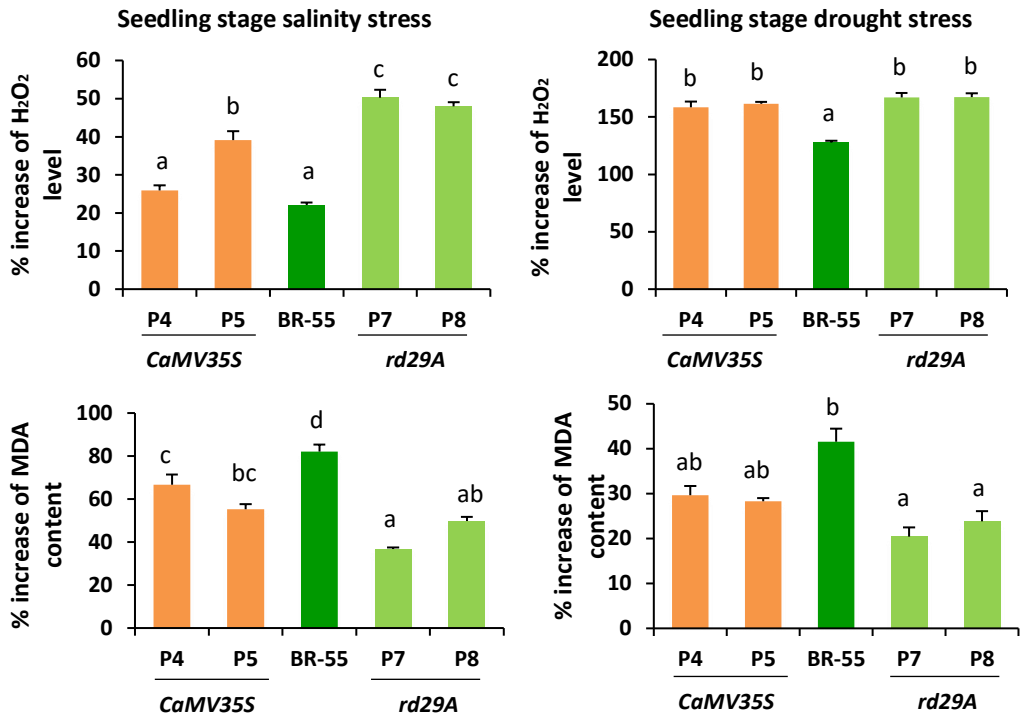


Figure 4.49: Comparative assay at seedling stage. (Left) percent increase of hydrogen peroxide (H_2O_2) level and percent increase of Malondialdehyde (MDA) content after salinity stress and (right) percent increase of hydrogen peroxide (H_2O_2) level and percent increase of Malondialdehyde (MDA) content after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–d) indicate significant differences ($P<0.05$, ANOVA and Duncan test).

Drought stress had more impact on shoot length and shoot weight than did salinity stress. As a result, the percent reduction in both shoot length and shoot weight under drought condition were greater than under salt stress. Transgenic lines showed better tolerance than wildtype BRRI Dhan-55 plants at both stress condition. Among transgenic lines rd29A_*SNAC*1_BR-55 performed better in both stress condition (Figure 4.50).

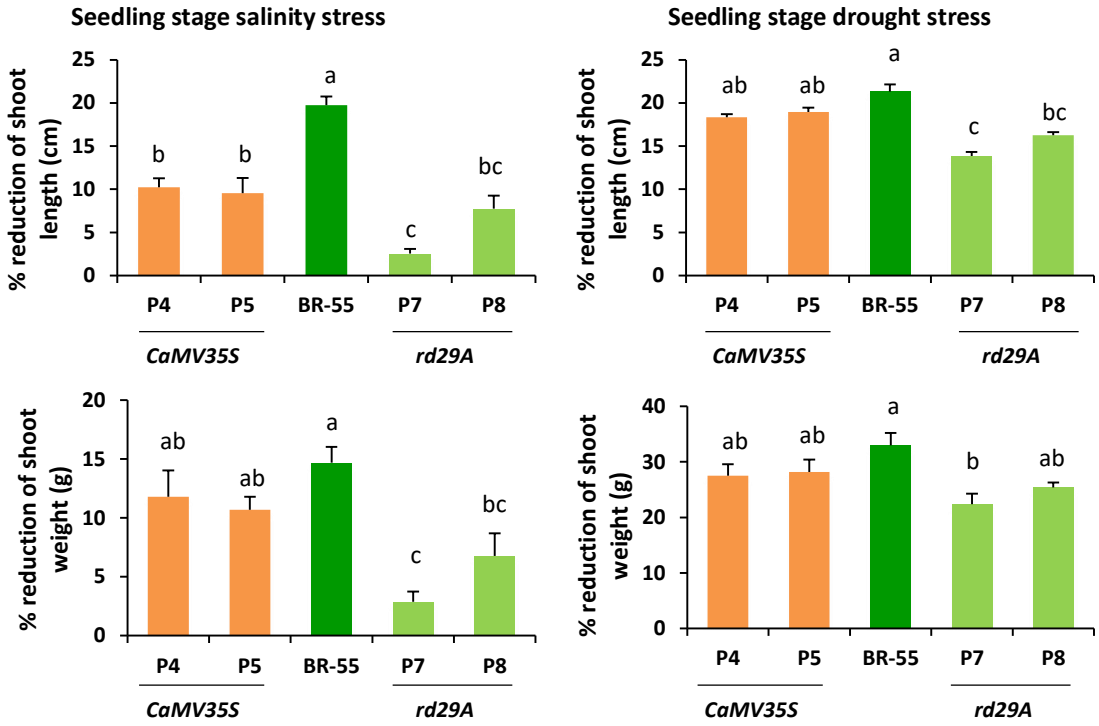


Figure 4.50: Comparative assay at seedling stage. (Left) percent reduction of shoot length (cm) and percent reduction of shoot weight (g) after salinity stress and (right) percent reduction of shoot length (cm) and percent reduction of shoot weight (g) after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–c) indicate significant differences (P<0.05, ANOVA and Duncan test).

b) Comparative analysis at reproductive stage:

Transgenic lines with both constitutive promoter *CaMV*35S and inducible promoter rd29A were subjected under salinity and drought stresses at reproductive stages. Between the two stresses, transgenic lines showed better drought tolerance than salinity tolerance. It seems that the transgenic lines with stress inducible promoter were more able to withstand drought and salinity stress than the transgenic plants with constitutive promoter.

All yield related parameters were measured during harvest. The rd29A_*SNAC*1_BR-55 transgenic lines possessed visibly better phenotype than *SNAC*1 overexpressed plants as well as wildtype plants (Figure 4.51 and 4.52). Line P7 showed better tolerance in all parameters.

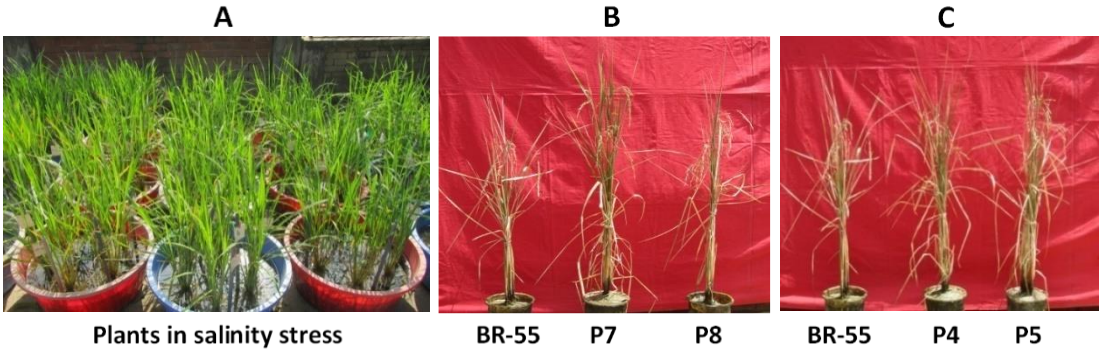


Figure 4.51: Reproductive stage salinity stress. **A)** Plants in 80 dS/m salt stress. **B)** and **C)** Phenotypes of rd29A_*SNAC*1_BR-55 and *CaMV*35S_*SNAC*1_BR-55 transgenic lines and wildtypes plants respectively after stress condition at maturity.

At reproductive stage, comparative assay was done in net house for both salinity and drought stresses. Both transgenic lines (rd29A_*SNAC*1_BR-55 and *CaMV*35S_*SNAC*1_BR-55) exhibited significant variation in their performance with regard to yield traits. In some parameters, line P8 (rd29A_*SNAC*1_BR-55) did similar tolerance with line P5 (*CaMV*35S_*SNAC*1_BR-55), But line P7 from rd29A_*SNAC*1_BR-55 transgenic lines differ significantly from transgenic lines with constitutive promoter as well as wildtype plants.

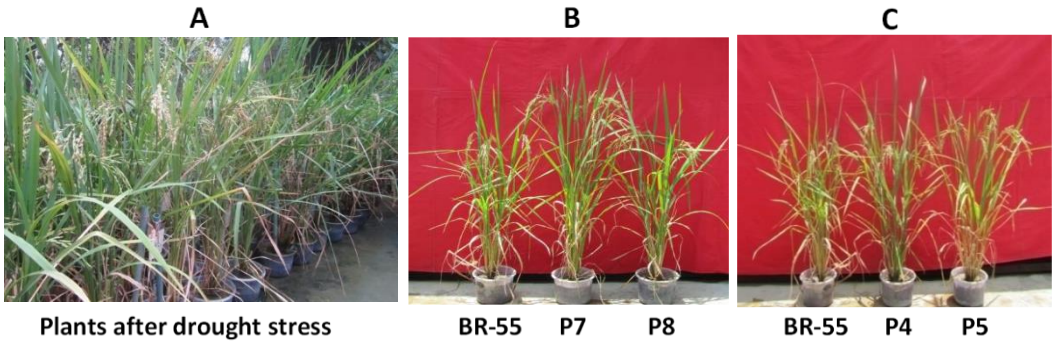


Figure 4.52: Reproductive stage drought stress. **A)** Transgenic and wildtype plants after drought stress. **B)** and **C)** Phenotypes of rd29A_*SNAC*1_BR-55 and *CaMV*35S_*SNAC*1_BR-55 transgenic lines and wildtypes plants respectively after stress condition at maturity.

*CaMV*35S_*SNAC*1_BR-55 lines showed 16-22% and 10-11% reduction of spikelet fertility in salinity and drought stress respectively whereas 8-10% and 7-8% reduction was found for rd29A_*SNAC*1_BR-55 lines in salt and drought condition. The percent reduction in yield for *CaMV*35S_*SNAC*1_BR-55 lines were 38-40 % in salt stress and 14- 16% in drought stresses compared to 31-35% in salt stress and 9-16% in drought condition for rd29A_*SNAC*1_BR-55 lines (Figure 4.53).

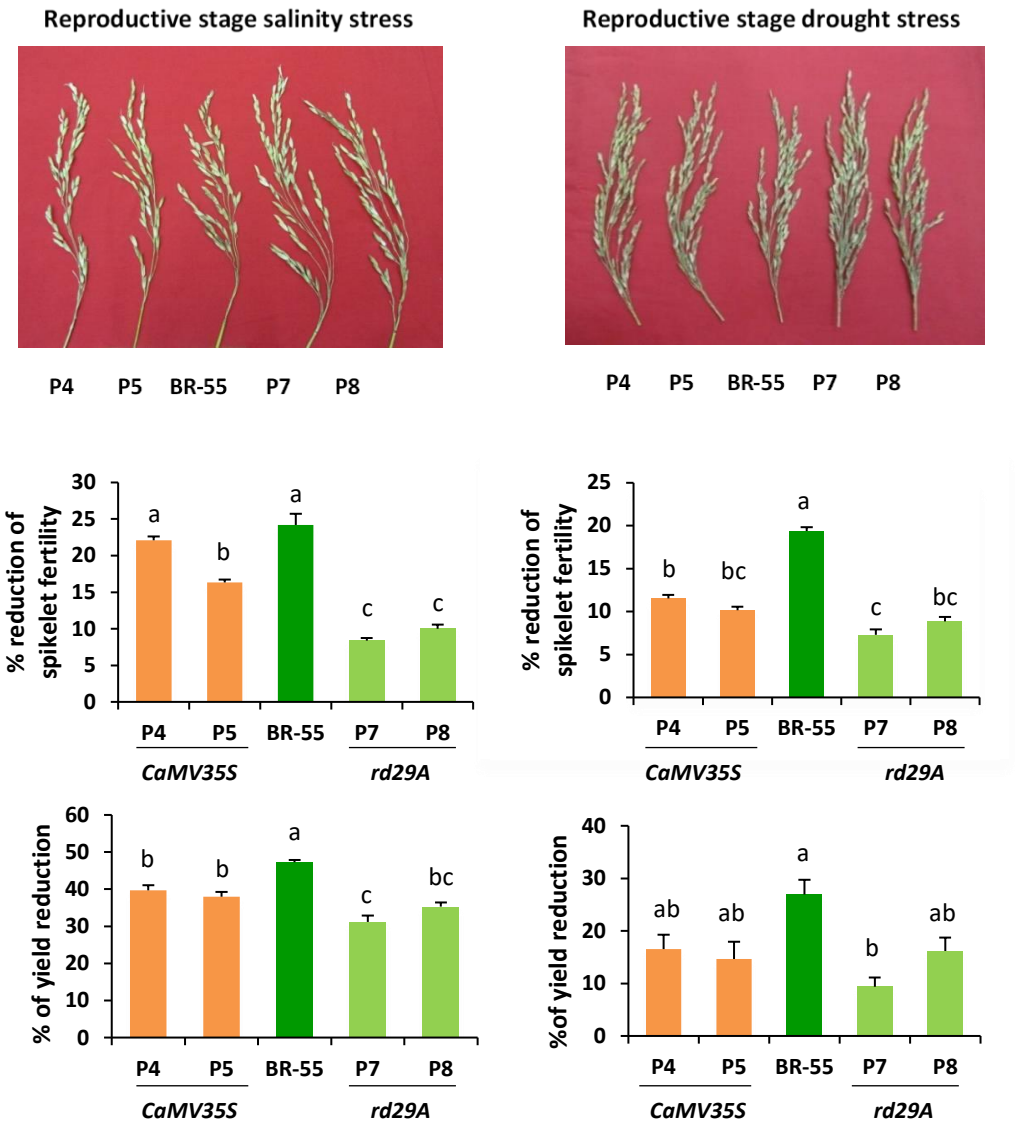


Figure 4.53: Comparative assay at reproductive stage. (left) the panicles of transgenic lines and wildtype, percent reduction of spikelet fertility and percent reduction of yield after salinity stress and (right) the panicles of transgenic lines and wildtype, percent reduction of spikelet fertility and percent reduction of yield after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–c) indicate significant differences (P<0.05, ANOVA and Duncan test)

Both transgenic lines *CaMV*35S_*SNAC*1_BR-55 and rd29A_*SNAC*1_BR-55 showed significant lower percent reduction of plant height and 1000 grain weight compared to their wildtype. Among these lines, P7 with inducible promoter showed better tolerance in both stresses (Figure 4.54).

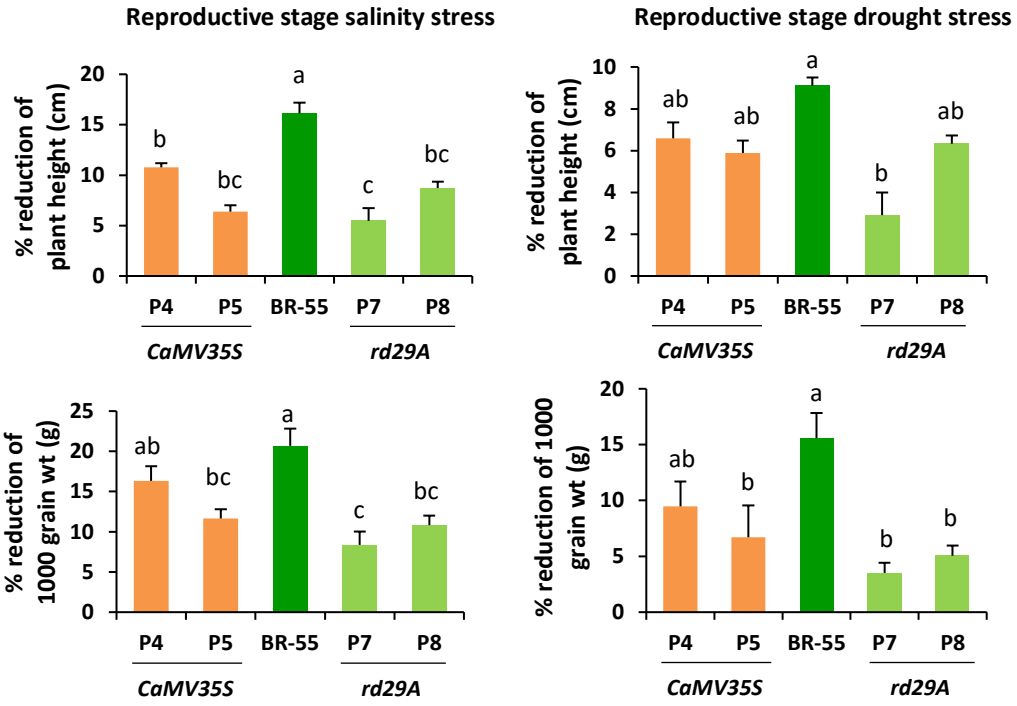


Figure 4.54 Comparative assay at reproductive stage. (left) percent reduction of plant height and percent reduction of 1000 grain weight after salinity stress and (right) percent reduction of plant height and percent reduction of 1000 grain weight after drought stress Error bar indicates the mean ±SE (n=5). Different letters (a–c) indicate significant differences (P<0.05, ANOVA and Duncan test).

The rd29A_*SNAC*1_BR-55 transgenic lines gave lowest value in percent reduction of panicle length as well as in percent reduction of flag leaf length under both saline and drought condition compared to *CaMV*35S_*SNAC*1_BR-55 transgenic lines and wildtype plants (Figure 4.55).

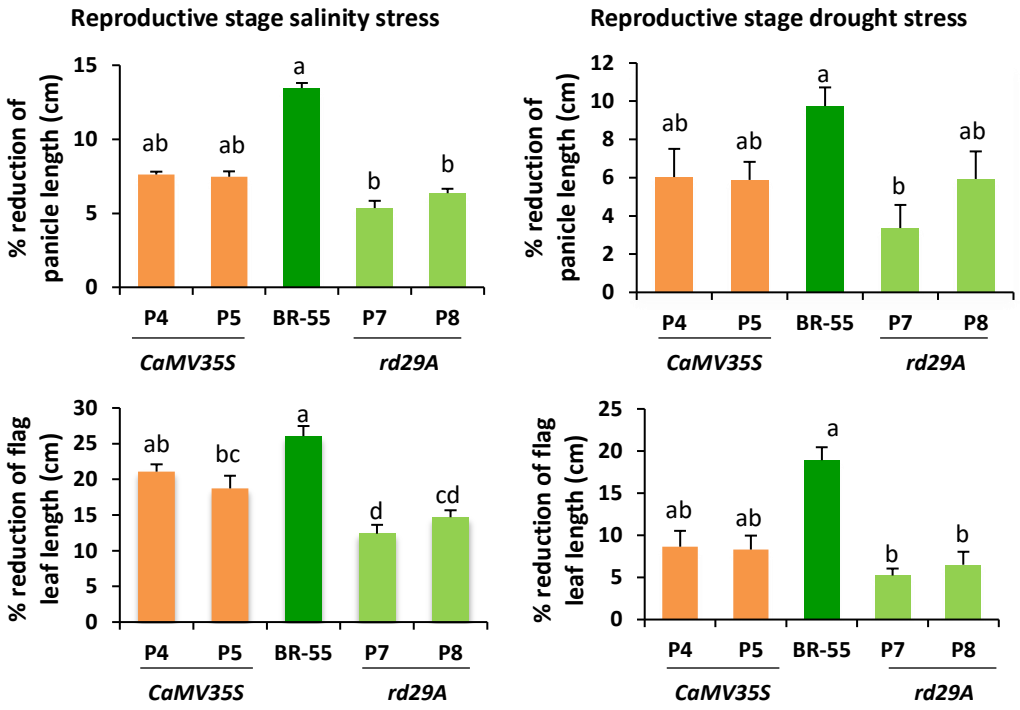


Figure 4.55: Comparative assay at reproductive stage. (left) percent reduction of panicle length (cm) and percent reduction of flag leaf length (cm) after salinity stress and (right) percent reduction of panicle length (cm) and percent reduction of flag leaf length (cm) after drought stress. Error bar indicates the mean ±SE (n=5). Different letters (a–d) indicate significant differences (P<0.05, ANOVA and Duncan test).

Among yield related parameters like total number of tillers, effective tillers and total grain, transgenic line rd29A_*SNAC*1_BR-55 showed better tolerance than *CaMV*35S_*SNAC*1_BR-55 transgenic lines. Total grain was more affected under salinity stress compared to drought stress (Table 4.11).

Table 4.11: Number of total tillers, effective tiller and total grain of *CaMV*35S_*SNAC*1_BR-55 and rd29A_*SNAC*1_BR-55 transgenic lines at salinity and drought stresses.

	Plants	Total no. of tiller		No. of effective tiller		No. of total grain	
		salt	Drought	salt	Drought	salt	Drought
Stress	BR-55	7.75 ± 0.20 ^{d}	7.75 ± 0.20 ^{c}	6.5 ± 0.23 ^{d}	5.5 ± 0.23 ^{e}	350.75 ± 5.3 ^{f}	543.75 ± 8.1 ^{d}
	P4	8.5 ± 0.23 ^{c}	8.75 ± 0.39 ^{b}	7.25 ± 0.20 ^{c}	5.75 ± 0.20 ^{d}	414.5 ± 2.3 ^{e}	662.75 ± 5.5 ^{c}
	P5	8.75 ± 0.20 ^{c}	8.75 ± 0.20 ^{b}	7.25 ± 0.20 ^{c}	6 ± 0.33 ^{d}	429.75 ± 5.2 ^{d}	674 ± 9.0 ^{c}
	P7	9 ± 0 ^{c}	9.25 ± 0.20 ^{b}	7.75 ± 0.20 ^{c}	6.5 ± 0.23 ^{c}	470.5 ± 5.5 ^{c}	705.75 ± 3.0 ^{b}
	P8	8.75 ± 0.20 ^{c}	8.75 ± 0.20 ^{b}	7.25 ± 0.20 ^{c}	6.25 ± 0.20 ^{c}	450.25 ± 3.2 ^{c}	692 ± 2.8 ^{b}
Control	BR-55	12.25 ± 0.25 ^{a}	10.5 ± 0.28 ^{a}	10.25 ± 0.25 ^{b}	8.5 ± 0.23 ^{b}	965.75 ± 3.1 ^{b}	858.75 ± 9.4 ^{a}
	P4	12 ± 0.40 ^{a}	10.75 ± 0.25 ^{a}	10.25 ± 0.47 ^{b}	8.75 ± 0.39 ^{ab}	978 ± 5.7 ^{b}	868.5 ± 4.3 ^{a}
	P5	11.75 ± 0.62 ^{b}	10.5 ± 0.28 ^{a}	10.5 ± 0.28 ^{b}	8.75 ± 0.20 ^{ab}	983 ± 3.2 ^{b}	870.75 ± 2.0 ^{a}
	P7	12 ± 0.40 ^{a}	10.5 ± 0.28 ^{a}	11.25 ± 0.25 ^{a}	9 ± 0.33 ^{a}	1046.75 ± 15.2 ^{a}	881 ± 7.3 ^{a}
	P8	11.5 ± 0.28 ^{b}	10.25 ± 0.47 ^{a}	10.5 ± 0.28 ^{b}	9 ± 0.33 ^{a}	1035.25 ± 13.1 ^{a}	878 ± 4.1 ^{a}

In summary, salinity and drought significantly inhibited plants growth at seedling stage as well as reproductive stage. However the performance of the transgenic lines was significantly better than wildtype, particularly where the *SNAC*1 transcription was driven by the stress-inducible promoter, rd29A. By measuring different parameters regarding to seedling level and others yield related parameters elucidated the inhibition level. In comparison, between different rice varieties rd29A_*SNAC*1_BR-49 and rd29A_*SNAC*1_BR-56, drought tolerant variety rd29A_*SNAC*1_BR-56 showed better salinity and drought tolerance than salt sensitive rd29A_*SNAC*1_BR-49 lines. It was also found that salinity had more adverse effect on plant growth than drought condition. Comparative assay was also done in between rd29A_*SNAC*1_BR-55 and *CaMV*35S_*SNAC*1_BR-55 transgenic lines. At both seedling and reproductive stages, transgenic lines with inducible promoter showed better tolerance than *SNAC*1 over-expressed plants in salinity and drought stresses.

4.4.Part four: Evaluation of the effect of *SNAC*1 expression on downstream genes

*SNAC*1 is one of the members of plant specific NAC (NAM, ATAF 1/2, and CUC2) protein family, showed increased expression predominantly in guard cells under drought condition and having transactivation activity (Hu et al., 2006). It was found that *SNAC*1 regulates the expression of other transcription factor like OsbZIP23 (Zong et al., 2016), also some genes are direct targets of *SNAC*1 such as OsSRO1c or OsPP2C18 (You et al., 2013, 2014). The higher level of expression of these gene provide tolerance to abiotic stresses such as salinity and drought in rice. DNA microarray analysis revealed that a large number of stress-related genes were up-regulated in the *SNAC*1 overexpressing rice plants. The features of *SNAC*1 as a transcription factor prompted the scientists to investigate the expression changes at the whole genome level using a rice DNA chip containing all putative genes in the rice genome. Compared with wildtype, >80 cDNA-supported genes showed 2.1-fold or higher up-regulation in the *SNAC*1 overexpressing *japonica* cultivar Nipponbare (Hu et al., 2006).

In this work, six genes were selected from *SNAC*1 up-regulated downstream genes from reported microarray data which were induced by abiotic stresses including drought and salinity. These selected genes are transcription factors, transporters and enzymes which are crucial for plant abiotic stress tolerance as well as growth and survival. In this study, downstream gene expression analysis was done in rd29A_*SNAC*1_BR-55 transgenic lines which showed better tolerance in both salinity and drought stress compared to *CaMV*35S_*SNAC*1_BR-55 transgenic lines (results Part three, comparative assay). It was also found that *SNAC*1 gene expression was two-fold higher in rd29A_*SNAC*1_BR-55 transgenic lines than *CaMV*35S_*SNAC*1_BR-55 lines (data not shown). Thus, rd29A_*SNAC*1_BR-55 lines were chosen as the best candidate to check the level of expression of the selected genes. These plants were subjected to 100 mM salt stress to test the induced effect of *SNAC*1 to its downstream genes.

Graphical Genotyping Tool (GGT2.0) helps to summarize all up-regulated genes in sequentially (Figure 4.56).

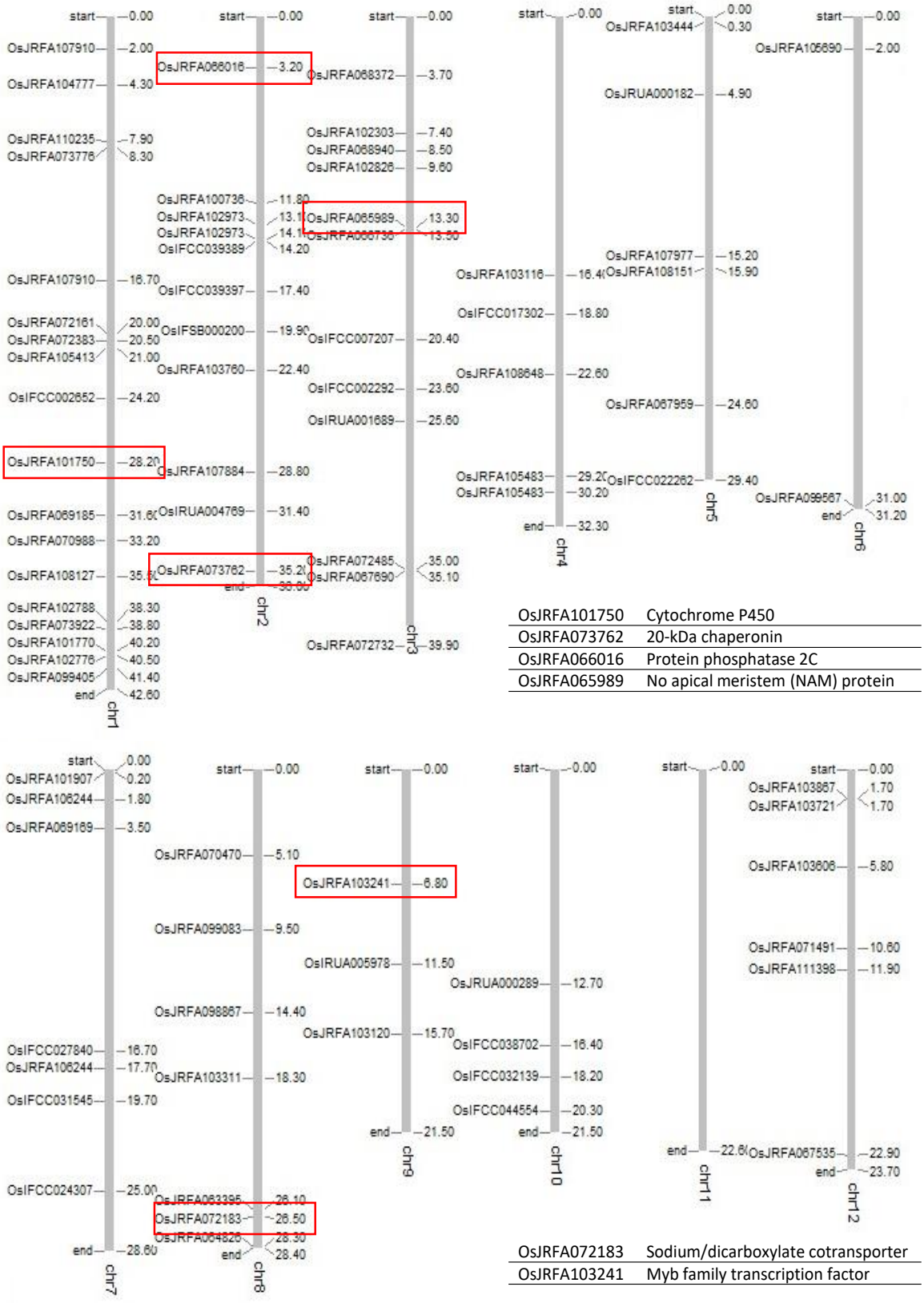


Figure 4.56: Chromosome map showing the positions of upregulated genes in *SNAC*1 transgenic plant, microarray data (Hu et al., 2006). Selected genes are indicated in box in chromosome specific location.

4.4.1.Workflow chart:

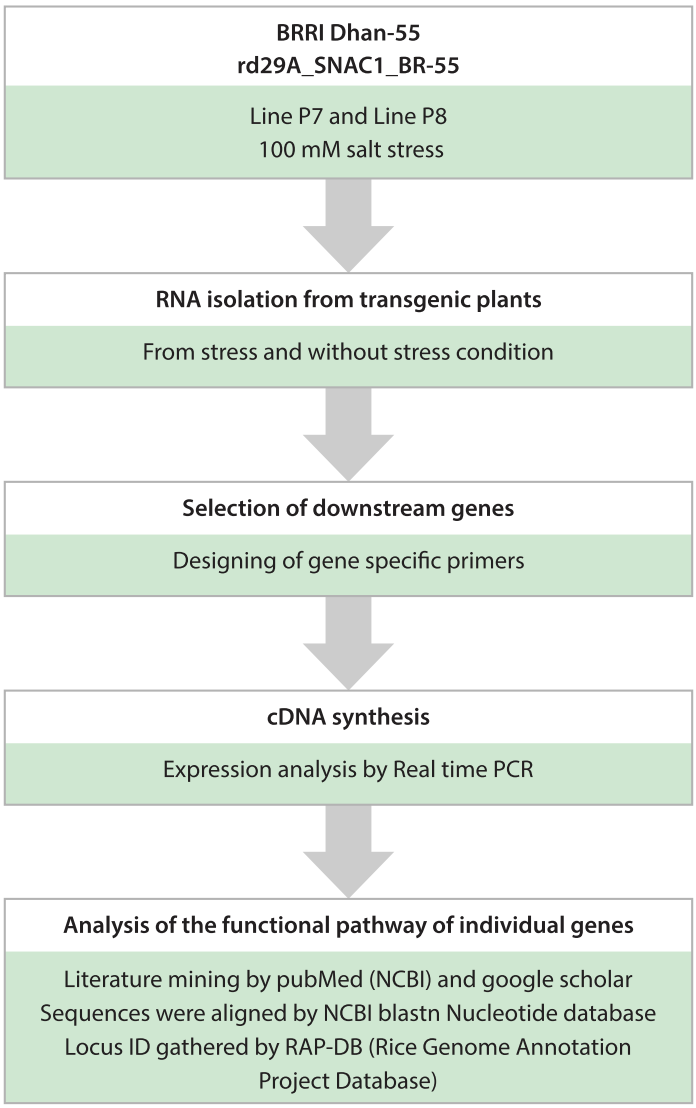


Figure 4.59: Workflow chart for expression pattern analysis of *SNAC*1 downstream genes from transgenic plants

4.4.2.Expression analysis of downstream genes:

For expression analysis, total RNA of *SNAC*1 transgenic plants were isolated at 0 hour and 24 hours after 100 mM salt stress. cDNAs were synthesized and selected gene expression were analyzed by real time PCR (qRT-PCR). The expression levels were normalized to Elongation Factor-α (EF- α) as endogenous control. Name and chromosome position of the genes are mentioned in Table 4.12.

Table 4.12: Expression analysis of the selected genes by qRT-PCR.

No. of genes	Name of the selected genes under study	Chromosome position	Accession No.
1.	Cytochrome P450	Chromosome 1	AK101750
2.	20-kDa chaperonin	Chromosome 2	AK060474
3.	Protein phosphatase 2C	Chromosome 2	AK066016
4.	No apical meristem (NAM) protein	Chromosome 3	AK067690
5.	Sodium/dicarboxylate cotransporter	Chromosome 8	AK072183
6.	Myb family transcription factor	Chromosome 9	AK103241

It was found that all the selected genes were upregulated in *SNAC*1 transgenic plants (rd29A_*SNAC*1_BR-55). Among them, under stress condition, the Myb transcription factor and sodium/dicarboxylate cotransporter gene expression differ significantly with respect to the gene expression in wildtype plants. There was much higher expression in transgenic lines compared to wildtype plants. The gene expression of No Apical Meristem (NAM) protein, 20-kDa chaperonin and Protein phosphatase 2C were statistically different in transgenic lines compare to wildtype plants at stress condition. There was no significant differences of Cytochrome P450 gene expression between transgenic lines and wildtypes plants (Figure 4.58).

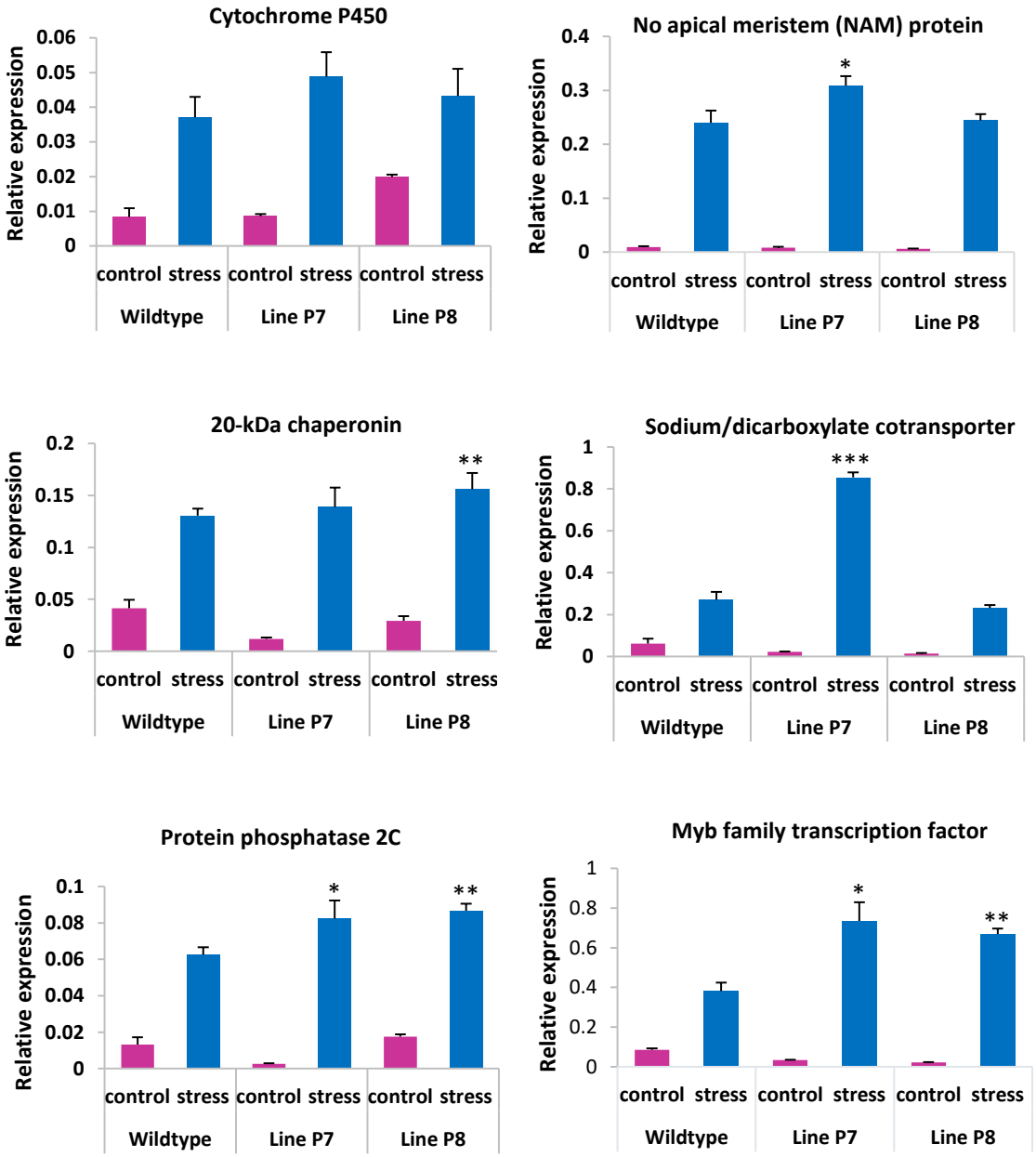


Figure 4.62: Expression analysis in induced *SNAC*1 transgenic plants. The transcript levels of 20-kDa Chaperonin, MYB transcription factor, Protein phosphatase 2C, Cytochrome P450, no apical meristem (NAM) protein, Sodium/dicarboxylate cotransporter genes were measured by qRT-PCR under normal condition and 100 mM salt treatment for 24hrs. Error bars indicate SE based on three replicates. *, **, *** student's *t* test, ($P<0.05$, $P<0.01$, $P<0.001$)

4.4.3.Regulatory pathway of the *SNAC*1 downstream up-regulated genes in rice by literature mining:

Expression of various genes depend upon *SNAC*1 transcription factor under different abiotic stresses. From the microarray data of *SNAC*1 overexpressing transgenic plants (Hu et al. 2006), it was clear that *SNAC*1 transcription factor is the master regulator of many stress related transcription factors and genes. The gene expression of the 6 genes analyzed here, in *SNAC*1 under rd29A promoter transgenic lines, all were also found expressed higher under stress condition. It can be assumed that the combined expression of these genes is important for plant defense during stress. I have explored the likely molecular mechanism of these genes and transcription factors upregulated by *SNAC*1 in such defense. The entire work in this section was based on literature mining to decipher the underlying pathway and relationship within these selected genes. Finally, individual pathways were integrated to try and understand their interactions under normal conditions and during abiotic stress.

a) Cytochrome P450:

Cytochrome P450 gene superfamily is widely involved in diverse processes of plant development and environmental responses including defence response to pathogens, secondary metabolism, detoxification of herbicides and chemical toxin (Wang Y. et al., 2004). The gene whose expression was studied here, has the Accession number AK 101750 and locus ID LOC_Os01g43740, namely cytochrome P450 72A. There are a total of 14 putative CYP72A members in the rice genome, with high diversity at the N-terminal sequences and high homology at C-terminal sequences of those 14 putative proteins. It was reported that CYP72A5 was involved in herbicide metabolism in corn (Persans et al., 2001). Other function of CYP72A5 subfamily in plant metabolism are still under the study.

In rice Blast, caused by *Magnaporthe oryzae, in planta* rice transcriptome analysis in leaf and neck tissues revealed that tissue-specific expression of rice cytochrome P450 gene was upregulated during neck infection (Mahesh et al., 2021). The gene expression pattern was analyzed in rice plants under tungro disease, caused by virus and transmitted by insect vector green leafhopper, showing that the P450 gene was upregulated compared to control (Mangrauthia et al., 2017). Therefore, Cytochrome P450 (CYP72A) was associated with plant defence in biotic stresses. Here, this gene is also upregulated in transgenic lines under stress condition. *SNAC*1 transcription factor plays a positive role in cytochrome P540 gene

expression which links both biotic and abiotic stresses. However, in transgenic plants the difference between control and stress was not significant.

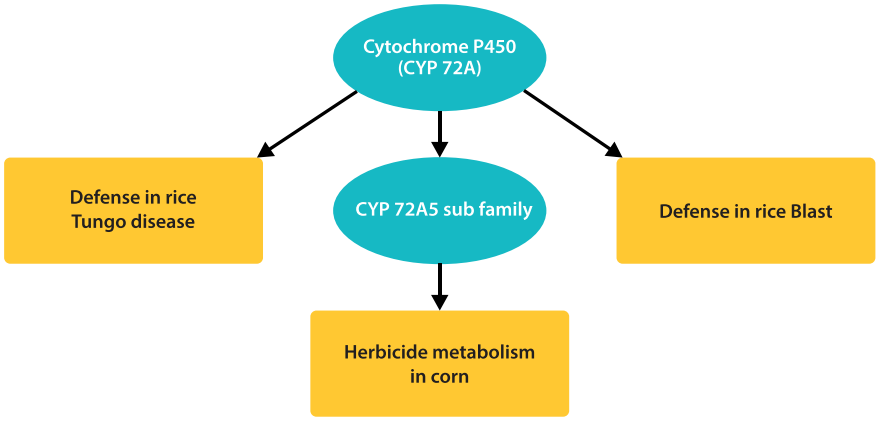


Figure 4.65: functions of cytochrome P450 in biotic stress (Persans et al., 2001; Mahesh et al., 2021; Mangrauthia et al., 2017).

b) 20-kDa Chaperonin (CPN20):

Abiotic stresses usually cause protein dysfunction. Maintaining proteins in their functional conformations and preventing their aggregation are particularly important for cell survival under stress. Many molecular chaperones are stress proteins and many of them are identified as heat shock proteins (Lindquist, S.1986). Direct support for Hsps/chaperones function in plant abiotic stress tolerance is rather limited (Wang W. et al., 2004). The gene studied here named Os02g781400, has a subcellular localization in the chloroplast, and in sequence similarity belongs to the GroES chaperonin. Plant chloroplasts harbor the 10-kDa GroES homologs, two CPN10s join head-to-tail to form CPN20 (20-kDa chaperonin). CPN20 mainly act as co-chaperones that helps CPN60 (chaperonin 60, GroEL homolog) in protein folding during stress. Chloroplast CPN60 was originally identified as Ribulose bisphosphate carboxylase (Rubisco) binding protein (Vitlin Gruber and Feiz, 2018). It was found that CPN20 mediates FeSOD activation in chloroplasts, a role independent of its known function in the chaperonin system (Kuo et al., 2013). Superoxide dismutase (SOD) acts as a primary defence against ROS by converting O^{-}_{2} to O_2 and H_2O_2, which requires a specific metal cofactor bound by CPN20. During seed germination under salt stress, comparative proteomic study showed that in *Brassica napus* salt tolerant cultivar Caravel upregulates 20-kDa chaperonin expression compared to the sensitive variety (Terzi and Yildiz, 2021). In this study the chaperonin gene was significantly upregulated under stress.

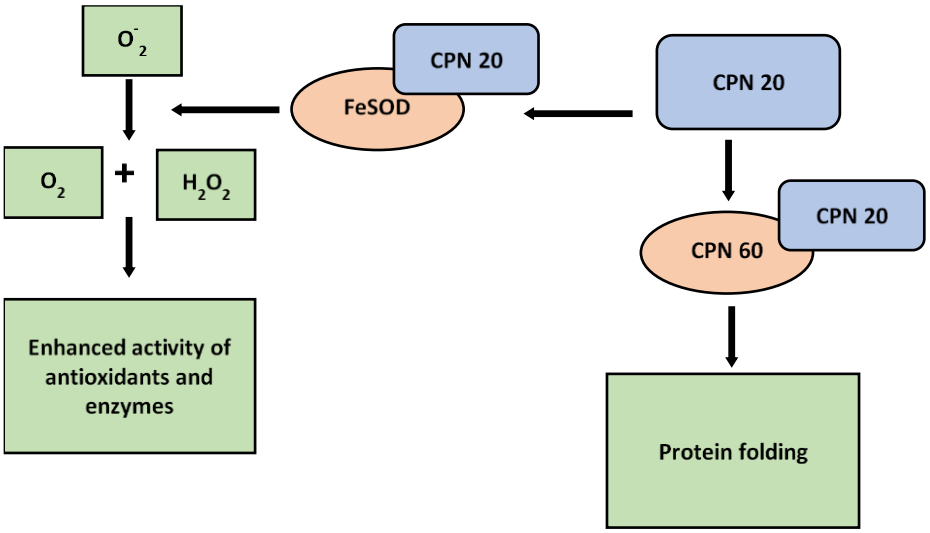


Figure 4.68: Mechanism of CPN20 functions in plant (Kuo et al.,2013; Vitlin Gruber and Feiz,2018)

c) Protein phosphatase 2C:

PP2C-type protein phosphatases are monomeric enzymes present in both prokaryotes and eukaryotes. It is one of the most important protein in ABA signaling pathway. Members of this family of phosphoprotein phosphatases are involved in the regulation of several signaling pathways (Schweighofer et al., 2004). It is reported that an ABA-dependent stress signal pathway plays an important role in plant stress response. Under normal condition, PP2C inhibits SnRK2s (Sucrose Non-fermenting Related Kinase2). During abiotic stresses, ABA is accumulated and sensed by PYR/PYL/RCAR proteins, resulting in PP2Cs inhibition. SnRK2s are then activated by self-phosphorylation, which in turn activate ABA-responsive element (ABRE) binding protein, (AREB)/ABRE-binding factor (ABF), transcription factors to regulate ABRE-dependent gene expression (Kline et al., 2010; Miyakawa et al., 2013; Feng et al.,2019).

Protein Phosphatase PP2C also plays a role in abscisic acid (ABA)-induced stomatal closure (ROS pathway). Inactivation of PP2C by ABA and receptor complex, activates the open stomata1 (OST1) kinase by phosphorylation, which in turn triggers events leading to K^+ ion efflux and stomata become close. Also, the activation of OST1 kinase phosphorylates NADPH oxidase to produce ROS and initiate events of stomata closure (Gahir et al., 2020). In *Arabidopsis*, PP2C promoter are bound with repressor, under salt

stress condition chromatin remodeling helps to release repressor and helps gene expression (Nguyen et al., 2019).

Interestingly it was found that the protein phosphatase PP2C (AK101750, LOC_Os02g05630), is significantly up-regulated in *SNAC*1 overexpressing plants, and probably acts through an ABA-independent pathway (You et al., 2014). Protein phosphatase PP2C (OsPP2C18) was characterized and found that its expression was down-regulated in the *SNAC*1- artificial microRNA transgenic plants, indicating that PP2C expression is positively regulated by *SNAC*1 transcription factor (You et al., 2014). PP2C is one of the target gene of *SNAC*1 transcription factor. It was found that its expression level strongly increased after drought stress, but not by abscisic acid (ABA). It positively affects drought and oxidative stress tolerance by regulating ROS homeostasis through ABA-independent pathway in rice (You et al., 2014). Here, in transgenic lines under inducible promoter, the PP2C gene expression was significantly higher than wildtype.

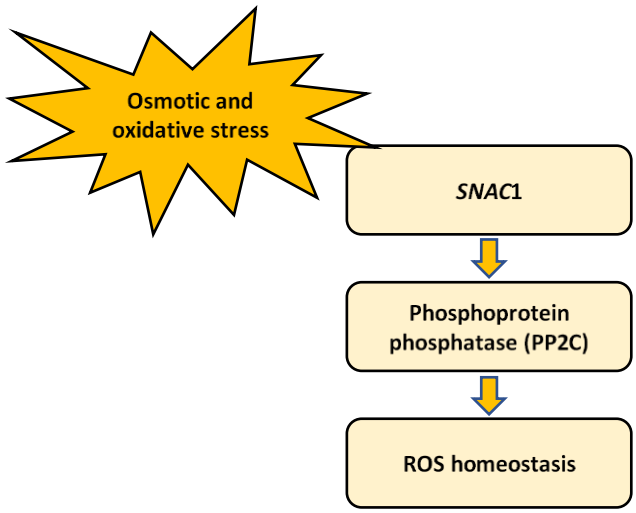


Figure 4.71: Functions of phosphoprotein phosphatase PP2C (OsPP2C18) in ABA-independent signaling pathways (You et al., 2014).

d) No apical meristem (NAM) protein

This is a member of no apical meristem (NAM) proteins family. These are transcription factors involved in organ formation and plant development. First it was reported that mutations in NAM result in the failure in development of a shoot apical meristem in petunia embryos (Souer et al., 1996). NAM is indicated as having a role in determining positions of meristems and primordia (Souer et al., 1996). It was mentioned that, in *Medicago truncatula* No apical meristem (NAM) protein, *MtNAM* regulates lateral organ separation

and floral organ identity (Cheng et al., 2012) and that compound leaf development is also regulated by this protein family (Ge et al., 2014). These protein family play key roles for plant organ development and boundary formation. NAM protein named RRF1, regulates in leaf dissection and initiation and *rrf1* mutant plant produce compound leaves with shorter rachis and fused leafets in mungbean (Jiao et al., 2019). In this study, the NAM gene was significantly upregulated in induced *SNAC*1 transgenic plants under stress.

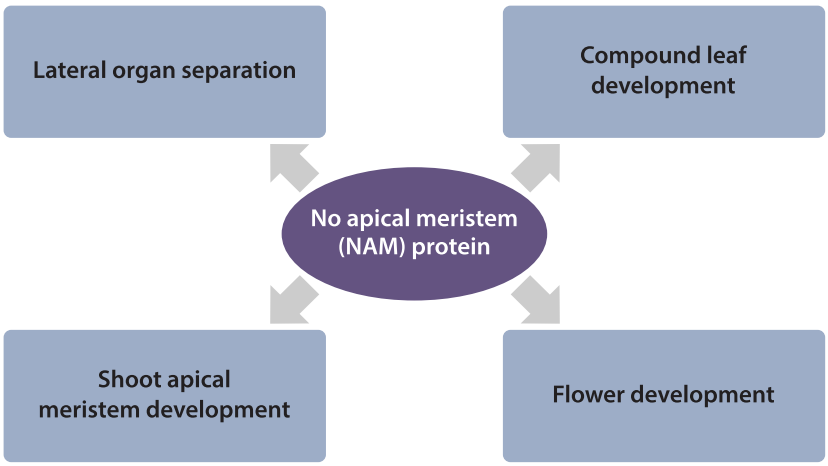


Figure 4.74: Functions of No apical meristem (NAM) protein (Cheng et al., 2012; Ge et al., 2014; Jiao et al., 2019).

e) Sodium/dicarboxylate cotransporter:

To find out the possible function of sodium/dicarboxylate cotransporter in rice, the sequence was aligned by NCBI blastn with nucleotide database and was found that it has highest sequence similarity with *A. thaliana* tonoplast dicarboxylate transporter (*At*tDT). *At*tDT is a putative carrier protein indirectly involved in the uptake of malate and fumarate into the vacuole, probably by regulating the energization across the tonoplast (Emmerlich et al., 2003). Malate, an intermediate in the Krebs and glyoxylate cycles, plays a central role in plant metabolism. Malate protects plants from aluminium toxicity, maintaining the osmotic pressure and charge balance. It is also involved in the regulation of stomatal aperture (Emmerlich et al., 2003). Malate is accumulated in the vacuole through the sodium/dicarboxylate cotransporter in tonoplast. Here, this cotransporter was significantly upregulated in induced *SNAC*1 transgenic rice under stress.

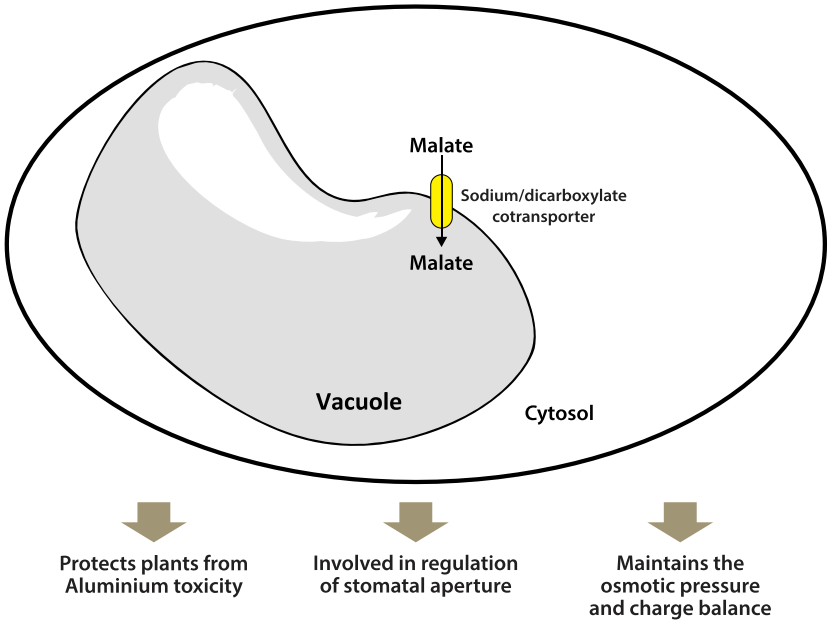


Figure 4.77: Accumulation of Malate in vacuole by sodium/dicarboxylate cotransporter (Emmerlich et al., 2003).

f) MYB transcription factor:

MYB TFs form one of the largest transcription factor families in plants. There are over 198 MYB genes in *Arabidopsis* and over 183 members in rice (Yanhui et al., 2006). MYB proteins are involved in many significant physiological and biochemical processes, including the regulation of primary and secondary metabolism, the control of cell development and the cell cycle, the participation in defence and response to various biotic and abiotic stresses, hormone synthesis and signal transduction (Rahaie et al., 2013). MYB TFs participate in the ABA-dependent pathway of stress signaling for the upregulation of the abiotic stress responsive genes (Lata et al., 2011). It was reported that rice MYB transcription factor OsMYB48-1 improves drought and salt tolerance by increasing LEA protein and proline content and reducing water loss in overexpressed plants (Xiong et al. 2014). It is also reported that rice MYB transcription factor OsMPS (MULTIPASS) targets genes in phytohormone biosynthesis and cell wall formation (Schmidt et al., 2013). *SNAC*1 directly activates MYB transcription factor which in turn interacts with many genes shown in the Figure 4.64.

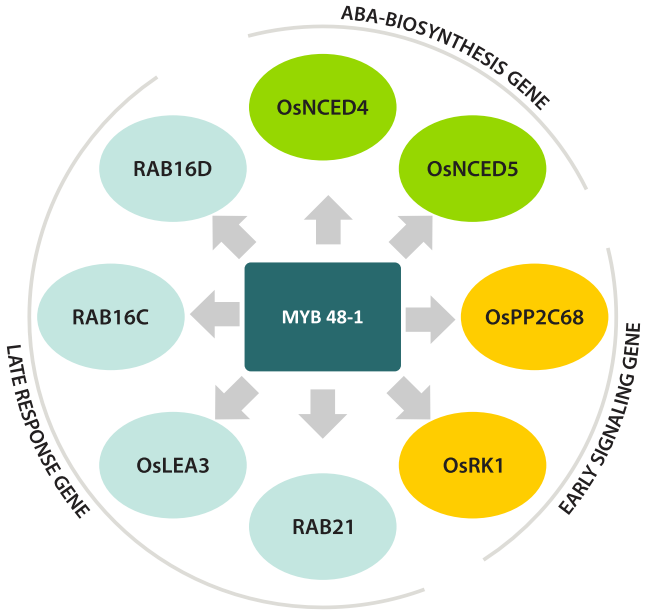


Figure 4.80: MYB transcription factor upregulates many genes in stress signaling pathways (Xiong et al., 2014).

4.4.4.Integration of functional pathway of individual genes:

Plant survival during stress depends on minimizing the effects of the stress by biosynthesizing hormones, production of different transcription factors and several stress related genes, as well as to maintain their normal phenomenon like shoot and root growth or flowering. The six selected genes work in different pathways, some are directly involved in stress related pathways like maintenance of ROS homeostasis and others in stomatal closure by oxidative pressure or charge balance (Protein Phosphatase PP2C, Sodium/dicarboxylate cotransporter). Transcription factors (MYB family) are involved with ABA biosynthesis, early and late gene expression in stress response. Cytochrome P450, 20-kDa chaperonin, No apical meristem (NAM) protein functions in plant growth and flowering. They are genes which are downstream of *SNAC*1 transcription, and therefore likely provide the best defense to plants during abiotic stress.

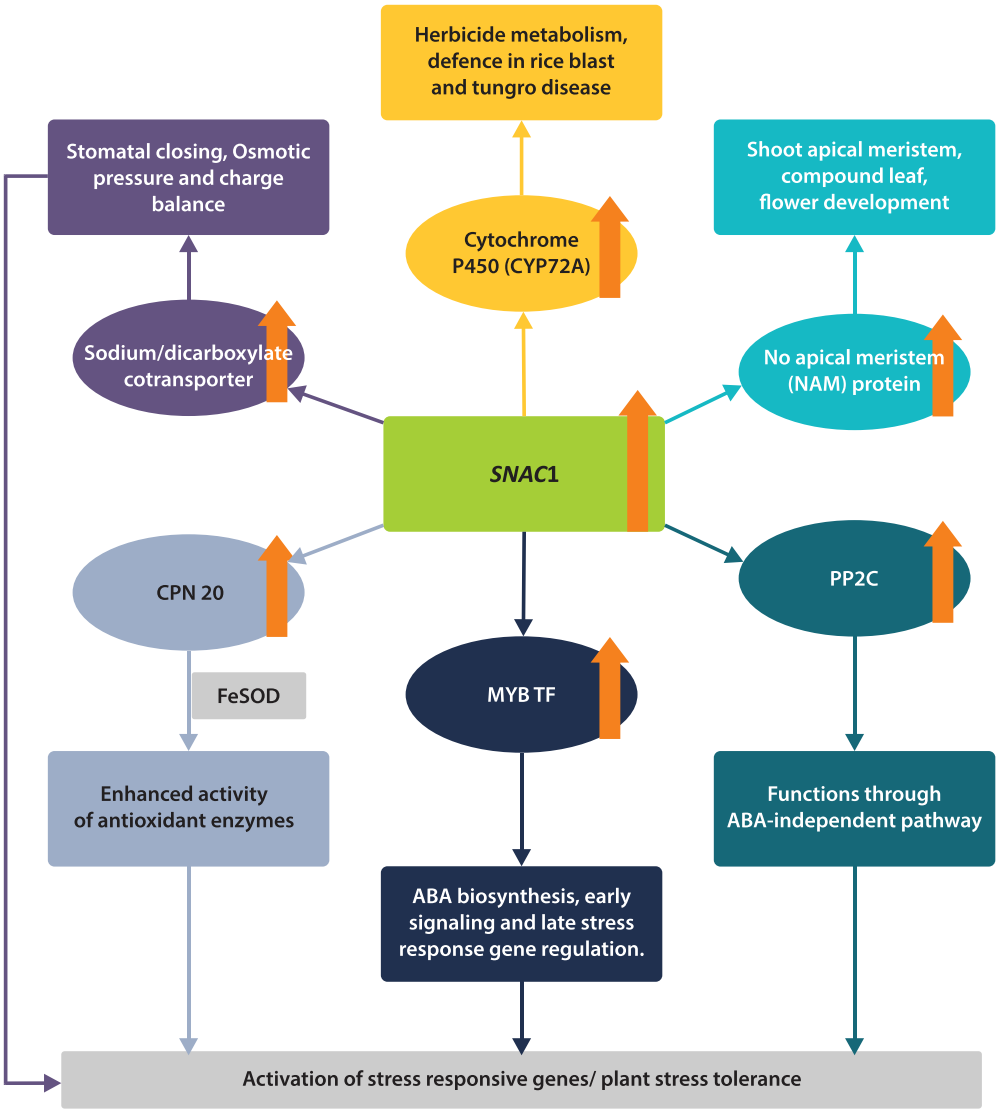


Figure 4.83: Representing master regulatory role of *SNAC*1. Sodium/dicarboxylate cotransporter, 20-kDa chaperonin (CPN20), MYB transcription factor, protein phosphatase (PP2C), No apical meristem (NAM) protein, cytochrome P450 (CYP72A) all of these are direct target of *SNAC*1 during stress. Upon activation by *SNAC*1 they play significant role in stress tolerance and plant growth.

5. Discussion

Salinity and drought are two major factors which are responsible for reducing rice productivity. Salinity affects rice growth in all stages starting from germination to maturity. Drought conditions also results crop yield reduction in plants. Hence, necessary approaches should be addressed to minimize the detrimental effects on rice production to ensure food security (Nozulaidi et al., 2015). However, plant adaptation to environmental stresses such as salinity and drought is dependent on the activation of cascades of molecular networks involved in stress perception, signal transduction, and the expression of specific stress-related genes and metabolites. Therefore, engineering genes that defend and preserve the function and structure of cellular components as well as enhances the expression of stress related gene can augment tolerance to abiotic stresses (Vinocur and Altman, 2005).

High yielding (HY) rice varieties, bred and released by BRRI (Bangladesh Rice Research Institute), such as BRRIdhan-55 (moderately salt and drought tolerant), BRRIdhan-56 (drought tolerant) and BRRIdhan-49 (salt sensitive) were used in this study to increase their stress tolerance. First two varieties possess certain level of tolerance in stress condition, but it is beneficial to make them more tolerant under salt and drought stress condition, because higher levels of stress tolerance are the crying need to the variable agroecological conditions in Bangladesh. For this reason, *SNAC*1 (Stress-Responsive NAC1) gene, under both constitutive *CaMV*35S promoter and stress inducible rd29A promoter, was transformed in high yielding *indica* rice *cv* by *in planta* transformation method to develop more stress tolerant rice varieties. It was reported that rd29A promoter showed higher GUS gene expression compared to the *CaMV*35S promoter in root and shoot in rice plant (Sarker et al., 2016). This study also includes the expression analysis of *SNAC1* up-regulated downstream genes by qRT-PCR in BRRI Dhan-55 transformed with rd29A_*SNAC*1 gene. The interplay of *SNAC*1 as a transcription factor was also found out by literature mining and networking of its downstream target genes under this work.

Part One: Characterization of transcription factor *SNAC*1 in *indica* rice variety Binnatoa for drought and salinity tolerance.

The *SNAC*1 transcription factor, which play important roles in the regulation of stress related genes in abiotic stress response, is one of the candidate genes for the genetic transformation to develop salinity and drought-tolerant varieties. Os*SNAC*1 was overexpressed in cotton, wheat or ramie (Liu et al.,2014; Saad et al., 2013; An et al., 2015) and found similar stress tolerance to abiotic stresses. Before transformed into high yielding

varieties, *SNAC*1 was first transformed in tissue culture responsive *indica* rice cv Binnatoa, as a proof of concept (Abdullah-Al-Emran et al., 2010). In this study, overexpressed *SNAC*1 transgenic lines were characterized at both seedling and reproductive stages. Among putative transformants three lines were selected according to their molecular analysis results as well as physiological screening. Transgenic lines showed stable gene insertion at Southern blot hybridization, also showed better gene expression by semi quantitative real time PCR. Phenotypically transgenic plants possess normal appearance even better in case of line P2, which contains more plant height and effective tiller compared to wildtype. It was reported that overexpression of *SNAC*1 gene provide growth positive effect through the increasing expression of its downstream gene (Cheng et al., 2012; Ge et al., 2014).

Transgenic lines did well in leaf disk senescence assay and contained significantly higher level of chlorophyll content in both 100 mM and 200mM salt condition than wildtypes. Transgenic lines showed 3:1 segregation ratio at T_1 generation. At survival assay Line P2 showed better survival rate at both salt and drought condition. At 200mM salt condition, about 80% P2 plants survived whereas only 19% wildtype plants survived. In drought condition, after 12 days of total withheld of water 50% P2 plants were survived compared to survival rate was 14% for wildtype plants.

At Seedling and reproductive stages, the rice plant is most susceptible to salinity stress (Moradi and Ismail, 2007). Screening was done at seedling stage in both drought and salt conditions at T_2 generation. At both stress condition, transgenic plants showed significantly ($P<0.01$ for salt condition and $P<0.05$ for drought condition) less reduction of chlorophyll content, showed better tolerance in stresses. Transgenic plants also showed better tolerance in terms of shoot length and root length. At saline condition transgenic plants showed significantly lower Na^+/ K^+ ratio in both shoot and root region.

At reproductive stages transgenic lines showed better tolerance in both salt and drought condition in yield related parameters. Number of panicle and number of filled grain per panicles was higher in transgenic lines and wildtype plants contains more unfilled grain than transgenic line. Significantly higher spikelet fertility ($P < 0.01$for salt condition and $P<0.001$ for drought condition) and yield per plant ($P < 0.01$ for both salt and drought condition) was observed.

Part Two: Transformation of high yielding BRRI rice varieties with transcription factor *SNAC*1 under stress-inducible promoter for conferring both salinity and drought tolerance.

Agrobacterium-mediated transformation is the method of choice to transform plants with genes of interest (Khanna and Raina, 1999). However, high yielding rice varieties are non-responsive to tissue culture methods. So, in the current study, a tissue culture independent transformation method (*in planta)* (Lin et al., 2009) has been applied to transform farmer popular high yielding rice varieties. This is an easy way for transformation with transformation efficiency was found about 20% in this study. This method is applicable for any rice genotypes which found restricted in the traditional tissue culture method.

The transformation events were successfully confirmed by PCR with rd29A promoter specific primers and hygromycin resistance assay. Transgenic plants from all three varieties showed correct sized bands and remained more greener and healthier in hygromycin solution than wildtype plants. Transgenic plants at T_2 generation showed better tolerance at leaf disk senescence assay followed by retaining significantly more chlorophyll at 100mM and 200mM salt condition. Segregation analysis revealed that most of the T_2 transformants showed Mendelian inheritance, indicating stable integration of *SNAC*1 genes into high yielding rice varieties.

Transgenic plants showed higher *SNAC*1 gene expression and in 150mM salt condition transgene expression significantly increased in transgenic lines compared to wildtype. Among the three variety, the gene expression value is higher in both stress and without stress condition in BRRI Dhan-49, indicates for adaptation sensitive variety require higher concentration of stress related genes in stress response.

Transgenic plants showed better phenotype compared to their respective wildtypes under normal condition. For rd29A_*SNAC*1_BR-55 and rd29A_*SNAC*1_BR-56 lines showed significantly better plant height, panicle length and also spikelet fertility and yield. All transgenic lines grew in net house condition, they are not directly under any stress like salinity or drought, but temperature and humidity always influence the growth, especially during the mid-day when temperature were so high in surrounding. As we know high temperature is another stress for plants and plants also activates stress responses at heat condition (Priya et al., 2019) and *SNAC*1 has the positive growth effect on plants through downstream genes (Jiao et al., 2019). We conclude that the better phenotype is the results

of *SNAC*1 gene expression and functions. But we always compared the transgenic lines with respective wildtypes to find the differences. Seed length, seed width, as well as 1000 grain weight were significantly increased in transgenic lines for all three varieties. Among the transgenic lines two lines were selected from each variety for further screening. These selected lines were also participated in comparative assay.

Survival rate at 200mM salt condition were measured after two weeks of stress and one week of recovery. The rd29A_*SNAC*1_BR-55 transgenic lines showed 64-68% survival rate whereas 60% for wildtype plants. rd29A_*SNAC*1_BR-55 and rd29A_*SNAC*1_BR-49 lines showed 73-74% and 37-55% of survival rate compared to 47% for BRRI Dhan-56 and 25% for BRRI Dhan-49. Transgenic lines showed significant differences from wildtype plants. Among the three varieties salt sensitive BRRI Dhan-49 lines showed the lowest survival rate at salt condition. Survival rate were also measured at 10 days of total water withdrawal and after two weeks recovery. Transgenic lines showed significantly higher survival rate than wildtype plants at drought condition.

The seedling stage salinity screening were done at 120mM salt condition. All transgenic plants showed significantly better results compared to their corresponding wildtypes. Transgenic lines showed significantly lower SES score than wildtypes. In salinity stress transgenic plants shower significantly lower percent reduction of chlorophyll content and lower percent increase of electrolyte leakage. In other parameters like percent reduction of shoot length, root length and fresh weight, transgenic lines showed significant better performance than respective wildtype plants. The seedling stage drought screening was done by total withdrawn of water for 8 days and different parameters were measured after stress. Selected transgenic lines showed significantly higher chlorophyll content and shoot length in stress condition compared to wildtype plants.

Part Three: Comparative assay in different rice genetic background on the ability of *SNAC1* to confer stress tolerance.

Comparative assay was done between two genotypes BRRI dhan-49 and BRRI Dhan-56, both were transformed with *SNAC*1 transcription factors under inducible promoter. We wanted to know how far their salt and drought tolerance levels will be changed. Both salinity and drought stress were applied in seedling and reproductive stages. Tolerant and sensitive varieties differ in their expression of different genes associated with abiotic

stresses such as salinity and drought (Goff et al., 2002). It was found that the unfavourable impact of salinity is much higher in plants than drought stress.

Rice varieties with different genotypes response differently under stress condition. At seedling stage sensitive lines BRRI Dhan-49 were more affected than tolerant variety BRRI Dhan-56. Both drought and salt stress showed similar damage in sensitive variety like chlorophyll content reduction or increase of electrolyte leakage. Tolerant variety also showed damage but in lower percentage than sensitive lines. Stress condition such as drought and salinity affected growth by suppressing cell enlargement and cell division, by reducing cell turgor, photosynthesis rate, water and nutrition uptake as well as the transportation of organic solutes from one organ to another (Safdar et al., 2019). The damage in sensitive variety indicates higher percent reduction of shoot weight and shoot length than tolerant variety. Both varieties did not show significant variation at normal condition compared to their wildtype. Transgenic lines for both varieties showed significant better tolerance than wildtype plants. At reproductive stage, under both salinity and drought condition, rd29A_*SNAC*1_BR-56 showed better tolerance than rd29A_*SNAC*1_BR-49 lines. Both varieties showed better spikelet fertility and filled grain per plant under drought condition compared to salinity stress.

Comparative assay was also done between *SNAC*1 overexpressed lines (previously developed in our lab, *CaMV*35S_*SNAC*1_BR-55 lines, Parvin et al., 2015) and *SNAC*1 under stress inducible promoter, rd29A_*SNAC*1_BR-55 lines, at both seedling and reproductive stages under salinity and drought stress. Here the comparison were done in the same genetic background BRRI Dhan-55, to understand the effect of constitutive promoter and stress inducible promoter at the level of stress tolerance. At seedling stage, in both salt and drought condition plants under stress inducible promoter showed significantly better tolerance like percent reduction of chlorophyll content and percent increase of electrolyte leakage than *SNAC*1 overexpressed plants. Transgenic lines with inducible promoter and constitutive promoter both showed higher percent increase of H_2O_2 in drought condition and higher percent increase of MDA in salt condition, indicates salt stress increase lipid peroxidation in cell more than in drought condition. In parameters like percent reduction of shoot length or shoot weight transgenic plants with stress inducible promoter showed better stress tolerance.

At reproductive stage, plants with rd29A_*SNAC*1_BR-55 lines showed less affected by salinity and drought stress than *SNAC*1 overexpressed lines. Saline condition showed greater impact on transgenic plants as well as wildtype plants than drought stress in transgenic lines. Among the yield related traits spikelet fertility, yield, 1000 grain weight rd29A_*SNAC*1_BR-55 lines showed significantly better results compare to overexpressing *SNAC*1 lines as well as wildtypes plants. rd29A_*SNAC*1_BR-55 lines also showed higher number of effective tiller and greater number of filled grain. In the same rice variety BRRI Dhan-55, plants under stress inducible promoter shower better tolerance at both salinity and drought condition than overexpressing plants.

There are distinctive differences in between genotype within rice varieties. After transformation Salt sensitive plants improved their tolerance to certain level but it will not compete with varieties which are tolerant genotype. To increase plant stress tolerance selection of tolerant variety is essential. Genes related to stress response pathway, needed to increase their expression during the time of stress, also very important. Existing tolerant varieties as well as gene expression under inducible promoter, will be target for molecular genetic plant transformation approach for the development of stress tolerant rice.

Part Four: Evaluation of the effect of *SNAC1* expression on downstream genes.

In this study, six *SNAC*1 up-regulated genes were selected from the reported microarray data to test expression pattern by RT-PCR in rd29A_*SNAC*1_BR-55 transgenic lines. All of the selected genes were upregulated in *SNAC*1 over-expressing *japonica* cultivar Nipponbare. In this study we found that the selected gene expression was upregulated in high yielding *indica* rice variety. Same signaling pathway involved in stress response in *indica* or *japonica* cultiver. We also found similar signaling mechanism present in different species like *Arabidopsis* and rice (Shinozaki and Yamaguchi-Shinozaki, 2014). This study suggested that stress tolerance is the combined effects of many genes like involvement of transcription factors, transporters, protein for dephosphorylation, growth and survival. And both biotic and abiotic pathways are also interconnected.

From published literature, it was found that most of the studied genes involved in the activation of different stress tolerance pathways. Some of the genes act in herbicide metabolism, defense against disease conditions while others involved in maintaining stomatal closure and activation of defense related genes. All of these genes were

upregulated by *SNAC*1 transcription factor under stress condition. If *SNAC*1 gene expression upregulated during stress in transgenic plants, these genes will more activated and showed much higher expression during stress. So, from these findings it can be concluded that *SNAC*1 transcription factor is a master regulator of many stress related genes and transgenic rice plants with *SNAC*1 gene under stress inducible promoter will help them to withstand various abiotic stresses.

Under this study, networking of the downstream genes was tried to elucidate but the published databases were not showing any interaction between the studied genes. These genes functions in different pathways. Altogether they provide a strong defense for plants to adopt with biological, biochemical changes though respective pathways. most of their function was similar. It can happen that the downstream targets of these studied genes may interact with themselves which yet to be identified.

6. Conclusion

*SNAC*1 transcription factor confers both drought and salt tolerance to plants. Stress tolerance in plants is regulated by multiple genes. It is established that *SNAC*1 is a master regulator in stress response signaling pathway, through ABA-independent way. Abscisic acid (ABA) is the main phytohormone which biosynthesize and accumulates under stress condition. In this present work, *SNAC*1 gene was transformed under stress inducible promoter rd29A, which is a downstream gene of ABA pathway. Now under rd29A promoter, *SNAC*1 can serve as a common intermediate of both ABA-dependent and ABA-independent pathways.

The main purpose of this work is to increase stress tolerance level in our existing farmer popular high yielding *indica* rice varieties. Before transformed into high yielding varieties, *SNAC*1 was first overexpressed and characterize in land race Binnatoa, tissue culture responsive *indica* rice. Transgenic plants showed stable gene insertion and higher gene expression than wildtype plants. Under stress condition (both salinity and drought) *SNAC*1 overexpressed plants showed better stress tolerance at seedling stage as well as at reproductive stage. Transgenic plants showed better yield than wildtype plants. *SNAC*1 overexpression makes transgenic lines more tolerant in abiotic stresses like salinity and drought.

High yielding variety BRRI Dhan-49, BRRI Dhan-56 and BRRI Dhan-55 were selected for transformation. Being tissue culture unresponsive, they were transformed by *in planta* method (Lin et al., 2009). Higher rate of transformation efficiencies indicates that the *in planta* method is quite an efficient method and applicable for any rice variety. High yielding rice varieties were transformed with *SNAC*1 under stress inducible promoter rd29A. Transgenic lines followed 3:1 segregation ratio at T_2 generation and showed higher expression of transgene under salt stress. High yielding varieties were also showed significantly better stress tolerance at seedling level at both salinity and drought condition. Among three varieties transgenic lines of BRRI Dhan-55 showed better tolerance. These results indicate that induced expression of *SNAC*1 enhanced stress response and tolerance against salinity and drought in high yielding varieties.

In this present work, comparative assay was done between *SNAC*1 induced transgenic lines of high yielding variety BRRI Dhan-49 (salt sensitive) and BRRI Dhan-56 (drought tolerant) to understand *SNAC*1 induced stress tolerance effect in two different genetic background. Comparative assay reveals that sensitive variety exhibited lower stress

tolerance at both salinity and drought stress condition than tolerant variety. Among salinity and drought stresses, salinity affects more in plant growth and yield related parameters than drought for both varieties. Sensitive varieties can increase their stress tolerance to certain level but cannot exaggerated than tolerant variety. Stress tolerance is something inherited by genotypes.

To understand the promoter effect comparative analysis was also done between *SNAC*1 induced BRRI Dhan-55 and *SNAC*1 overexpressed BRRI Dhan-55 (previously developed in our lab) lines at seedling and reproductive stages. Both salinity and drought condition, transgenic lines with *SNAC*1, under stress inducible promoter showed significant drought and salt tolerance than *SNAC*1 overexpressed transgenic lines. To enhance stress tolerance in plants inducible promoter is ultimate choice than constitutive promoter.

It was found that *SNAC*1 transcription factor positively regulates the downstream genes in transgenic plants under stress condition. The downstream gene expression was found significantly higher than gene expression in wildtype plants at stress condition. Higher level of gene expression of different transcription factors, cotransporters or stress- related proteins altogether provide better stress tolerance in plants. They are possible to be interconnected in underling pathways yet to be identified.

In future, other farmer popular high yielding rice varieties can be transformed with *SNAC*1 gene under stress inducible promoter by *in planta* method for the development of salt and drought tolerant varieties. This approach will help to produce rice varieties suitable for salt and drought prone areas in Bangladesh.

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Appendix

Appendix 01

SNAC 1 sequence:

AGAAGCAAGCAAGAAGCGATGGGGATGAGGAGGGAGAGGGACGCGGAGGCGGAGCTGAACCTGCCGCCGGGG
TTCAGGTTCCACCCCACGGACGACGAGCTGGTGGAGCACTACCTGTGCAGGAAGGCGGCGGGGCAGCGCCTG
CCGGTGCCGATCATCGCCGAGGTGGATCTCTACAAGTTCGACCCGTGGGATCTGCCCGAGCGCGCGCTGTTC
GGCGCCAGGGAGTGGTACTTCTTCACCCCGCGGGATCGCAAGTATCCCAATGGGTCACGCCCCAACCGCGCC
GCCGGCAACGGGTACTGGAAGGCCACCGGCGCCGACAAGCCCGTCGCGCCGCGTGGGCGCACGCTTGGGATC
AAGAAGGCGCTCGTGTTCTACGCCGGCAAGGCGCCGCGAGGGGTCAAGACTGATTGGATCATGCATGAGTAC
CGGCTCGCCGATGCTGGCCGCGCCGCCGCGGGCGCCAAGAAGGGATCTCTCAGGTTGGATGATTGGGTGCTG
TGTCGGCTGTACAACAAGAAGAACGAGTGGGAGAAGATGCAGCAGGGGAAGGAGGTGAAGGAGGAGGCGTCC
GACATGGTTACGTCGCAGTCGCACTCGCACACCCACTCGTGGGGCGAGACGCGCACGCCGGAGTCGGAGATC
GTGGACAACGACCCCTTCCCGGAGCTGGACTCGTTCCCGGCGTTCCAGCCTGCGCCGCCGCCGGCGACGGCG
ATGATGGTGCCCAAGAAAGAATCGATGGACGACGCCACCGCGGCCGCCGCCGCCGCCGCCACCATCCCCAGG
AACAACAGCAGCCTGTTCGTGGACCTGAGCTACGACGATATCCAGGGCATGTACAGCGGCCTCGACATGCTG
CCGCCGGGCGACGACTTCTACTCGTCGCTCTTCGCGTCGCCGCGGGTGAAGGGGACGACGCCACGCGCCGGC
GCCGGCATGGGCATGGTCCCGTTCTGAGGTGACGGCGACGCGATCGAACAGGTGGTGATCGATGCTGCAACG
TGTGTAAATATACAGCGCCGGCTGGGTCAAGAGATGGCTCGGG

Appendix 02

Promoter sequence of rd29A gene (*Arabidopsis thaliana*)

CGACTCAAAACAAACTTACGAAATTTAGGTAGAACTTATATACATTATATGTGTAATTTTTTGTAACAAAAT
GTTTTTATTATTATTATAGAATTTTACTGGTTAAATTAAAAATGAATAGAAAAGGTGAATTAAGAGGAGAGA
GGAGGTAAACATTTTCTTCTATTTTTTCATATTTTCAGGATAAATTATTGTAGAAGTTTAAAAGATTTCCAT
TTGACTAGTGTAAATGAGGAATATTCTCTAGTAAGATCATTATTTCATCTACTTCTTTTATCTTCTACCAGT
AGAGGAATAAACAATATTTAGCTCCTTTGTAAATACAAATTAATTTTCGTTCTTGACATCATTCAATTTTAA
TTTTACGTATAAAATAAAAGATCATACCTATTAGAACGATTAAGGAGAAATACAATTCGAATGAGAAGGATG
TGCCGTTTGTTATAATAAACAGCCACACGACGTAAACGTAAAATGACCACATGATGGGCCAATAGACATGGA
CCGACTACTAATAATAGTAAGTTACATTTTAGGATGGAATAAATATCATACCGACATCAGTTTGAAAGAAAA
GGGAAAAAAAGAAAAAATAAATAAAAGATATACTACCGACATGAGTTCCAAAAAGCAAAAAAAAAGATCAAG
CCGACACAGACACGCGTAGAGAGCAAAATGACTTTGACGTCACACCACGAAAACAGACGCTTCATACGTGTC
CCTTTATCTCTCTCAGTCTCTCTATAAACTTAGTGAGACCCTCCTCTGTTTTACTCACAAATATGCAAACTA
GAAAACAATCATCAGGAATAAAGGGTTTGATT

Appendix 03

Primers used in this study.

***SNAC*1 primers (full length)**

Primers	Sequences	Annealing temperature
*SNAC*1_F	5' AGAAGCAAGCAAGAAGCGAT 3'	55 ºC
*SNAC*1_R	5' CCGAGCCATCTCTTGAC 3'	52.5 ºC

***SNAC*1 primers (Internal)**

Primers	Sequences	Annealing temperature
*SNAC*1_int_F	5' GTGGGCGCACGCTTGGGATC 3'	59.2 ºC
*SNAC*1_int_R	5' CGTGGCGTCGTCCCCTTCAC 3'	61.4 ºC
*SNAC*1_P_F	5' GCCGAGGTGGATCTCTACAA 3'	63.3 ºC
*SNAC*1_P_R	5' GTTGTCCACGATCTCCGACT 3'	62 ºC

Appendix 04

Media composition used in *in planta* transformation.

Table 1.1: Composition of YM media.

Components	Volume (for 100mL)
Yeast extract	0.04g
Mannitol	1.0g
NaCl	0.01g
MgSO4	0.02g
Dry K_2HPO_4	0.036g
Agar (for solid)	1.5g

*pH is adjusted to 7.0 before adding agar.

Table 1.2: Composition of Bacterial Re-suspension Media.

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

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

Table 1.3: Composition of hydroponic solution.

	Element	Reagent (AR grade)	Preparation (g/4L solution)
Macronutrient	N	Ammonium nitrate ($NH_4NO_{3)}$	365.6
	P	Sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$)	142.4
	K	Potassium sulfate ($K_2SO_{4.}$)	285.6
	Ca	Calcium chloride dehydrate ($CaCl_{2.}2H_2O$)	469.4
	Mg	Magnesium sulfate. Seven-hydrate($MgSO_4.7H_2O$)	1296.0
Micronutrient	Dissolve each reagent separately and mix in 2 L distilled water then add 200mL H_2SO_4 and make up volume to 4 L		
	Mn	Manganous chloride, 4-hydrate ($MnCl_{3.}4H_2O$)	6.000
	Mo	Ammonium molybdate, 4-hydrate [$(NH_4)_6Mo_7O_{24}.4H_2O$]	0.296
	Zn	Zn-Sulfate, 7-hydrate ($ZnSO_4.7H_2O$)	0.140
	B	Boric acid (H_3BO_3)	3.736
	Cu	Cupric sulfate, 5-hydrate ($CuSO_4.5H_2O$)	0.124
	Fe	Ferric chloride, 6-hydrate ($FeCl_{3.}6H_2O$)	30.800
		Citric acid monohydrate ($C_6H_8O_7$)	47.600

Appendix 05

Isolation of plant DNA by CTAB method

The CTAB method provides a less expensive procedure and is characterized by high yields of DNA from a small amount of tissue (Doyle and Doyle, 1990). The main drawbacks of this procedure are less pure DNA will obtain, time consuming and laborious. The procedure of CTAB method is outlined below:

0.01-g-1.0 g of leaf tissue was grinded to a very fine powder in a mortar containing liquid nitrogen. The powdered tissue was transferred directly into the screw capped tube containing 5 ml of preheated (65˚C) CTAB buffer and 12µL of beta mercapto ethanol and was swirled gently to mix.

[CTAB (Cetyl trimethylammonium bromide) is a detergent, as well as an anion-binding reagent. Any negatively charged molecule may be bound with CTAB. It also precipitates some negatively charged proteins and polysaccharides and beta *mercapto ethanol was added to bind and precipitate polyphenolics]*

The sample was incubated at 65˚C in water bath continued for 20 min with occasional vigorous shaking. Five ml mixture of Phenol:chloroform:IAA (25:24:1) was added, and was gently mixed. *[Phenol was used to precipitate proteins and for purification]*. The tube was centrifuged at 4000 *rpm* for 15 minutes to resolve phases. The aqueous phase was transferred to a fresh tube. 2/3 volume of ice-cold isopropanol was added, mixed gently and then kept overnight at 4˚C to precipitate DNA. [*Isopropanol increases the concentration of DNA*]. Tube was then centrifuged at 4000 *rpm* for 15 minutes to collect the precipitate.

The supernatant was discarded, and the tube was washed with 70% ice cold ethanol. After that, the tube was centrifuged at 4000 *rpm* for 10 minutes and the supernatant was discarded, and pellets were allowed to dry completely. TE buffer was added according to the concentration of precipitate and the precipitate was dissolved carefully. RNase A (100 µg/mL) was added and the tube was kept in 37˚C for 30 to 40 minutes. After adding equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), the tube was centrifuged at 4000 *rpm* for 15 minutes. Aqueous phase was taken into 1.5 mL Eppendorf tubes and then equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The sample was centrifuged at 12000 *rpm* for 15 minutes. The aqueous phase was transferred into fresh tube

and equal volume of phenol: chloroform:IAA (25:24:1) was added. The sample was shaken and centrifuged at 12000 *rpm* for 10 minutes. The aqueous phase was transferred into fresh tube. One tenth volume of 3M Na-acetate (pH 5.2) and double volume of 99% ice cold ethanol was added and was shaken. The DNA should be observable at this step. [*Precipitation of the protein is aided by the addition of salts such as sodium acetate.*] The tube was kept for overnight incubation at -20ºC. The tube was centrifuged at 12000 *rpm* for 15 minutes. 1 ml of 70% ice cold ethanol was added to wash any salt. The tube was centrifuged for 5 minutes at 12000 *rpm* and then the supernatant was discarded, and the pellet was allowed to dry completely. Finally, the PCR graded TE buffer was added according to the concentration of the pellet (Doyle and Doyle 1987).

Quality assessment and quantification of DNA

The quality of DNA is very important to obtain good results and for long-term storage. Degradation often occurs due to careless handling. It is also important to know the exact concentration of the DNA for correct PCR amplification used later.

Using Nanodrop spectrophotometer (ND-1000)

Nanodrop spectrophotometer (ND-1000) can measure the concentration of nucleic acid (both DNA and RNA), protein samples and others with only one microliter of sample. It also shows the Standard curve of the sample for quality assurance. The spectrophotometer was selected to measure nucleic acid sample. The wavelength was fixed with 260 and 280 nm for nucleic acid analysis. The nozzle of the machine was first cleaned with soft cotton bud after lifting its lid and was initialized with PCR grade water. After initialization, the blank was set with appropriate buffer according to the buffer of the DNA dissolved. [Optical density (OD) of buffer was taken as blank]. One microliter of sample nucleic acid was sufficient and was loaded onto the nozzle, the lid was then closed, and the OD was measured. The machine showed the concentration of the sample in ng/µl, its Standard curve with the absorbance ratio of 260 nm to 280. A ratio value of 1.8 suggested highly pure preparation.

Comparison of sample DNA with λ DNA standard

Stock DNA preparations were diluted to 10X. 1-2 µl of diluted samples (10X) were loaded in the wells of 0.8% agarose gel followed by 25, 50, 100, 200 ng of λ DNA standard. Electrophoresis and staining with ethidium bromide was carried out. DNA concentration

was estimated by visually comparing the fluorescence in any of the standard with the fluorescence of diluted DNA sample preferably under UV light. This method is helpful to estimate both the quality and quantity of the DNA as well as to check RNA contamination.

Appendix 06

Plant DNA isolation by Short Method (IRRI):

Leaves of the T_0 plants of high yielding varieties were collected and DNA was extracted as following methods.

Materials and different solutions used:

Liquid nitrogen, DNA extraction buffer, Chloroform: isoamyl alcohol (24:1v/v), RNase solution 10µg/ml (DNase free), Ethanol Isopropanol, TE.

Protocol for Isolation of isolation of genomic DNA:

1. Around 1.0g of leaf tissue was ground to a very fine powder in a mortar containing liquid nitrogen. The powder was transferred directly into the 2.0 mL Eppendorf tubes using a spatula.
2. 700µL pre-heated extraction buffer was added to each tube and was mixed well by a vortex machine. The samples were incubated at 65^0C in water bath continued for 20 min with occasional vigorous shaking.
3. 700µL of the mixture of chloroform and isoamyl alcohol (24:1) was added to each tube and was mixed gently. The tubes were centrifuged at 10000 rpm for 10 min.
4. 350µL of the upper aqueous layer was transferred to a new 1.5mL tube. 10µL RNase was added to the tubes and tubes were incubated at 37^0C for 40 min.
5. 1000µL of 99% ice cold ethanol was added to each tube and mixed gently. DNA should be observable at this step. The tubes were kept at -20^0C for 30 min/overnight. The tubes were centrifuged at 10000 rpm for 10 min.
6. The supernatant was discarded.1000µL of 70% ice cold ethanol was added to each tube. The tubes were centrifuged at 10000 rpm for 5 min and the supernatant was discarded. This step was repeated again. The pellet was dried for 2-3 hours. The PCR graded TE buffer was added according to the DNA concentration of the pellet.

Appendix 07

Total RNA isolation from rice tissue by TRIZOL method:

1. ~ 1g of rice tissue were ground in liq-N_2 by morter and pestle.1 ml of TRIZOL was added for 100-200 mg of grind tissue and was homogenized thoroughly (these two steps done in laminar).
2. After grinding immediately TRIZOL was added then ups and down. Otherwise, sample will thaw). The tube was incubated at room temperature for 5 mins or more until all samples were homogenized.
3. The tube was then spinned at 10,000 rpm for 15-20 mins in cold (4° c). The supernatant was transferred to a clean tube and 0.2 ml of chloroform (without IAA) was added for each 1 ml TRIZOL.
4. The tube was vigorously shaken by hand for 15 seconds and was incubated at room temperature for 2-3 mins. The tube was spinned at 10,000 rpm for 15-20 mins in cold (4°c).
5. The aqueous phase was transferred to a clean tube. It was about 60% of initial vol of TRIZOL. 0.25 ml of isopropanol and 0.25 ml of 2M NaCl (DEPC treated) was added per 1 ml of TRIZOL.
6. The tube was mixed by inversion and was incubated for 10 mins upto 1 hr at room temperature. The tube was spinned at 10,000 rpm for 15-20 mins in cold (4°c).
7. The supernatant was removed, and the pellet was washed with 75% ethanol (made by DEPC treated water) for 1 ml of TRIZOL and shaken well by hand.
8. The tube was spinned at 10,000 rpm for 15-20 mins in cold (4°c).
9. The supernatant was removed. The pellet was dried briefly (avoiding hardening the pellet by over drying). DEPC treated water was added depending on the amount of precipitate. Once the RNA is dissolved, it is quantified using nanodrop spectrophotometer.