Investigations on abiotic stress tolerance of transgenic banana plants overexpressing *MusaNAC042* and *MusaMPK5*

By

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Himanshu Tak

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Himanshu Tak

List of Publications arising from the thesis

Journal

a. Published

1. **Tak H**, Negi S, Ganapathi TR (2017) Banana NAC transcription factor *MusaNAC042* is positively associated with drought and salinity tolerance. *Protoplasma*. 254: 803-816.

2. **Tak H**, Negi S, Alka Gupta, Ganapathi TR (2018) A stress associated NAC transcription factor MusaSNAC67 from banana is involved in regulation of chlorophyll catabolic pathway. Plant Physiology and Biochemistry. 132: 61-71.

b. Manuscript under preparation:

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Himanshu Tak

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SYNOPSIS OF Ph. D. THESIS

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Introduction

Stress in agricultural perspective has been defined as any external factor that threatens the survival of the plant along with reduction in growth, overall productivity and reproductive capacity (Rhodes and Nadolska 2001). High salinity and water deficiency are major hurdles for growth and yield of many plant species (Mahajan and Tuteja 2005). High salinity is an environmental stress resulting in hyperionic and hyperosmotic conditions causing reduction in growth and photosynthesis, membrane disorganization, generation of reactive oxygen species and overall toxicity to cell metabolism (Niu et al. 1995; Greenway and Munns 1980). Drought stress results in cell shrinkage leading to cellular membrane damage, impaired function of membrane associated enzymes, broad level peroxidation of cellular membrane, protein denaturation by

excessive production of ROS, extensive protein aggregation and reduced photosynthetic activity (Bowler et al. 1992; Hoekstra et al. 2001).

Plants being sessile need to withstand environmental stresses and hence to adapt and survive under stress conditions, plants have developed various mechanisms including enhanced expression of stress protective genes which include regulatory factors like transcription factors and protein kinases known to regulate expression and activity of many functional genes involved in abiotic or biotic stress tolerance. One of these plant specific transcription factor families is the NACs (NAM, ATAF and CUC). NAC proteins have a DNA binding domain at N-terminus and a regulatory region towards C-terminus. NAC domain containing proteins are present in large number in plants with almost 110 members in *Arabidopsis* and 151 members in rice (Olsen et al. 2005; Nuruzzaman et al. 2010). Few of NAC proteins from different plant species have been characterized for their functions in plant development and stress responses. *Arabidopsis thaliana NAC042/JUB1* has been proposed as central longevity regulator and its overexpression lowers intracellular H_2O_2 levels and increase tolerance to abiotic stress conditions besides delaying senescence (Wu et al. 2012) and enhancing heat tolerance in *NAC042/JUB1* overexpressing transgenic plants (Shahnejat-Bushehri et al. 2012). Moreover, stress is associated with senescence, a process wherein the leaves undergo programmed cell death involving chlorophyll degradation and tissue degeneration facilitating redistribution of nutrients to support reproduction and growth of other organs (Thomas et al., 2002; Quirino et al., 2000). Factors responsible for leaf senescence can be hormones, age, stress conditions such as high salinity, drought, biotic stress and darkness (Moore et al., 2003; Sakuraba et al., 2012). Many transcription factors (TFs) play important role in leaf senescence by modulating transcript level of other SAGs (senescence-associated genes) (Lohman et al., 1994; He and Gan, 2002). In

Arabidopsis nearly 40 transcription factors (senTFs) are associated with the process of leaf senescence and important among them are members of *NAC* (*NAM, ATAF* and *CUC2* family (Balazadeh et al., 2008; Gregersen and Holm, 2007). *ANAC032* is a transcriptional activator which positively regulates stress and age-dependent senescence by up-regulating expression of *SAGs* such as *NYE1, SAG113* and *SAUR36*/*SAG201* (Mahmood et al., 2016). *ANAC029* (*AtNAP*) promotes senescence and its T-DNA insertion mutants showed delayed senescence phenotype (Guo and Gan, 2006).

Low temperature stress is a major abiotic stress condition limiting the plant growth and final productivity. Banana (*Musa x paradisiaca*) is highly susceptible to cold stress and at temperature below 12 °C, the growth is severely affected. Moreover, even at slightly lower temperature of 8 °C, irreparable damage to banana plants can occur (Yang et al. 2015). The cold induced damage arises because of changes in membrane structure and fluidity, protein denaturation, and production of reactive oxygen species. Plants have developed methods including signal transduction pathways to sense change in environmental conditions. *Mitogen-activated protein kinases* (*MAPKs*) are important proteins involved in plant growth, development, signalling and biotic as well as abiotic stress responses (Hamel et al. 2006). A MAPK signal works in a cascade fashion consisting of mitogen-activated protein kinase kinase kinase (MAPKKK/MEKK), mitogen-activated protein kinase kinase (MAPKK/ MEK) and mitogen-activated protein kinase (MAPK) and the signal transduce in sequential phosphorylation (MAPKKK -> MAPKK -> MAPK) event (Samajova et al. 2013). *Arabidopsis MPK4* is involved in responses to cold, salinity and drought stress (Droillard et al. 2004). Maize *MPK4* has been demonstrated to be involved in low temperature stress tolerance as transgenic plants overexpressing *MPK4* show better growth associated with higher proline and elevated soluble

sugar content (Zhou et al. 2012). Rice *MPK5* (*OsMPK5*) is induced in both biotic and abiotic stress and its overexpression improved tolerance to cold, high-salinity and drought stress (Xiong et al. 2003). Banana is sensitive to water stress due to features like shallow roots and a permanent green canopy (Xu et al. 2014). Therefore, studies on regulation of stress responses by NAC transcription factors and MAP kinase coding genes will provide a way to generate transgenic banana plants which can withstand the adverse effects of undesirable changes in soil and environmental conditions

Objective of the thesis

1. Identification of putative homologues of *MAP kinase* and *NAC* transcription factors from banana.
2. Molecular characterization of *MAP kinase* and *NAC* transcription factors from banana.
3. Determination of drought and salinity tolerance in transgenic banana plants over expressing *MAP kinase* and *NAC* transcription factors.

The work carried out to address the above objectives will be presented in the thesis in following chapters.
Chapter 1: General introduction and review of literature
Chapter 2: Material and Methods
Chapter 3: Results
Section 3.1 Studies on *MusaNAC042* gene from banana
Section 3.2 Studies on *MusaSNAC67*, a stress associated NAC transcription factor from banana
Section 3.3 Studies on *MusaMPK5*, a stress associated MAP kinase coding gene from banana
Chapter 4: Conclusion and future prospective

Chapter I [General introduction and review of literature]

This chapter deals with the general introduction and prior reports published on the structure, functions and roles of *NAC* transcription factors and MAP kinase proteins in plants. This chapter will be mainly highlighting the roles of stress associated *NAC* transcription factors and MAP kinase proteins in abiotic stress responses in plants. The importance of characterizing abiotic stress related genes for genetic improvement of banana will also be provided in this chapter.

Chapter II [Material and Methods]

This chapter will describe the various materials and methods utilized for achieving the aims and objectives of the current thesis. Information on identification of stress associated *NAC* transcription factors and *MAP kinase* coding genes in banana, generation of binary vectors and other recombinant vectors, procedure to generate and further molecular analysis of transgenic banana lines, various biochemical estimations, stress tolerance analysis, protein overexpression and characterization will be presented in this chapter.

Chapter III [Results]

Section 3.1 [Studies on *MusaNAC042* gene from banana]

This chapter will discuss the characterization of *MusaNAC042* gene from banana. The chapter describes the results obtained with expression analysis, analysis of *MusaNAC042* promoter region, sub-cellular localization and stress tolerance assays on transgenic banana lines over expressing *MusaNAC042*.

A putative stress responsive *NAC* transcription factor, *MusaNAC042* was identified from banana genome database. Salinity and drought challenge studies indicated that *MusaNAC042* is a stress inducible gene as its expression increases during high salinity and drought conditions. Banana plants harboring P_{NAC042}*::GUS* and subjected to drought and salinity stress were analyzed for GUS activity using 4-methylumbelliferyl-b-D-glucuronide (MUG) and total protein extract. Under both salinity and drought stress, the activation of P_{NAC042} was observed in terms of elevated GUS activity. Elevation was observed in all the organs, however, strong elevation was observed in corm following the stress challenge. Transgenic banana plants over expressing *MusaNAC042* were successfully regenerated and their transgenic nature was confirmed by PCR and Southern blot. Leaf disc assay and imposing salinity (250mM NaCl) and drought conditions

on hardened plants suggested that transgenic lines could tolerate these stress conditions better than control and they retain higher photosynthesis capacity (Fv/Fm) and proline content. MusaNAC042 is a nuclear localized protein was demonstrated by transiently overexpressing MusaNAC042-GFP in banana embryogenic cells. Probable involvement of *MusaNAC042* in stress response pathways in banana was monitored by analyzing expression of members of *CBF/DREB, LEA* and *WRKY* family by quantitative RT-PCR. Total of 55 members of *CBF/DRE*B, 30 members of *LEA* and 50 members of *WRKY* gene family were analyzed by quantitative RT-PCR. At least expression of 9 *CBF/DREBs*, 5 *WRKY* and 3 *LEA* coding genes was found to be altered in transgenic lines suggesting that *MusaNAC042* might be directly or indirectly affecting their expression. These results suggests the underlying mechanism of high salinity and drought stress responses mediated by *NAC042* transcription factor in banana.

Section 3.2 [Studies on *MusaSNAC67,* a stress associated *NAC* transcription factor from banana]

The present chapter discusses the outcomes of *MusaSNAC67* overexpression in transgenic banana plants.

Process of senescence includes multiple steps involving break-down of chlorophyll to degrade photosynthetic machinery. In this chapter, the results obtained on overexpression analysis of a stress-associated *NAC* transcription factor *MusaSNAC67* and the regulation of senescence by *MusaSNAC67* through chlorophyll-catabolic genes will be discussed. Expression of *MusaSNAC67* showed positive responses to multiple abiotic stress conditions suggesting that *MusaSNAC67* is a stress associated *NAC* transcription factor. Transgenic banana lines overexpressing *MusaSNAC67* showed highly senesced phenotype including yellowing and de-greening of leaves similar to etiolated leaves. Transgenic leaves possessed low chlorophyll

content and fails to retain normal chloroplast morphology including loss of granum thylakoid, non-uniform chloroplast membrane and increased number as well as size of plastoglobulins. In a gel shift assay MusaSNAC67 could retard the mobility of chlorophyll catabolic genes such as *PAO*-like (*Pheophorbide-a-oxygenase*), *HCAR*-like (*hydroxymethyl chlorophyll-a-reductase*), *NYC*/*NOL*-like (*Chlorophyll-b-reductase*) as well as *ORS1*-like (a *SenNAC*). Expressions of these genes were highly elevated in transgenic lines which indicate that *MusaSNAC67* is a positive regulator of senescence in banana and exercise its effect by regulating the expression of chlorophyll catabolic genes and *ORS1*.

Section 3.3 [Studies on *MusaMPK5*, a stress associated *MAP kinase* coding gene from banana]

This chapter will discuss the results obtained during the characterization of *MusaMPK5* gene from banana.

Mitogen activated protein kinases (*MAPKs*) are known to play important functions in stress responses of plants. The functional characterization of a *MAPK*, *MusaMPK5* from banana and its function in cold tolerance response in banana will be discussed in this chapter. Expression of *MusaMPK5* showed positive responses to cold, methyl-jasmonate and salicylic acid treatment. Transgenic banana harbouring $P_{MusaMPK5}$*::GUS* showed strong induction of *GUS* in cells surrounding central vascular cylinder of corm and cortical cells of pseudostem after exposure to cold stress (8 °C). Transgenic banana overexpressing *MusaMPK5* were raised and four different transgenic lines were confirmed for T-DNA insertion by Southern blot and PCR analysis. *In-vitro* growth assay under cold stress indicated that elevated cold tolerance ability in transgenic lines than control plants, as transgenic lines gain better shoot length and fresh weight during recovery from cold stress. Leaf disc assay (4°C and 8°C) showed that leaf discs of

transgenic lines bleached less and retain lower MDA content than control plant leaf discs after cold stress. Cold stress tolerance analysis with two month old plants suggested that elevated cold tolerance ability of transgenic lines might be associated with increased proline and reduced MDA content. MusaMPK5 gets localized in cytoplasm as evidenced in onion epidermal cells transiently overexpressing either MusaMPK5-GFP or MusaMPK5-GUS fusion protein. MusaMPK5 is a functional kinase as it autophosphorylate itself and phosphorylate myelin basic protein (MBP) in an *in vitro* reaction. Purified MusaMPK5 can phosphorylate banana NAC042 and SNAC67 transcription factors which are important regulators of stress tolerance in banana. This study has not only pointed towards a useful gene for cold stress engineering in plants but will also expand the current knowledge regarding the stress responses regulated by *MAP kinases* in plants.

Chapter IV [Conclusion and future prospective]

The major conclusions of the present thesis are summarized as below:

1. *MusaNAC042* is a stress responsive *NAC* transcription factor as it is rapidly induced under high salinity and drought stress conditions. MusaNAC042 is a nuclear localized protein was demonstrated by transiently overexpressing MusaNAC042::GFP in banana embryogenic cells. Further, the study dealt with morphological and physiological features of *MusaNAC042* overexpression in banana plants. Role of *MusaNAC042* in increasing salinity and drought tolerance was demonstrated in transgenic banana plants and results were supported with elevated proline and reduced MDA content in transgenic lines. Expression pattern of abiotic stress responsive genes suggests potential molecular mechanism of *MusaNAC042* function as well as its transactivation activity. The analyses were corroborated with results from expression profiling, stress tolerance analyses and analysis of promoter region fused to GUS reporter

gene. Present study will expand our knowledge about the roles of *NAC* transcription factors in understanding the mechanisms involved in abiotic stress responses in banana. The present analysis can be further substantiated by results obtained from down regulation of *MusaNAC042* using either *RNAi* mediated suppression of gene expression or recently emerged CRISPR/Cas9 technology.

2. *MusaSNAC67* induces senescence in banana by regulating chlorophyll catabolic genes and by promoting expression of *ORS1*-like by binding to their promoter. It is possible that regulation of age-dependent as well as environmental induced senescence and chlorophyll catabolic genes is mediated by multiple transcription factors acting in a coordinated fashion during and their precise function and interactions need to be studied. The present study has characterized a stress-induced NAC transcription factor, *MusaSNAC67* with a role in promoting precocious senescence in banana. Future work focusing on application of *MusaSNAC67* for potential manipulation of banana to enhance crop productivity can be carried out by using the recent genome editing techniques such as CRISPR/Cas9 technology.
3. *MusaMPK5* is a cold stress responsive *MAP kinase* which positively regulate cold stress tolerance in banana. This study indicated the physiological responses of *MusaMPK5* overexpression in cold stress responses and the data was corroborated by biochemical estimations of total chlorophyll, MDA and proline. Phosphorylation of two stress associated NAC transcription factors by MusaMPK5 indicated its potential to regulate stress responses through transcription factors and provides directions for future studies. These results will increase our knowledge about the cold stress responses in plants and will facilitate engineering plants with improved cold stress tolerance. Further work in the identification of global targets

of MusaMPK5 can be carried out to understand the downstream targets of *MusaMPK5* in plants.

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Chapter 1: General introduction and review of literature

Plants constantly encounter stress situations due to ever changing environmental conditions (such as drought, high salinity, cold stress) or by exposure to biotic agents such as pathogenic microorganism, herbivorous agents like insects. These stress situations reduce the growth, induce senescence, diminish the reproductive ability of plants and thus limiting the overall productivity of plants. Plants sense the perturbation in homeostasis by stress in the form of signal transduction pathways. These signaling pathways instigate changes at physiological, biochemical and molecular levels to counteract the adverse effects of the stress on plants. One of the important component of these signaling pathway are proteins belonging to kinase family including MAP kinases (MAPKs) (Zhou et al. 2012). Mitogen activated protein kinase signaling pathway is a highly conserved signaling pathway in eukaryotes involved in various physiological functions such as developmental regulation, responses to stress conditions (Sinha et al. 2011).

The MAPK pathway is a phosphorylation cascade composed of three kinases acting downstream of sensor of signal. These kinases are MAPK kinase kinase kinase (MAPKKK), MAPK kinase kinase (MAPKK) and MAPK acting in sequential phosphorylation to transude the signal downstream protein (Samajova et al. 2013). MAPKs are members of serine/threonine kinases and are activated by phosphorylation of threonine and tyrosine of TXY motif (Chen et al. 2012). Activated MAPKs then phosphorylate downstream targets such as transcription factors, proteins kinases, cytoskeleton proteins (Popescu et al. 2009; Ishihama and Yoshioka 2012).

A number of MAPKs have been identified in plants: 20 MAPKs in *Arabidopsis*, 21 in poplar and 17 MAPKs in rice (Colcombet et al. 2008; Reyna and Ynag 2006; Nicole et al. 2006). Plant MAPKs are divided into two categories by the presence of either TEY motif or

TDY phosphorylation motif. MAPKs harboring TEY motif are further classified into groups A, B and C. MAPKs of group A, B and C are mostly involved in abiotic and biotic stress conditions, cell cycle regulation and hormonal regulation (Zhou et al. 2012; Ouaked et al. 2003; Seo et al. 1999). Maize *MPK4* improves cold stress tolerance in transgenic plants by increasing the levels of proline and soluble sugars (Zhou et al. 2012). Maize *MPK5* is involved in recovery from cold stress and in regulation of antioxidant enzymes suggesting its role in ROS detoxification (Berberich et al. 1999; Ding et al. 2013). *Arabidopsis MPK3* and *MPK6* are stress inducible genes and activation of MPK3 and MPK6 is a critical step for generating stress tolerance or resistance in plants (Beckers et al. 2009). Rice *MPK5* (*OsMPK5*) is induced in both biotic and abiotic stress and its overexpression improved tolerance to cold, high-salinity and drought stress (Xiong and Yang 2003). *Arabidopsis* MPK6, a group-A MAPK phosphorylate MYB15 reducing its affinity towards promoter of *CBF3* (*C-repeat-binding factor*) and thus relieving the repression of MYB15 on the expression of *CBF3* improving cold tolerance (Kim et al. 2017).

MAPKs phosphorylate downstream proteins such as transcription factors to regulate their activity. Among putative targets of MAPKs for phosphorylation are proteins belonging to family of NAC transcription factors. NAC domain containing proteins are one of the largest family of plant specific transcription factors containing either of the DNA binding domain: NAM (no apical meristem), CUC2 (cup shaped cotyledon2) and ATAF1/2 (*Arabidopsis thaliana* activation factor1 and 2) (Ooka et al. 2003). Genomic analysis has indicated the presence of 117 NAC domain containing transcription factors coding genes in *Arabidopsis*, 79 in grapes, 152 in soybean, 151 in rice genome and 163 in poplar genome (Le et al. 2011; Nuruzzaman et al. 2010, 2012; Hu et al. 2010).

Structurally NAC domain containing proteins are composed of two regions: a NAC domain which is highly conserved and located towards N-terminus and a variable regions for transcriptional activity regulation towards C-terminus. This conserved NAC domain has been further divided into five subdomains classified as A-E subdomains. Sub domains A, C and D are highly conserved while sub domains B and E are divergent (Ooka et al. 2003; Olsen et al. 2005).. Sub domains D and E are involved in DNA binding activity owing to a net positive charge as they are rich in basic amino acids. Subdomain A is involved in formation of heterodimer and homodimer. While the NAC domain is responsible for nuclear localization, the C-terminal divergent region sometimes contain a transmembrane motif for anchoring in either plasma membrane or endoplasmic reticulum as in case with membrane localized NAC domain containing proteins (Mathew et al. 2016). A genome wide analysis of NAC domain coding genes in *Arabidopsis* has categorized them into two groups on the basis of NAC domain sequence identities. These two groups are further divided into 18 subgroups with group I containing 14 subgroups (TERN, ONAC022, SENU5, NAP, AtNAC3, ATAF, OsNAC3, NAC2, ANAC011, TIP, OsNAC8, OsNAC7, NAC1, and NAM) and group II containing 4 subgroups (ANAC001, ONAC003, ONAC001, and ANAC063) (Ooka et al. 2003).

NAC domain containing proteins have been functionally characterized for their roles in shoot apical meristem formation (Souer et al. 1996), secondary wall formation and tracheids development (Zhong et al. 2010), separation of embryonic and floral organs (Aida et al. 1997), adventitious shoot formation (Hibara et al. 2003), leaf senescence (Guo et al. 2006), lateral root formation and auxin signaling (Xie et al. 2000), flower development (Sablowski and Meyerowitz 1998), stress responses (Tran et al. 2004) and nutrient remobilization (Uauy et al. 2006). NAC transcription factors are regulated by presence of cis-elements such as *AuxRE* (*Auxin responsive*

element), *ABRE* (*ABA responsive elements*) etc, which alter the expression of *NAC* genes under changing physiological and environmental conditions (Negi et al. 2016). The transcripts of *NAC* transcription factors are subjected to regulation by alternative splicing and microRNA mediated gene regulation (Puranik et al. 2012). NAC transcription factors regulate the expression of multiple genes by binding to cis-elements in their promoter. The binding site for reorganization by NAC proteins has been determined experimentally as CACG and CGT[A/G] as multiple NAC proteins have been shown to bind either of these sequences (Tran et al. 2004; Hao et al. 2011). However, some NAC proteins can also recognize sequences other than CACG and CGT[A/G] such as a calmodulin-binding NAC protein from *Arabidopsis* (CBNAC) could recognize GCTT site (Kim et al. 2007). Moreover, a subset of NAC domain containing proteins involved in the regulation of secondary cell wall and tracheids development specifically recognize the *SNBE*-motif (a 19bp consensus sequence comprising of (T/A)NN(C/T) (T/C/G)TNNNNNNNA(A/C)GN(A/C/T) sequence) (Zhong et al. 2010) indicating that different NAC transcription factors can recognize different DNA sequences in the promoter of their target genes. These target sequences can be categorized broadly into genes involved in regulation of other genes, genes with protective functions, or genes coding for proteins of signal transduction pathways (Puranik et al. 2012).

Multiple studies have established the functions of *NAC* transcription factors in abiotic stress responses in different plant systems. *SNAC1* from rice is involved in the closures of stomata under drought conditions thus imparting elevated drought tolerance (Hu et al. 2006). Two more *NAC* transcription factors from rice, *SNAC2* and *SNAC3* have been shown to improve drought tolerance of transgenic lines after overexpression (Hu et al. 2008; Fang et al. 2015). In soybean, functions of *NAC20* and *NAC11* have been established in salinity and cold responses

(Hao et al. 2011). Improved drought tolerance was observed in transgenic *Arabidopsis* overexpressing three stress associated *NAC* transcription factors, *ANAC019, ANAC055* and *ANAC072* (Tran *et al*. 2004). Some of the *NAC* transcription factors are involved in biotic stress regulation as well. In wheat two *NAC* transcription factors, *GRAB1* and *GRAB2* are involved in response to geminivirus infection by inhibiting its DNA replication by interaction with viral Rep A protein (Xie et al. 1999). Functional analysis has suggested that some of the *NAC* transcription factors are involved in metabolism of reactive oxygen species (ROS) which are important signaling molecules in stress conditions. In *Arabidopsis* a *NAC* transcription factor, *NTL4* is involved in leaf senescence and ROS metabolism by regulating the *Atrboh* (*respiratory burst oxidase homolog*) genes (Lee et al. 2012). Another *NAC* transcription factor from *Arabidopsis*, *AtJUB1* is positive regulator of plant longevity and delays senescence by dampening intracellular H_2O_2 concentration and directly regulating the expression of *DREB2A* gene (Wu et al. 2012). In rice *SNAC1* regulates the expression of *SRO1c* which is involved in stomatal closure by increasing H_2O_2 accumulation in guard cells thus reducing water loss under drought conditions (You et al. 2013).

Some of the *NAC* transcription factors are also involved in the process of stress induced senescence. Senescence is a nutrient redistribution process by which old leaves undergo tissue degeneration and chlorophyll degradation to support the reproductive growth (Thomas et al. 2002). Many stress conditions induce senescence which is then regulated at molecular level by transcription factors among which roles of *NAC* transcription factors are prominent (Balazadeh et al. 2008). Some of the senescence associated *NAC* transcription factors upon overexpression induces precocious senescence in transgenic plants. In *Arabidopsis*, *AtNAC016* has been linked with senescence and is strongly induced in high-salinity and oxidative stress conditions (Kim et

al. 2013). Some of the senescence associated *NAC* transcription factors regulates the expression of chlorophyll catabolic pathway genes and senescence associated markers for induction of senescence. In *Arabidopsis*, *ANA032* induces expression of *NYE1* (*non-yellowing 1*), *SAG113* (*senescence associated gene*) among others for regulation of stress induced senescence (Mahmood et al. 2016). Positive regulation of senescence associated genes such as *NYC1* (*chlorophyll-b-reductase)*, *SGR1/2* (*STAY-GREEN*) and *PAO* (*Pheophorbide-a-oxygenase*) by *ANAC046*, an *Arabidopsis NAC* transcription factor has been demonstrated for precocious senescence in plants (Oda-Yamamizo et al. 2016). Inhibition of the expression of *ANAC092*/*AtNAC2*/*ORE1* (*ORESARA 1*) and *ANAC059*/*ORESARA1 SISTER1* (*ORS1*) (a paralog of *ORE1*/ *ANAC092*/*AtNAC2*) delays development of senescence (Kim et al. 2009; Balazadeh et al. 2010a, 2010b, 2011). Hence, NAC domain containing transcription factors are important regulators of tolerance towards stress conditions as well as stress induced senescence. Thus, in the present study two NAC domain containing protein coding genes were identified after initially analyzing their expression pattern under stress conditions and functionally characterized them after overexpression in transgenic banana lines. As MAPKs are also important modulators of stress conditions in plants, a putative *MAP kinase* coding gene was also chosen for its functional characterization in banana plants.

The specific aims and objective of the present study are as follows:

1. Identification of putative homologues of *MAP kinase* and *NAC* transcription factors from banana.
2. Molecular Characterization of *MAP kinase* and *NAC* transcription factors from banana.
3. Determination of drought and salinity tolerance in transgenic banana plants over expressing *MAP kinase* and *NAC* transcription factors.

Chapter II: Material and Methods

***In silico* analysis of MusaNAC042**

MusaNAC042 was employed for pBLAST search at NCBI and NAC sequences (with high BLAST score) from different plant species were chosen for generating a phylogenetic tree. Neighbor joining tree (bootstrap value of 1000 replicates) was constructed with Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA6 software (Kumar et al. 2008). For multiple sequence alignment, MusaNAC042 was aligned with *Arabidopsis thaliana* ANAC042 (NP_181828.1), *Theobroma cacao* TcNAC042 (XP_007048954.1), *Oryza brachyantha* ObJUB1 (XP_006651871.1) and *Vitis vinifera* VvJUB1 (XP_002283251.1) using Clustal omega and online available box shade server (http://www.ch.embnet.org/software/BOX_form.html).

Stress treatments and expression analysis of *MusaNAC042*

Expression analysis of *MusaNAC042* was carried out using *in vitro* grown and green house hardened plants of banana cv. *Karibale Monthan* plants. Similar aged plants (plants with growth of two month in green house) were used in all the stress treatments. Individual banana plants were imposed with different stress treatments and RNA was isolated from leaves for expression analysis at different time points (5, 12 and 48 hours). For each time point three plants were utilized for stress imposition and top most leaves were harvested at the defined time. Tissue of three separately treated plants was mixed in equal amount for the isolation of total RNA. The qPCR was performed at least three times for each treatment. For salinity stress, 20 ml of 250 mM NaCl was irrigated to the plants. For drought treatment, plants were dried on a blotting paper in a laminar air flow hood (Negi et al. 2015b). Total RNA isolated from leaves using Concert plant RNA reagent (Invitrogen, USA; Cat. No. 12322012) was subsequently cleaned using RNeasy spin column supplied with the RNeasy plant mini kit (Qiagen, Germany; Cat. No. 74904) following the manufacturer instructions.

During RNA isolation, the genomic DNA contamination was eliminated using Qiagen on column DNAase digestion (Cat. No.79254) following manufacturer's instructions. Synthesis of cDNA was carried out with 2µg total RNA using thermoscript AMV-RT by following manufacturer's instructions (Invitrogen: Cat. No.12236-014). Quantitative RT-PCR was carried out with 1:100 diluted cDNA and SYBR Green Jump Start Taq Ready Mix (Sigma, USA; Cat. No. P2893) following the supplier instructions. Q-PCR running condition used was: 94°C for 4 minutes followed by 30 cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 20 seconds followed by a melting curve analysis. Expression of *Musa EF1α* (housekeeping gene) was also analyzed for normalization of different Ct values. Calculations were carried out as follows: ΔCt (Target gene) = Ct (Target gene) −Ct (*EF1α*) and fold change was calculated by dividing $2^{-\Delta Ct}$ value of target gene in stressed and in control condition.

Construction of *MusaNAC042* overexpression vector

Complete coding sequence of *MusaNAC042* was amplified from leaf cDNA of banana cv. *Karibale Monthan*. PCR was carried out in a 50 µl volume containing forward and reverse primer (10 pmol each), Taq DNA polymerase (1 unit), 0.5 µl dNTP (10mM stock), 1x PCR buffer, 2mM $MgCl_2$ and 1µL of cDNA (1:100 diluted). PCR running conditions were: 94°C for 5 minutes for initial denaturation followed by 35 cycles with each cycle of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 1 minute after which final extension was done at 72 °C (for 10 minutes). Binary vector *pCAMBIA1301* was digested with *Nco*I and *Pml*I to release the *GUS* coding sequence and the resultant vector backbone was gel purified (Roche, Germany, catalogue number. 11732676001). *MusaNAC042* coding sequence was digested with *Bsp*HI and *Pml*I and ligated with above generated vector backbone. Recombinant plasmid (*pCAMBIA1301- MusaNAC042*) was confirmed

by restriction digestion as well as by sequencing of cloned insert. *Agrobacterium tumefaciens* strain *EHA105*(Hood et al. 1993) was electroporated with the binary vector.

Construction of *pCAMBIA1302-MusaNAC042* for sub-cellular localization

Full length coding sequence of *MusaNAC042* was cloned in *pCAMBIA1302* employing *Bgl*II and *Spe*I restriction sites, generating a translational fusion of *MusaNAC042* and *GFP*. Embryogenic cells of banana cultivar *Rasthali* were transiently transformed using *Agrobacterium tumefaciens* (*EHA105*) harboring *pCAMBIA1302-MusaNAC042*. GFP fluorescence was monitored after five days of transformation with the help of fluorescent microscope (Eclipse 80i, Nikon, Japan). Hoechst 33258 staining was carried out to identify position of nuclei in the cells. Binary vector was sequenced to confirm the cloning of *MusaNAC042* in correct coding frame.

Generation of transgenic banana plants overexpressing *MusaNAC042*

Agrobacterium mediated transformation of banana cv *Rasthali* embryogenic cells was carried out as described previously (Ganapathi et al. 2001). Briefly, 0.5ml PCV (packed cell volume) of embryogenic cells was co-cultivated with *A. tumefaciens* strain *EHA105* for 30 minutes and later aspirated onto glass fiber filter which was incubated on semi-solid M2-medium in dark for three days (Cote et al. 1996). Following this, *Agrobacterium* was eliminated by culturing the cells in light on M2-medium added with antibiotic Cefotaxime (400mg/l). Selection of transformed cells and growth of embryos was carried out on embryo development medium (BEM) with cefotaxime (400 mg/l) and selection agent hygromycin (5 mg/l). Conversion of somatic embryos into shoot was carried on MS-medium supplemented with BA (0.5 mg/l).

Confirmation of transgenic lines overexpressing *MusaNAC042*

Genomic DNA of putative transgenic lines was isolated using Plant Genomic DNA Kit (Sigma, USA; G2N350) and subjected to PCR amplification of *hpt-II* (*hygromycin phosphotransferase*)

using PCR conditions: 94°C for 5 minutes (initial denaturation), 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 45 seconds and final extension at 72 °C (10 min). Southern blotting was carried out to determine copy number of T- DNA transferred in different transgenic lines. Genomic DNA isolated as described above was digested (20µg) with *Nco*I for 16 hours at 37 °C. Digested genomic DNA was resolved on 0.8% agarose TAE (pH 8.0 tris acetate buffer consisting of tris-acetate (0.04 M) and EDTA (0.001 M)) gel. Transfer of digested DNA onto Hybond-N nylon membrane (Amersham, Cat. No. RPN.203N) was carried out with 10x SSC (0.45M NaCl and 0.045M tri- sodium citrate with final pH 7.0) buffer by capillary transfer. DNA was fixed onto membrane by baking at 120°C for 30 min. DIG labeled probe against *hpt-II* coding sequence was prepared using DIG labeling kit (Roche, Germany, Cat. No. 11585614910) as per manufacturer's instruction. In brief, 1µg hpt-*II* (heat denatured) was mixed with 4µl of DIG high prime (supplied with the kit) in a reaction of 20µl and incubated at 37°C for 3 hours. Hybridization of probe with membrane bound DNA was carried out overnight at 42°C using DIG Easy Hyb granules. Excess and non-specific binding of probe was washed by stringency washes at room temperature (with 2X SSC with 0.1 % SDS) and at 65°C (with 0.5X with 0.1 % SDS). Binding of probe was visualized by anti-DIG antibody (1:5000) labeled with alkaline phosphatase. Chemiluminescence signal was detected as per manufacturer's protocol (Roche, Germany). Transcript level of *MusaNAC042* in confirmed transgenic lines was analyzed by quantitative RT-PCR as described above. Amplification of banana *EF1α* was used for normalization of the Ct values.

Leaf disc assay with transgenic lines overexpressing *MusaNAC042*

Leaf discs (1.0 cm diameter) excised from healthy leaves of control and transgenic lines were incubated on different concentrations of NaCl (0, 100, 200 and 250 mM) for imposing salinity

stress. Treatment was carried out for six day under continuous white light at 27±1°C after which visual injury to discs was recorded. Total chlorophyll estimation was carried out as per method described (Arnon 1949). Briefly, leaf disc homogenized in 80% acetone was centrifuged at 5000 rpm (10 min) and the absorbance was recorded at 652 nm. Total chlorophyll content was expressed as µg per mg fresh weight. Thiobarbituric-acid method was used for analyzing the MDA content as described earlier (Negi et al. 2015b). Briefly, leaf tissue was homogenized and incubated at 95°C for 15 minutes with 0.5% TBA in 20% TCA. Reaction was terminated by cooling on ice and subsequently supernatant was collected by centrifugation. Absorbance was recorded at 600nm and 532nm and the reading of 600nm was subtracted with reading at 532nm. MDA content was estimated with the help of extinction-coefficient of $155mM^{-1}cm^{-1}$ as reported earlier (Heath and Packer 1968). Experiment was carried out in triplicate. MDA and chlorophyll content analysis was done in triplicate after mixing the tissue of at least three separate treatments.

Drought and high salinity tolerance assay with transgenic lines overexpressing *MusaNAC042*

Two month old control and transgenic lines maintained in the green house were used for stress tolerance analysis. Plants were exposed to salinity stress by irrigating plants with 20ml of 250mM NaCl on every alternate day up to 15 days. For drought imposition, water was withheld for 14 days and stress symptoms were recorded. Recovery potential of transgenic and control plants was monitored after one month of regular re-watering with tap water. MDA, F_V/F_M ratio and proline content were calculated for stressed plants during treatment. Photosynthetic efficiency was monitored in terms of Fv/Fm ratio using plant efficiency analyzer (Hansatech Instruments, United Kingdom; Model: Handy-Pea). MDA content was estimated as described above. Proline content was estimated using ninhydrin reaction procedure. Stress assay were carried out with three biological replications. Tissues of the three replications were mixed in equal amount and three

technical replications were performed for biochemical estimation of MDA and proline. Relative water content in the leaves of control and transgenic lines was performed as described earlier (Gaxiola et al. 2001). For relative water content, youngest fully expanded leaves were used and three biological replications were tested.

Quantification of stress related gene expression in transgenic banana plant overexpressing *MusaNAC042*

Multiple members of *CBF*/*DREB*, *WRKY* and *LEA* family were identified from NCBI and banana genome sequence database (http://banana-genome.cirad.fr/). Total RNA was isolated from leaves of control and transgenic lines under non-stress conditions as described above. The cDNA synthesis was performed as described earlier. Quantitative RT-PCR with 1:50 diluted cDNA as template was carried out and the data obtained was used for calculations of fold change in expression as described above. The quantitative RT-PCR analysis was carried out in triplicate.

Cloning of 5' upstream sequences (promoter region) of *MusaNAC042* upstream of *GUS* in *pBI121*

To study the tissue specific expression and stress inducible nature of *MusaNAC042* the upstream sequence (promoter) was cloned in *pBI121* upstream of *GUS* coding sequences. The *pBI121* was digested with *Hin*dIII and *Xba*I to release the *CaMV35S* promoter and the resulting digested vector backbone was used for ligation with *Hin*dIII and *Xba*I digested promoter of *MusaNAC042*. The ligation was confirmed with PCR analysis and sequencing.

RNA isolation and quantitative RT-PCR analysis of *MusaMPK5*

Total RNA was isolated from the roots and leaves of banana plants with the help of RNeasy plant mini kit (Qiagen, Germany; Cat. No.74904). In Brief, total RNA was isolated using Concert plant RNA reagent (Invitrogen, USA; Cat. No. 12322012) and cleaned using RNeasy plant mini kit

(Qiagen, Germany; Cat. No. 74904) as per suppliers instructions. RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific; Cat. No. K1622) was utilized for generation of cDNA using the kit protocol. In separate experiments, RNA was isolated from banana plants subjected to cold stress (4±2 °C) and applications of methyl jasmonate (200 µM MeJA) and salicylic acid (2mM). Complementary DNA was diluted to 1:50 and utilized for quantitative RT-PCR with JumpStar Taq ReadyMix (2X) (Sigma, USA; Cat. No. P2893). PCR was set as follows: 94°C for 4min, 30 cycles of 94°C for 20 sec, 56°C for 20 sec, 72°C for 20 sec ending with a melting curve analysis. The data obtained was normalized by the expression of banana reference gene, *EF1α* and represented as fold change using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Schmittgen and Livak 2008). Primer utilized for quantitative RT-PCR of *MusaMPK5* were FP: 5'- AAAGCGGACACTTCGAGAGA-3' and RP:5'-TTGCATTCAGCAGAAGGTTG -3' while for *EF1α*, FP:5'-CCGATTGTGCTGTCCTCATT-3' and RP:5'- TTGGCACGAAAGGAATCTTCT -3' were used.

Sequence analysis of MusaMPK5

NCBI database was searched for related MAP kinase proteins utilizing MusaMPK5 as a query source in pBLAST search. A neighbor joining tree (with boot strap replications of 1000) was constructed utilizing clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA software (Kumar et al. 2008). MusaMPK5 and related MAPK kinase proteins were aligned using multiple sequence alignment at clustal-omega and conserved residues among them were shaded in black at box-shade server (http://www.ch.embnet.org/software/BOX_form.html). Theoretical molecular weight and pI (isoelectric point) of MusaMPK5 was determined at expasy server (www.expasy.org).

PCR amplification and cloning of coding and 5' up-stream region of *MusaMPK5*

MusaMPK5 (genbank accession number KU507535) was amplified from the cDNA of banana cv. *Karibale Monthan* leaf. Total RNA isolation followed by cDNA synthesis was carried out as described above. The PCR amplification was performed as follows: 20 pmoles of specific primers for amplification of complete coding sequence (FP:5'-AACCATGGACGGGGCTCCGGTG-3' *Nco*I underlined; RP:5'- AACACGTGCTAGTATGCTGGATTGAATGCTATAGCTTCTTTG-3' *Pml*I underlined), 0.5 unit of Taq DNA-polymerase, 5 µL of 1:50 diluted cDNA, 1µl of dNTP from 10 mM stock and 1x reaction buffer with $MgCl_2$ were mixed in a reaction volume of 50 µL. PCR running condition was 94°C for 10 min followed by 35 cycles of 94°C (1 min), 55°C (50 sec), 72 °C (1.5 min) ending with a final extension of 72°C (10 min). Binary vector *pCAMBIA1301* was digested with *Nco*I and *Pml*I to release *GUS* coding sequence and similarly digested *MusaMPK5* coding sequence was cloned under the control of *CaMV35S* promoter to generate the binary vector *pCAMBIA1301-*$P_{CaMV35S}$*-MusaMPK5*. Genomic DNA was isolated from leaves of banana cv. *Karibale Monthan* with the help of GenElute Plant Genomic-DNA Miniprep-Kit (Sigma, USA). For amplification of 5' up-stream regulatory region of *MusaMPK5*, 20 pmols of primers (FP: 5'- TTAAGCTTCACGTGGAATTTTCCTCTTTC-3' *Hind*III underlined; RP:5'-TTTCTAGACGTGACAGAACTCGAGCAGA -3' *Xba*I underlined), was mixed with 5 µL of genomic DNA, 1µl of dNTP from 10 mM stock, 1x reaction buffer with $MgCl_2$ and 0.5 unit of Taq DNA-polymerase in reaction volume of 50 µL. PCR conditions for amplification of *MusaMPK5* promoter region was: 98°C for 10 min followed by 35 thermal cycles comprised of 98 °C (60 sec), 55 °C (60 sec), 72 °C (1.5 min) ending with a final extension of 72 °C of 10 min. Binary vector *pBI121* was digested with *Hind*III and *Xba*I to release *CaMV35S* promoter and the resulting

vector backbone was used for ligation with *Hind*III and *Xba*I digested promoter of *MusaMPK5* to generate *pBI121-$P_{MusaMPK5}$-GUS.*

Generation and confirmation of transgenic banana plants overexpressing *MusaMPK5*

ECS (Embryogenic cell suspension) of banana cultivar *Rasthali* was generated and maintained as described earlier (Ganapathi et al. 2001). Transformation of ECS was carried out essentially as described previously (Ganapathi et al. 2001). Binary vectors *pCAMBIA1301-$P_{CaMV35S}$-MusaMPK5* and *pBI121-$P_{MusaMPK5}$-GUS* were transformed into *Agrobacterium tumefaciens* strain *EHA105* (Hood et al. 1993). and the recombinant bacterium was induced at the OD_{600} 0.6 with 100 µM ACS (acetosyringine). In brief, ECS (0.5 ml packed cell volume) was co-cultivated with induced bacterium for 30 minutes with intermittent shaking. The ECS was filtered onto a glass fiber filter and incubated in semi-solid M2-medium for three days in dark (Cote et al. 1996). Selection of the embryos was carried on banana embryo development medium (BEM) supplemented with cefotaxime (400 mg/L) and hygromycin (5 mg/L). Embryos to shoot conversion was achieved on MS-medium (Murashige and Skoog) supplemented with 0.5 mg/L BAP (6-Benzylaminopurine). Generation of multiple shoots and rooting of explants was carried out on MS-medium supplemented with 2 mg/L BAP and 1mg/L NAA respectively. T-DNA insertions in the genome of transgenic lines generated for overexpression of *MusaMPK5* was analyzed by PCR and Southern blot. The genomic DNA of transgenic lines was isolated as described earlier and subjected to PCR amplification of *hygromycin phosphotransferase-II* coding sequence with gene specific primers (FP: 5'-GTCCTGCGGGTAAATAGCTG-3'; RP:5'- ATTTGTGTACGCCCGACAGT-3'). PCR reaction was set by making master mix as described above. PCR running condition was 94°C (5 min), 35 cycles of 94°C (45 sec), 55°C (45sec), 72°C (45 sec) ending with final extension of 5 minutes at 72°C. Southern blot analysis with *Nco*I digested genomic DNA and digoxigenin labeled

probe (DIG labeling kit, Roche; Cat. No. 11585614910) against *hpt-II* was performed as described earlier (Negi et al. 2016). The fold change in transcript level of *MusaMPK5* in transgenic lines was carried out by quantitative RT-PCR analysis as described above.

Cold stress tolerance analysis

For *in vitro* cold tolerance assay, equal size shoots of control and transgenic lines were cultured on rooting medium (MS medium supplemented with 1 mg/L NAA) and exposed to 8 °C for 12 days and later the shoots were recovered on 27±2°C. Fresh weight gain was calculated by monitoring the fresh weight of shoots before stress exposure and after recovery. For leaf disc assay, discs of approximately 1cm were excised with cork-borer and floated on $1/10^{th}$ MS-medium at either 4 °C and 8 °C in a growth chamber maintained at 16/8 light /dark hour system for 10 days.

The visual injury in the form of bleaching was recorded along with estimation of MDA and total chlorophyll content. MDA content was analyzed by thiobarbituric-acid method as described earlier (Srinivasan et al. 2009). In brief, tissue was homogenized, mixed with 0.5% TBA prepared in 20% TCA and heated at 95°C for 15min. Reaction was terminated on ice and the supernatant was subjected to measurement of absorbance at 600nm and 532nm. The MDA content was estimated with extinction-coefficient of $155mM^{-1}cm^{-1}$ after reducing the absorbance of 600nm with absorbance of 532nm (Heath and Packer 1968).

Total chlorophyll content was estimated essentially as described earlier (Arnon 1949). For cold tolerance assay with hardened plants, uniform plants of two month age were exposed to 8 °C for 12 days in a growth chamber maintained at 70% relative humidity and later recovered in green house conditions. At the end of the stress period, percentage of chilling injury, MDA content and proline content was estimated. Chilling injury area was monitored as ratio of injured leaf area and total leaf area (Dou et al. 2016). Leaf area was calculated by formula leaf area: 0.83 (length *

breath). Proline content estimation was performed by ninhydrin reaction procedure and MDA content was estimated as discussed above.

Transgenic banana plants transformed with *pBI121-P$_{MusaMPK5}$-GUS* were stressed at 8°C for 24 hours and the GUS activity in different organs after protein extraction was monitored by 4-methylumbelliferyl-b-D-glucuronide (MUG) assay as described earlier (Jefferson1987). Histological sections of corm and pseudostem were stained with GUS staining buffer composed of 1mM X-Gluc (5-Bromo-4-chloro-3-indolyl Glucuronide) in a solution comprising of 0.5mM potassium ferrocyanide and 0.5mM potassium ferricyanide in sodium phosphate buffer (100mM, pH 7). Triton X-100 at a final concentration of 0.1% was added to the GUS staining buffer and staining of the tissue was carried out overnight. Images were recorded in Eclipse 80i microscope (Nikon make).

Subcellular localization of MusaMPK5

MusaMPK5 was fused with *GFP* coding sequence using *Bgl*II and *Spe*I restriction sites in binary vector *pCAMBIA1302*. The primer utilized for generation of *pCAMBIA1302-MusaMPK5::GFP* were FP: 5'-AAGGATCCGGACGGGGCTCCGGTGAA-3' (*Bam*HI site underlined) and RP: 5'-AAACTAGTGTATGCTGGATTGAATGCTATAGCTTC-3' (*Spe*I site underlined). *MusaMPK5* was also fused with *GUS* coding sequence in *pCAMBIA1301* utilizing restriction sites *Nco*I and *Bam*HI. Primers used for generation of *pCAMBIA1301-MusaMPK5::GUS* were FP: 5'-AACCATGGACGGGGCTCCGG-3' (*Nco*I site underlined) and RP: 5'-AAGGATCCGTATGCTGGATTGAATGCTATAGC-3' (*Bam*HI site underlined). *Agrobacterium tumefaciens* strain *EHA105* harboring *pCAMBIA1302-MusaMPK5::GFP* and *pCAMBIA1301-MusaMPK5::GUS* were used for transient transformation of onion epidermal cells as described earlier (Sun et al. 2007). Five days after transformation of onion epidermal cells, the epidermal

peeling was either stained with GUS staining buffer or observed under fluorescent microscope (Eclipse 80i, Nikon, Japan) after staining with nuclear stain Hoechst-33258. After five days of transformation, GFP-fluorescence was monitored with fluorescent microscope (Eclipse 80i, Nikon, Japan) and nuclei was identified by staining with Hoechst-33258.

Purification of MusaMPK5 protein

Complete coding sequence of *MusaMPK5* was PCR amplified (FP: 5'-AAACATATGGACGGGGCTCCGGTGAA-3' *Nde*I underlined and RP: 5'-AAAGGATCCCTAGTATGCTGGATTGAATGCTATAGC-3' *Bam*HI underlined) and cloned in *Nde*I and *Bam*HI sites of *pET28a*-vector. Recombinant *E.coli* stain *BL21(DE3)* harboring *pET28a-MusaMPK5* was grown till OD_{600} is 0.6 and then induced with 1mM IPTG for three hours. Cells were sonicated in Tris buffer (25 mM) containing lysozyme (5 mg), PMSF (1 mM) and 10% glycerol. The soluble fraction containing MusaMPK5 was used for affinity purification on nickel-NTA column. The nickel-NTA resin was washed with 10 bed volume of binding buffer (25 mM Tris buffer with 250mM NaCl and 10 % glycerol) and then was loaded with the soluble fraction obtained after sonication. Flow through was collected and the column was washed with 50 ml of binding buffer containing 20 mM imidazole. Elutions were collected by binding buffer containing 250mM imidazole and different fractions were analyzed on 10% SDS-PAGE. Elution fractions containing only MusaMPK5 were dialyzed against dialysis buffer (25 mM Tris-buffer and 10% glycerol) and stored at -80 °C for further applications.

Kinase activity of MusaMPK5

Purified MusaMPK5 from different elutions were incubated for 30 minutes at 37 °C with MBP (myelin basic protein) in a 10 µl reaction containing γ-^{32}P-ATP and ATP. In a separate experiment, MusaNAC042 and banana SNAC67 were incubated with MusaMPK5, γ-^{32}P-ATP and ATP in 10 µl

reaction for 30 minutes at 37 °C. The reactions were separated on 10% SDS-PAGE and the result was analyzed by autoradiography.

Stress treatments and expression analysis of *MusaSNAC67*

In separate experiments, two month old plants of banana cv. *Karibale Monthan* were subjected to 250mM NaCl, 4±2 °C for cold, drying on blotting paper for drought, sprayed with 2mM H_2O_2 and 2mM Salicylic acid. For each treatment, at-least three plants were treated and their tissues were mixed in equal amount before RNA isolation. RNA isolation at separate time points was carried with Concert plant RNA-reagent (Invitrogen, USA; Cat. No. 12322012) as described earlier (Negi et al. 2015a). On-column DNAase digestion by Qiagen DNAase-digestion (Cat. No.79254) set to remove contaminating DNA was performed. First strand cDNA synthesized using thermoscript AMV-RT (Invitrogen: Cat. No.12236-014) was diluted to 1:100 and utilized for quantitative RT-PCR using SYBR Green Jump Start Taq Ready Mix (Sigma, USA; Cat. No. P2893). *Musa EF1α* was used for normalizing expression data obtained from quantitative RT-PCR. Quantitative RT-PCR was operated as follows: 95°C(5min), 30-cycles of 95°C(20sec) , 55°C(20sec), 72°C (20sec) ending with a melting curve analysis.

Amplification of coding sequence and 5' up-stream region of *MusaSNAC67*

RNA isolation and synthesis of cDNA from leaf of banana cv. *Karibale Monthan* was carried out as described above. PCR conditions for amplification of *MusaSNAC67* was as follows: 20pmols of primers, 0.5unit of Taq DNA-polymerase, 1µl of 10mM dNTP stock, 1x buffer with $MgCl_2$ and 5µL cDNA. PCR operating conditions were: 95°C(8min), 36-cycles of 95°C(40sec), 58°C (45sec), 72°C(60sec), 72°C(20min). Leaf genomic-DNA was extracted with the help of GenElute Plant Genomic-DNA Miniprep-Kit (Sigma, USA; Cat. No. G2N70) and further used for amplification of 5' up-stream region of *MusaSNAC67*. PCR was set up as described above, except that instead of

cDNA, 3µL of purified genomic-DNA was used. PCR operating conditions was: 98°C(10min), 36-cycles of 98°C(60sec) , 58°C(45sec), 72°C(60sec), 72°C(20min). Sequences generated were submitted to NCBI databank as MF347599 (for *MusaSNAC67* coding sequence) and MF347600 (for 5' up-stream regulatory region of *MusaSNAC67*).

Construction of binary vectors for analysis of *MusaSNAC67*

Complete coding sequence of *MusaSNAC67* was digested with *Pst*I and *Kpn*I and cloned under *Zea mays* polyubiquitin-promoter (*pZmUbi*) in *pCAMBIA1301* as follows. A terminator, *nos 3'-UTR* was first inserted in multiple-cloning-site (MCS) using *Sac*I and *Eco*RI sites and the vector (*pCAMBIA1301-nosT*) was confirmed by sequencing. Both *pZmUbi* and *MusaSNAC67* was ligated with *pCAMBIA1301-nosT* in a three-way-ligation using *Hind*III and *Kpn*I as descried earlier (Tak et al. 2017) to generate *pCAMBIA1301-pZmUbi-MusaSNAC67-nosT*. Correct ligation and construction of binary vector was confirmed by DNA-sequencing. The 5' up-stream region of *MusaSNAC67* ($P_{MusaSNAC67}$) was digested with *Pst*I and *Nco*I and ligated with *Pst*I and *Nco*I digested *pCAMBIA1301*(which released *CaMV35S*-promoter located upstream of *GUS*) to generate *pCAMBIA1301-*$P_{MusaSNAC67}$*-GUS*. PCR and cloning integrity was confirmed by sequencing analysis.

Sequence analysis of *MusaSNAC67*

MusaSNAC67 and other related NAC proteins from various plant species were aligned using clustal-omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/) and a neighbor-joining tree with stringency of 1000 bootstrap-replicates was built with MEGA6-software (Kumar et al. 2008). Different NAC-proteins (with known function) and MusaSNAC67 were aligned by clustal-omega and identical residues were shaded in black by box-shade server (http://www.ch.embnet.org/software/BOX_form.html). 5' up-stream region of *MusaSNAC67* was analyzed for presence of cis-elements by JASPAR-database (http://jaspar.genereg.net/), PantPan2

(Plant Promoter Analysis Navigator; http://plantpan2.itps.ncku.edu.tw/) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Generation of transgenic banana lines overexpressing *MusaSNAC67*

Binary vectors (*pCAMBIA1301-P$_{MusaSNAC67}$-GUS* and *pCAMBIA1301-pZmUbi- MusaSNAC67 -nosT*) were transformed into *Agrobacterium*-strain *EHA105* and used for transformation of ECS (embryogenic cell suspension) of banana cv. *Rasthali* following previously published protocol (Ganapathi et al. 2001). Around 2ml packed-cell-volume of embryogenic cells and recombinant *Agrobacterium* were co-cultivated (30min) and then cultured on M2-medium (3days) in dark (Cote et al. 1996). Embryo-development was carried on embryo-development-medium (BEM) which was added with cefotaxime (400mg/l) and hygromycin (5mg/l). Germination of embryos into shoots was carried out with cytokinin supplemented medium (BA 0.5mg/l).

Protein induction and purification of *MusaSNAC67*

Complete coding sequence of *MusaSNAC67* was inserted in *pET28a*-vector using *Nde*I and *Bam*HI sites and the construct was sequenced to verify ligation. Recombinant *E.coli* cells *BL21(DE3)* growing at 37 °C were induced at 0.6 O.D. by 1mM IPTG. Pellet containing inclusion-bodies was solubilized with 0.2% N-lauroylsarcosine for 24hours (Tao et al. 2010). In solubilized fraction, 0.4% triton-X100 and 0.4%CHAPS was added and the lysate was incubated for 2 hours. Soluble fraction then obtained was used for purification of MusaSNAC67 on Ni-NTA column. Column washing was carried out by binding-buffer (50mM Tris-buffer, pH 8 with 250mM NaCl, 50mM imidazole and 10%glycerol) and different-fraction (flow-through and wash) was collected. Elution of column bound protein was carried out using binding-buffer containing 250mM imidazole which was later removed by dialysis in dialysis buffer (50mM Tris-buffer and 10% glycerol). All fractions collected were analyzed on 12% SDS-PAGE. MusaSNAC67 protein was detected by western-

blotting using primary-antibody against polyhistidine and later with a secondary-antibody linked with alkaline-phosphatase enzyme. Color development was carried out using NBT/BCIP substrate as per manufacturer instructions (Sigma USA; 11681451001).

Gel-shift assay with MusaSNAC67

Purified protein was incubated with different DNA-substrates in a reaction buffer (10mM Tris-buffer, pH8, 5mM $MgCl_2$ and 10mM KCl). Binding was allowed for 20minutes at 25°C, after which reaction was resolved on 1% agarose-gel which was stained by ethidium bromide post-run. Oligonucleotides containing core-binding site CGT[A/G] or *SNBE/TERE*-like motif and mutated-sequences were synthesized by out-sourcing. Different promoter fragments containing CGT[A/G] sites were amplified from genomic-DNA of banana and utilized for gel shift assay with MusaSNAC67.

Transmission electron microscopy

Leaves incubated in cacodylate-buffer (50mM , pH7.4) were fixed in glutaraldehyde (2.5%) and para-formaldehyde (0.5%) solution for 12hours (4°C). After repeated washing with cacodylate-buffer, leaf pieces were dehydrated in ethanol series for 30minutes each. Ethanol was substituted with propylene oxide and then leaf pieces were infiltrated for 2hours using different ratios (1:3, 1:1, and 3:1).of Spurr-resin and propylene oxide. Embedding was carried out for 3 days (60°C), and then 70nm thin sections prepared in a ultramicrotome were stained with 1.5% uranyl acetate, dried and then observed in Libra 120 Plus TEM (Carl Zeiss).

Chapter III: Results

Section 3.1: Studies on *MusaNAC042* gene from banana

Introduction

Stress in agricultural perspective has been defined as any external factor that threaten the survival of the plant along with reducing its growth, overall productivity and reproductive capacity (Rhodes and Nadolska 2001). High salinity and water deficiency in the soil are major hurdles for growth and yield of many plant species (Mahajan and Tuteja 2005). High salinity is an environmental stress resulting in hyperionic and hyperosmotic conditions causing reduction in growth and photosynthesis, membrane disorganization, generation of reactive oxygen species and overall toxicity to cell metabolism (Niu et al. 1995; Greenway and Munns 1980; Yeo 1998). Drought stress results in cell shrinkage leading to cellular membrane damage, impaired function of membrane associated enzymes, broad level peroxidation of cellular membrane and protein denaturation by excessive production of ROS, extensive protein aggregation and reduced photosynthesis activity (Bowler et al. 1992; Hoekstra et al. 2001). Banana and plantain are grown worldwide with a production of around 106.7 million tonnes per year (FAO,2013). Banana is sensitive to water stress due to features like shallow roots and a permanent green canopy (Xu et al. 2014; van Asten et al. 2011). Banana needs nearly 2000–2500 mm rainfall evenly distributed throughout the year for optimal yield output (Vanhove et al. 2012). In India 51.1 Mha of agricultural land is drought prone while 6.73 Mha is affected by salinity (NRCB newsletter, 2010). Moreover, report suggest that 25% of ground water used for irrigation in India is saline and by 2025 nearly 11.7 Mha of agricultural land may get affected by salinity (NRCB newsletter, 2010). In Tamil Nadu (India), banana production has been reported around 4.64 million tones which was declined due to drought during

2001-2004 (Surendar et al. 2013). Field studies conducted at the NRCB (National Research Centre for Banana, India) suggested a reduction of up to 42% in bunch weight of banana by imposition of drought for one month during flowering (Ravi et al. 2013).

Plants being sessile need to withstand environmental stresses and hence to adapt and survive under stress conditions, plants have developed various mechanisms including enhanced expression of stress protective genes which include regulatory factors like transcription factors known to regulate expression of many functional genes involved in abiotic or biotic stress tolerance. One of these plant specific transcription factor families is the NACs (NAM, ATAF and CUC). NAC proteins have a DNA binding domain at N-terminus and a regulatory region towards C-terminus. NAC domain containing proteins are present in large number in plants with almost 110 members in *Arabidopsis* and 151 members in rice (Olsen et al. 2005; Nuruzzaman et al. 2010). Few of NAC proteins from different plant species have been characterized for their function in plant development and stress responses.

Rice *SNAC1* is stress inducible and its overexpression resulted in elevated drought tolerance of transgenic plants (Hu et al. 2006). Soybean *NAC* transcription factors, *GmNAC20* and *GmNAC11* have been characterized as important regulators of salinity and cold stress (Hao et al. 2011). Overexpression of rice *SNAC2* and *SNAC3* resulted in improved drought tolerance in transgenic plants (Hu et al. 2008; Fang et al. 2015). *Hordeum vulgare NAC* factor, *HvSNAC1* is induced strongly by abiotic stress conditions and transgenic lines showed improved drought tolerance at various growth stages (Al Abdallat et al. 2014). Wheat NAC transcription factors *TaGRAB1* and *TaGRAB2* inhibits DNA replication of wheat dwarf geminivirus in wheat cells by interacting with Rep A protein of the virus (Xie et al. 1999). Wheat *NAC* transcription factor, *TaNAC4* is induced by multiple stress conditions like methyl jasmonate, infection of stripe rust

pathogen, ABA, ethylene, high salinity, wounding and low-temperature indicating that *TaNAC4* is involved in response to multiple stress conditions (Xia et al. 2010). *TaNAC69* expression was positively associated with stress responses of wheat and transgenic plants overexpressing *TaNAC69* produced higher shoot biomass and longer roots than control plants (Xue et al. 2011; Baloglu et al. 2012).

A comprehensive analysis of *NAC* transcription factors in banana (*Musa acuminata*) was reported recently resulting in identification of nearly 167 potential NAC transcription factor coding genes (Cenci et al. 2014). In the recent years, there has been growing interest on the characterization of the *NAC* transcription factors in banana with emphasis on secondary wall development and stress responses. *MusaVND1*, a banana *NAC* domain containing transcription factor was demonstrated to convert banana embryogenic cells into tracheary elements–like cells indicating its role in xylem development (Negi et al. 2015a). Further *MusaVND2* and *MusaVND3* were reported to exhibit ability to develop ectopic secondary wall development in transgenic banana (Negi et al. 2016). Expression of banana *NAC* transcription factors, *MaNAC1* and *MaNAC2* was elevated in the fruit by ethylene and their interaction with ethylene insensitive 3 (EIN3)-like protein which is a component of ethylene signaling suggested that MaNAC1/2 may be involved in fruit ripening (Shan et al. 2012). While MaNAC1, is also involved in cold tolerance of banana fruits and is involved in interaction with ICE1-CBF cold signaling pathway (Shan et al. 2014). *MaNAC5* in cooperation with *WRKY* transcription factor is involved in pathogen resistance and *PR*-genes regulation (Shan et al. 2015). Another banana *NAC* transcription factor, *MusaNAC68* is induced by multiple stress conditions and positively regulates drought and salinity tolerance in banana (Negi et al. 2015b).

However, exact role of *NAC* transcription factors in imparting tolerance to abiotic and biotic stresses needs to be elucidated in banana. Among the other *NAC* transcription factors, *Arabidopsis thaliana NAC042/JUB1* has been proposed as central longevity regulator and its overexpression lowers intracellular H_2O_2 levels and increase tolerance to abiotic stress conditions besides delaying senescence (Wu et al. 2012) and enhancing heat tolerance in *NAC042/JUB1* overexpressing transgenic plants (Shahnejat-Bushehri et al. 2012). In another study, *Arabidopsis ANAC042* was shown to be involved in regulation of camalexin (a phytoalexin) which was substantiated by lower levels of camalexin in the T-DNA insertion mutants (Saga et al. 2012).

To test whether banana *NAC042* can also elevate the drought and high salinity tolerance of transgenic banana and to promote the utilization of *NAC* genes in genetic improvement of banana, we have characterized *MusaNAC042* and shown its probable involvement in drought and high salinity tolerance. We have chosen *Musa* cv. *Rasthali* for the generation of transgenic plants as it is one of the economically important banana cultivars in India and is highly susceptible to drought as well as high salinity stress conditions resulting in considerable yield reduction. Quantitative RT-PCR indicated that expression of *MusaNAC042* was elevated during multiple stress conditions suggesting its potential role in their regulation. Transgenic banana plants showed better tolerance to drought and high salinity than control plants. *MusaNAC042* encodes a nuclear localized protein and transgenic banana overexpressing *MusaNAC042* displayed altered expression of multiple stress related genes. The present study suggests that *MusaNAC042* is a stress related transcription factor and can be useful for engineering abiotic stress tolerance in banana.

Results

Phylogenetic analysis of MusaNAC042

MusaNAC042 was identified employing pBLAST in banana genome sequence database (http://banana-genome.cirad.fr/) using *Arabidopsis* NAC042 (ANAC042: At2g43000.1 in *Arabidopsis* Genome Initiative) as search query. Sequence with the highest pBLAST score was designated as MusaNAC042. Coding sequence of *MusaNAC042* contained 861 bases and encoded a 287 amino acids long protein with predicted molecular weight of 32.7 kDa and a theoretical pI of 6.4. MusaNAC042 shared high sequence similarity with other NAC042/JUNGBRUNNEN1 proteins from different plant species. MusaNAC042 exhibited identities of 52% with GrJUB1, 50% with CsJUB1, 54% with JcJUB1, 52% with VrJUB1, 52% with NsJUB1, 55% TcNAC042, 56% with NnJUB1, 55% with VvJUB1, 62% with PdJUB1, 60% with BdJUB1, 68% with ObJUB1, 54% with SiJUB1, 51% with ZmJUB1, 51% with ANAC042, 50% with BrJUB1, 34% with AtNST1, 23% with AtVND6 and 31% with AtSND1 (Fig. 3.1.1). N-terminal end of MusaNAC042 possessed a conserved NAC domain while the C-terminal end was relatively divergent (Fig. 3.1.2).

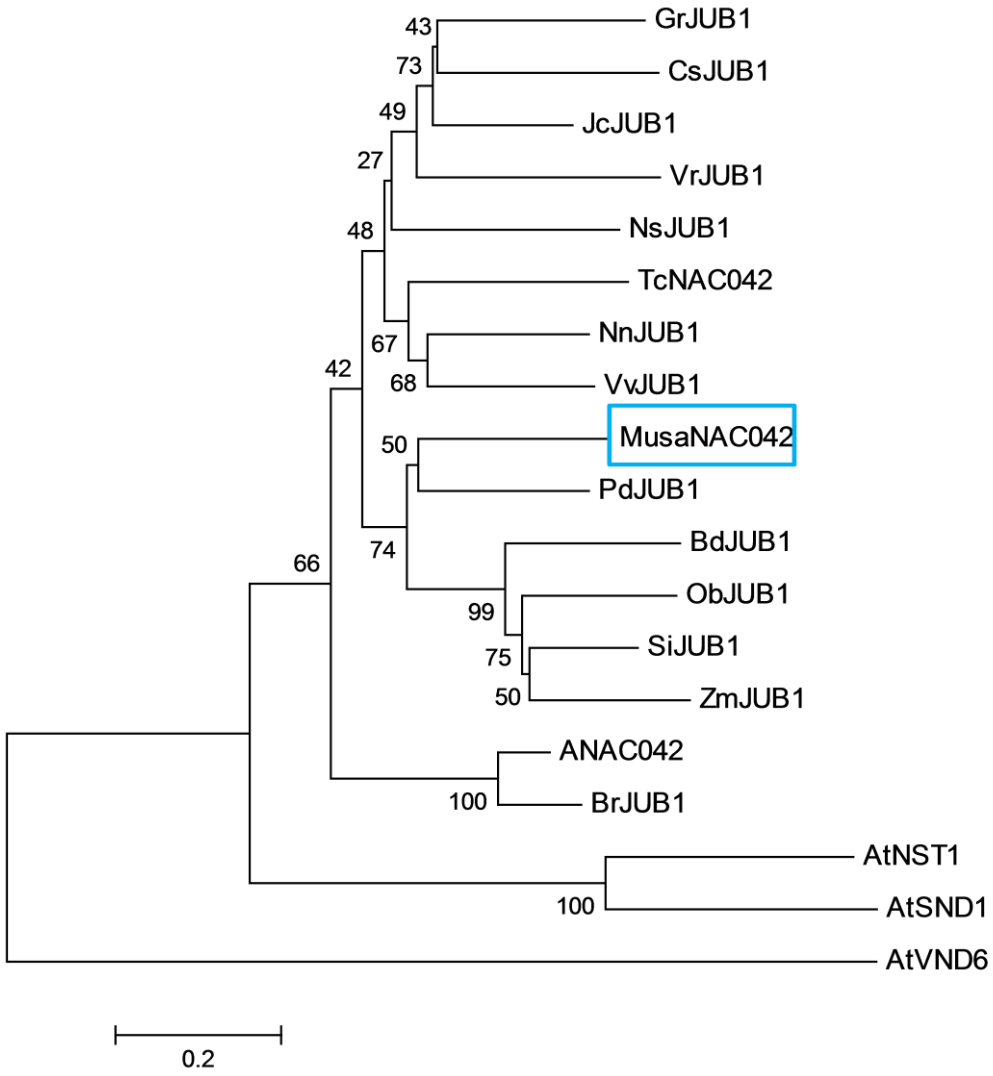


Fig. 3.1.1 Phylogenetic analysis of banana MusaNAC042 with selected NAC proteins from different plant species. Amino acid sequences of NAC proteins in the tree are: *Gossypium raimondii* GrJUB1 (XP_012441089.1), *Citrus sinensis* CsJUB1 (XP_006475534.1), *Jatropha curcas* JcJUB1 (XP_012087398.1), *Vigna radiata* VrJUB1 (XP_014500826.1), *Nicotiana sylvestris* NsJUB1 (XP_009778771.1), *Theobroma cacao* TcNAC042 (XP_007048954.1), *Nelumbo nucifera* NnJUB1 (XP_010272267.1), *Vitis vinifera* VvJUB1 (XP_002283251.1), *Phoenix dactylifera* PdJUB1 (XP_008806688.1), *Brachypodium distachyon* BdJUB1 (XP_003559373.1), *Oryza*

brachyantha ObJUB1 (XP_006651871.1), *Setaria italica* SiJUB1 (XP_004981565.1), *Zea mays* ZmJUB1 (XP_008644461.1), *Arabidopsis thaliana* ANAC042 (NP_181828.1), *Brassica rapa* BrJUB1 (XP_009142897.1), *Arabidopsis thaliana* AtNST1 (NP_182200.2), *Arabidopsis thaliana* AtSND1 (AEE31527.1) and *Arabidopsis thaliana* AtVND6 (AED96207.1). Neighbor joining tree was built with bootstrap value of 1000. MusaNAC042 is boxed in blue.

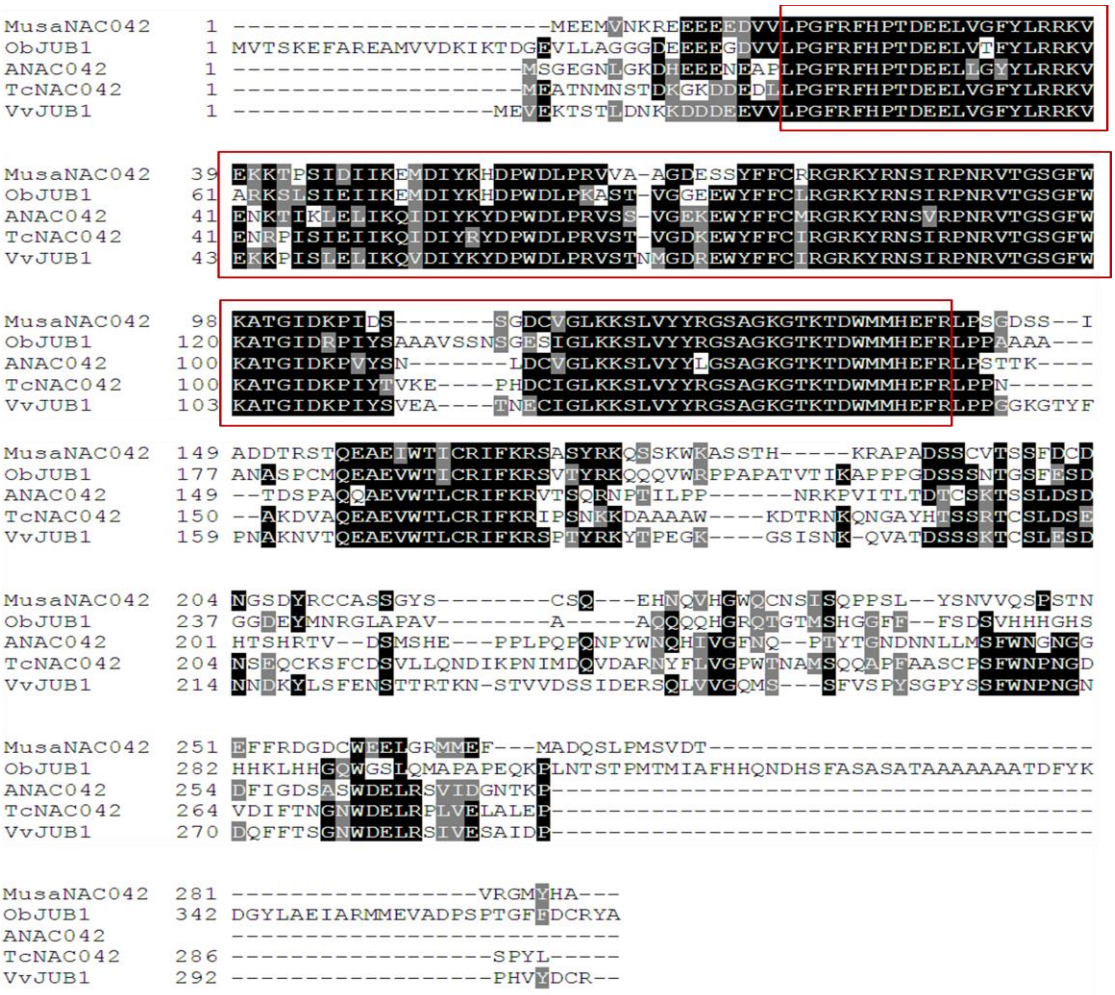


Fig. 3.1.2 Multiple sequence alignment of MusaNAC042 with other selected NAC proteins from *Arabidopsis thaliana* ANAC042 (NP_181828.1), *Theobroma cocao* TcNAC042 (XP_007048954.1), *Oryza brachyantha* ObJUB1 (XP_006651871.1) and *Vitis vinifera* VvJUB1 (XP_002283251.1). The highly conserved NAC domain is red boxed.

Expression analysis of *MusaNAC042*

MusaNAC042 was induced in the leaves of banana cultivar *Karibale Monthan* under high salinity and drought. During drought (drying of the plant on blotting paper), expression of *MusaNAC042* was maximum at 5 hour and then it was decreased at 48 hours but remain higher than control level (Fig. 3.1.3a). Similarly, upon application of 250mM NaCl (Fig. 3.1.3b) expression of *MusaNAC042* peaked at 12 hours and then reduce towards 48 hours but remain higher than the expression value in control.

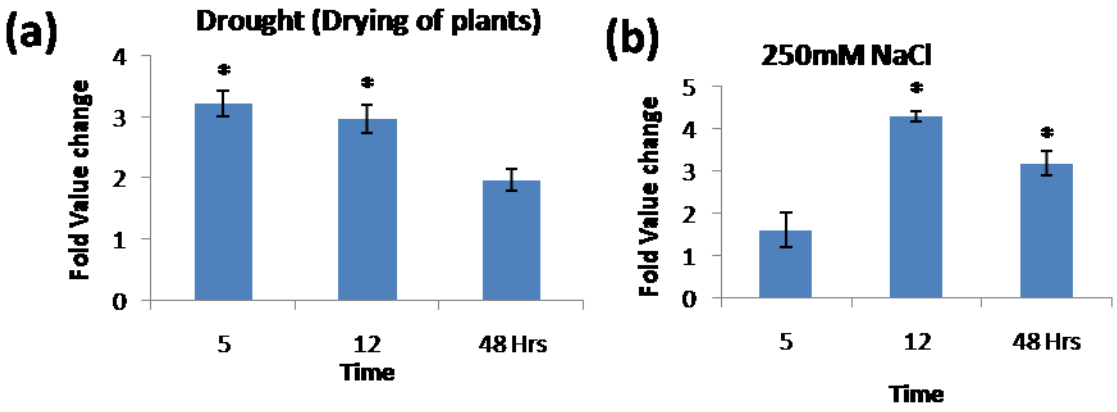


Fig. 3.1.3 Expression profiles of *MusaNAC042*. Expression of *MusaNAC042* after exposure to (a) drought (drying of plants) and (b) 250mM NaCl. Values indicated are mean ± SD of three technical replicates. Statistical significant events at 5% are represented with an asterisk (*).

Analysis of GUS activity (histochemical and biochemical estimation) in banana plants harboring *P_{NAC042}::GUS*

Banana plant harboring *P_{NAC042}::GUS* and subjected to drought and salinity stress were analyzed for GUS activity using 4-methylumbelliferyl-b-D-glucuronide (MUG) and total protein extract. Under both salinity and drought stress, the activation of *P_{NAC042}* was observed in terms of elevated GUS activity. Elevation was observed in all the organs, however, strong elevation was observed in corm following the stress challenge (Fig. 3.1.4).

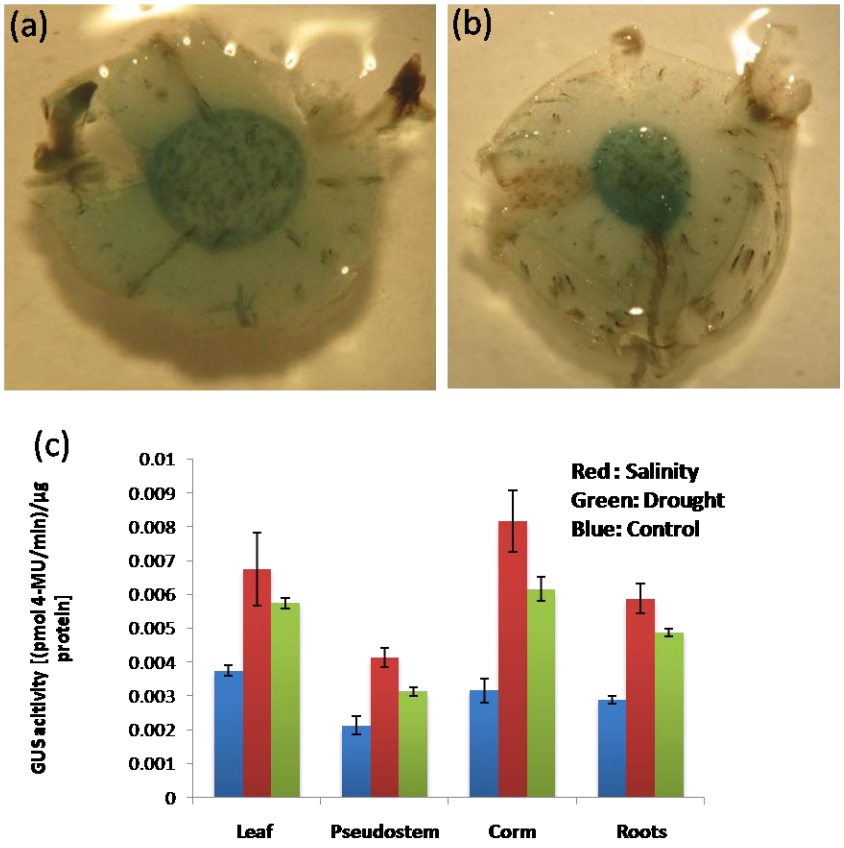


Fig. 3.1.4 GUS assay of banana shoots transformed with P_{NAC042}*::GUS*. (a) GUS staining in corm of banana transformed with P_{NAC042}*::GUS* and subjected to drought stress.(b) GUS staining in corm of plants harboring P_{NAC042}*::GUS* corm under salinity stress. (c) MUG assay to determine the activity of P_{NAC042} under drought and salinity in different organs of banana.

Sub-cellular localization of MusaNAC042

MusaNAC042 was fused with *GFP* in *pCAMBIA1302* and transiently overexpressed in banana cultivar *Rasthali* embryogenic cells displayed uniform distribution of green fluorescence (Fig. 3.1.5a) indicating GFP distribution throughout the cell. Cells transformed with *pCAMBIA1302-MusaNAC042* displayed preferential localization of green fluorescence in nucleus suggesting nuclear localization of MusaNAC042 (Fig. 3.1.5c). Position of nucleus in cells was determined after staining with Hoechst 33258 (Fig. 3.1.5b, 3.1.5d).

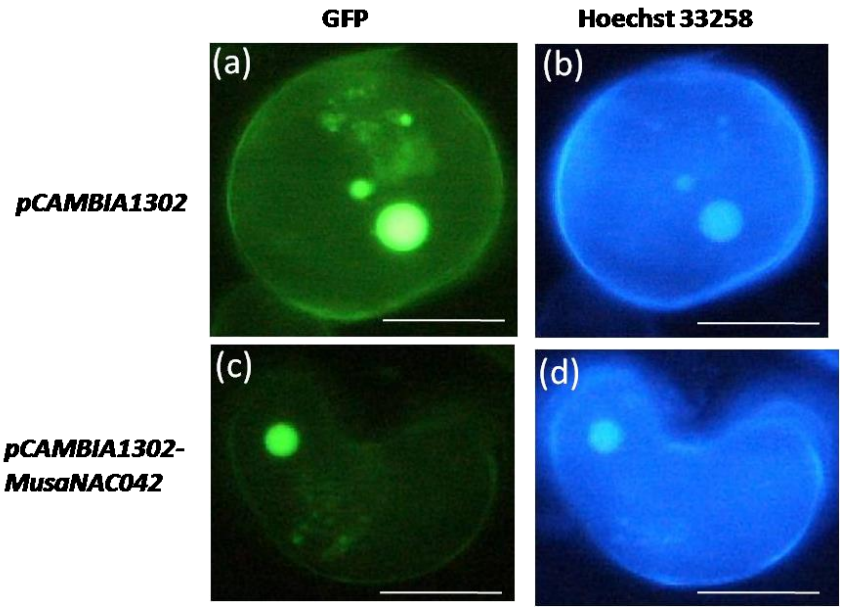


Fig. 3.1.5 Subcellular localization of MusaNAC042 in banana embryogenic cells. (a) GFP fluorescence observed in *pCAMBIA1302* transformed cells.(b) Nuclear position in the cells detected by Hoechst 33258 staining. (c) Green fluorescence observed in cells transformed with *pCAMBIA1302-MusaNAC042*. (d) Hoechst 33258 staining of cell transformed with *pCAMBIA1302- MusaNAC042*. The scale bar measures 50µm.

Regeneration of transgenic banana lines

Complete coding sequence of *MusaNAC042* was cloned downstream of *CaMV35S* promoter in *Nco*I and *Pml*I sites of *pCAMBIA1301* for achieving constitutive expression of *MusaNAC042* in transgenic lines (Fig. 3.1.6a). Embryogenic cells of banana cultivar *Rasthali* were transformed with *pCAMBIA1301-MusaNAC042* by *Agrobacterium* mediated method and further growth was monitored (Fig. 3.1.6b). Growth of transformed embryogenic cells on hygromycin selection medium resulted in white and globular embryos (Fig. 3.1.6c). Further culturing of embryos on shoot development medium resulted in the conversion into putatively transformed shoots (Fig.

3.1.6d). Individual shoots were clonally propagated on shoot multiplication medium (Fig. 3.1.6e) and rooting was carried out on MS-medium supplemented with 1mg/l of NAA (Fig. 3.1.6f) which were subsequently hardened in the green house (Fig. 3.1.6g).

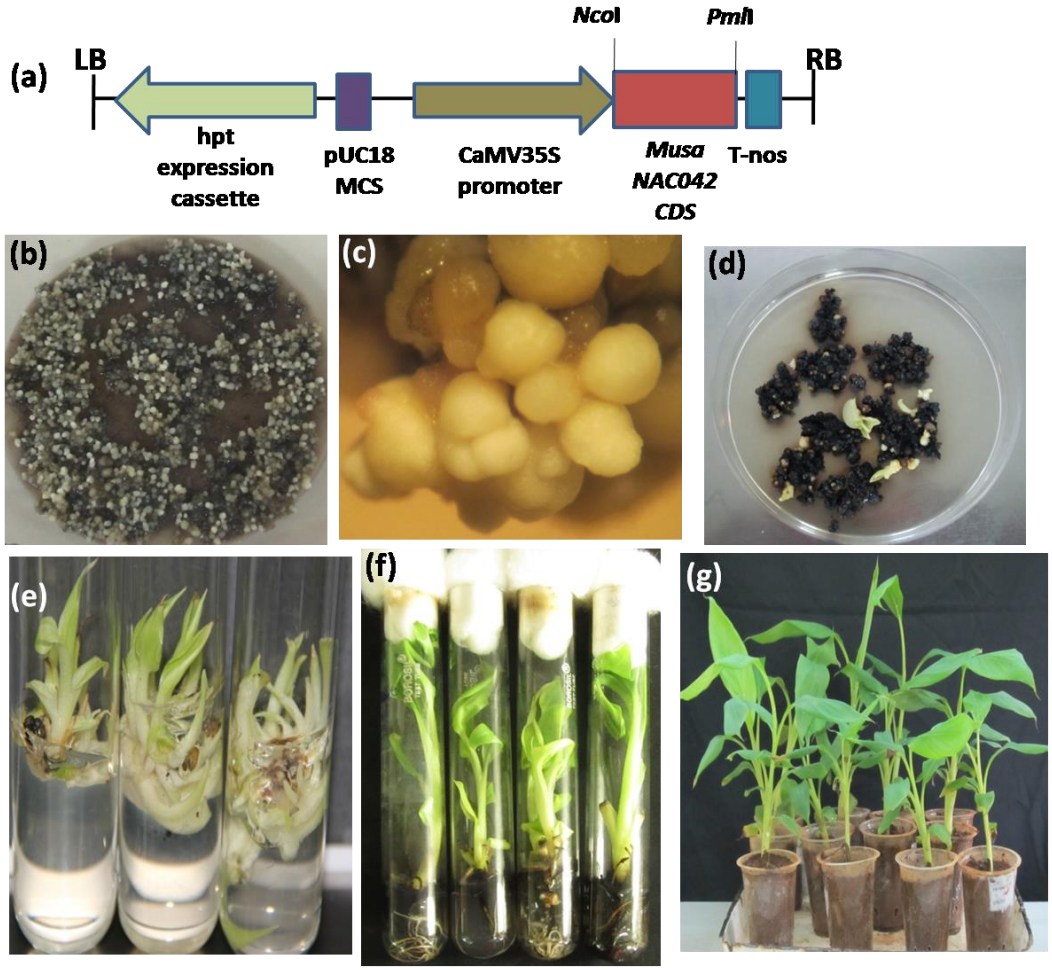


Fig. 3.1.6 Regeneration of transgenic banana lines. (a) T-DNA region of *pCAMBIA1301-MusaNAC042* designed to overexpress *MusaNAC042*. (b) Development of embryogenic cells of banana cultivar *Rasthali* transformed *with pCAMBIA1301-MusaNAC042* on glass fiber filter. (c) Close up of developing embryos on selection medium. (d) Shoot emergence on selection medium. (e) Multiple shoots of different putatively transgenic banana lines. (f) Rooting of different putative transgenic lines. (h) Hardened plants of different transgenic lines in the green house.

Molecular confirmation of T-DNA integration in transgenic lines

Genomic DNA of putative transgenic lines was analyzed for PCR amplification of *hpt-II* (*Hygromycin phosphotransferase*). PCR amplification of a 788bp band from coding region of *hpt-II* indicated successful integration of T-DNA in genome of transgenic lines (Fig. 3.1.7a) while no amplification was observed in the wild type (control, non transformed) plants. Southern blot analysis carried out with digoxin labeled *hpt-II* probe revealed 2-5 copies of T-DNA transferred in different transgenic lines (Fig. 3.1.7b). Further, overexpression of *MusaNAC042* due to T-DNA copies analyzed by quantitative RT-PCR indicated that lines L2, L4, L7 and L11 accumulated 8.18, 6.12, 12.88 and 9.23 fold of *MusaNAC042* transcript respectively over control value (Fig. 3.1.7c).

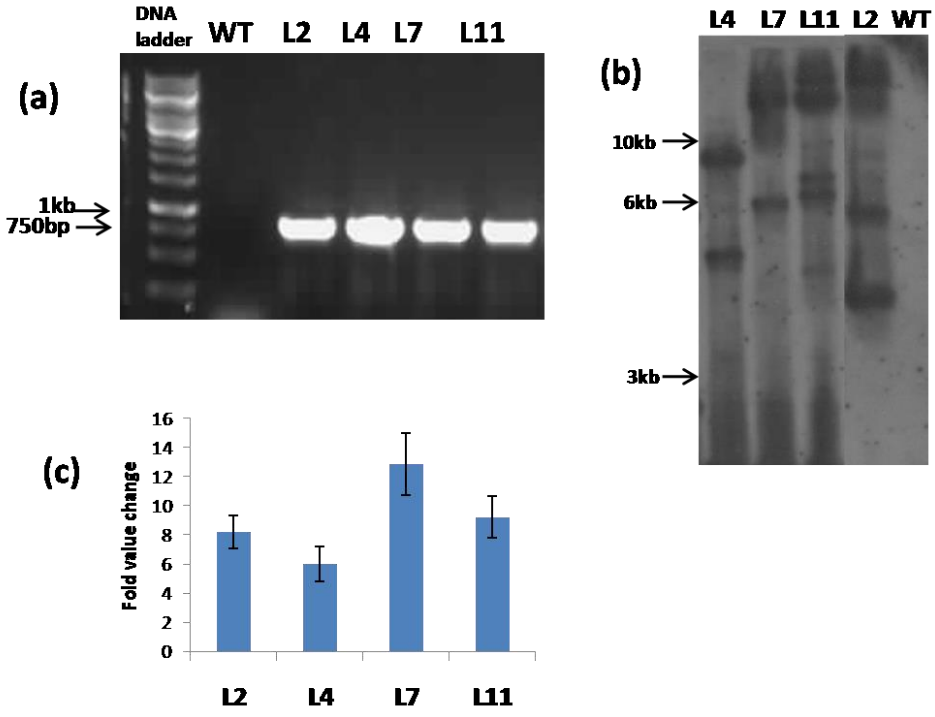


Fig. 3.1.7 Molecular confirmation of transgenic lines. (a) PCR amplification of *hpt-II* from genomic DNA of different lines . (b) Analysis of number of T-DNA insertions in different lines by Southern blot analysis. The blot was probed by digoxin labeled probe against *hpt-II* present within the T-DNA region. (c) *MusaNAC042* transcript analysis in different transgenic lines by quantitative RT-

PCR. The C_t-value of *EF1α* was utilized for normalization of *MusaNAC042* C_t-values. Values indicated are mean ± SD of three technical replicates. (WT: wild type; L2,L4,L7and L11: transgenic lines).

Leaf disc method for analysis of salinity tolerance

Tolerance of transgenic banana lines towards salinity stress was analyzed by leaf disc method. For this, leaf disc punched using cork borer were incubated in different concentrations of NaCl. Visual injury to leaf disc due to chlorophyll degradation was lower in transgenic lines than control suggesting better tolerance to salinity in the transgenic lines. While bleaching in the control discs was profound even in 100mM NaCl, leaf discs of transgenic lines showed remarkably less bleaching and senescence even in 200 mM NaCl (Fig. 3.1.8a) after 6 days of treatment. Higher tolerance of transgenic lines in leaf disc method was confirmed by estimation of total chlorophyll and MDA content. Transgenic leaf discs retained significantly more chlorophyll than control at different concentrations of NaCl (Fig. 3.1.8b). Extent of oxidative damage in term of MDA content was significantly lower in transgenic discs than the control plant leaf disc suggesting enhanced salinity tolerance of transgenic lines (Fig. 3.1.8c).

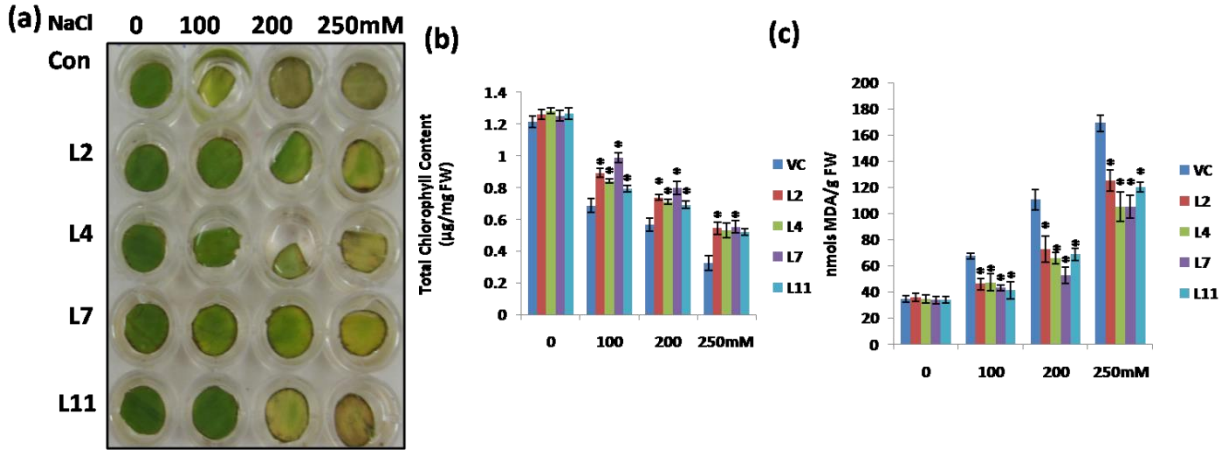


Fig. 3.1.8 Salinity tolerance test by leaf disc method. (a) Chlorophyll loss from leaf disc of control and transgenic lines incubated on different concentration of NaCl (0, 100, 200, 250 mM). (b) Total

chlorophyll content (µg/mg FW) in leaf disc after salinity treatment. (c) MDA (malondialdehyde) content (nmols MDA/g FW) in leaf disc after NaCl treatment. Values indicated are mean ± SD. (VC: Vector control; L2,L4,L7and L11: transgenic lines). Statistical significant events at 5% are represented with an asterisk (*).

High salinity and drought tolerance analysis of transgenic banana lines

Superior stress tolerance of transgenic banana lines as observed in leaf discs assay was further confirmed by imposing salinity and drought stress on hardened plants (Fig. 3.1.9a, 3.1.10a). Salinity (250 mM NaCl) and drought stress (Soil gravimetric water content was 0.21 and 0.15 on 7^{th} and 14^{th} day respectively) was imposed on plants and symptoms appeared in the form of leaf yellowing and wilting. However, the stress symptoms were less prominent in transgenic lines than control suggesting that overexpression of *MusaNAC042* could reduce the stress induced damage (Fig. 3.1.9b, 3.1.10b). Recovery of transgenic lines was much better than control in case of both salinity and drought stress. Compared to recovery in NaCl stressed plants, control plants failed to recover and ceased to grow while majority of transgenic lines recovered and resumed normal growth (Fig. 3.1.9c). Transgenic lines stressed by drought treatment recovered better than control indicating superior drought tolerance ability of transgenic lines overexpressing *MusaNAC042* (Fig. 3.1.10c). Biochemical responses of control and transgenic lines were recorded during and after stress treatment in terms of photosynthetic efficiency (Fv/Fm) and content of proline and malondialdehyde (MDA). Transgenic lines displayed better photosynthetic efficiency than control as measured by significantly higher Fv/Fm ratio during imposition of salinity (Fig. 3.1.9d) and drought (Fig. 3.1.10d). Stress induced oxidative damage was significantly lower in transgenic lines as shown by lower MDA (marker for membrane damage) content in transgenic lines under salinity (Fig. 3.1.9e) and drought stress (Fig. 3.1.10e). Level of osmoprotectant proline was higher in

transgenic lines than control which further suggested that performance of transgenic lines was better under salinity (Fig. 3.1.9f) and drought stress (Fig. 3.1.10f). Relative water content of all the transgenic lines was remarkably higher than control plants at the same level of drought stress indicating that transgenic lines could survive better than control under such stress conditions (Fig. 3.1.10g). Stress assay were carried out with three biological replications.

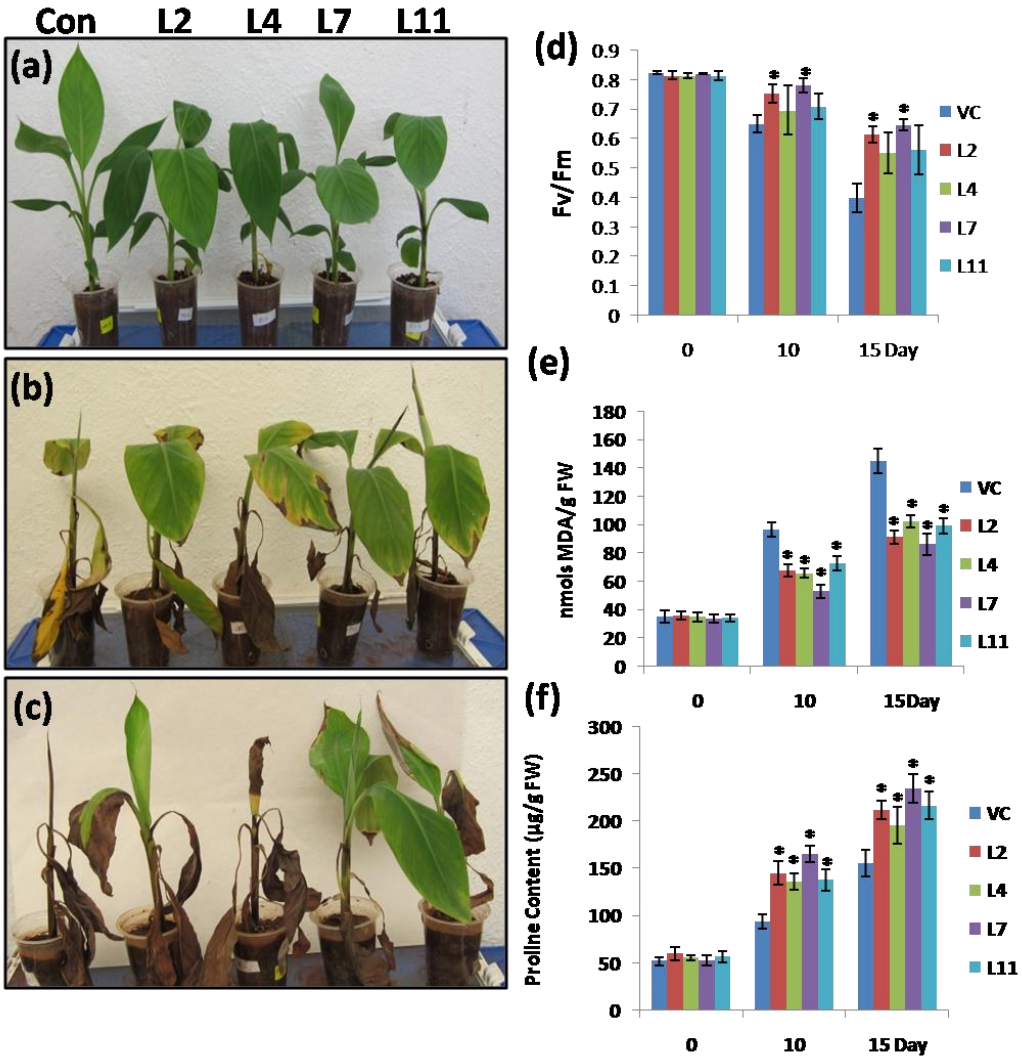


Fig. 3.1.9 Salinity tolerance analysis of transgenic banana lines. (a) Two month old wild plants and transgenic lines used for imposing salinity stress. (b) Control and transgenic lines subjected to 250mM NaCl after 15 days of stress initiation displaying the salinity induced stress symptoms. (c)

Wild plants and transgenic lines after watering with tap water for one month. (d) Photosynthetic efficiency (Fv/Fm) of control and transgenic lines at initiation and end of stress treatment. (e) MDA content (nmols MDA/g FW) of control and transgenic lines at different time period. (f) Proline content of control and transgenic lines at different time period. Values indicated are mean ± SD. (VC: Vector control; L2,L4,L7and L11: transgenic lines). Statistical significant events at 5% are represented with an asterisk (*).

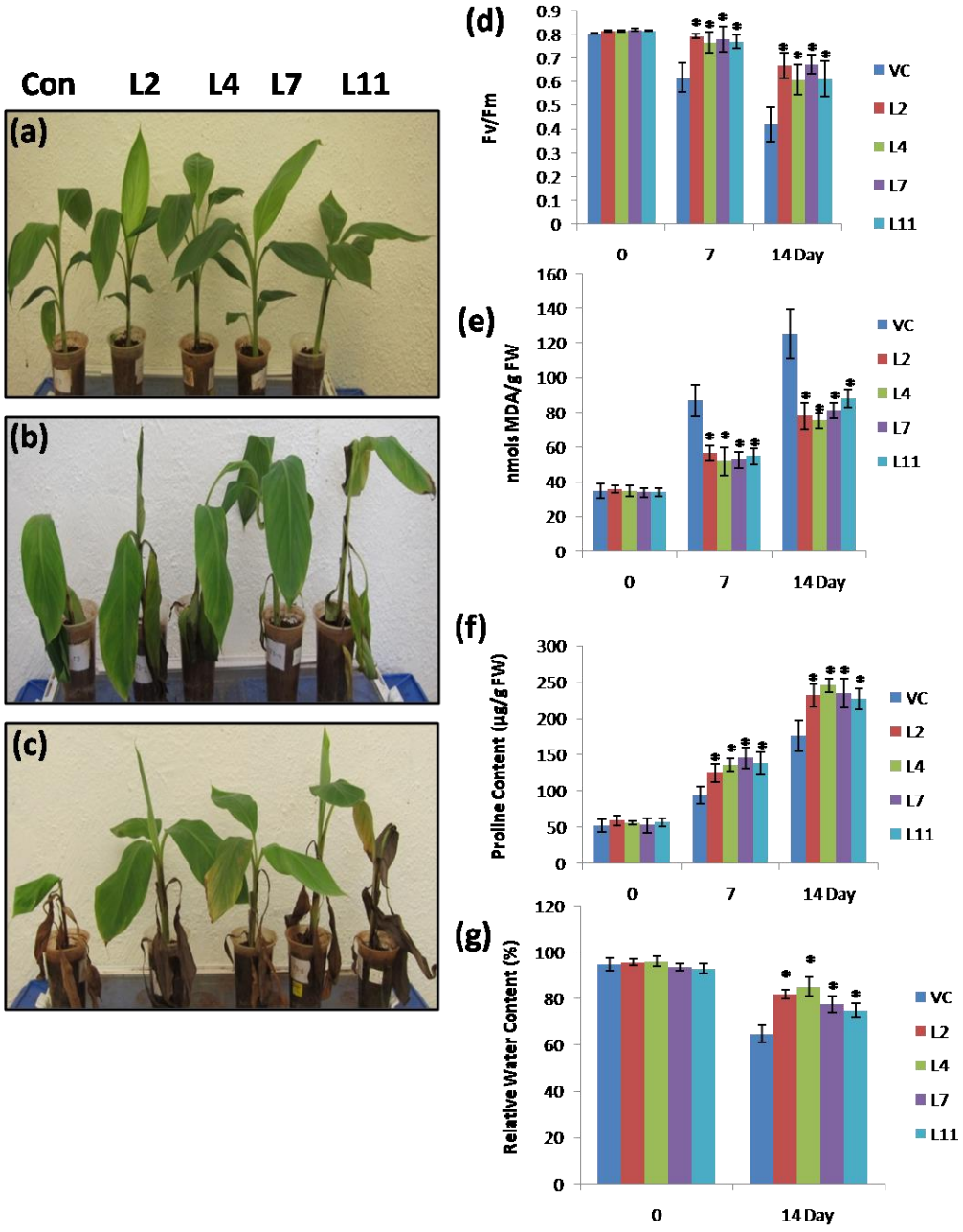


Fig. 3.1.10 Drought tolerance analysis of transgenic banana lines. (a) Two month old control and transgenic lines used for imposing drought. (b) Control and transgenic lines subjected to drought displaying the drought symptoms. (c) Control and transgenic lines after watering with tap water for one month. (d) Photosynthetic efficiency (Fv/Fm) of control and transgenic lines subjected to drought at indicated time points. (e) MDA content (nmols MDA/g FW) of control and transgenic

lines at different time period. (f) Proline content of control and transgenic lines during drought. (g) Percentage relative water content of transgenic lines and control plants before and after the drought stress treatment. Values indicated are mean ± SD. (VC: Vector control; L2, L4, L7 and L11: transgenic lines). Statistical significant events at 5% are represented with an asterisk (*).

Expression of stress related genes in transgenic lines

Probable involvement of *MusaNAC042* in stress responses pathways in banana was monitored by analyzing expression of members of *CBF*/*DREB*, *LEA* and *WRKY* family by quantitative RT-PCR. A total of 55 members of *CBF*/*DREB*, 30 members of *LEA* and 50 members of *WRKY* gene family were analyzed by quantitative RT-PCR in control plants and transgenic lines under non-stressed condition. At least expression of nine CBF/*DREBs*, five *WRKY* and three *LEA* coding genes was found to be altered in transgenic lines suggesting that *MusaNAC042* might be directly or indirectly affecting their expression. The quantum of expression change among these nine *CBF*/*DREBs* was as follows: FL659594 (3.3 fold), ES433029 (5.1 fold), FF561178 (3.2 fold), GSMUA_Achr11T15810_001 (14.2 fold), GSMUA_Achr6T28760_001 (2.3 fold), GSMUA_Achr4T26080_001 (7.1 fold), GSMUA_Achr11T03210_001 (9.9 fold), GSMUA_Achr8T09110_001 (3.4 fold) and GSMUA_Achr1T15350_001 (3.1 fold). While the expression alteration for 5 *WRKY* are: GSMUA_Achr5G04870_001 (2.9 fold), GSMUA_Achr10G23420_001 (2.5 fold), GSMUA_Achr6G13630_001 (3.9 fold), GSMUA_Achr3G05670_001 (2 fold) and GSMUA_AchrUn_randomG17570_001 (3.1 fold) . Fold change in transcript level of three *LEA* was: GSMUA_Achr11T05130_001 (4.6 fold), GSMUA_Achr10T21680_001 (7.4 fold) and GSMUA_Achr5T16860_001 (2 fold) (Fig. 3.1.11).

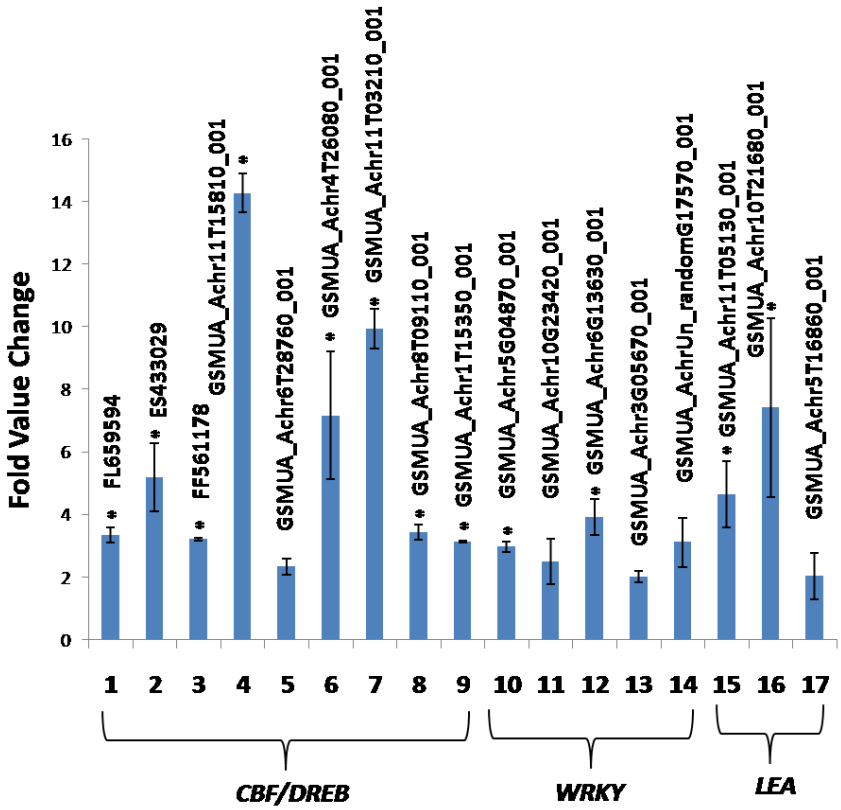


Fig. 3.1.11 Transactivation activity of *MusaNAC042*. Quantitative RT-PCR of stress related genes in transgenic banana overexpressing *MusaNAC042*. Change in expression of at least 9 *CBF*/*DREB*, 5 *WRKY* and 3 *LEA* coding genes was observed in transgenic banana plants under non-stressed condition. Corresponding banana genome database locus identifier or NCBI accession number is indicated on top of graph. Values indicated are mean ± SD). Statistical significant events at 5% are represented with an asterisk (*).

Discussion

In this study we have characterized *NAC042* transcription factor from banana and investigated its involvement in salinity and drought stress responses. Only few reports (Wu et al. 2012; Shahnejat-Bushehri et al. 2012; Saga et al. 2012) are available on the functions of *NAC042*/*JUB1* suggesting the need for further research into functions of this important NAC protein. Expression analysis and cloning of *MusaNAC042* was carried out in *Musa cv. Karibale Monthan* (ABB genome) while

transformation for generation of transgenic banana lines was carried out with *Musa cv. Rasthali* (AAB genome). Banana cultivars with more copies of B-genome (cultivars with ABB genome) have been documented to be more robust towards tolerance of stress conditions compared to banana cultivars with single B-genome or having only A-genome i.e. cultivars with AAB or AAA genome (Placide et al. 2012; Robinson and Sauco 2010). The higher stress tolerance ability of banana cultivars with B-genome than cultivars with A-genome has been experimentally tested using molecular techniques (Vanhove et al. 2012). *MusaNAC042* is induced by abiotic stress conditions like drought and high salinity. Abiotic stress conditions lead to generation of harmful reactive oxygen species (ROS) which also acts as signaling molecules for triggering stress responses (Choudhury et al. 2013). *Arabidopsis JUB1* is induced by many abiotic stress conditions like high salinity, stress induced by methyl viologen, hydrogen peroxide (H_2O_2), ozone, *Alternaria alternata* fungal toxin and other conditions leading to generation of H_2O_2, a type of ROS (Wu et al. 2012). Induction of *MusaNAC042* by high salinity indicates that *Arabidopsis NAC042/JUB1* and *MusaNAC042* may be sharing similar function of conferring abiotic stress tolerance.

The data obtained from Q-PCR experiments are generally represented after $2^{-\Delta\Delta Ct}$, $2^{-\Delta Ct}$, or 2^{-Ct} transformation (Schmittgen and Livak 2008; Livak and Schmittgen 2001). The comparative Ct method ($2^{-\Delta\Delta Ct}$) gives the "fold change" in expression of a particular gene while the $2^{-\Delta Ct}$ transform the QPCR readings into "normalized expression" relative to expression of an internal control (Schmittgen and Livak 2008). We have calculated fold change in the expression of genes analyzed in this study by dividing the normalized expression ($2^{-\Delta Ct}$) under treatment and control conditions. Expression of *Musa EF1α* was monitored for normalization of Ct values. One of the earlier reports has evaluated different reference genes in banana suggesting that banana *EF1α* is one the most optimal reference genes among others for quantitative RT-PCR experiments as it shows

minimum variation in different tissues including leaves of greenhouse plants (Podevin et al. 2012). The suitability of *EF1α* as a reference gene has been well documented in many other plant systems supporting the utilization of *EF1α* as a reference gene in banana (Ray and Johnson 2014; Silveira et al. 2009; Galeano et al. 2014; Reid et al. 2006).

Functional analysis of *MusaNAC042* was carried out by generating transgenic banana lines overexpressing *MusaNAC042*. Four transgenic lines were generated by transformation of embryogenic cells of banana cultivar *Rasthali* and confirmed by PCR and Southern blot. Genomic DNA was restricted with *Nco*I for Southern blot analysis as *Nco*I digest the T-DNA of *pCAMBIA1301-MusaNAC042* only once, resulting in a direct interrelationship between number of bands observed during autoradiography and copies of T-DNA transferred to genome of transgenic lines. Moreover, difference in band size in autoradiograph suggested that each transgenic line has originated by an independent transformation event. Data regarding the overexpression of *MusaNAC042* could not be correlated with copies of T-DNA in transgenic lines indicating that different copies of T-DNA might have been incorporated into regions of genomic DNA with differences in transcriptional activities. Some of NAC domain proteins are characterized as positive regulators of senescence. Notable among these are *ORS1* (Balazadeh et al. 2011), *AtNAP* (Guo and Gan 2006) and *ANAC092* (*ORE1*) (Balazadeh et al. 2010a, 2010b). However, many of the NAC domain factors are known to repress the senescence process. *Arabidopsis NAC* genes *ANAC019*, *ANAC055* and *ANAC072* overexpression resulted in superior drought tolerance (Fujita et al. 2004;Tran et al. 2004). *Arabidopsis JUB1* delayed senescence in overexpression lines while *jub1-1* mutant plants showed precocious senescence indicating that *JUB1*/*ANAC042* can be an important candidate gene for stress responses (Wu et al. 2012). In our studies, transgenic banana plants overexpressing *MusaNAC042* were significantly more tolerant to drought and high salinity than

control, which is further supported by the induction pattern of *MusaNAC042* in response to high salinity and drought. Overexpression of some of the transcription factors like rice *NAC6* and *DREB1,* resulted in growth retardation (Ito et al. 2006; Nakashima et al. 2007) which can lead to reduction of productivity. However, such retardation was not observed in case of *MusaNAC042* indicating that *MusaNAC042* can be useful for increasing salinity and drought tolerance of banana. Abiotic stress conditions generally lead to proline accumulation (Surender Reddy et al. 2015) which is known to act as molecular chaperon preventing protein denaturation along with maintaining osmotic balance (Székely et al. 2007). Similarly in our study, transgenic banana lines overexpressing *MusaNAC042* contained higher proline level after salinity and drought stress indicating that elevated proline can be one of the possibilities for enhanced stress tolerance of transgenic lines. Transgenic lines retained higher relative water content than control plants at the same level of drought stress suggesting better drought tolerance in transgenic lines. Relative water content has been commonly employed to measure the water content in plants and is a useful parameter to indicate the turgidity of plants under water scarcity conditions (Richard and Gail 1974). Possible molecular mechanisms by which *MusaNAC042* overexpression confers elevated salinity and drought tolerance was studied by probing expression of multiple stress related genes. Elevated expression levels were detected for at least 9 *CBF*/*DREB* (*C-repeat-binding* /*dehydration-responsive element-binding*), 5 *WRKY* and 3 *LEA* (*Late Embryogenesis Abundant*) coding genes. *CBF*/*DREB* transcription factors are important class of transcription factors involved in stress responses and regulate expression of multiple stress-responsive genes (Akhtar et al. 2012). *WRKY* transcription factors are one of the major regulators of disease responses, abiotic stress responses, development and senescence in plants (Banerjee and Roychoudhury 2015). LEA proteins accumulate during seed maturation for acquisition of desiccation tolerance in embryo and during

dehydration in vegetative organs suggesting their protective functions during abiotic stress conditions mainly drought stress (Rorat 2006). Thus *MusaNAC042* regulates high-salinity and drought stress responses in banana and its over expression improves the drought and high-salinity stress tolerance by increasing proline content and reducing MDA content.

Table 3.1.1 Primer sequences used for cloning and other molecular work in the study of *MusaNAC042*

		Forward Primer (5' - 3')	**Reverse Primer (5' -3')**
1	Primers for cloning *MusaNAC042* in *pCAMBIA1301*	AATCATGAAGGAGATGGTGAACAAGAGG	AACACGTGCTATGCATGATACATCCCTCTAACT
2	Primers for cloning *MusaNAC042* coding sequence in *pCAMBIA1302*	aaggatccgAAGGAGATGGTGAACAAGAGGG	aaactagtTGCATGATACATCCCTCTAACTG
3	Hygromycin phosphotransferase (*hpt)* gene	GTCCTGCGGGTAAATAGCTG	ATTTGTGTACGCCCGACAGT
4.	*Musa EF1α* real time PCR primers	CCGATTGTGCTGTCCTCATT	TTGGCACGAAAGGAATCTTCT
5.	Primers for cloning of promoter region of *MusaNAC042* upstream of *GUS* in *pBI121*	ttaagcttTGCCATGGTGCCTGTTGT	tttctagaCACTATCTTCTCTCCTCCCTTCTC

Table 3.1.2 LEA genes and their real time primer sequences used in the study of *MusaNAC042*

S.No	**NCBI accession/ Banana genome locus identifier**	**Forward Primer (5' - 3')**	**Reverse Primer (5' -3')**
1	GSMUA_Achr10T21680_001	GCAATAGAGGCAGCCAAGAG	ACGCCAATGGTTTCCAGTAG
2	GSMUA_Achr4T19200_001	GGAGAAGGTGAAGGACATGG	GACTGGTAAGGGGAACACGA
3	GSMUA_Achr4T14830_001	TGTCTTCATGGACCGACAAG	CAGCTTGGTCTTGTCCTCGT
4	GSMUA_Achr5T16860_001	CCAAGGACAAGGGATCTGAG	AGCTCTTCACAGCATGAGCA
5	GSMUA_Achr7T15580_001	CGAGACTGTCGTCCCTGGT	GCTCTCCTCCCGACTCCTC
6	GSMUA_Achr10T04300_001	CAATAGCAAACCTCCACCTGA	CCCTTCCCCTGATCATATCC
7	GSMUA_Achr3T30940_001	GATCGCAACGACGACAAAAC	CAACGTTGTTGGTTAAGCCTTG
8	GSMUA_Achr1T17700_001	CAAGTTCTTTGGGCTGTCG	CTCATCTTGCTGGTGCAGTC
9	GSMUA_Achr6T20420_001	TGGTTAACCGGAGGGGATA	TTGCTTGTGGGAGAGAGTCA
10	GSMUA_Achr5T09440_001	CATCTGCGAGATCTCCTACACC	GAGAGGGGGATGGTGAAGTC
11	GSMUA_Achr11T18110_00	CTCTCTCTCGGGTGCTGCT	AGTTGGCGGGCCTGTAGTA

	1		
12	GSMUA_Achr1T16570_001	AGTCCGGCATGGACAAGAC	CCCTCGACAGGGTAGAATCC
13	GSMUA_Achr5T02900_001	AGACTGACCTCCCCGGTCAA	CCCAGTCCACCTGCGAGAG
14	GSMUA_Achr7T27190_001	CAACGACGGCTGGTAACAA	TCCCTCGTTGCTTCTTTGTC
15	GSMUA_Achr3T25520_001	ACAGGGGTAAATACCGTGCTG	GGACGCATCTCCAGGTTTG
16	GSMUA_Achr1T13650_001	GATCCGGCTACCGGGTACTA	TGACAGCATCGTTCGTTCAT
17	GSMUA_Achr9T16350_001	CCATCTGCCAGCTCTCCTAC	TAGAGAGCGGGATGGTGAAG
18	GSMUA_Achr7T15490_001	GACGTTCCTGGAAGTGATTT	ACCAACACATATAAGGCGTAAA
19	GSMUA_Achr9T09720_001	TCCCAGACTGGTTGGAAATG	CGCCAGATAGGGTAAACCAA
20	GSMUA_Achr9T26430_001	TTCCCTGACTGGTTGGAGAT	CGCCAGATAGGGTAAACCAA
21	GSMUA_Achr11T16760_001	TGGAGAAGATGCAGGAGGAT	TTTCTCCTCGGTCTTGTGGT
22	GSMUA_Achr6T34630_001	AGATGCCCGGAGCACTAAT	ATGCAGGAATAGGTGCCAGA
23	GSMUA_Achr11T05130_001	CGCAAAAGGCTAATCTGGAG	TTGTAGCGAGCGGCAATAGT
24	GSMUA_Achr10T12880_001	GGGCTATCACTTGCTCACATT	CAAGCCTGCAATTACCATCA
25	GSMUA_Achr4T11310_001	CATAACCGGCATGCTCCAC	ACCTTCTCCACGAATCCCTTC
26	GSMUA_Achr4T32740_001	GGATTCTCTTGCATGCCTTC	CCTGCCGAATAAGGGTCATA
27	GSMUA_Achr1T19670_001	GCCGAGATCAACAAGCAAGA	GAGCCGACGTAGGGGTTGT
28	GSMUA_Achr5T01540_001	CGGGGGTGGAAGAAACTATT	AGATAGTGGCCCAACAGCAT
29	GSMUA_Achr8T30100_001	CTGGAGCCTAAAGCTACAAAGG	TGTTGAGCTTCTTCAGCCTTC
30	GSMUA_Achr9T26840_001	GGAAAATCCAACAGGAGGAAC	GCAAGTGAATCACCTTCTCGT

Table 3.1.3 *WRKY* genes and their real time PCR primers sequences used in the study of *MusaNAC042*

S.No	NCBI accession/ Banana genome locus identifier	Forward Primer (5' - 3')	Reverse Primer (5' -3')
1	GSMUA_Achr7G00610_001	AGCGGGTCTAATGGTTCAAA	GATGCTGATTGGTTGTTTCTGA
2	GSMUA_AchrUn_randomG17930_001	CCAAGTGCAACGTGAAGAAG	TTTTCGAGAGTAGGGACGATG
3	GSMUA_Achr7G26450_001	AATCCTAACCTAGGCTCTCTGGA	TCTGAGAAGAAGCTGCCATGT
4	GSMUA_Achr3G15690_001	CAGAACCGCCTACCTCCTC	CCGAGGTAGTGGAAGCAGAA
5	GSMUA_Achr7G15930_001	GTTCATGTCGTCCGTCACC	TCACCCGTGATTTCCTTCTC
6	GSMUA_Achr10G08720_001	CACTCCAACTCCTCCCAAGA	AGCTGGCAACACTCCTATCC
7	GSMUA_Achr6G02330_001	AGCAGAAGCAGCAGCAAATC	TCCAAAGTTGCTCCAGTGTG
8	GSMUA_Achr11G17620_001	TGACGCAGTCCGTGAACC	GGACTTGGGAAGCTGAGGAG
9	GSMUA_Achr3G29850_001	ACCTTGGTGTTGGCATCAG	GCGGCCGAATAGTACATATCA
10	GSMUA_Achr8G20660_001	GCTGGCCATGGAAGAGTTAG	GATCAGATGATGCTGGTCCAC
11	GSMUA_Achr7G14140_001	CCTTCTCCTTCCCTTCGACT	AATGATCTCGGTGAGGTCAGA
12	GSMUA_Achr9G06750_0	GTGGTGATGGCGGTAAGAAC	CCTTTCTTCCTCGGCTTGTT

	01		
13	GSMUA_Achr4G02800_001	CACACACCTAGTGGCACCTC	TGCCGTGATCAAAAGAGAATAGT
14	GSMUA_Achr6G27090_001	GTAGTCGCAGGAGCAAGACC	TCCCTCTCCCTCTTCTCTCC
15	GSMUA_Achr7G24840_001	GAACCACCACCACAAGCAC	GCGAACAAAGAGTCCTCCTC
16	GSMUA_Achr9G21560_001	CCTTCACTTCCTCCAACAGC	TTCATCGAGTCGGACAACTG
17	GSMUA_Achr8G10740_001	TGAACACGAAGGAGTTCCAA	ACTCCTCCTTTCTTATCTCTGACG
18	GSMUA_Achr11G10010_001	CTCCAACACAGCACCCATC	GGACCCAGTCCAGCCATAG
19	GSMUA_Achr5G04870_001	TTGACCCTCGACTTCACGA	GAGGAGAGCGGAGGCTTG
20	GSMUA_Achr10G06050_001	TGGCTGATCTGATATCCAAGG	GCTTCGAAACTTTGGGCTTA
21	GSMUA_Achr3G27880_001	GGTGTCGTCGGCCCATAAG	TTTTTCCCGGTCCTTCCTGT
22	GSMUA_Achr4G14990_001	CAACAGTCAGAGGGAGCACA	GCCGTGAATGAAGGAAGGA
23	GSMUA_Achr7G25400_001	TCATCAGAACCCTGCAACAG	AAGGAAGGAACGATGTCCTG
24	GSMUA_Achr6G05710_001	CCCGATGAGCACTTTGTGTA	CCACTGAGTCGAGCATGAAG
25	GSMUA_Achr8G19810_001	TCCTCTCCTGCCTCTGTCTC	GCCCATGAAGAGCAGGTC
26	GSMUA_Achr9G05460_001	CGAGGAGGAGGAAGAAGAGG	ATTGCTGTTAGCCAGCCAAG
27	GSMUA_Achr6G13630_001	ACCGACGGACTGATGAACTC	GCATCACCAAGTGGGTCTTT
28	GSMUA_Achr4G07230_001	AGTAATTTGCCCTGTGGACTG	TCCTAGGCTCAGTCAATGTCC
29	GSMUA_Achr10G23420_001	CCATGGAGCACCTCATCAGT	TGAAATCCACCGTCAACGTC
30	GSMUA_Achr4G15540_001	CTGATCACCGAGGAGGAGTC	GAACAGAGAGTCGTCGTCACC
31	GSMUA_Achr3G05670_001	CTGATCTCACCGTCTCCAAGTT	CGATCTTGCCGTTGGAGAT
32	GSMUA_Achr7G19340_001	TGAGCACAACCATGGACATC	TTAGTTTTGAGCCGGTGGAT
33	GSMUA_Achr2G15200_001	CGTTCCCTGGTATGCCTGTA	TTATTGGCGCTGCTCTTGTT
34	GSMUA_Achr8G14610_001	GGTCGGATGCTCTTTTCGTA	AACATGTGTGACGGATCCAA
35	GSMUA_Achr4G03660_001	AGACCCAAGACCGAGCTTTAG	TCATGCCACTGTTGCTTCTC
36	GSMUA_AchrUn_randomG17570_001	CCAGATTCCGTCCACCTTT	GTTGGCAGTGTCGGATGAC
37	GSMUA_Achr3G09940_001	AGCCTCTGTCCACCAGAAAG	GGAGTCTGCACCACCACTCT
38	GSMUA_Achr5G16460_001	ATTTAGAAGCCGAGCTGAACC	TCGTGTTCTTCGGGTCGAT
39	GSMUA_Achr1G17160_001	CCTCGTCAGGTTCACAGGTC	GGTCGTCGTTCTTCCTTCTCT

40	GSMUA_Achr11G08290_001	CCAATCCTCTCGTCCTGAAG	TTGGAGGAGGAGATGTCTCAG
41	GSMUA_Achr4G01600_001	CAAACCTTGCCATAGCTGGA	AGCTGAGGTCCTAGAGGAAGC
42	GSMUA_Achr1G04770_001	TCTCCTTCTACGCCGAGAGA	ACTGAAGAGCTCGGTGATGC
43	GSMUA_Achr6G22160_001	TCATCAAAGCCCCGCAACT	CTCGCTGTACTGCGTGAAGG
44	GSMUA_Achr5G16750_001	CAACTTCCTAGCGAGGACCA	GATTGGGTTTGGAGCGTCT
45	GSMUA_Achr4G14270_001	TCAACGTCCGACACCAAATA	TCAACCTCGCTTGTTGTCTG
46	GSMUA_Achr10G22020_001	AGTTACCCTCCCCTGAAGGA	CCACCAAGAGCAAGTCGTC
47	GSMUA_Achr9G22950_001	GGAGGATGGACGACCAGAT	CTCTACCGGGGGTACGAAG
48	GSMUA_Achr10G15290_001	CACGTTGGATCTCACCCAAA	AAGTTCGGGTCGGCAGTTAT
49	GSMUA_Achr4G20600_001	TGACGGTTTCCAAGTTCAGG	CCGAGACTCAGGGTCGTTG
50	GSMUA_Achr6G31840_001	CAGCACTCGCCAGAAGTTT	ATGTCTCCCACCGTCAACA
51	GSMUA_Achr6G16760_001	AAATGGCATGACTGCTGCTAC	ACCTCGCTTGTTGTTTGCAC

Table 3.1.4 *DREB* genes and their real time primer sequences used in the study of *MusaNAC042*

S.No.	NCBI accession/ Banana genome locus identifier	Forward Primer (5' - 3')	Reverse Primer (5' -3')
1	FF561432	CGCCCACAAGAAAGTCTCAA	ACAAAGGAGTTGCTTCCCTGA
2	ES435892	CAGTTCACAGAAACAGGGGAAG	TCCCATGATGGATACTTGTGC
3	FF560746	GGCTTCTTGGAAGAGAAGGTG	TGTCTGAGGGTTATCGTGTCC
4	FF561371	TCAAATGTAGCCAAAGCACCT	GAAAGAGTTGCTTCCCTGGTC
5	FL662828	ACGGGTCGAAGAAGAAGAAGA	ACTGCAGGTCCATCAGATTCA
6	ES432514	CCAGTAGTCAGAGCAGCACCT	GACTTCGCGTCGACAAAGA
7	ES437095	GAGCGATCATCTCTGACTTCG	TTCCTCGGGTCTCTTATCTCC
8	ES435265	GGCCGCGGAGATAAGAGA	GCGTAGTCGCTGGGGTTC
9	FF561178	ATCAGGAGATTTTAGTGGATCTGC	ATAGGCTCTTGCAGCCTCTTC
10	ES433424	CAGGGATAAAACCAGCTCCAC	CCCAGCAGCACTTTCCTTAGT
11	ES431610	GGCTAAGACCAACTTCCCAATC	AAGCAGCGCCTAGGAACAAG
12	FF560353	GGAGGTGTGAGGAGGGATTC	CCACCCTCATCTTCTAATTCCAC
13	ES433029	GAGGAGAAGGAAGGAGATCAGC	TGAGATCTGGATGGCTTGTTTC
14	ES436780	GACACCGCGCTCTTCTACC	GAACCGTTTCTGATCTCCTTCC

15	FF559848	CCACAAGAGATGCTCAATGTCA	TTGGGGGTTTTAACCTCACTTT
16	FL659594	AGGCCCCTTCCTCCTGAT	ATCAGCGCCCTCCAGTTTA
17	ES432749	AAGCAGCACGGTGGAGTC	GTCAGACGGTGGTGGTTCAT
18	ES436343	CTCTGATGACCCGGTGGT	CTGCTGATTGAGATCAGGGTTT
19	GSMUA_Achr10T04860_001	ATCATCAAACGCAGCAAACA	GTTGGGTCACCACCAGTTTT
20	GSMUA_Achr1T02070_001	CCTTTCGGGAAGACTGACAC	TCGGTGGACTAGAGGAATGC
21	GSMUA_Achr9T28840_001	GGACCAACTCGTCGACATCT	AGCCTCGATCGGGAATAAGT
22	GSMUA_Achr1T15350_001	ACCTGGGGAGGAGGAGGAG	CACCGGGGAAGTTGAGGAC
23	GSMUA_Achr11T02840_001	GCTGAGGCAAGCAAAAACAG	GTGCAATGGAGCATCATCAG
24	GSMUA_Achr8T09110_001	TGGTAACATGGCCAGCAGTA	GCAGCCATGGTGTTAGACCT
25	GSMUA_AchrUn_randomT23040_001	CTGCTCACTCTCCACCAACA	CCCGTAACCATTACCACCAA
26	GSMUA_Achr2T05880_001	GCAACACCCAGACCTTCTTC	TACACGTTCCTGCCACCATA
27	GSMUA_Achr8T14110_001	GACAACAACACCCGAAATGG	TAGAAGACGCGTCAGCATCC
28	GSMUA_Achr8T14110_001	GACAACAACACCCGAAATGG	TAGAAGACGCGTCAGCATCC
29	GSMUA_Achr11T03210_001	CTCAACTTCCCCGACGAGAT	CGGCATGTCGAAGATTTCAT
30	GSMUA_Achr4T26080_001	AACATTCCATCTGCCGTAGC	CCTGACATGCTGGTGAAGAA
31	GSMUA_Achr6T28760_001	TTCGGAAAGTCGGACACAG	CTTTGAGCAATTCTGCGTGT
32	GSMUA_AchrUn_randomT26940_001	GACGAGATCCTAGCGGAAGA	AACTGCTGTTGTTCGAGCTG
33	GSMUA_Achr6T28550_001	TTCCAGCCATCTATGATCCA	GTCGACTGCTGTGGCAGATA
34	GSMUA_Achr4T06170_001	CCTCAGTAGCACCGTGGAAT	CTGGTGGGAGGAGATTGAGA
35	GSMUA_Achr8T33590_001	CCCTAAGGCCAAGACCAACT	ACACCGGCGCTCTTAAATCT
36	GSMUA_Achr3T15150_001	AGGCAACTGATGCAGGAAAA	TTCCCTAACCTGTCCGTGTC
37	GSMUA_Achr11T15810_001	GTCAACTTCCCCAACGAGGT	CGCCTCAATCCCAATATTCC
38	GSMUA_AchrUn_randomT10890_001	AGCATGGGTGAATCGGTTAG	TGGCTTCACACCTATGTTGC
39	GSMUA_Achr11T22020_001	GAGCCGAAGGAGAAGTCGTA	CTGCTGCTCGTCTCACTCTC
40	GSMUA_Achr8T12230_001	TCATGGCTGGTCTAGCCTCT	CTATTTTGCCTCGAGTTGTGC
41	GSMUA_Achr3T08170_001	GCAAGAAGGCAAAGGTCAAC	CAAAACTCCAGAGCATCTCCA
42	GSMUA_Achr8T03550_001	AGTAGCCAGAGCAGCACCAT	GAAGAGTCGGAGTCGCTTTG
43	GSMUA_Achr2T19400_001	GTGGGTTAACAGGCTTGCAG	TAGTTAGCTGCTCCCCACCA
44	GSMUA_Achr5T05160_001	GCCGACTCCAGTTGACTCTC	AAAGGAATTGGACCCGAAGT
45	GSMUA_Achr4T28640_001	AAGTGTACAGGCCGTGCTTT	AGCGTATCTGAAGGGTCGTG
46	GSMUA_Achr2T20760_001	CTCGGAACCCAAGGACATC	CACAGAAAGGGATCCCACAA
47	GSMUA_Achr5T04670_001	ACGGGATTAGCATGTTGGAG	CCTCAGCTTGCCTCCTTTTA
48	GSMUA_Achr4T23620_001	CTCTTCGACACGATCGTCAG	GGGAAGGTTGAGGTCGAGAT
49	GSMUA_Achr5T09470_001	TCCAAGATCCAGGCCATCT	CGAGCACTTCCCAGATCAAT
50	GSMUA_Achr9T07890_001	TCGTGAACCTGGAATCATCA	ACATTGTTCCCTCCATTTGC
51	GSMUA_Achr2T05810_001	GCCAAGAAGTCAAAGGGACA	TATGGAGCACTCAGCACCTG
52	GSMUA_Achr4T12060_001	CGGAGATCAGGGACTCGAC	TGACCTCCTCCTCTTCCTGA
53	GSMUA_Achr5T25620_001	ACACCCACAGCTTCTTCCAG	ATGCAGTTTGACCTCCATGC
54	HN253812	GGTTTGATGGATCTGCTGCTA	GATGATGCAGTTGCTGCTGT
55	HN240556	CGGCCTTCGACTACACCTT	AATCGCCAAACTCCACCTC
56	HN248835	ATTACCGAGGGGTGAGGAAG	TGCGGGAAGTTGGTCTTG

Section 3.2: Studies on *MusaSNAC67*, a stress associated *NAC* transcription factor from banana

Introduction

Senescence is a process wherein the leaves undergo programmed cell death involving chlorophyll degradation and tissue degeneration facilitating redistribution of nutrients to support reproduction and growth of other organs (Thomas et al. 2002; Quirino et al. 2000). Factors responsible for leaf senescence can be hormones, age, stress conditions such as high salinity, drought, biotic stress and darkness (Moore et al. 2003; Sakuraba et al. 2012; Jiang et al. 1993. Balazadeh et al. 2010a, 2010b; Bohnert et al. 1995). Many transcription factors (TFs) play important role in leaf senescence by modulating transcript level of other SAGs (senescence-associated genes) (Lohman et al. 1994; He and Gan 2002). In *Arabidopsis* nearly 40 transcription factors (senTFs) have been associated with the process of leaf senescence and important among them are members of *NAC* (*NAM, ATAF* and *CUC2* family (Balazadeh et al. 2008; Gregersen and Holm 2007). Recent years have seen elucidation of function of some of these *SenNACs* (Balazadeh et al. 2008; Balazadeh, et al. 2011; Kim et al. 2013). *ANAC029* (*AtNAP*) promotes senescence and its T-DNA insertion mutants showed delayed senescence phenotype (Guo and Gan 2006). *ANAC092*/*AtNAC2*/*ORE1* have been reported as a *senNAC* with roles in salinity stress, auxin signaling, lateral roots formation (He et al. 2005) and senescence (Kim et al. 2009). Further work has shown that *ore1* mutation slows down the senescence and *EIN2* (*ETHYLENE INSENSITIVE2*) increase expression of *ORE1*, thus promoting senescence by repressing *miRNA164*, which is a negative regulator of *ORE1* expression (Kim et al. 2009). Downstream

targets of *ORE1* identified after induction of *ORE1* indicated that approximately half of the up-regulated genes were associated with senescence (Balazadeh et al. 2010a). Expression of another *SenNAC*, *ANAC059*/*ORESARA1 SISTER1* (*ORS1*) (a paralog of *ORE1*/ *ANAC092*/*AtNAC2*) is responsive to H_2O_2 and age dependent senescence, and its induction increased expression of genes involved in salinity stress, H_2O_2 response and senescence (Balazadeh et al. 2011). *ors1-1* mutant and *ORS1* RNAi line showed delayed senescence while its overexpression reduced chlorophyll content and induced precocious senescence (Balazadeh et al. 2011). Expression of *AtNAC016*, another *senNAC* recently identified in *Arabidopsis* showed positive response to salt and oxidative stress conditions and *AtNAC016* overexpressing plants showed precocious senescence (Kim et al. 2013). *ANAC046* can bind to promoter of *NYC1*, *SGR1* (*STAY-GREEN*) *PAO* and *SGR2* and its overexpression reduced chlorophyll content causing an early-senescence phenotype (Oda-Yamamizo et al. 2016). *ANAC032*, another *senNAC* is a transcriptional activator which positively regulates stress and age-dependent senescence by up-regulating expression of *SAGs* such as *NYE1*, *SAG113* and *SAUR36*/*SAG201* (Mahmood et al. 2016).

Chlorophyll catabolic pathway has been identified and well characterized in the past. The conversion of chlorophyll-b to end products like nonfluorescent chlorophyll catabolite occur by the mode of multiple steps involving many enzymes like *7-hydroxymethyl chlorophyll-a-reductase* (*HACR*), *chlorophyll-b-reductase* (*NYC1*/*NOL*), *pheophytinase* (*PPH*) and *Pheophorbide-a-oxygenase* (*PAO*) (Masuda and Fujita 2008) (Fig. 3.2.1).

In this chapter, we have identified a *senNAC* from banana, *MusaSNAC67* and showed its positive regulation over genes of chlorophyll degradation pathway to promote senescence. We showed that *MusaSNAC67* expression was induced by stress conditions like drought, high-salinity and H_2O_2 application. 5' up-stream region of *MusaSNAC67* drives expression of *GUS* in

vascular elements and harbors stress associated cis-elements. *MusaSNAC67* alters the morphology of chloroplast resulting in loss of grana thylakoids and increase in number of plastoglobulins. Moreover, *MusaSNAC67* could retard mobility of promoter of chlorophyll catabolic pathway genes like *NYC*/*NOL*-like, *PAO*-like, *HCAR*-like as well as *ORS1*-like (a *SenNAC*). Our work indicated involvement of a stress-responsive transcription factor in leaf senescence suggesting a crosstalk between senescence and stress pathways mediated by such *senNAC*.

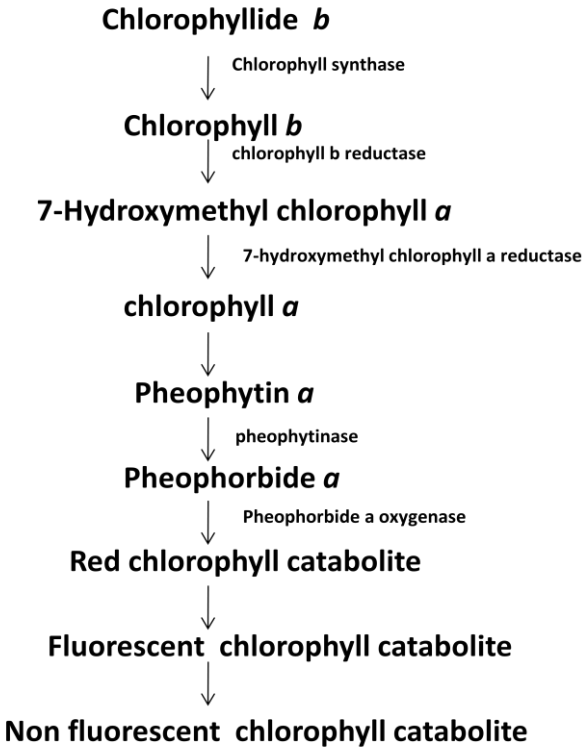


Fig. 3.2.1 Chlorophyll catabolic pathway in higher plants. Genes involved in chlorophyll degradation. Abbreviation of important enzymes are as follows: *HCAR*: 7-hydroxymethyl chlorophyll-a-reductase ; *NYC1*/*NOL*: chlorophyll-b-reductase; *PPH*: pheophytinase and *PAO*: Pheophorbide-a-oxygenase.

Results

MusaSNAC67 is a stress responsive *NAC* transcription factor

To investigate the expression of *MusaSNAC67* during stress-conditions, we performed quantitative RT-PCR analysis using cDNA isolated from leaves of two month old *Musa* cv *Karibale Monthan* subjected to various stress-conditions. Expression of *MusaSNAC67* got rapidly elevated during drought and remained higher till 48 hours post stress initiation (Fig. 3.2.2a). However, during high salinity stress (250 mM NaCl) transcript levels of *MusaSNAC67* showed elevation only post 24 hours after stress exposure suggesting that salinity-stress may not be the early trigger factor for *MusaSNAC67* induction (Fig. 3.2.2b). Exposure to cold resulted in maximum elevation of *MusaSNAC67* expression during 24 hours indicating probable involvement of *MusaSNAC67* in cold stress-responses in banana (Fig. 3.2.2c). We have also tested the expression of *MusaSNAC67* after application of peroxide (2mM H_2O_2) and salicylic acid (2mM) and *MusaSNAC67* was induced in both stress conditions (Fig. 3.2.2d, 3.2.2e). Sequence analysis showed MusaSNAC67 to be 293 amino acid long protein with presence of a conserved DNA-binding NAM domain at N-terminal and a relatively variable C-terminal. Moreover, a WVLCR-motif (Nuruzzaman et al. 2013) identified to be conserved among members of stress associated NAC transcription factors (SNAC) was also detected in MusaSNAC67 (Fig. 3.2.3). A Neighbor-joining -tree (stringency of 1000 bootstrap-replicates) constructed with MusaSNAC67 and other NAC proteins from different plant species suggested clustering of MusaSNAC67 in SNAC group (Fig. 3.2.4). It was named MusaSNAC67 because of close evolutionary relationship of MusaSNAC67 with BdNAC67 (XP_003557366.1) and TaNAC67 (AHB32901.1).

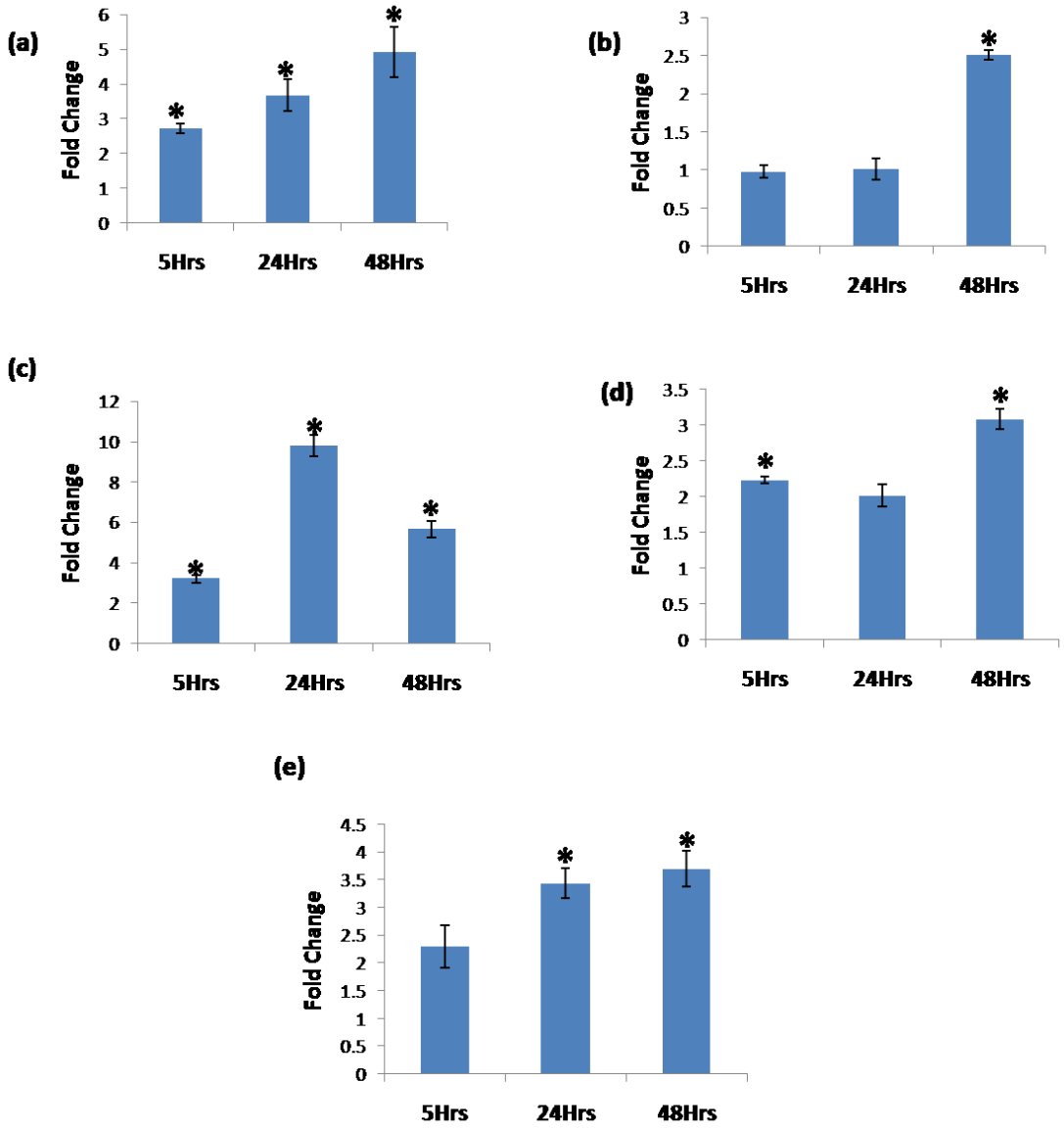


Fig. 3.2.2 Expression pattern of *MusaSNAC67* under abiotic stress conditions and chemical treatments. Two month old green house grown and treated plants were analyzed by quantitative RT-PCR. Expression of *MusaSNAC67* in response to (a) dehydration, (b) 250mM NaCl, (c) cold, (d) 2mM H_2O_2, (e) 2mM Salicylic acid. Values shown are mean of three replicates and is represented as mean±SD. Statistical significance at 5 % is shown by *.

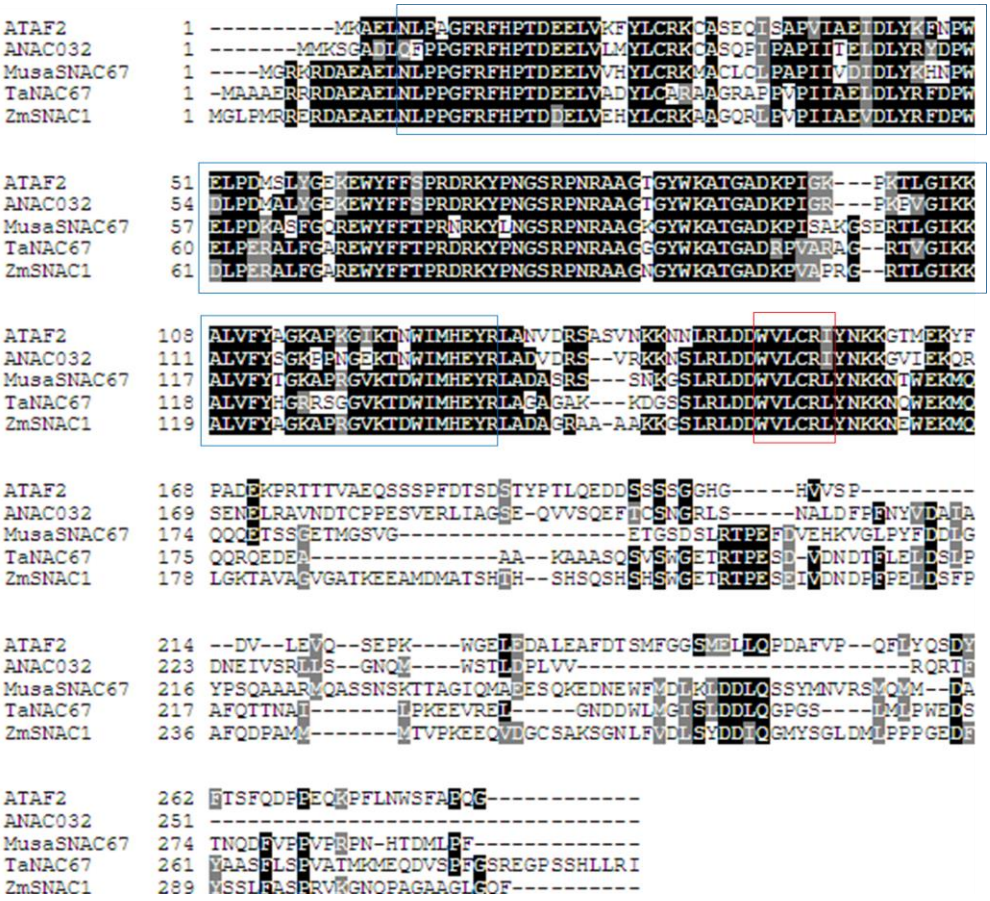


Fig. 3.2.3 Sequence analysis of MusaSNAC67. Protein sequences of characterized NAC domain proteins and MusaSNAC67 was aligned with clustal- omega software and identical residues are shaded in black. Length of the alignment at a position is indicated by the numbers at the left. Highly conserved NAC domain is boxed in blue and the 'WVLCRL' motif found in SNAC group is in red box.

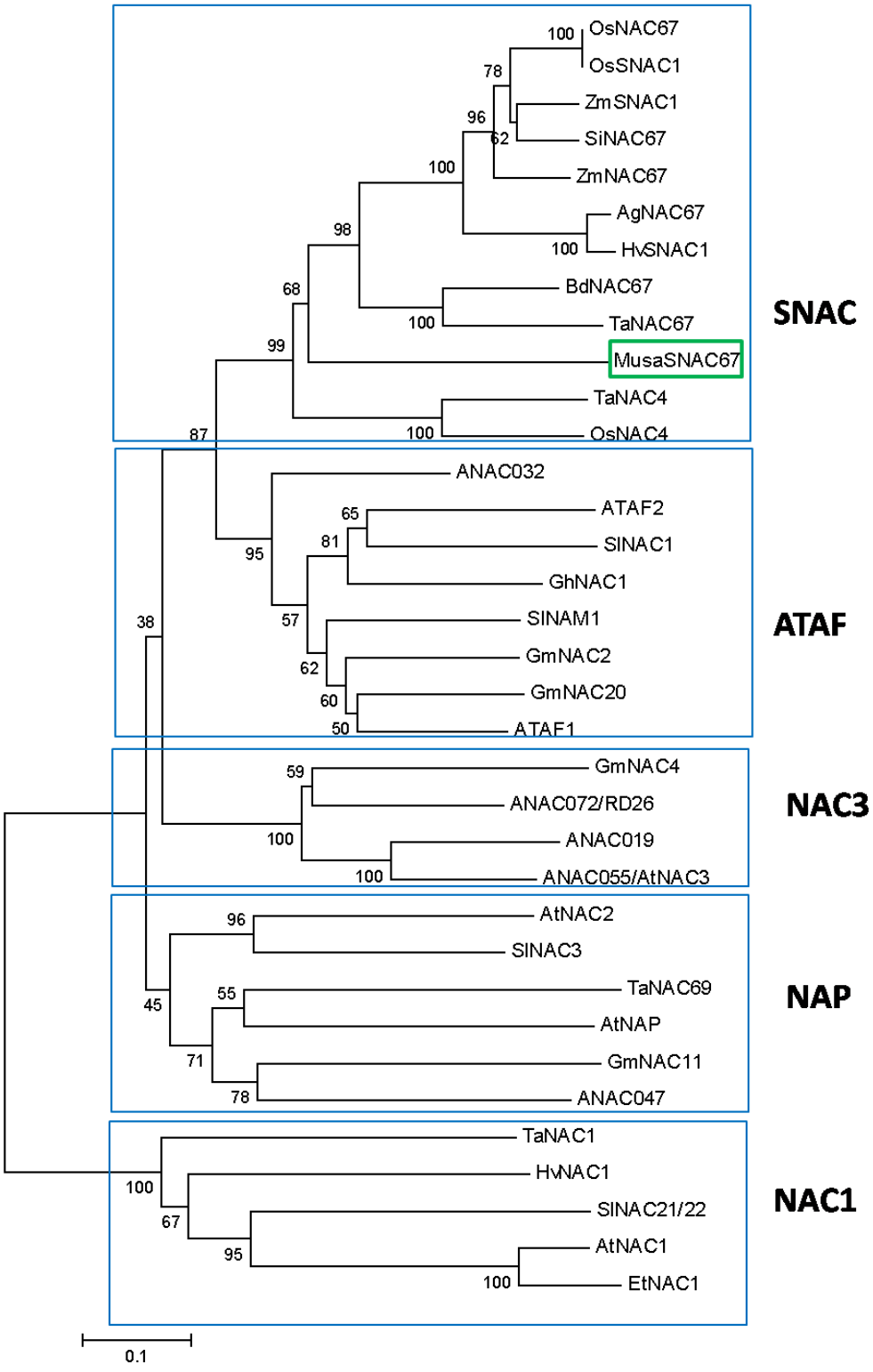


Fig. 3.2.4 Phylogenetic relation of MusaSNAC67 with NAC-proteins from diverse plants. Neighbor-joining-tree with stringency of 1000 bootstrap-replicates was built with MEGA6-

software. MusaSNAC67 is clustered in SNAC-subgroup and showed close evolutionary relationship with BdNAC67 and TaNAC67.

Drought and salinity activate transcription from *MusaSNAC67* promoter

To study tissue and stress-inducible nature of *MusaSNAC67* , we cloned approximately 1kb promoter region of *MusaSNAC67* up-stream of *GUS* in *pCAMBIA1301* and generated transgenic banana plants harboring the T-DNA with $P_{MusaSNAC67}$*::GUS* (Fig. 3.2.5a). The GUS staining of un-stressed transgenic banana indicated expression of GUS under the control of $P_{MusaSNAC67}$ was chiefly observed in vascular tissues of leaves (Fig. 3.2.5b), corm (Fig. 3.2.5f) and pseudostem (Fig. 3.2.5i). No expression of GUS under the control of $P_{MusaSNAC67}$ was observed in roots (Fig. 3.2.5e). To analyze whether salt and drought stress can initiate the transcription of *GUS* under the control of $P_{MusaSNAC67,}$ we stressed these transgenic banana lines with dehydration or 250 mM NaCl stress. It was observed that salt stress could enhance the GUS staining observed in leaves (Fig. 3.2.5d), corm (Fig. 3.2.5h) and pseudostem (Fig. 3.2.5k) suggesting that salinity stress could control the expression of *MusaSNAC67* at transcriptional level. Similar to salinity stress, drought could also control expression of *MusaSNAC67* at transcriptional level as it has elevated the GUS staining in leaves (Fig. 3.2.5c), corm (Fig. 3.2.5g), and pseudostem (Fig. 3.2.5j). Furthermore, under salinity and dehydration stress, expression of GUS under the control of $P_{MusaSNAC67,}$ was also visible in guard cells of pseudostem but not in leaves, indicating differential regulation of $P_{MusaSNAC67}$ in different organs of banana (shown by arrow in Fig. 3.2.5j and 3.2.5k). This differential regulation of $P_{MusaSNAC67}$ was also observed in corm. In control condition, expression of GUS in corm was mainly observed in vascular tissue while under drought and salinity stress it spreads to central cylinder and cortex region (Fig. 3.2.5g, 3.2.5h).

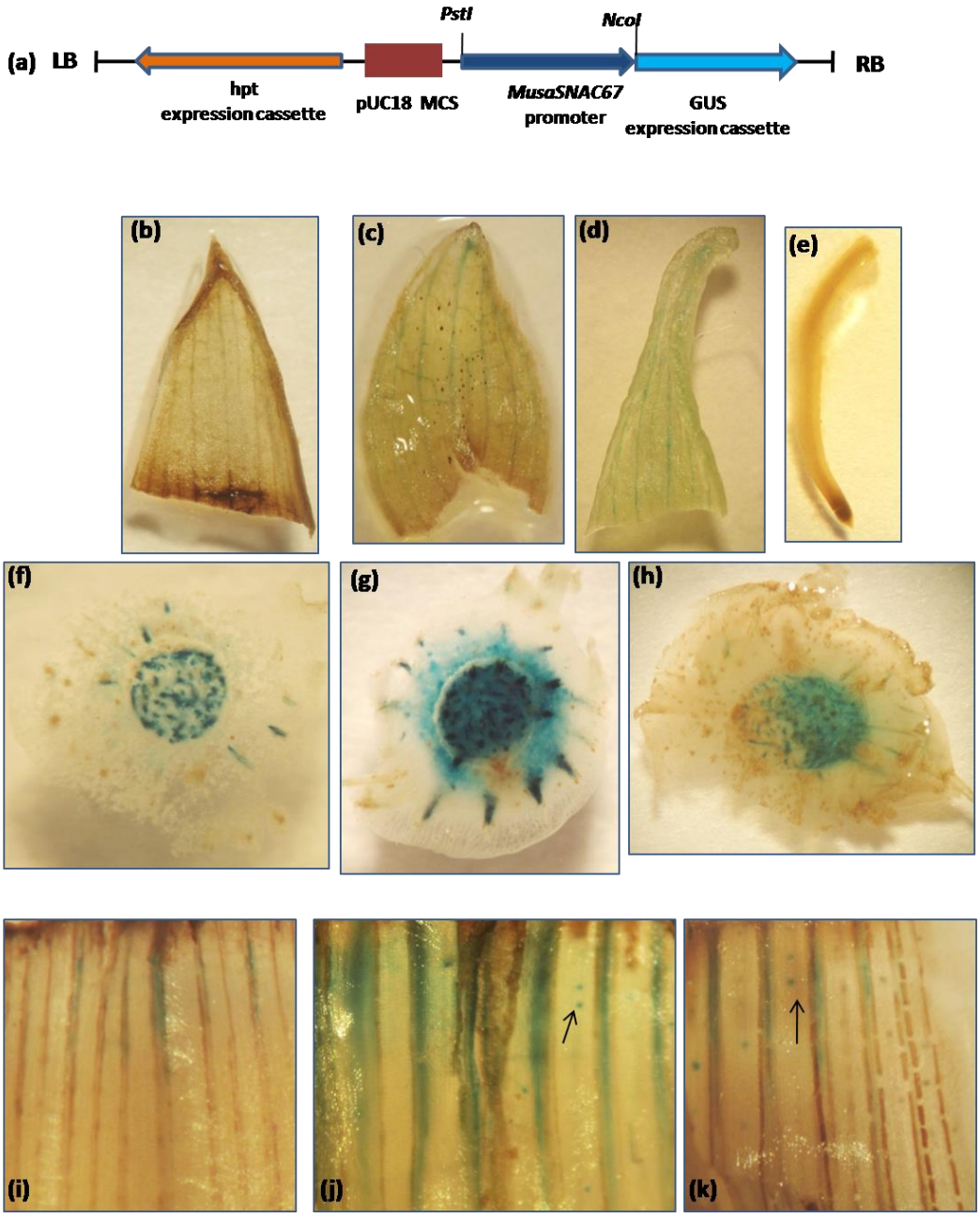


Fig. 3.2.5 Tissue specific and stress inducible activity of *MusaSNAC67* promoter. (a) Diagrammatic representation of the T-DNA with $P_{MusaSNAC67}$*::GUS* used for generation of transgenic banana plants for analysis of *MusaSNAC67* promoter activity. Expression of GUS under the control of $P_{MusaSNAC67}$ in control conditions (b,f,i) or either under dehydration stress (c,g,j) or high salinity stress (d,h,k). The tissues observed were leaves (b,c,d), corm (f,g,h) and

pseudostem (i,j,k). No expression of GUS driven by $P_{MusaSNAC67}$ was observed in roots (e). The GUS staining was carried out after exposure of transgenic banana plants to dehydration for 24hours and 250mM NaCl for 48hours in separate experiments.

$P_{MusaSNAC67}$ harbors stress related cis-elements

Analysis of 5' up-stream region of *MusaSNAC67* indicated presence of many stress related cis-elements (boxed in green) along with binding site for vessel-related NAC transcription factors (Fig. 3.2.6a). We have detected two *SNBE/TERE*-like sites (boxed in blue color) in the $P_{MusaSNAC67}$ indicating that the $P_{MusaSNAC67}$ might be driven by VNDs in banana in vascular tissues.

```
-1126 ACATAGGCAG CAATTCTTGG CCTAATGTCC TAATAATTTT TTTAAAAAAA
-1076 ATTATTACAT TAAATGATGT ATATGATGTT CACACGTGAA TTCGAACTTA
-1026 CGTTTACATA TGGGACACGA ACGCTTATCA CTTGTATCAT ACAACAATAC
 -976 ACTAATCAAC CCATGTCTCA ACAAAAAACC AGACATCCTA ATAATTAACA
 -926 CATGTGCATA GTCAATAGAT CAGGACTGTA GCGTCTAATC TCAATATAAT
 -876 GCCGACATGG AATCTAAGAA GAAGAGAAAA CATAGGTATG GAATGACTTA
 -826 TTGAGGAGTT GAATTACTAA GACTAGTAGG ATGGAAGATT AAATGTGTTT
 -776 CTTAAAAATG AGGAATCGTG CGAAACATTC GGTGGTTGAA GTGTCACATC
 -726 GGGATTAGTT GTTGGCTCAT TTCATCTCCT TAATTGGTCA ATTATGTTGA
 -676 ATGGCAGAAG CTAGACAAAT CTAATTTTCC ACATAAATTG GAGGGCTTGA
 -626 AAACTAATTG AGCCAACCTT GACGTAACAC ATAGCCACCG CTACATTAAT
 -576 CACCACATGA TCCATCCACT TTGGTGATCA CAAGGCAAGA TTAATGAATA
 -526 TACCAAAAAG ATTAAAAATT AATACCTAAT AAAAGGAAAG AATATATATC
 -476 AACAGAAAAA TATGATTTAA CATAGCAGAA TCATTTTTGC ATTCTACTAT
 -426 TAAAGAGATC TGTGTCGGTG GCTACGACTT TTCTAAGCCG CCGTAGTTAA
 -376 TCCAATCACA TCACTTCTTC GTTCCATTTC TACTGTTCGA ATAATTTGAT
 -326 CTAATTCATT TGTTTTTTTT TTTTTTGGGT TAGGTTTAAA CTGAGAGCCG
 -276 TCCCTCTTTC TTCCAACTTT AAATTAATTA TAATAAGAAA AATCATTATA
 -226 ATCAAAATAT TACTCTTCCA AGTAGGCCAA TGGTCGTCAG CCGCCTCCGA
 -176 AGCGCACACG AAATTTCCTG GGTCCCACGT GCGGCCGATG GGGTCCACTC
 -126 AAGAACCCAC ACAACTCCTC TCATGTCCCA CAGGTCCACA CACGTACATG
  -76 TTCTACCAAT CGTTACCGGT GCAACTCTAA TAAATACATA TAGTAATAAC
  -26 AGGGAGAGAG AGAGAGATCG GAAGGAGGAA GATAGAAAGC TTACTTTGGC
                                +1 TSS

   25 TGCCATCTGA GAGAGAGTGG AGAGCTTTCT TGACTTGTGA GCCAGCGAAG
   75 GAAACGAGGA TGGGGAGGAA AAGAGA
```

Fig. 3.2.6 Cis-elements in the promoter of *MusaSNAC67*. (a) Sequence analysis of 5' up-stream region of *MusaSNAC67* indicated presence of cis-elements for stress-responses and expression in

vascular-tissue. The position of different nucleotides with respect to transcription start-site (+1 TSS) is shown in the left. TSS is underlined and the TATA-box and start-codon are red-boxed. Different cis-elements for induction in abiotic stress-conditions are green-boxed. Important among them are TgGTCAAtt(-692) , LTRECOREATCOR15(-100 and -121,CCCAC), Gccgac(-876 and -413) and tgcCGACAtg(-877), ttgACGTAac(Binding site of VND4), tgggtcccACGTGc (-158), ACGTATERD1(-85 ACGTA), AGCCGCC (-391), CATGTg (-926), Gccgac(-413, -876), MYBATRD22(CACATg at -572,-928), ggtTAGGTtt(-299) and tgcCGACAtg (-877). Two putative *SNBE/ TERE*-like sites in 5' up-stream region of *MusaSNAC67* detected are boxed in blue-color.

Generation of transgenic banana plants overexpressing *MusaSNAC67*

To ascertain the roles of *MusaSNAC67* in stress response in banana, we generated transgenic banana lines overexpressing *MusaSNAC67* after transforming banana embryogenic cells with a T-DNA containing *MusaSNAC67* coding sequence under control of *pZmUbi* (maize polyubiquitin promoter) (Fig. 3.2.7a). Transformation of banana embryogenic cell suspension (ECS) resulted in the development of white embryos (Fig. 3.2.7b) with globular to torpedo shape (Fig. 3.2.7c). Germination of these embryos on shoot induction medium resulted in emergence of putatively transformed shoots (Fig. 3.2.7d). Further multiplication of shoots and rooting was carried out (Fig. 3.2.7e, 3.2.7f) on appropriate medium. However, rooted plants could not be hardened in the glass house or growth chamber condition suggesting inability of these lines to survive under these conditions. T-DNA insertion in these lines was confirmed by GUS staining and PCR amplification of *hpt-II* (*hygromycin phosphotransferase-II*) coding sequence from genomic DNA (Fig. 3.2.7g). Overexpression of *MusaSNAC67* in four transgenic lines (S1, S2, S3 and S4) quantified by quantitative RT-PCR analysis indicated elevated expression level of

MusaSNAC67 ranging from 52 fold in S1, 38 fold in S2, 27 fold in S3 and 46 fold in S4 over control value (Fig. 3.2.7h).

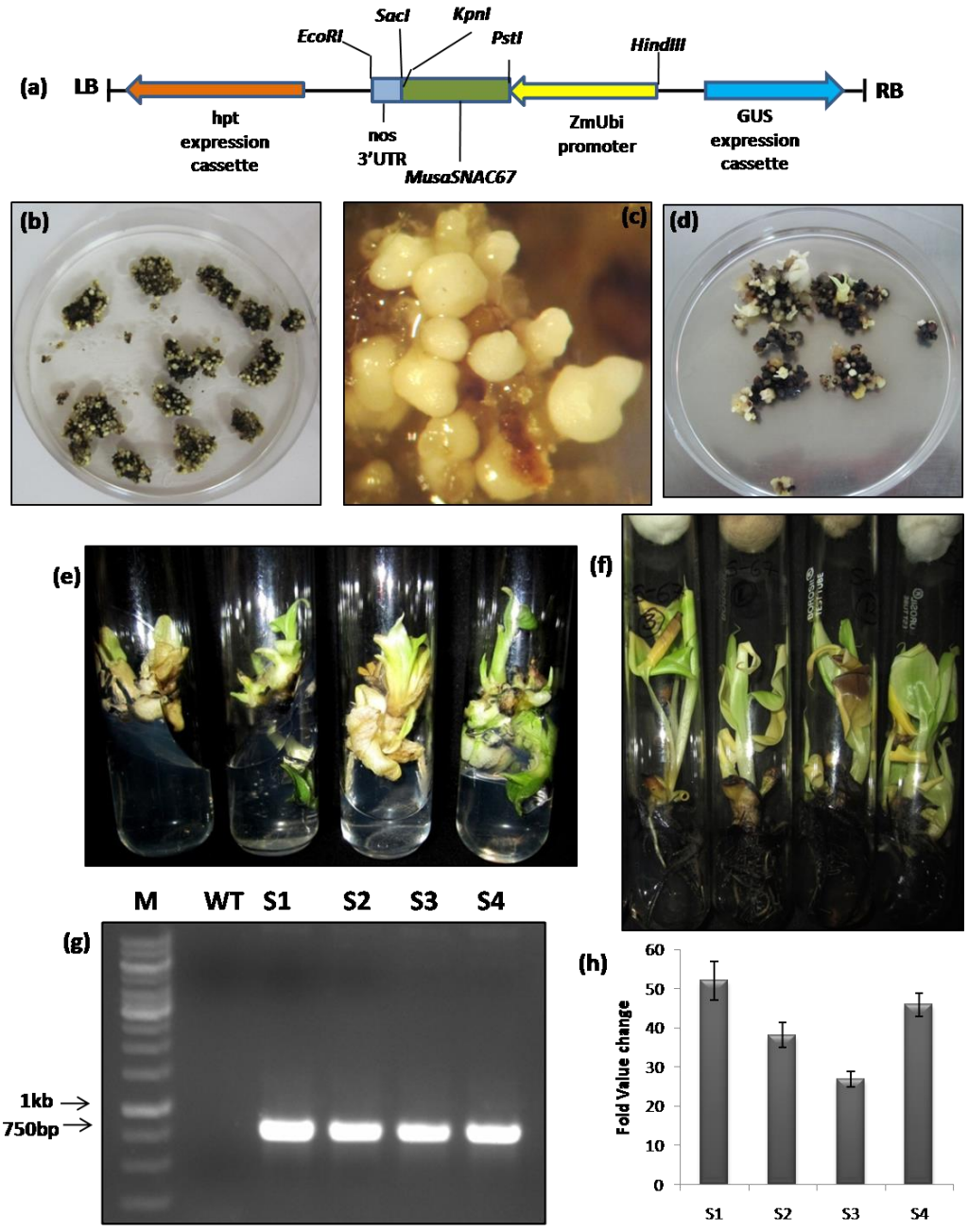


Fig. 3.2.7 Generation of transgenic banana lines overexpressing *MusaSNAC67*. (a) T-DNA region used for transformation of banana embryogenic cells. *MusaSNAC67* cloned under the control of *ZmUbi* promoter in binary vector *pCAMBIA1301* drives high level of expression in

transgenic lines. (b) Putatively transformed embryos appeared on selection medium after transformation. (c) Globular to torpedo shaped whitish embryos. (d) Picture of shoot emergence medium showing emergence of shoots on selection medium. (e) Multiplication of different transgenic shoots on shoot multiplication medium. (f) Elongation and rooting of transgenic lines on MS-medium supplemented with NAA (1mg/ml). (g) PCR from genomic DNA of putatively transformed shoots showing amplification of *hpt-II* coding sequence for confirmation of transgenic lines for integration of T-DNA. M: 1 kb DNA ladder, WT: wild type banana, S1-S4: different transgenic lines. Marker positions of 750bp and 1kb is shown by arrows. (h) Result of quantitative RT-PCR analysis showing fold-value change in expression of *MusaSNAC67* in different transgenic lines. Values shown are mean of three replicates and represented as mean±SD.

Overexpression of *MusaSNAC67* resulted in precocious senescence

Overexpression of *MusaSNAC67* resulted in transgenic banana with yellowish leaves showing de-greening phenotype even under control culture conditions (Fig. 3.2.8a). Transgenic shoots showed reduced growth than control plants. Relative to control plants, leaves of transgenic lines of equal age were smaller and showed highly senescent phenotype (Fig. 3.2.8b). As de-greening is associated with loss of chlorophyll content (a physiological senescence marker), we estimated chlorophyll content of transgenic lines under control conditions, which indicated remarkably reduced chlorophyll content in transgenic lines (Fig. 3.2.8c). As *MusaSNAC67* appeared to induce senescence by reducing chlorophyll content, we analyzed ultra-structure of chloroplast by transmission electron microscopy (TEM). Chloroplast of control plants appeared normal and oval-shaped, having abundance of grana thylakoids, presence of regular chloroplast membrane and smaller and few plastoglobuli (Fig. 3.2.9a). Transgenic lines chloroplast were abnormal in

shape, lacking a complete chloroplast membrane (breaks in membrane) and devoid of regular grana thylakoid arrangement along with appearance of multiple and large plastoglobuli structures and starch granules (Fig. 3.2.9b, 3.2.9c, 3.2.9d).

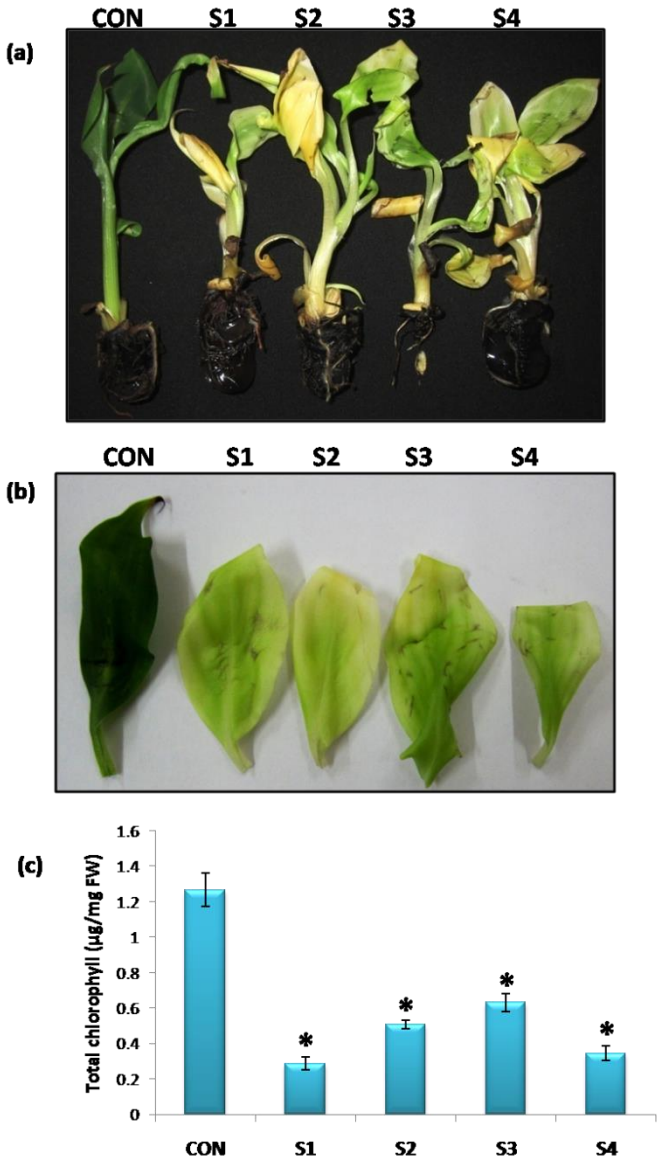


Fig. 3.2.8 Phenotype of transgenic lines overexpressing *MusaSNAC67*. (a) Shoots of transgenic lines showing the yellowing in leaves and pseudostem observed due to overexpression of *MusaSNAC67*. (b) Detached leaves of control and transgenic lines. Note the yellowing in transgenic leaves compared to control leaves. (c) Total chlorophyll content (µg/mg FW) of

transgenic lines and control plants. All the transgenic lines showed significantly reduced chlorophyll content than control plant. Values shown are mean of three replicates and represented as mean±SD. Statistical significance at 5 % is shown by * .

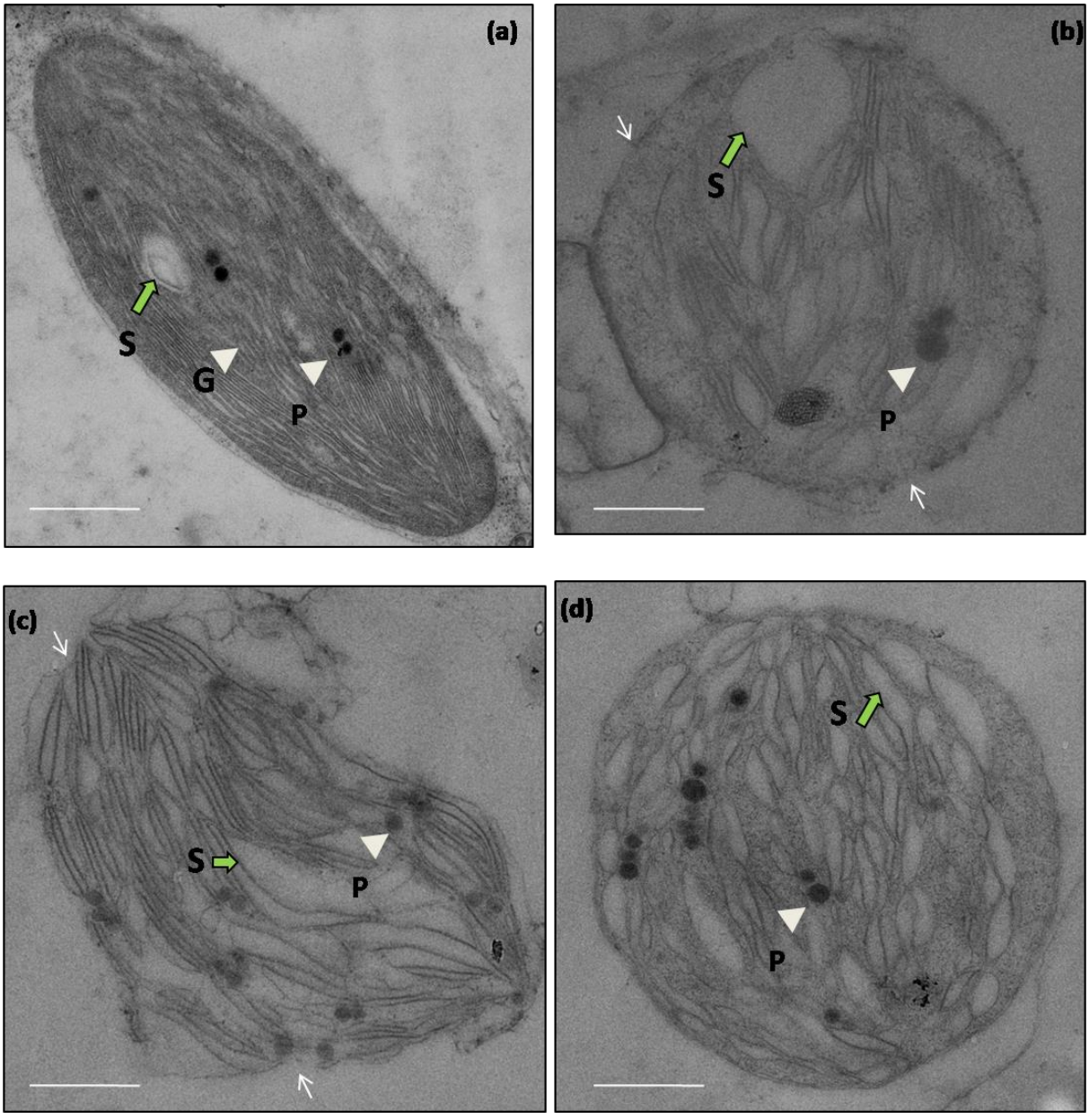


Fig. 3.2.9 Transmission electron microscopy analysis of chloroplast ultra-structure. Morphological changes in chloroplast and granum arrangement in transgenic lines S1 (b), S2 (c) and S3 (d). Note the absence of regular grana thylakoid arrangement and appearance of multiple

and large plastoglobuli and starch-granules (green-arrow) due to overexpression of *MusaSNAC67*. Also note the absence of regular chloroplast shape and a complete chloroplast membrane (gaps in membrane shown by white-arrows). G: granaum, P: plastoglobuli. White bar corresponds to 500nm.

MusaSNAC67 bind to core binding sequence (CGT[A/G]) of NAC proteins

Core binding site of NAC domain protein has been reported in earlier work (Olsen et al. 2005). To analyze whether MusaSNAC67 regulate promoter of its target genes by binding to this core site, we performed gel-shift analysis using repeated sequence of CGT[A/G] and purified MusaSNAC67. *MusaNAC67* was cloned in *pET28a*, transferred to *E.Coli BL21(DE3)* and later induced by 1mM IPTG. Inclusion bodies solubilized by N-lauryl sarcosine was purified on Ni-NTA column. Purification of MusaSNAC67 in elution fraction was analyzed on 12% SDS poly acrylamide gel electrophoresis (PAGE) along with other fractions (Fig. 3.2.10a). Further, the purified MusaSNAC67 protein was detected in western blot using antibody against polyhistidine (Fig. 3.2.10b). A 36bp double-stranded DNA containing two copies of both core binding sequence CGTA as well as CGTG was employed for gel shift assay with MusaSNAC67 protein. Binding of MusaSNAC67 protein was also checked with another 36bp double stranded DNA containing mutated core site (CGCA instead of CGTA and CGCG in place of CGTG). Our gel-shift analysis suggested that MusaSNAC67 has the ability to bind the core sites CGT[A/G] as it retard the mobility of this sequence in gel-shift assay. Retardation of CGT[A/G] increased with increasing amount of MusaSNAC67 protein suggesting that binding occur in dose dependent manner (Fig. 3.2.10c). Moreover, MusaSNAC67 protein failed to bind the mutated core sequence (CGC[A/G]) even at increasing protein concentration suggesting that binding of MusaSNAC67 protein to core CGT[A/G] occurs in a very specific manner (Fig. 3.2.10c).

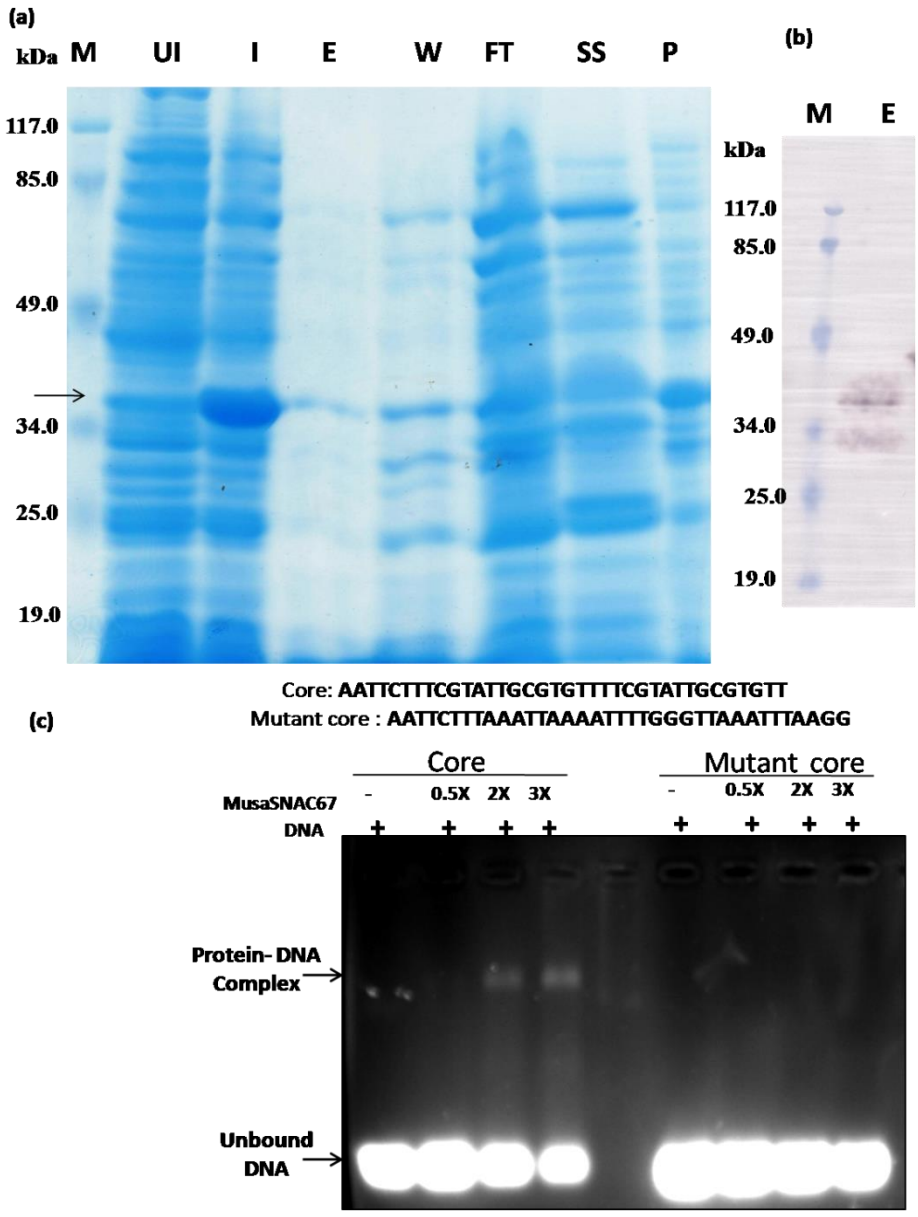


Fig. 3.2.10 Purification and DNA binding activity of MusaSNAC67. (a) Purification profile of MusaSNAC67 protein. *E.Coli BL21(DE3)* having *pET28a- MusaSNAC67* was induced by 1mM IPTG and the induced protein (Indicated by an arrow) was purified on Ni-NTA column. Different fractions analyzed in 12% SDS PAGE showing purification of MusaSNAC67 protein. Lane M: Molecular weight marker, UI: un-induced culture, I: induced culture, E: eluted MusaSNAC67 from Ni-NTA column, W: wash, FT: flow through, SS: sonicated supernatant, P:

pellet. (b) Detection of MusaSNAC67 protein by western blot analysis using antibody against polyhistidine. (c) Binding of MusaSNAC67 to core binding sequence of NAC-protein. The sequence of core and mutated core used for gel-shift assay is indicated on top of the gel. Free DNA and protein-DNA complex is indicated by arrow.

MusaSNAC67 regulate the expression of genes involved in chlorophyll catabolism

Precocious senescence with severe symptoms of de-greening and yellowing coupled with diminished chlorophyll content due to overexpression of MusaSNAC67 prompted us to analyze whether MusaSNAC67 controlled genes that are involved in chlorophyll degradation. Sequence analysis of promoter regions of genes involved in chlorophyll degradation suggested presence of CGT[A/G] motifs indicating possibility of their regulation by binding of MusaSNAC67 to these motifs. The gel-shift analysis suggested that MusaSNAC67 retarded the mobility of genes such as *PAO*-like (*Pheophorbide-a-oxygenase*), *HCAR*-like (*hydroxymethyl chlorophyll-a-reductase*), *NYC*/*NOL*-like (*Chlorophyll-b-reductase*), *PPH*-like (*pheophytinase*) as well as *ORS1*-like (a *SenNAC*). MusaSNAC67 retarded a 200bp DNA fragment (-88 to -288) of *pPPH*-like, a 180bp DNA fragment (-400 to -580) of *pPAO*-like (Fig. 3.2.11a), a 180bp upstream DNA fragment of *pHCAR*-like, a 213bp DNA fragment (-279 to -492) of *pORS1*-like (Fig. 3.2.11b), a 161bp DNA fragment (-382 to -543) of *pNYC1*-like and a 154bp DNA fragment (-10 to -164) of *pNOL*-like (Fig. 3.2.11c) in a gel shift assay. All of these fragments contained CGT[A/G] motifs to which MusaSNAC67 did show binding specifically. Furthermore, retardation of DNA-protein complex increased with increasing protein concentration suggesting that MusaSNAC67 could efficiently bind to these promoter. To ascertain whether, MusaSNAC67 could elevate expression of these chlorophyll catabolic genes, quantitative RT-PCR was performed which suggested markedly higher expression of these genes in MusaSNAC67 overexpressing transgenic banana line (Fig.

3.2.12). These results imply that *MusaSNAC67* promote senescence by regulating genes involved in chlorophyll catabolism.

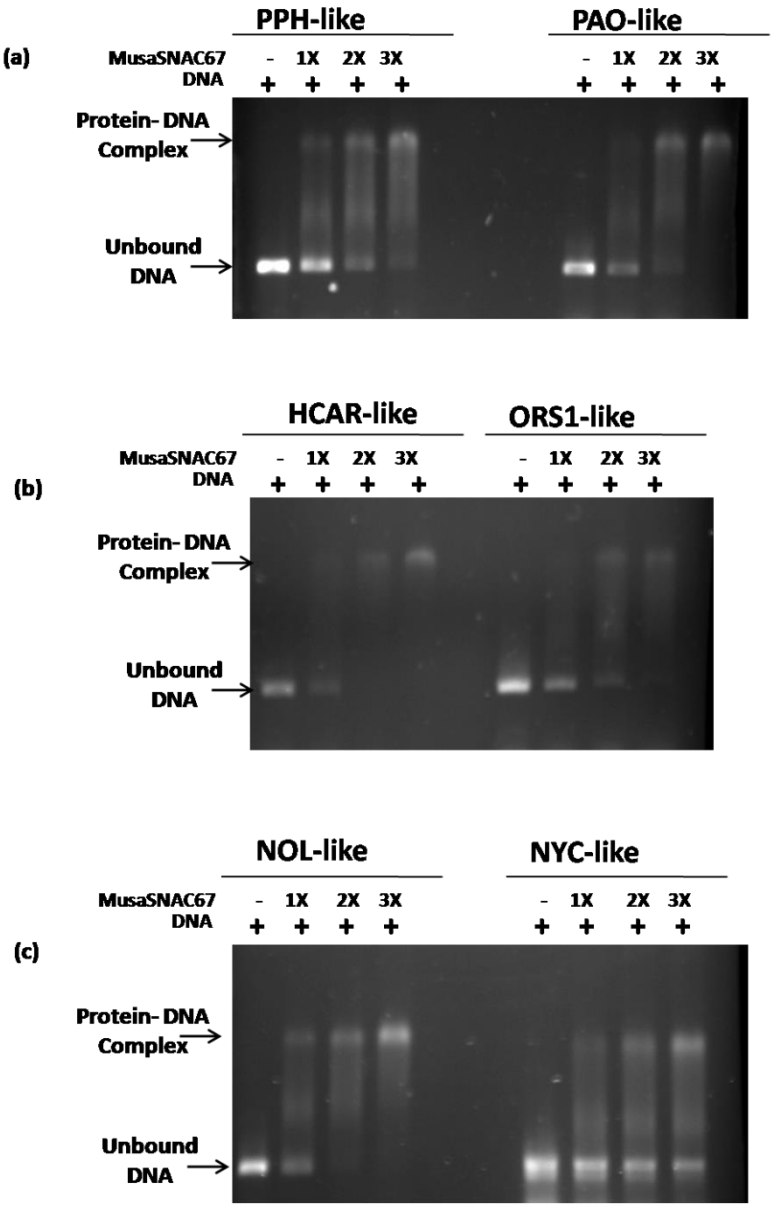


Fig. 3.2.11 MusaSNAC67 could bind to promoters of genes involved in chlorophyll catabolism. Binding analysis of MusaSNAC67 to promoter sequences of *PPH*-like (GSMUA_AchrUn_randomT14790_001), *PAO*-like (GSMUA_Achr4T25150_001), *HCAR*-like

(GSMUA_AchrUn_randomT03050_001), *ORS1*-like (GSMUA_Achr6T08600_001), *NOL*-like (GSMUA_Achr2T03080_001) and *NYC*-like (GSMUA_Achr7T10870_001). Un-bound DNA and protein-DNA complex are indicated by arrow.

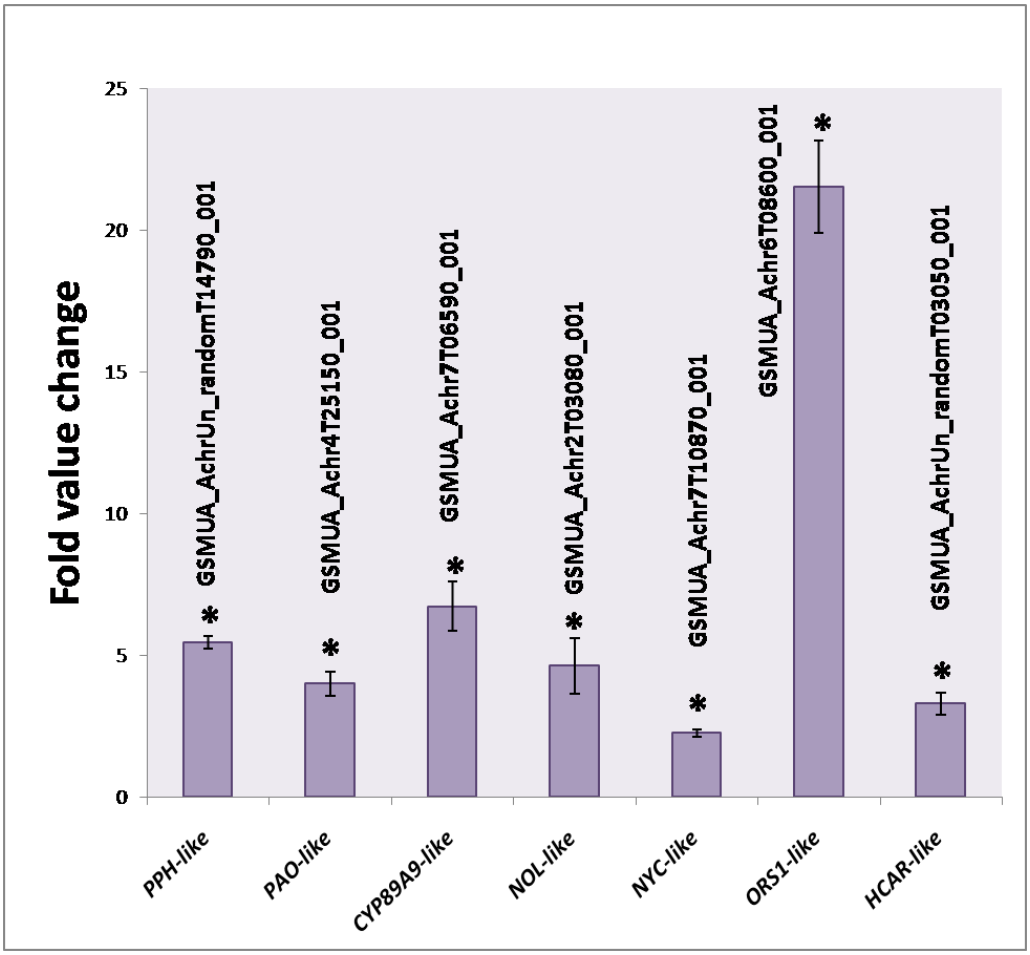


Fig. 3.2.12 Transgenic banana overexpressing *MusaSNAC67* showed higher transcript levels of genes involved in chlorophyll catabolism. Quantitative real time RT-PCR analysis of *PPH*-like (GSMUA_AchrUn_randomT14790_001), *PAO*-like (GSMUA_Achr4T25150_001), *HCAR*-like (GSMUA_AchrUn_randomT03050_001), *ORS1*-like (GSMUA_Achr6T08600_001), *NOL*-like (GSMUA_Achr2T03080_001) and *NYC*-like (GSMUA_Achr7T10870_001) genes indicated higher transcript levels in transgenic banana than control. The values were normalized by expression of banana EF1α and data was represented as mean±SD of three replicates. The

banana genome locus identifier is indicated on the top of each bar. Statistical significance at 5 % is shown by *.

Discussion

In the present study we investigated the functions of a stress-related NAC domain gene from banana and showed its role in regulating leaf senescence. Senescence occurs due to reasons like age-dependent senescence and stress inducing factors like salinity, drought, darkness, pathogen and attack (Munns 2005; Lutts et al. 1996).

Expression of *MusaSNAC67* showed positive response to multiple stress-conditions like drought, high-salinity, cold, peroxide, and salicylic acid. Salinity is one of the important factor influencing senescence by affecting expression of many *SAGs* and *senNACs* including *ANAC092*/*AtNAC2*/*ORE1* (Balazadeh et al. 2010a) and *MusaNAC042* (Tak et al. 2016) and *Arabidopsis JUB1* (Wu et al. 2012) among others. Short term salinity-stress (24 hours) did not increase transcript levels of *MusaSNAC67* however, prolonged salinity-stress caused an elevation in expression of *MusaSNAC67*. *ANAC092*/*AtNAC2*/*ORE1* showed similar response to short term salinity-stress and its transcript level got elevated only after exposure to long term salinity-conditions (Balazadeh et al. 2010a).

Certain plant hormones can also affect process of senescence (Jibran et al. 2013) and salicylic acid (SA) is one of positive regulator of plant senescence. One of the previous work has shown that salicylic acid and jasmonic acid positively regulate expression of many genes during senescence and age-related leaf senescence is hampered in mutant defective in SA signaling pathway (Buchanan-Wollaston et al. 2005). SA could induce expression of *MusaSNAC67* suggesting either a probable crosstalk of SA dependent signaling-pathway and senescence process or involvement of *MusaSNAC67* downstream of SA signaling-pathway.

Stress induction of many *senNACs* has been documented however, tissue specific transcriptional regulation of many *senNACs* has not been reported. In the present work, $P_{MusaSNAC67}$ showed major activity in vascular tissue of corm and leaves, which was elevated under salinity and drought (in line with quantitative RT-PCR expression of *MusaSNAC67*). Under salinity and drought, in corm but not in leaves the activity of $P_{MusaSNAC67}$ apart from vascular elements spreads to other tissue regions like central cylinder and cortex-region suggesting differential transcriptional regulation of *MusaSNAC67* in these organs.

SNBE-like sequence in 5'up-stream region of MusaSNAC67 indicated that $P_{MusaSNAC67}$ drives the expression of *MusaSNAC67* in vascular tissue. *SNBE* sequences has been documented as consensus binding site for *Arabidopsis* VND proteins and this *SNBE* (*secondary wall NAC binding element* or *TERE, tracheary element-regulating cis-element*) is a 19bp sequence comprising of (T/A)NN(C/T)(T/C/G)TNNNNNNNNA(A/C)GN(A/C/T)(A/T) consensus site (Zhong et al. 2010). *VNI2/NAC083* is also a *senNAC* negatively regulating xylem formation and its expression is also predominantly observed in vascular tissues (Yamaguchi et al. 2010). *MusaSNAC67* is a positive regulator of senescence while *VNI2/NAC083* is negative regulator and also promoter of *MusaSNAC67* do not show any activity in roots while that of *VNI2/NAC083* does, suggesting that these two genes do not have overlapping functions. Also *MusaSNAC67* differ from other reported *senNACs* as its overexpression causes precocious senescence even under control-conditions. Induction of *ANAC092*/*AtNAC2*/*ORE1* (under estradiol -inducible promoter) expression in transgenic lines showed senescence after 3 days of estradiol treatment and did not showed any senescence even after 16 hours (Balazadeh et al. 2010a).

Mesophyll chloroplasts of *MusaSNAC67* overexpressing transgenic lines contained larger and more numerous plastoglobuli and starch-granules than control plants. Increase in size and

number of plastoglobuli in chloroplast are considered as markers of senescence and aging in plants (Tevini and Steinmüller 1985) and our observation due to overexpression of *MusaSNAC67* suggested that *MusaSNAC67* is an efficient inducer of senescence. Number and size of starch-granules were also increased in the chloroplast suggesting the inefficiency of transgenic banana lines to utilize the carbon source which possibly resulted in reduction in growth and biomass. Moreover, accumulation of starch has been attributed to aging and senescence process in plants (Diaz et al. 2005; Oda-Yamamizo et al. 2016), suggesting that increased number and size of starch grains in transgenic lines is due to senescence caused by overexpression of *MusaSNAC67*.

Consensus binding site of NAC proteins has been found to be CGT[A/G] after observing the binding of multiple NAC-proteins like ANAC019, ANAC092 (Olsen el at. 2005), ANAC055 (Tran et al. 2004) and TaNAC69 (Xue 2005) to this consensus site. MusaSNAC67 could also bind CGT[A/G] site and failed to bind a mutated version of this site, suggesting that MusaSNAC67 might regulate its target genes by biding to this element in their promoter.

Gel-shift assay with fragment containing CGT[A/G] site in promoter suggested that MusaSNAC67 protein was able to retard the mobility of promoter of at least five chlorophyll catabolic genes (*PAO*-like, *HCAR*-like, *NYC*-like, *NOL*-like and *PPH*-like) and *ORS1*-like gene. Some of the senNACs reported in past can bind to the promoters of chlorophyll catabolic genes. ANAC046 could bind to the promoter region of *NYC1*, *SGR1* (*STAY-GREEN1*), *SGR2* and *PAO* and transcript abundance of these genes were higher in plants after overexpression of *ANAC046* (Oda-Yamamizo et al. 2016). Yeast-one-hybrid assay showed that ANAC016 binds to the promoter of SGR1 and increased its expression in overexpressing transgenic lines (Sakuraba et al. 2016). MusaSNAC67 bind to the promoter fragment of *PAO*-like , *HCAR*-like, *NYC*-like ,

NOL-like and *PPH*-like as well as increase their transcript levels in transgenic lines along with transcript of *ORS1*-like indicating that MusaSNAC67 that is a efficient regulator of their expression. Above results suggested that *ORS1*-like in banana is a downstream regulator of *MusaSNAC67* and possibly could be co-regulated during senescence induced by *MusaSNAC67*. Earlier reports on some *senNACs* also suggested cross-regulation of different *senNACs* during senescence process. *ANAC046* overexpression increased expression of *ANAC055*, *ANAC019*, *ATAF1* and *ANAC072* among others (Oda-Yamamizo et al. 2016). ANAC016 binds to the promoter of NAP and ORS1 and increase their expression in overexpressing transgenic line (Kim et al. 2013). *Arabidopsis ORS1* was induced by salinity and H_2O_2 stress conditions and overexpressing plants displayed increased senescence (Balazadeh et al. 2011).

Thus the results suggest that *MusaSNAC67* regulates senescence in banana through regulation of chlorophyll catabolic pathway.

Table 3.2.1 Primers for cloning and other molecular work carried out in the study of *MusaSNAC67*

S.No	Gene	Forward Primer (5' - 3')	Reverse Primer (5' -3')
1	**Primers for cloning *MusaSNAC67* in *pCAMBIA1301***	**Aactgcag**ATGGGGAGGAAAAGAGACGC	AaggtaccTCAGAATGGCAGCATGTCCG
2	***MusaSNAC67* real time PCR primers**	AGACAGCTTGAGGACACCAG	TTTGTTGCATCCATCATTTG
3	**Hygromycin phosphotransferase (*hpt*) gene**	GTCCTGCGGGTAAATAGCTG	ATTTGTGTACGCCCGACAGT
4.	***Musa EF1α* real time PCR primers**	CCGATTGTGCTGTCCTCATT	TTGGCACGAAAGGAATCTTCT
5.	**Primers for cloning of $P_{MusaSNAC67}$ upstream of GUS in *pCAMBIA1301***	TTCTGCAGACATAGGCAGCAATTCTTGG	TTCCATGGCCTCGTTTCCTTCGCTG
6.	**Primers for cloning *MusaSNAC67* in *pET28a***	**aaaCAT**ATGGGGAGGAAAAGAGACG	aaa**GGATCC**TCAGAATGGCAGCATGTCC

Table 3.2.2 Primer sequences for expression analysis of chlorophyll catabolic pathway genes and other genes used in the study of *MusaSNAC67*

Gene	Banana genome locus identifier	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
NYC-like	GSMUA_Achr7T10870_001	TTACCTGACACCACCTCGAA	CCGGAACTGGAGAGGATTAT
NOL-like	GSMUA_Achr2T03080_001	CACAACAAAACAGGCCAAAT	CGAGATATCTGTTCCGTCGT
HCAR-like	GSMUA_AchrUn_randomT03050_001	AGCAGTGGTAACCGAAAACC	TTCTTGGCGTATGAGGGAAT
PPH-like	GSMUA_AchrUn_randomT14790_001	TCCGAGGGTGGATTAAGAAC	GAGGCGTAGCTTCAAGGATG
PAO-like	GSMUA_Achr4T25150_001	GCTAGCCATCAGCCTTTACC	GGAGTTCACGCAAAGCATAA
ORS1-like	GSMUA_Achr6T08600_001	GCTCAAAGCCCTTACTGCTC	GTCCACATGAAGGATTGTCG

Table 3.2.3 Primer sequences for gel shift assay used in the study of *MusaSNAC67*

	Name	Forward Primer (5' - 3')	Reverse Primer (5' -3')
1	**pPPH-like (-88 to -288)**	ATTCAATTGATCCCGAATTGT	TCTTATTCCCTCGAAAAGCA
2	**pPAO-like (-400 to -580)**	CGAGAGACCTCGAGCAAAG	TTTAATTGTAGCATCTATGTTAAT
3	**pHCAR-like**	ATTCATAAAAAGAAAAGGTTTTTAG	CTACTGTAATATAGAACAGAATAAATG
4	**pORS1-like (-279 to -492)**	GAGCTATGTGCTTGTCCAGAG	ATATGAATGTCAACTTTATTGATT
5	**pNYC1-like (-382 to -543)**	ATGCCACATGAAGGACAGAT	TGAAAGCAATCATCATGTCAA
6	**pNOL-like (-10 to -164)**	TTTTTACAAAGGATTCCGACA	GTTATGGCTTCTCGGCTCA
7	**SNBE-like in $P_{MusaSNAC67}$**	AATTCTAGTGTAGTGAAGAAGCAACACTAT	ATAGTGTTGCTTCTTCACTACACTAGAATT
8	**Mutated SNBE**	AATTCGAGAAGAGTGAAGTGCCGCCACTAT	ATAGTGGCGGCACTTCACTCTTCTCGAATT
9	**Core binding sequence**	AattcTTTCGTATTGCGTGTTTTCGTATTGCGTGTt	ctagaACACGCAATACGAAAACACGCAATACGAAAg
10	**Mutant core**	AATTCTTTAAATTAAAATTTTGGGTTAAATTTAAGG	CCTTAAATTTAACCCAAAATTTTAATTTAAAGAATT

Section 3.3: Studies on *MusaMPK5*, a stress associated *MAP kinase* coding gene from banana

Introduction

Low temperature stress is a major abiotic stress condition limiting the plant growth and final productivity. Banana (*Musa x paradisiaca*) is highly susceptible to cold stress and the growth is severely affected at temperature below 12 °C. Moreover, even at slightly lower temperature of 8 °C, irreparable damage to banana plants can occur (Santos et al. 2009; Yang et al. 2015). The cold induced damage arises because of changes in membrane structure and fluidity, protein denaturation, and production of reactive oxygen species.

Plants have developed methods including signal transduction pathways to sense change in environmental conditions. *Mitogen-activated protein kinases* (*MAPKs*) are important proteins involved in plant growth, development, signalling and biotic as well as abiotic stress responses (Hamel et al. 2006).

A MAPK signal works in a cascade fashion consisting of mitogen-activated protein kinase kinase kinase (MAPKKK/MEKK), mitogen-activated protein kinase kinase (MAPKK/ MEK) and mitogen-activated protein kinase (MAPK) and the signal transduce in sequential phosphorylation (MAPKKK -> MAPKK -> MAPK) event (Samajova et al. 2013). MAPK activation requires phosphorylation of tyrosine and threonine residues of TXY/TDY motif by MAPKKs, which themselves are activated by phosphorylation of serine/threonine in the S/T-X3–5-S/T motif by MAPKKKs (Chen et al. 2012). MAPK phosphorylate downstream proteins such as transcription factors and effector proteins thus regulating their activity for generation of certain response (Colcombet et al. 2008).

Some of the *MAPKs* have been reported in past for their roles in different physiological functions. *Arabidopsis* AtMPK3 and AtMPK6 are induced by abiotic and biotic stress conditions and their activation is a critical step for generating stress tolerance or resistance in plants (Beckers et al. 2009). *Arabidopsis MPK4*, (*AtMPK4*) is involved in response to cold, salinity and drought stress (Teige et al. 2004; Droillard et al. 2004). Maize *MPK4* has been demonstrated to be involved in low temperature stress tolerance as transgenic plants overexpressing *MPK4* showed better growth associated with higher proline and elevated soluble sugar content (Zhou et al. 2012). Rice *MPK5* (*OsMPK5*) is induced in both biotic and abiotic stress and overexpression of *OsMPK5* improved tolerance to cold, high-salinity and drought stress (Xiong et al. 2003).

Since banana is a crop of tropical origin, low temperature stress severely impacts its production. Temperature below 10°C results in bunch malformation, blackening of the fruit skin, improper and delayed ripening, yellowing and death of the leaves (Ravi et al. 2016). In this regard, studies on genetic improvement of banana for cold stress tolerance has great relevance and development of cold tolerant banana will boost its cultivation in low temperature climate.

In this chapter, we have shown that a *Mitogen activated protein kinase*, *MusaMPK5* positively regulates cold stress tolerance in banana. Expression of *MusaMPK5* is responsive to multiple stress conditions and $P_{MusaMPK5}$*::GUS* displayed strong induction of *GUS* after exposure to cold stress (8 °C). Stress analysis with transgenic banana lines overexpressing *MusaMPK5* showed superior cold stress tolerance ability of transgenic lines than control plants. MusaMPK5 is a cytoplasm localized protein and encodes for a functional kinase. MusaMPK5 can phosphorylate two stress associated NAC transcription factors, MusaNAC042 and banana SNAC67 indicating its direct role in regulating the activity of these proteins. These results will

increase our knowledge about the cold stress responses in plants and will facilitate engineering plants with improved cold stress tolerance.

Results

Sequence analysis of MusaMPK5

MusaMPK5 shared high sequence identity with other characterized and annotated MAPKs. MusaMPK5 shared identity of 84% with OsBIMK1 (AAK01710), 83% with ZmMPK5 (NP_001288546), 82% with ZmMPK4 (BAA74733) (Fig. 3.3.1). The results showed that MusaMPK5 was clustered with group-A MAP kinases and is named after maize MPK5 with which it share high percentage identity (Fig. 3.3.1). MusaMPK5 has an open reading frame of 1104 bases and encodes a protein of 367 amino acids (Fig. 3.3.2). MusaMPK5 has a theoretical pI of 5.59 and molecular weight of 42.5 kDa. MusaMPK5 and other group-A MAP kinases shared the eleven consensus subdomains along with a phosphorylation motif TEY located in between subdomain VII and VIII and a common docking motif at c-terminus end for docking of MAPKKs, substrates and other regulatory proteins (Fig. 3.3.2).

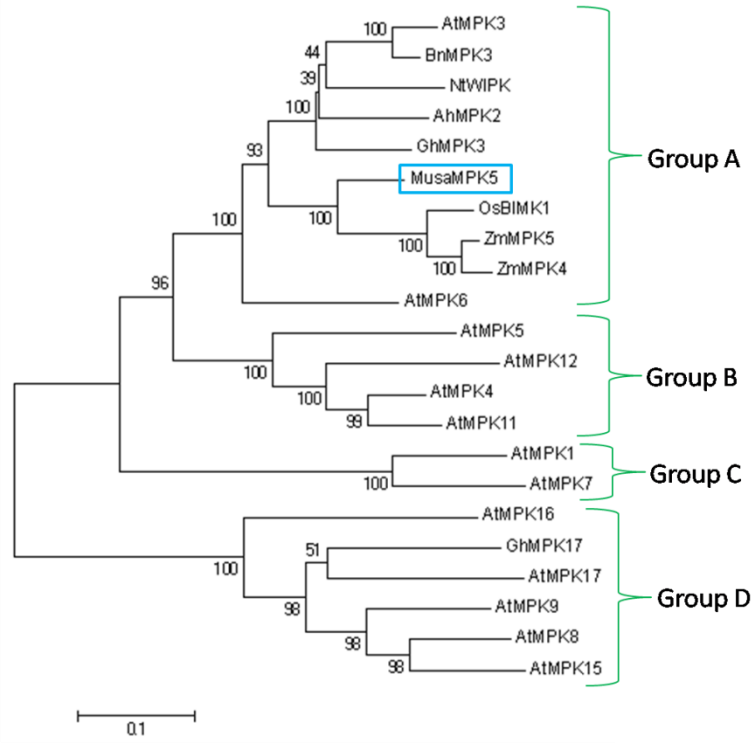


Figure. 3.3.1 Phylogenetic analysis of MusaMPK5 with other MAP kinase proteins. Neighbor joining tree (Boot strap value of 1000) indicated MusaMPK5 in relation with MPK from other plants. MusaMPK5 was clustered with ZmMPK5 and ZmMPK4 and also shared high % identity with them. The clustering of MAP kinase proteins into different groups (A-D) has also been indicated. The accession number used to built the phylogenetic tree were: MusaMPK5 (ANR02350), OsBIMK1 (AAK01710), ZmMPK5 (NP_001288546), ZmMPK4 (BAA74733), AtMPK3 (NP_190150), AtMPK1 (NP_172492), AtMPK4 (NP_192046), AtMPK5 (NP_567378), AtMPK6 (NP_181907), AtMPK7 (NP_179409), AtMPK8 (NP_173253), AtMPK9 (NP_566595), AtMPK11 (NP_001117210), AtMPK12 (NP_182131), AtMPK15 (NP_565070), AtMPK16 (NP_197402), AtMPK17 (NP_001030941), NtWIPK (BAA09600), GhMPK17 (AHW85276), GhMPK3 (ADI52626), AhMPK2 (AAZ23129) and BnMPK3 (AAV34677).

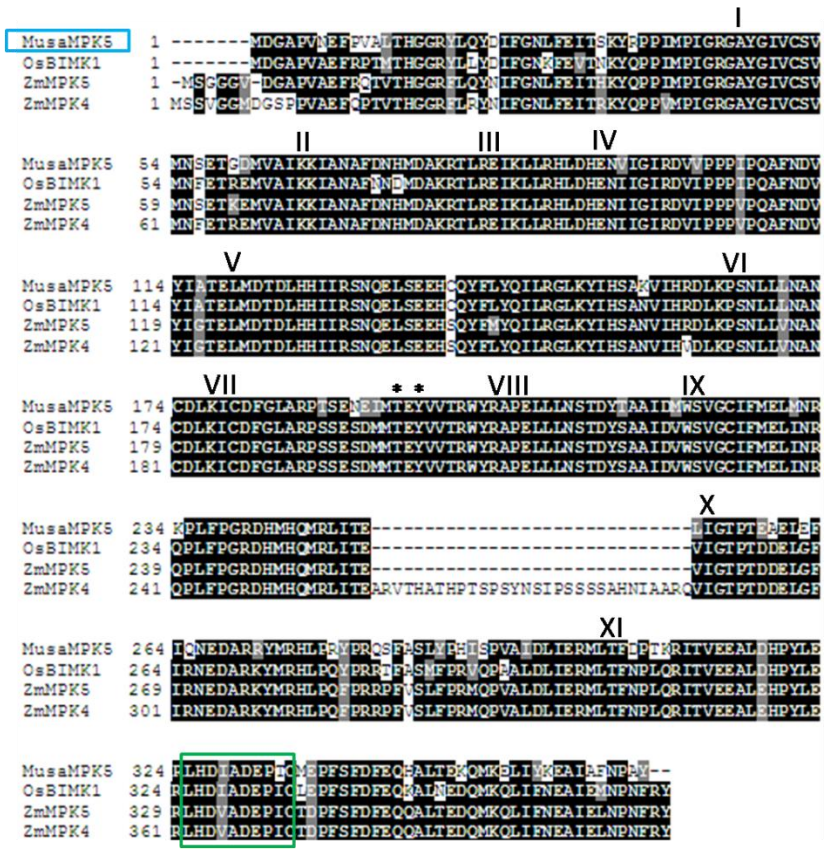


Figure. 3.3.2 Multiple sequence alignment of MusaMPK5. MusaMPK5 shared high sequence identity with other well characterized and annotated MAPK. *MusaMPK5* shared identity of 83% with OsBIMK1, 84% with ZmMPK5 and 84% with ZmMPK4. Threonine (T) and Tyrosine(Y) residues whose phosphorylation is reported to be essential for MAPK activation are indicated by asterisks. The eleven consensus subdomains of the protein kinases are indicated above the sequences by the roman numerals. The common docking motif is boxed in green. The accession number used for multiple sequence alignment were: MusaMPK5 (ANR02350), OsBIMK1 (AAK01710), ZmMPK5 (NP_001288546) and ZmMPK4 (BAA74733).

Expression profiling of *MusaMPK5*

The expression of *MusaMPK5* was analyzed by quantitative RT-PCR in the plants of banana cultivar *Karibale Monthan* subjected to various treatments. *MusaMPK5* was induced in response

to cold and application of salicylic acid (2mM) and methyl jasmonate **(**200 µM MeJA). During cold stress (4±2 °C), expression of *MusaMPK5* was maximum at 5 hours post stress and then reduced towards 24 and 48 hours but remained higher than control value (Fig. 3.3.3a). This early elevation of *MusaMPK5* after cold stress exposure was also observed in roots where the expression was maximum after 5 hour of stress imposition (Fig. 3.3.3d). After application of methyl jasmonate **(**200 µM MeJA) slight elevation in transcripts of *MusaMPK5* was observed in leaves of banana plants (Fig. 3.3.3b). Application of salicylic acid (2mM) does not increase the expression of *MusaMPK5* in leaves till 24 hours after which it peaked around 48 hours (Fig. 3.3.3c). However, exposure of salicylic acid remarkably increased the expression of *MusaMPK5* in roots after five hours of exposure and peaked at 24 hours post stress (Fig. 3.3.3e).

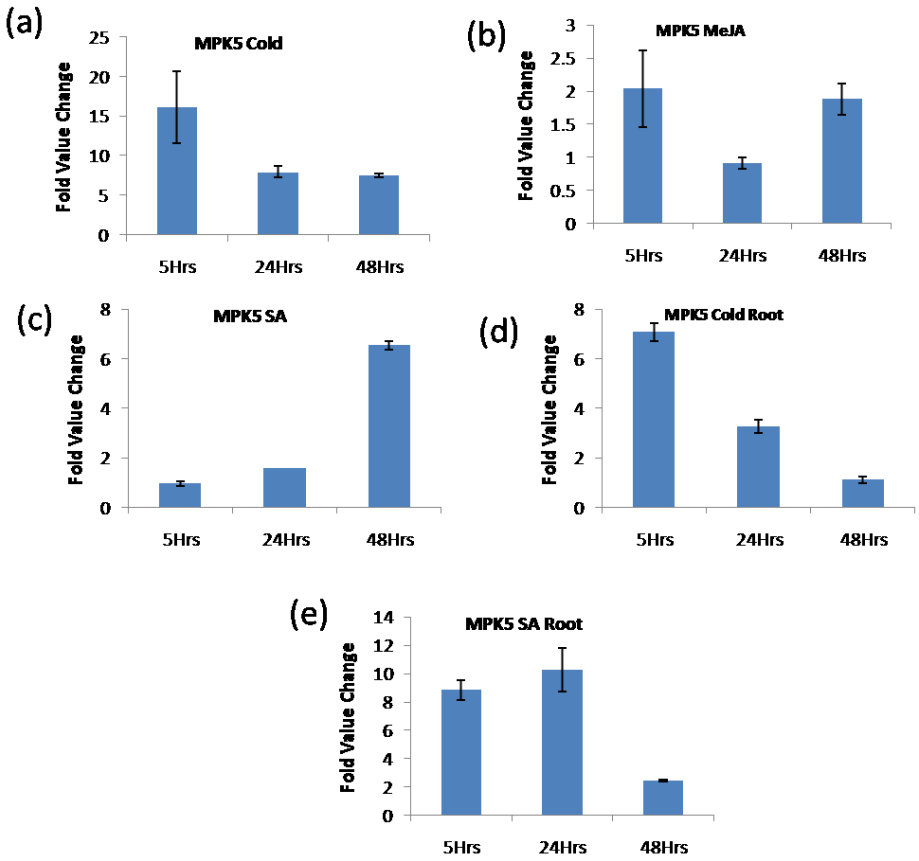


Figure. 3.3.3 Expression patterns of *MusaMPK5*. Banana cv. *Karibale Monthan* were subjected to cold (4±2 °C) and applications of methyl jasmonate (200 µM MeJA) and salicylic acid (2mM) and the expression of *MusaMPK5* was estimated by quantitative RT-PCR analysis. (a) Expression in leaves of plants subjected to cold stress. (b) Expression pattern of *MusaMPK5* in leaves after application of MeJA . (c) *MusaMPK5* expression in leaves after application of SA. (d) Expression of *MusaMPK5* at different time points in roots of plants subjected to cold stress. (e) Expression of *MusaMPK5* in roots after plants subjected to application of SA.

Analysis of *MusaMPK5* promoter

Highest and early elevation of *MusaMPK5* was observed during cold stress both in leaves and roots of banana suggesting it may have important roles in cold stress responses. To observe the tissue specific nature and analyze the cold inducible activity of *MusaMPK5*, we cloned approximately 1kb 5'-upstream region of *MusaMPK5* upstream of *GUS* in binary vector *pBI121* (Fig. 3.3.4a). Transgenic banana plants harboring $P_{MusaMPK5}$-*GUS* were generated and subjected to cold stress (8 °C) for analyzing the GUS activity. GUS activity under the control of $P_{MusaMPK5}$ was observed in all the organs of banana however, strong activity of GUS was observed in corm and pseudostem (Fig. 3.3.4b, 3.3.4e). In corm, strong activity of *MusaMPK5* promoter was observed in the cells surrounding the central vascular cylinder upon induction with cold stress (8 °C) (Fig. 3.3.4c, 3.3.4d). Cold stress induced the expression of *GUS* from $P_{MusaMPK5}$ in the cortical cells of pseudostem which was visible in form of increased GUS staining in cells of pseudostem. To estimate the relative activity of $P_{MusaMPK5}$ in terms of GUS activity, MUG assay was done after exposing the plants to cold stress (8 °C) which indicated significant elevation in GUS activity over control value after exposure to cold stress (Fig. 3.3.4g).

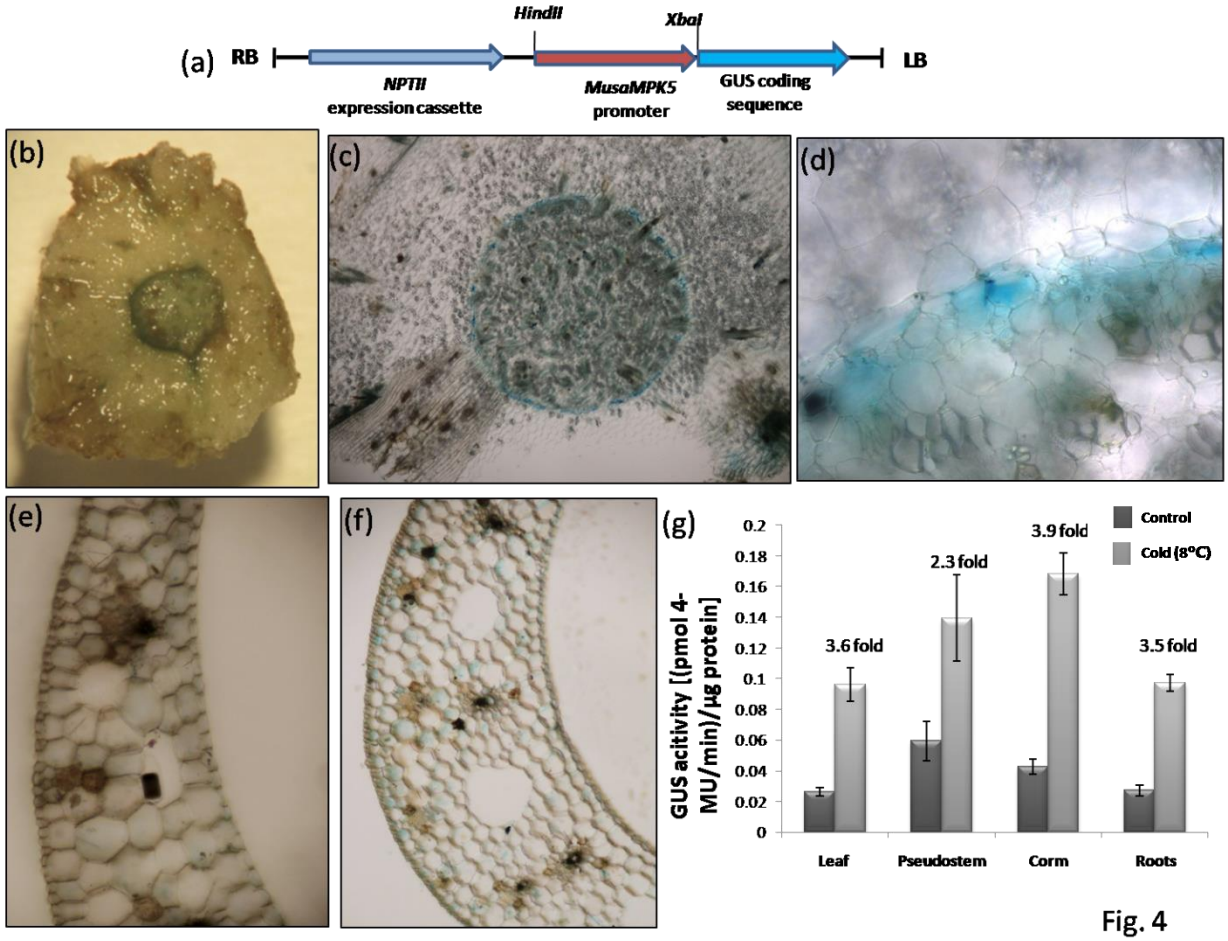


Figure. 3.3.4 Tissue specific and stress inducible nature of *MusaMPK5* promoter. (a) T-DNA region harboring P_{MPK5}*::GUS* was transferred to transgenic banana for analysis of *MusaMPK5* promoter activity. (b) GUS staining in corm of banana transformed with P_{MPK5}*::GUS.* Strong activity of *MusaMPK5* promoter was observed in the cells surrounding the central vascular cylinder upon induction with cold stress (8 °C). (c, d) Close-up view showing the GUS staining in the cells surrounding the central vascular cylinder of corm. (e) Activity of P_{MPK5}*::GUS* in pseudostem under control conditions. (f) Induction of *MusaMPK5* promoter in cortical cells of the pseudostem after exposure to cold stress (8 °C). (g) MUG assay to determine the GUS activity under the control of *MusaMPK5* promoter after exposure to cold stress (8 °C). GUS

activity in different organs was estimated and the fold change over control is indicated over the corresponding bars. Data is represented as mean±SD.

Sub-cellular localization of MusaMPK5

In separate experiments, *MusaMPK5::GFP* and *MusaMPK5::GUS* were transiently over expressed in onion epidermal cells and the cells were observed under a fluorescent microscope. An *in silico* analysis of MusaMPK5 subcellular localization at 'wolf psrot' server (https://www.genscript.com/wolf-psort.html) predicted MusaMPK5 to be associated with cytoskeleton. Onion epidermal cells overexpressing only GFP indicated the distribution of fluorescence throughout the cells (Fig. 3.3.5a, 3.3.5b) while in cells overexpressing MusaMPK5::GFP the fluorescence was predominantly localized in cytoplasm (Fig. 3.3.5c, 3.3.5d). Cytoplasmic localization of MusaMPK5 was confirmed by overexpressing MusaMPK5::GUS in onion epidermal cells which suggested association of MusaMPK5 with the cytoskeleton portion of the cells (Fig. 3.3.5e).

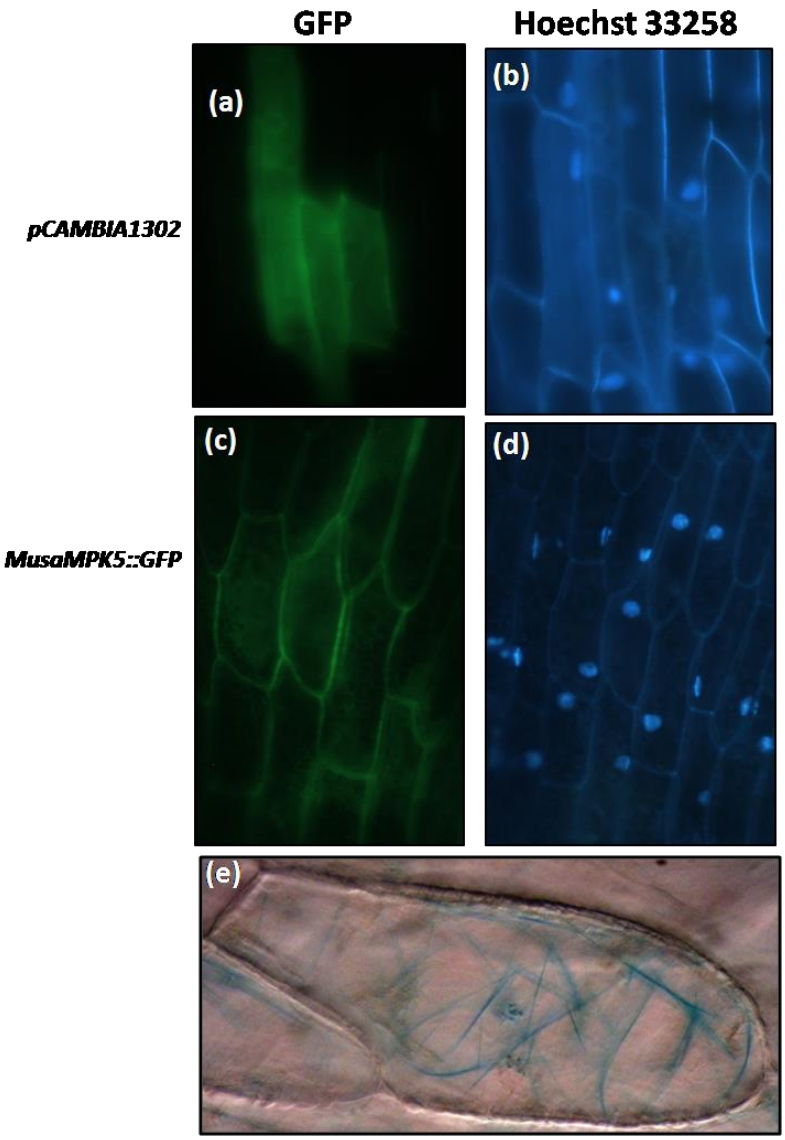


Figure. 3.3.5 Sub-cellular localization of MusaMPK5. (a) GFP fluorescence in onion epidermal cells due to activity of $P_{CaMV35S}$ ::GFP. (b) Nuclear position in banana cell (for a) observed after Hoechst-33258 staining. (c) Cytoplasmic localization of GFP-fluorescence in *pCAMBIA1302-MusaMPK5* transformed cell. (d) Hoechst-33258 staining to detect nuclei (for c). (e) Cytoplasmic localization of MusaMPK5-GUS fusion in cells of onion epidermis after transient transformation.

Generation and confirmation of transgenic banana plants overexpressing *MusaMPK5*

Agrobacterium strain *EHA105* was used to transfer T-DNA (*MusaMPK5* cloned under the control of *CaMV35S* promoter for overexpression of *MusaMPK5*) in embryogenic cells of banana cv *Rasthali* (Fig. 3.3.6a). Putatively transformed banana embryogenic cells were developed into embryos on selection medium containing hygromycin (5mg/l) (Fig. 3.3.6b). These developing embryos were then converted into shoot by supplementing the culture medium with 0.5mg/l BAP (Fig. 3.3.6c). Individual shoots emerging on shoot conversion medium were then clonally propagated on banana shoot multiplication medium (Fig. 3.3.6d). Different putatively transformed transgenic lines were then rooted with the help of culture medium supplemented with 1mg/L NAA (Fig. 3.3.6e). Different lines of banana overexpressing *MusaMPK5* were then hardened in the controlled conditions of green house for further analysis (Fig. 3.3.6f). Transgenic lines were confirmed for T-DNA integration by PCR analysis and Southern blot. Genomic DNA of different transgenic lines was analyzed for amplification of *hpt-II* (*Hygromycin phosphotransferase-II*) coding sequence (788bp PCR product) which is present in T-DNA region. Four transgenic lines with normal growth on selection medium tested positive for *hpt-II* amplification and were named as L1, L3, L6 and L14 (Fig. 3.3.7a). Further, the genomic DNA was digested with *Nco*I and probed for *hpt-II* coding sequence (using digoxigenin labeled probe) by Southern blot analysis. Bands on autoradiograph indicated that different transgenic lines possessed 1-2 copies of T-DNA in their genome (Fig. 3.3.7b). Quantitative RT-PCR analysis indicated that fold change in expression of *MusaMPK5* over control was 6 to 14 folds indicating the suitability of these transgenic lines for further analysis (Fig. 3.3.7c).

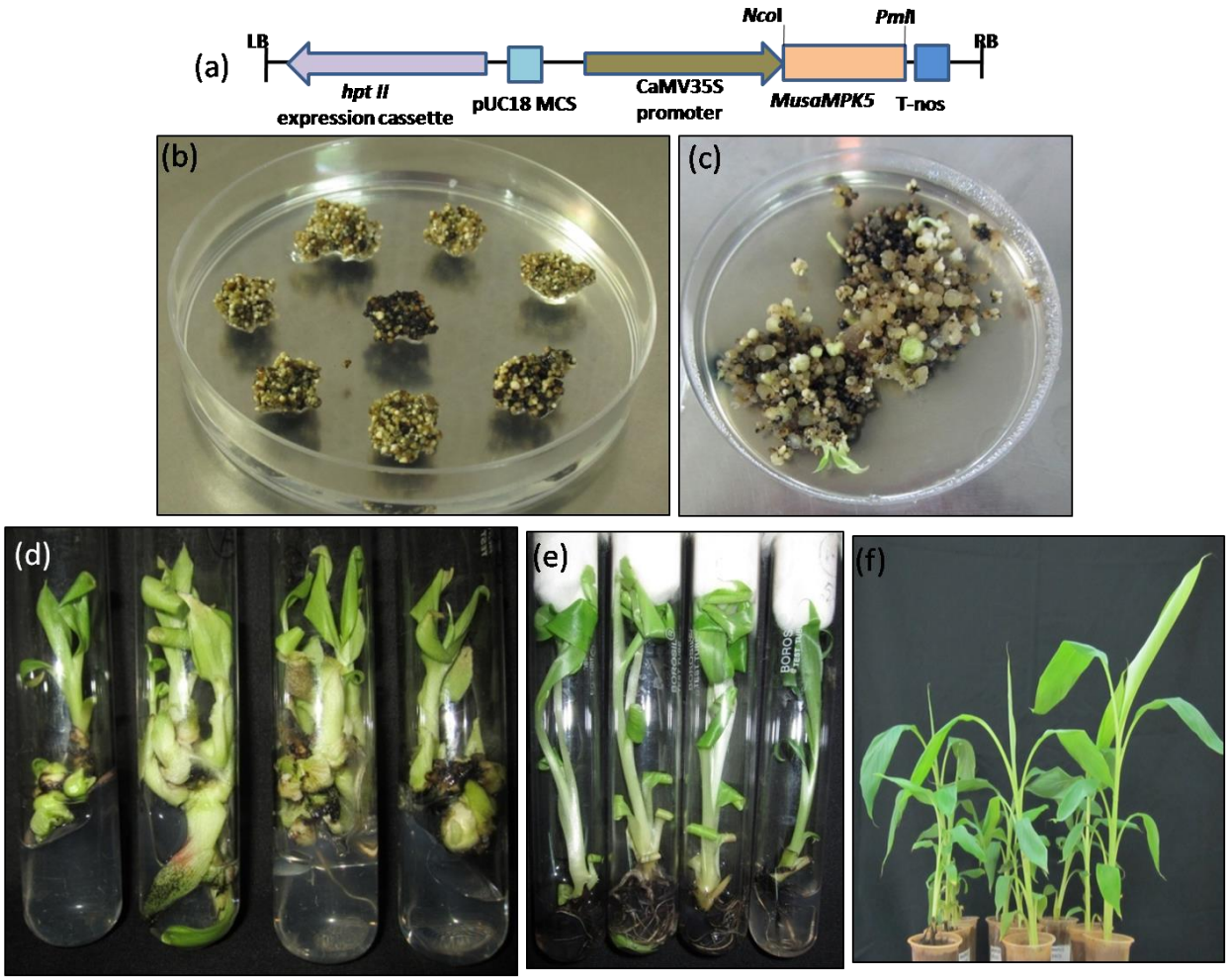


Figure. 3.3.6 Generation of transgenic lines of banana cultivar *Rasthali* overexpressing *MusaMPK5*. (a) T-DNA harboring $P_{CaMV35S}$-*MusaMPK5* was used to generate transgenic banana plants.(b) *Agrobacterium* strain *EHA105* was used to transfer T-DNA in embryogenic cells of banana cv *Rasthali* and transformed cells were developed into embryos on selection medium containing hygromycin (5mg/l). (c) Embryo to shoot conversion was carried out on medium supplemented with BAP (0.5mg/l). (d) Multiple shoots of putatively transformed transgenic plants were raised on banana multiplication medium. (e) Rooted transgenic lines overexpressing *MusaMPK5*. (f) Rooted transgenic lines were further hardened in green house conditions.

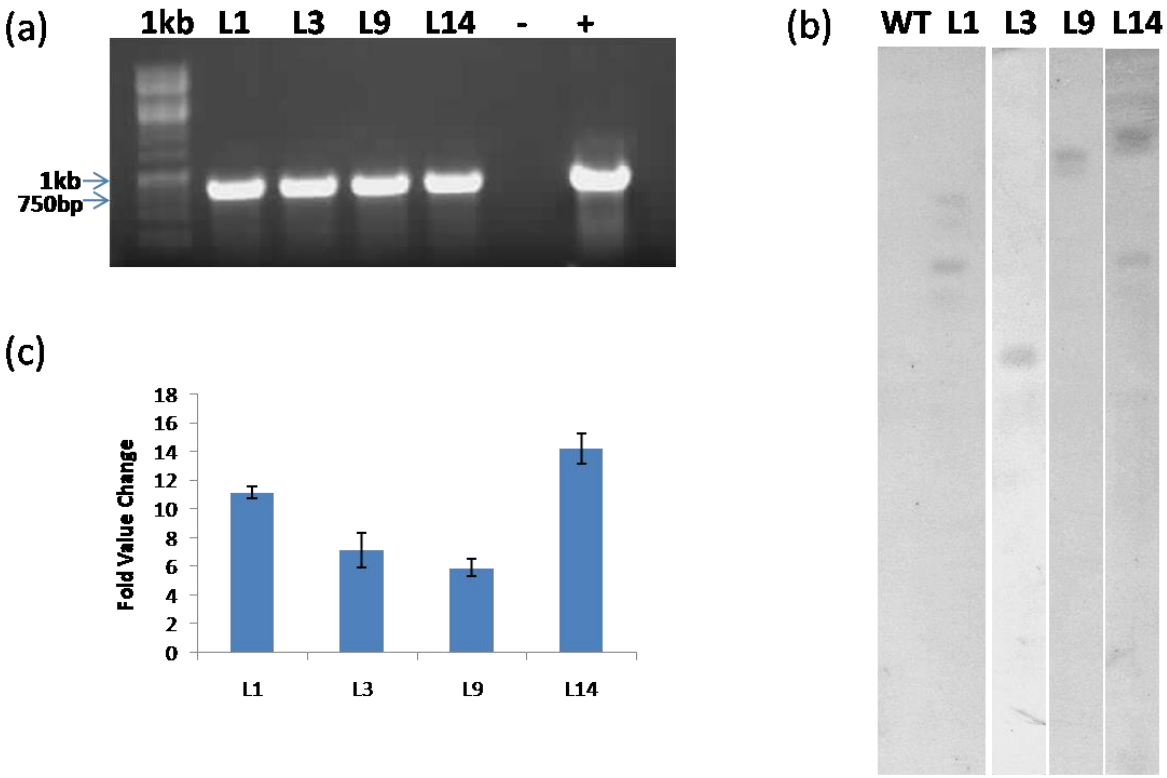


Figure. 3.3.7 Molecular confirmation of transgenic lines. Transgenic lines were analyzed by PCR, Southern blot and quantitative RT-PCR. (a) PCR analysis. PCR amplification of *hpt-II* coding sequence (788bp) from genomic DNA of different transgenic lines. Negative and positive controls are indicated by – and + sign. (b) Copy number determination of T-DNA transferred to transgenic lines by Southern blot. The blot was probed with a digoxin labeled probe against *hpt-II*. (c) Quantitative RT-PCR analysis of *MusaMPK5* overexpression in different transgenic lines. Fold change in expression was calculated after normalization with expression of banana *EF1α*. Data is represented as mean±SD.

Cold stress tolerance assays of transgenic lines

The expression of *MusaMPK5* showed early and profound elevation during cold stress indicating a probable function of MusaMPK5 in cold stress responses of banana. We analyzed the transgenic lines for cold stress tolerance by different assays involving *in vitro* growth assay, leaf

discs assay and cold stress tolerance assay by using green house hardened plants. For *in vitro* growth assay, equal age and size shoots of control and transgenic lines were subjected to 8 °C for 12 days and their recovery growth was monitored (Fig. 3.3.8a). After cold stress, transgenic lines showed remarkably better recovery than control plants by displaying better growth (fresh weight gain) and longer shoot length (Fig. 3.3.8b, 3.3.8c, 3.3.8d). Further, transgenic lines were assayed by leaf discs assay for cold stress tolerance. Leaf discs of transgenic lines exposed to 8 °C and 4°C stress for 10 days showed lower bleaching than discs from wild type plants indicating better tolerance of transgenic discs (Fig. 3.3.8e, 3.3.8f). Superior tolerance of transgenic leaf discs was corroborated by higher total chlorophyll (µg/mg FW) and lower MDA (nmols MDA/gm FW) content than control leaf discs after exposure to 8 °C and 4°C stress (Fig. 3.3.8g, 3.3.8h). We further analyzed transgenic and control plants growing in green house for cold stress tolerance by exposing these plants to 8°C for 12 days in a growth chamber at 70% relative humidity. After the period of cold stress exposure, the symptoms of cold stress in terms of bleaching and wilting of leaves appeared on leaves of plants however, the symptoms were more pronounced in control plants as indicated by reduced percentage of chilling induced injury in transgenic lines (Fig. 3.3.8i, 3.3.8k). Recovery growth of these cold stressed plants under ambient conditions in green house indicated that all the transgenic lines recovered better than control plants (Fig. 3.3.8j). Biochemical parameter such as lower MDA and higher proline content indicated that transgenic lines can tolerate cold stress better than control plants (Fig. 3.3.8l, 3.3.8m).

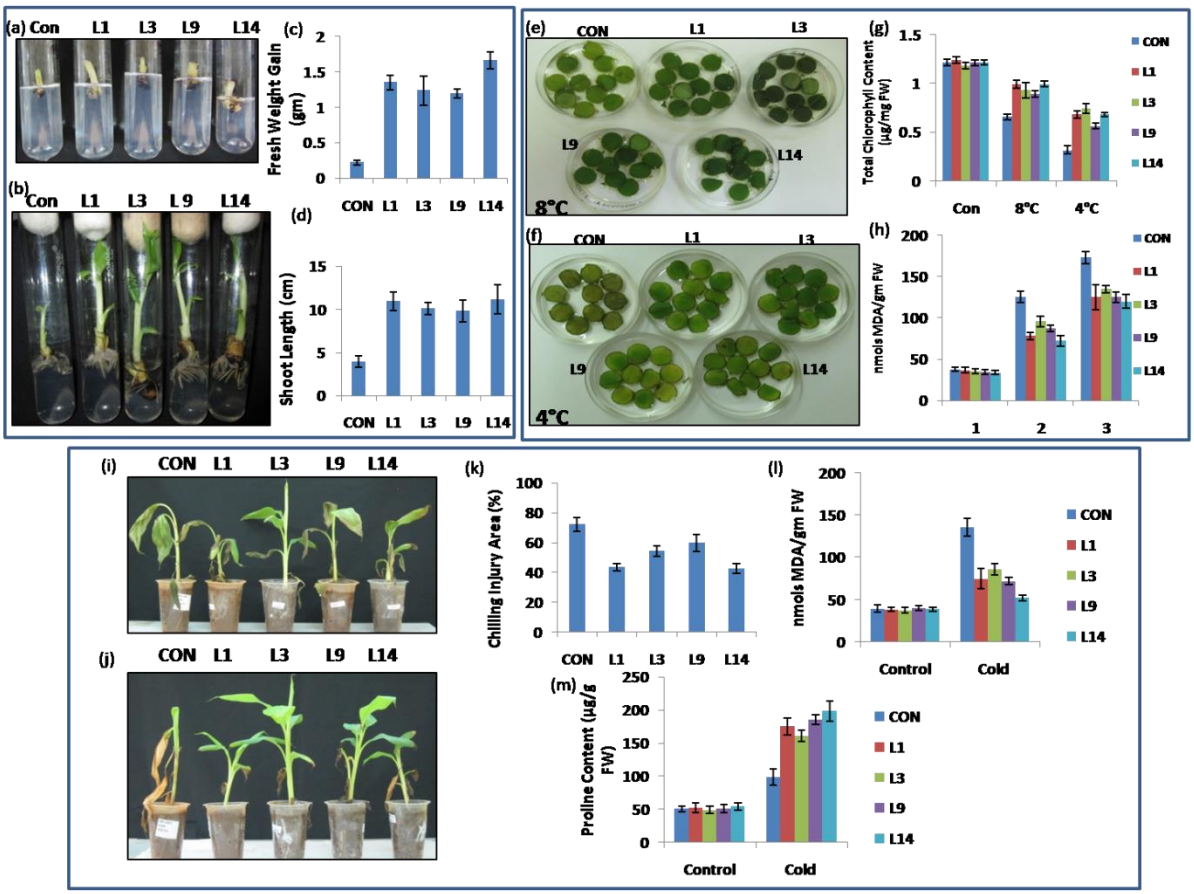


Figure. 3.3.8 Improved cold stress tolerance after overexpression of *MusaMPK5*. *In vitro* cold tolerance assay. (a) Equal size shoots cultured on rooting medium were stressed to cold (8 °C) for 12 days. (b) Recovery growth of stressed plants at 27±2°C after transferring to fresh rooting medium. (c) Fresh weight gain in control and transgenic lines during recovery after stress at 8 °C. (d) Shoot length gained by control and transgenic lines during recovery after stress at 8 °C. Leaf disc analysis for stress tolerance. (e) Bleaching of wild type and transgenic leaf discs after getting stressed at 8 °C. (f) Bleaching of wild type and transgenic leaf discs after being stressed at 4°C. Note the better tolerance of transgenic leaf discs in terms of lower bleaching than wild type plants discs. (g) Total chlorophyll content (µg/mg FW) of cold stressed (8 °C and 4°C) wild type and transgenic leaf discs. (h) Total MDA content (nmols MDA/gm FW) of cold stressed (8 °C and 4°C) wild type and transgenic leaf discs. Note the higher chlorophyll content and lower

MDA content in transgenic leaf discs than wild type leaf discs. Cold tolerance analysis with hardened plants. (i) Cold stress symptoms in control and transgenic lines stressed to 8 °C for 12 days. (j) Recovery of control and transgenic lines stressed to 8 °C for 12 days. Note the better recovery of transgenic lines than control plants. (k) Percentage of chilling injury area in transgenic lines and control plants after cold stress. (l) Total MDA content (nmols MDA/gm FW) of cold stressed control and transgenic plants. (m) Proline content (µg/g FW) in leaves of control and transgenic lines stressed to 8 °C for 12 days. Note the higher proline content and lower MDA content in transgenic lines than control plant indicating better recovery of transgenic plants. Data is represented as mean±SD.

Purification and kinase activity of MusaMPK5 protein.

To analyze the putative kinase activity of MusaMPK5, its protein was over expressed in *E. coli* strain *BL21(DE3)* after cloning in *pET28a* vector. Complete lysis by sonication of IPTG induced log phase culture indicated that majority of induced MusaMPK5 protein was in soluble fraction (sonicated supernatant) (Fig. 3.3.9a). Sonicated supernatant (soluble fraction) was used for purification of MusaMPK5 by affinity chromatography using Ni-NTA column (Invitrogen, USA; Cat. No. R90101). An analysis of different fractions on 10% SDS PAGE indicated that MusaMPK5 can be efficiently purified by this procedure as all of the elutions contain only MusaMPK5 (Fig. 3.3.9b). We analyzed the kinase activity of MusaMPK5 using γ-^{32}P-ATP and MBP (myelin basic protein) as a substrate in an *in vitro* reaction. Different elutions of MusaMPK5 used in phosphorylation reaction were able to autophosphorylate themselves and successfully phosphorylate MBP indicating that MusaMPK5 is a functional protein kinase (Fig. 3.3.10a). As MAP kinases are known to regulate the activity of downstream proteins such as transcription factors by phosphorylation, we analyzed whether in an *in vitro* reaction MusaMPK5

can phosphorylate two transcription factors, MusaNAC042 and banana SNAC67. The results suggested that MusaMPK5 was able to phosphorylate both MusaNAC042 and banana SNAC67 indicating that MPK5 probably regulate the transcriptional activity of these NAC domain containing proteins (Fig. 3.3.10b).

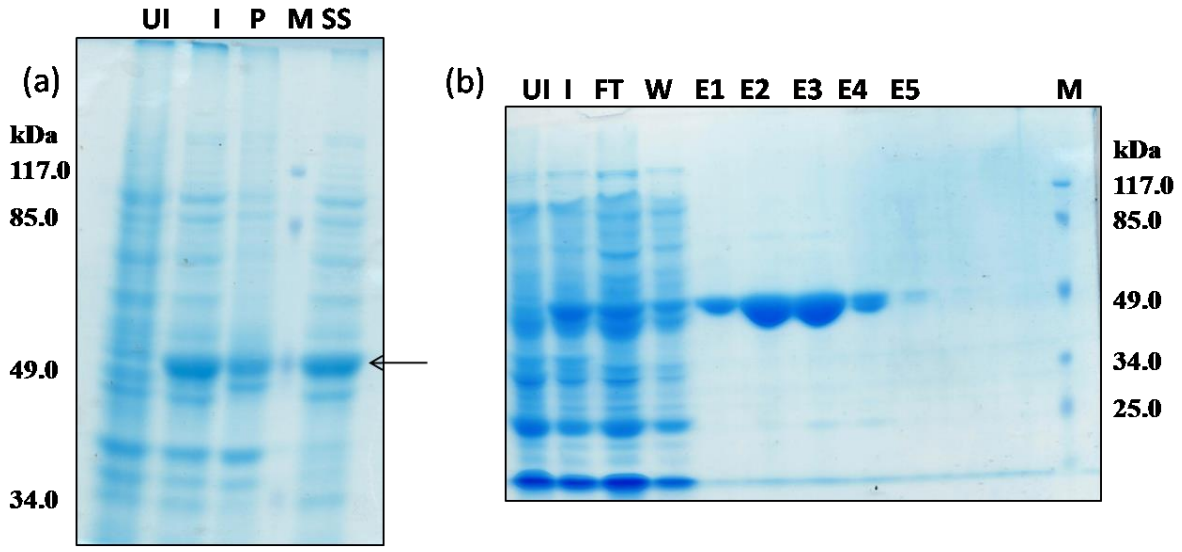


Figure. 3.3.9 Purification of MusaMPK5 protein. (a) Induction and solubility of MusaMPK5 protein. SDS PAGE analysis showing the different fractions obtained after induction of MusaMPK5 in *E.coli BL21 (DE3)* strain (UI: Un induced culture; I: culture induced by 1mM IPTG; P: Pellet obtained after sonication; SS: Soluble supernatant obtained after sonication; M: marker). Note the extent of MusaMPK5 protein in the soluble supernatant fraction (indicated by an arrow). (b) Purification profile of MusaMPK5 after Ni-NTA affinity column chromatography. (FT: Flow through; W: wash; E1-E5:different elutions).

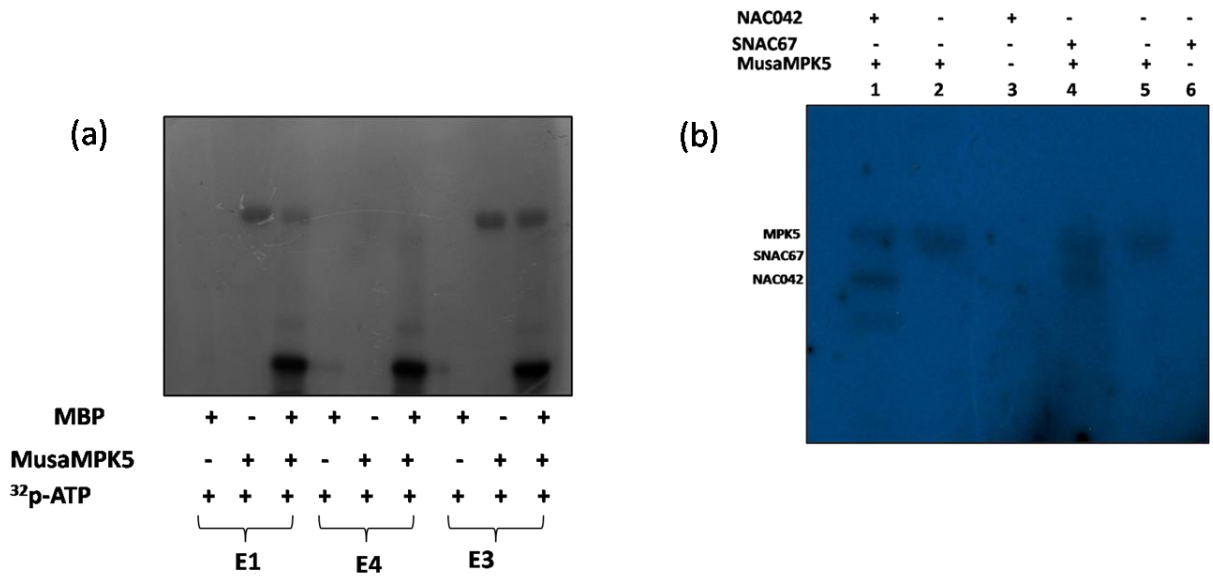


Figure. 3.3.10 Kinase activity of MusaMPK5. (a) Phosphorylation of myelin basic protein (MBP) by MusaMPK5 . Different fractions of purified MusaMPK5 was used for assessing the kinase activity towards MBP using γ-^{32}P-ATP as a phosphate source. (b) Phosphorylation status of NAC042 and SNAC67 by MPK5. MPK5 can phosphorylate banana NAC042 (lane 1) and banana SNAC67 (lane 4) in an in vitro reaction using γ-^{32}P-ATP as a phosphate source. The approximate position of banana NAC042, banana SNAC67 and MusaMPK5 is indicated on the side of the autoradiograph. + and –signs indicates the presence and absence of corresponding component of the reaction.

Discussion

Plants being sessile have to withstand numerous biotic and abiotic stress conditions. Cold is an important abiotic stress condition limiting the cultivation and growth of plants resulting in diminished crop productivity (Chinnusamy et al. 2007). To protect against these adverse conditions, plants have evolved certain protective mechanisms involving changes at physiological, biochemical and molecular levels. At molecular level, expression of a plethora of genes including members of transcription factor families such as *NAC* transcription factors,

Mitogen activated protein kinases, effectors protein such as *dehydrins* get altered in response to diverse stress conditions.

In the present study we have identified a *MAP kinase*, *MusaMPK5* from banana and analyzed its role in regulation of cold stress tolerance. *MusaMPK5* belongs to group-A of *MAPKs* having TEY-motif. Plant *MAPKs* have been divided into two categories, that is either containing TEY- motif or TDY motif. The category containing TEY motif has been divided into A, B and C groups while *MAPKs* having TDY motif have been placed in group D (Zhang et al. 2014).

Not much information is available on the functional analysis of *MAP kinases* in regulation of plants stress responses. However based on the available information, *MAPKs* belonging to group A and B are mainly involved in hormonal, abiotic and biotic stress responses (Jonak et al. 2004; Seo et al. 1999; Ouaked et al. 2003). Moreover, in the phylogenetic analysis MusaMPK5 is clustered with maize MPK5, ZmMPK4 and rice BIMK1 owing to high sequence identity with them. Maize *MPK4* has been reported as a positive regulator of cold stress tolerance by reducing reactive oxygen species and increasing expression of osmolytes such as proline and soluble sugars (Zhou et al. 2012). *MPK5* has been implicated in recovery from cold stress in maize as its expression showed significant early elevation after shift from 5°C to 25°C (Berberich et al. 1999). Moreover, *ZmMPK5* is involved in ABA signaling pathway by regulating antioxidant enzymes indicating its strong role in ABA dependent antioxidant defense mechanism for ROS homeostasis (Lin et al. 2009; Ding et al. 2013). These studies implicate that close homologues of MusaMPK5 have important roles in regulation of cold stress tolerance in plants.

Expression analysis of *MusaMPK5* suggested its positive correlation with stress conditions like cold, application of methyl jasmonate and salicylic acid. Both salicylic acid and methyl jasmonate are involved in abiotic and biotic stress conditions and increased expression of

MusaMPK5 under their influence indicates a possible involvement of *MusaMPK5* in jasmonic acid and salicylic acid signaling (Yi et al. 2016; Khan et al. 2015). Our work has indicated involvement of *MusaMPK5* in cold stress tolerance of banana but its roles in biotic stress signaling remains to be examined. However, *OsBIMK1* a close homologue of *MusaMPK5* is indicated to have a potential role in disease responses as its expression is positively correlated with *Pseudomonas syringae pv. syringae* and chemicals involved in resistance responses (Song and Goodman 2002).

Tissue specific transcriptional regulation of many *MAPKs* has not been documented. $P_{MusaMPK5}$ was active in all the organs of banana however, its activity was maximum in corm and pseudostem. In line with our quantitative RT-PCR analysis, the expression of *GUS* under the control of $P_{MusaMPK5}$ was elevated in different organs of banana after cold stress. Hence, $P_{MusaMPK5}$ can be an important regulatory element for regulated expression of transgene in plants.

We carried out the expression pattern analysis and cloning of *MusaMPK5* from banana cultivar *Karibale Monthan* which posses ABB genome and performed the functional analysis of *MusaMPK5* in banana cultivar *Rasthali*. Banana cultivars with higher number of B genome like cultivar *Karibale Monthan* (harbors ABB genome) are demonstrated to be more stress tolerant than cultivars with less number of B-genomes such as cultivar *Rasthali* (Vanhove et al. 2012; Rukundo et al. 2012).

Our laboratory have a well established transformation and regeneration protocol of banana cultivar *Rasthali*, which we have been utilizing to functionally analyze the stress responsive genes from banana cultivar *Karibale Monthan* (Ganapathi et al. 2001; Tak et al. 2016; Negi et al. 2016). We generated four transgenic banana lines overexpressing *MusaMPK5* and characterized them for stable T-DNA integration by Southern-blot analysis. As this T-DNA

contains a unique site of *Nco*I, hence genomic DNA (digested with *Nco*I) analyzed with a probe against T-DNA directly yields the copy number of T-DNA on autoradiograph. Variation between T-DNA copy number and fold change in transcript abundance of *MusaMPK5* was probably due to different site of integration of T-DNA in transgenic lines, commonly referred to as "position effect". However, all the transgenic lines were morphologically similar to control plants indicating that overexpression of *MusaMPK5* does not alter the growth and phenotype of banana plants further suggesting that *MusaMPK5* can be a potential target to engineer cold stress tolerance in banana.

Alteration in expression of *MusaMPK5* by cold stress treatment, prompted us to analyze the cold stress tolerance ability of different transgenic lines of banana, a highly cold susceptible fruit crop. Cold stress tolerance of transgenic lines by *in-vitro* growth assay, leaf disc assay and stress assay with hardened plants indicated that overexpression of *MusaMPK5* positively regulates the cold tolerance in banana. These observations were based on results indicating better fresh weight gain, higher retention of chlorophyll content and reduced chilling injury area in transgenic lines than control plants. Multiple stress conditions leads to accumulation of proline in plants which have protective actions against protein denaturation and as osmolytes (Surender et al. 2015). The better cold tolerance of transgenic lines than control plants can be attributed to increased proline content and reduced accumulation of MDA content.

Transcriptome analysis of *Fen Jiao*, a significantly higher cold tolerant banana cultivar than *BaXi Jiao* has indicated that transcripts of *MusaMPK5* were significantly enriched in *Fen Jiao* than *BaXi Jiao* in response to cold further corroborating the imperative role of *MusaMPK5* in cold tolerance of banana (Hu et al. 2017). Moreover, there is dearth of information regarding improvement of cold tolerance in banana by genetic engineering approaches. A recent report has

indicated involvement of a banana *MYB* transcription factor, *MpMYBS3* in cold tolerance as transgenic banana lines overexpressing *MpMYBS3* showed improved cold endurance and retained higher proline content during stress exposure (Dou et al. 2016).

MAP kinases regulate their downstream target proteins by phosphorylation hence, we analyzed the putative kinase activity of MusaMPK5 after overexpressing and purification in *E. coli* cells. MBP (Myelin basic protein) has been utilized as a generic kinase substrate for assaying the activity of multiple protein kinases such as LRRK2 (Leucine Rich Repeat Kinase 2) and *Arabidopsis* MPK6 (Lewis 2012; Wang et al. 2016). MusaMPK5 successfully phosphorylate MBP indicating that it indeed encodes a functional kinase capable of regulating the activity of downstream proteins such as transcription factors by phosphorylation.

An analysis of *Arabidopsis* MAPKs target has indicated enrichment of transcription factors belonging to families of *bZIP*, *WRKY*, *homeobox* etc. in the results suggesting that many MAP kinases regulate the physiological and stress responses through these transcription factors (Popescu et al. 2009). Some of the target transcription factors of MAPKs have been reported in the past. Regulation of *WRKY33* and *ethylene response factor6 (ERF6)* has been shown by phosphorylation through *Arabidopsis* MPK3/MPK6 cascade, involved in phytoalexins biosynthesis and expression of defense related genes (Mao et al. 2011; Meng et al. 2013). However, not much information is available on the regulation of *NAC* transcription factors by *MAPKs*. An *in silico* analysis has identified multiple NAC transcription factors as potential target for regulation by *Arabidopsis* MPK3/MPK6 cascade (Avashthi et al. 2014). Furthermore, a recent report indicated phosphorylation of maize NAC84 transcription factor by a calcium/calmodulin-dependent protein kinase, CCaMK for regulation of drought stress tolerance

(Zhu et al. 2016). These reports indicated that *NAC* transcription factors can be potentially regulated by different protein kinases including *MAPKs*.

Our lab has been working on functional analysis of stress responsive *NAC* transcription factors in banana and in previous work it has been established that *MusaNAC042* positively regulates high salinity and drought stress tolerance in banana (Tak et al. 2016). Moreover, study on another stress associated *NAC* transcription factor, *SNAC67* suggested its role in regulation of chlorophyll degradation in banana (Tak et al. 2018). Further, we analyzed whether MusaMPK5 could phosphorylate MusaNAC042 and banana SNAC67 for their functional regulation. Our results showed that MusaMPK5 can phosphorylate banana NAC042 and SNAC67, suggesting a direct regulation of these NAC transcription factors by MusaMPK5. However, further studies in this direction are warranted to shed more insights into the regulation of *NAC* transcription factors by *MAPKs*.

In conclusion, the results have revealed that *MusaMPK5* is a cold stress responsive *MAP kinase* which positively regulates cold stress tolerance in banana. This study indicated the physiological responses of *MusaMPK5* overexpression under cold stress and the data was corroborated by biochemical estimations of total chlorophyll, MDA and proline. Phosphorylation of two stress associated NAC transcription factors by MusaMPK5 indicated its potential to regulate stress responses through transcription factors and provides directions for future studies. This study has not only pointed towards a useful gene for cold stress engineering in plants but will also expand the current knowledge regarding the stress responses regulated by *MAP kinases* in plants.

Chapter 4: Conclusion and future prospective

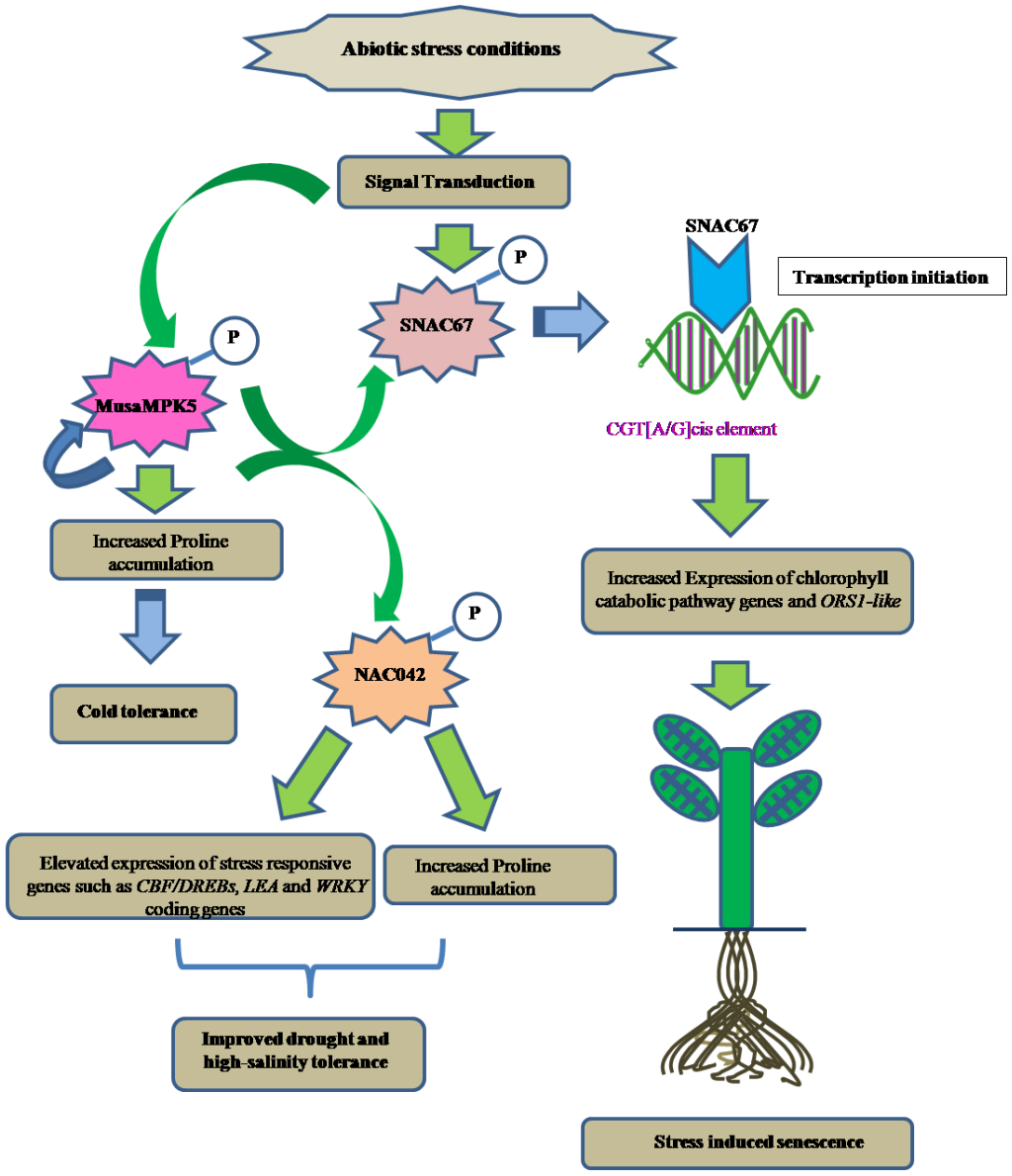
The major conclusions of the present thesis are summarized as below:

1. *MusaNAC042* is a stress responsive *NAC* transcription factor as it is rapidly induced under high salinity and drought stress conditions. MusaNAC042 is a nuclear localized protein and it was demonstrated by transiently overexpressing MusaNAC042::GFP in banana embryogenic cells. Further, the study dealt with morphological and physiological features of *MusaNAC042* overexpression in banana plants. Role of *MusaNAC042* in imparting salinity and drought tolerance was demonstrated in transgenic banana plants and results were supported with elevated proline and reduced MDA content in transgenic lines. Expression pattern of abiotic stress responsive genes suggested potential molecular mechanism of *MusaNAC042* function as well as its transactivation activity. The analyses were corroborated with results from expression profiling, stress tolerance analyses and analysis of promoter region fused to GUS reporter gene. Present study will expand our knowledge about the roles of *NAC* transcription factors in understanding the mechanisms involved in abiotic stress responses in banana. The present analysis can be further substantiated by results obtained from down regulation of *MusaNAC042* using either RNAi mediated suppression of gene expression or recently emerged CRISPR/Cas9 technology.
2. *MusaSNAC67* induces senescence in banana by regulating chlorophyll catabolic genes and by promoting expression of *ORS1*-like by binding to their promoter. It is possible that regulation of age-dependent as well as environmental induced senescence and chlorophyll catabolic genes is mediated by multiple transcription factors acting in a coordinated fashion during and their

precise function and interactions need to be studied. The present study has characterized a stress-induced NAC transcription factor, *MusaSNAC67* with a role in promoting precocious senescence in banana. Future work may be focused on using the candidate gene *MusaSNAC67* for potential manipulation of banana for improved plant productivity via genome editing techniques such as CRISPR/Cas9 technology.

3. *MusaMPK5* is a cold stress responsive *MAP kinase* which positively regulates cold stress tolerance in banana. This study has indicated the physiological role of *MusaMPK5* overexpression in cold stress responses and the data was substantiated by biochemical estimations of total chlorophyll, MDA and proline. Phosphorylation of two stress associated NAC transcription factors by MusaMPK5 indicated its potential to regulate stress responses through transcription factors and provides directions for future studies. These results will increase our knowledge about the cold stress responses in plants and will facilitate engineering plants with improved cold stress tolerance. Further work in the identification of global targets of MusaMPK5 can be carried out to understand the downstream targets of *MusaMPK5* in plants.

   Based on the above conclusions, a model depicting the mode of action and their overexpression effect is postulated below.



Here, it is shown that various abiotic stress conditions leads to activation of banana stress related genes such as *MusaMPK5*, *MusaSNAC67* and *MusaNAC042*. MusaMPK5 phosphorylate MusaSNAC67 and MusaNAC042 for their probable regulation besides improving cold tolerance by increasing the proline content. Banana SNAC67 binds to CGT[A/G] cis-elements in the promoter of chlorophyll catabolic pathway genes and *ORS1-like* gene for transcriptional activation leading to increased stress induced senescence. Overexpression of banana *NAC042* elevated the proline content and transcript level of stress related genes such as *CBF/DREBs, LEA* and *WRKY* coding genes which results in improved drought and high salinity tolerance.

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