Molecular studies on *NAC* transcription factors regulating secondary wall deposition and abiotic stress responses in banana

By

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Recommendations of the Viva Voce Committee

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Sanjana Negi

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.

Sanjana Negi

List of Publications arising from the thesis

Journal

Published

1. **Negi S**, Tak H, Ganapathi TR (2018) A banana NAC transcription factor (MusaSNAC1) impart drought tolerance by modulating stomatal closure and H_2O_2 content. Plant Molecular Biology doi: 10.1007/s11103-018-0710-4
2. **Negi S**, Tak H, Ganapathi TR (2017) Native vascular related NAC transcription factors are efficient regulator of multiple classes of secondary wall associated genes in banana. Plant Science 265: 70-86
3. **Negi S**, Tak H, Ganapathi TR (2016) Expression analysis of *MusaNAC68* transcription factor and its functional analysis by overexpression in transgenic banana plants. Plant Cell Tiss Organ Cult. 25:59–70
4. **Negi S**, Tak H, Ganapathi TR (2015) Functional characterization of secondary wall deposition regulating transcription factors *MusaVND2* and *MusaVND3* in transgenic banana plants. Protoplasma. DOI:10.1007/s00709-015-0822-5
5. **Negi S**, Tak H, Ganapathi TR (2015) Cloning and functional characterization of *MusaVND1* using transgenic banana plants. Transgenic Res. 24:571-85

Other Publications:

Conference/Symposium

1. **Sanjana Negi**, Himanshu Tak, T R Ganapathi (2018) *VND1-VND3* encodes xylem specific transcription factors and regulates multiple classes of secondary wall associated genes in banana. In: DAE-BRNS LIFE SCIENCES SYMPOSIUM 2018. Bhabha Atomic Research Centre, Mumbai, India, 26-28 April, 2018. Pp56.

2. **Sanjana Negi**, Himanshu Tak, T R Ganapathi 2016. *MusaNAC68* transcription factor positively regulates salinity and drought tolerance in banana. In: 37th annual meeting of Plant tissue Culture Association (PTCA-India) & National Symposium on Plant Biotechnology for Crop Improvement. CSIR-National Botanical Research Institute, Lucknow, India, 25-27 February, 2016. Pp46.
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Sanjana Negi

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Sanjana Negi

CONTENTS

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SYNOPSIS OF Ph. D. THESIS

1. **Name of the Student:** Sanjana Negi
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Introduction

The deposition of secondary wall in xylem vessel elements requires coordinated activation of cellulose and lignin biosynthetic pathways. Besides helping in development of water transport channels in plants, secondary wall deposition also provides mechanical strength to support plant mass thus allowing their vertical growth. A systematic analysis of transcription factors regulating lignocellulosic mass and secondary wall deposition improves the understanding of cell wall dynamics including the co-regulation of lignin and other cell wall components. Such understanding could provide novel targets for engineering plant biomass (Yamaguchi and

Demura 2010). The plant biomass is mainly composed of secondary cell wall; hence studies aiming at increasing secondary wall deposition can increase plant biomass (Yang et al. 2013). Furthermore, such improvements in secondary wall deposition can be a potential source to reduce the problem of lodging (Ma 2009; Li et al. 2009; Ookawa et al. 2010). Towards this, characterization of potential genetic factors regulating secondary wall deposition is necessary. Characterization and further manipulation of master regulators of secondary cell wall deposition presents the potential to enhance desired properties of lignocellulosic biomass through altering the expression of a large number of genes. Secondary wall deposition is regulated by a complex network of genetic factors (Zhong and Ye 2007a) including a group of *NAC* domain-containing transcription factors. This group include members like secondary wall associated *NAC* domain protein1 (*SND1*), *NAC* secondary wall thickening promoting factors (*NST1* and *NST2*) and vascular related *NAC* domain factors (*VND6* and *VND7*) which are master regulators of secondary wall deposition. Transdifferentiation of various kinds of cells into tracheary elements was observed in transgenic plants overexpressing either of *VND6* and *VND7* (Kubo et al. 2005). The secondary wall deposition in anther endothecium is regulated by *NST1* and *NST2* (Mitsuda et al. 2005) while that in xylem fibers is regulated by SND1 (Zhong et al. 2006; Zhong et al. 2007b). The banana crop can be a sustainable source for biofuel production because of the fact that banana bears fruit only once in its life cycle, leaving behind a remarkably high amount of unused lignocellulosic biomass (Krishna 1999). Considering that banana can be a potential second-generation biofuel crop, the understanding of regulation of secondary wall deposition in banana is essential.

Productivity of plants is threatened by stress-conditions like high salinity and drought. Stress conditions such as drought and high salinity are external factors reducing the growth and

yield of plants. (Mahajan and Tuteja 2005). High salinity and drought causes hyperosmotic conditions causing cell membrane damage and protein aggregation and denaturation due to excessive production of reactive oxygen species (Greenway and Munns 1980; Hoekstra et al. 2001). Banana is an economically important fruit crop with production of around 106.7 million tonnes per year (FAO,2013) worldwide. Due to morphological features like shallow root and permanent green canopy (Xu et al. 2014; van Asten et al. 2011) banana is sensitive to water stress conditions and requires nearly 2000–2500 mm rainfall throughout the year for optimal productivity (Vanhove et al. 2012). One of plant specific transcription factor family, which is explored for involvement in stress-responses is of *NAC* (*NAM*, *ATAF* and *CUC*) proteins (Fujita et al. 2004; Nakashima et al. 2007). The influx of CO_2 for photosynthesis and transpiration for water loss is regulated by the opening and closing of stomatal aperture formed by guard cells (Hetherington and Woodward, 2003). Stress in plants causes synthesis of abscisic acid (ABA) which induce variety of effects including stomatal closure (Giraudat et al. 1994). ABA induced stomatal closure in leaves is achieved by influx of Ca^{+2} coupled by solute efflux in guard cells (Blatt and Grabov, 1997). H_2O_2 is known to be a second messenger in ABA induced stomatal closure in plants as it resulted in increase in Ca^{+2} content of guard cells by activating Ca^{+2} channels (McAinsh et al. 1996; Pei et al. 2000). ABA induced H_2O_2 generation in guard cells is achieved by activation of NADPH-oxidase through phosphorylation by SnRK2/OST1 (Kwak et al. 2003; Sirichandra et al. 2009). However, other mechanisms regulating H_2O_2 concentration in guard cells impacting the stomatal opening and closing are mostly unknown. Hence, knowledge of NAC transcription factors with roles in regulation of stomatal closure may results in development of transgenic plants with better drought tolerance. Moreover, some of the NAC factors besides involved in drought and high salinity tolerance are known to regulate the auxin

signaling and roots development. *Arabidopsis NAC1* (Xie et al. 2000) and *NAC2* (He et al. 2005) are involved in auxin-signaling and root development. Rice overexpressing *NAC5* have enlarged root and improved drought tolerance with higher grain yield (Jeong et al. 2013). Hence, studies on stress responses regulated by NAC transcription factor will result in elucidation of the mechanisms of the *NAC* factors in banana. Such studies will also lead to development of transgenic banana lines with better potential than wild type banana for mitigating the adverse effects of abiotic stress conditions.

Objective of the thesis

1. Understanding the mechanism of secondary cell wall deposition in banana.

2. Development of transgenic banana with enhanced drought and salinity tolerance.

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: Studies on *MusaVND1*, *MusaVND2* and *MusaVND3* genes from banana
Chapter 4: Studies on *MusaSNAC1*, a stress associated NAC transcription factor from banana
Chapter 5: Studies on *MusaSNAC68*, a stress associated NAC transcription factor from banana
Chapter 6: Conclusion and future prospective

Chapter I [General introduction and review of literature]

This chapter will provide a brief introduction about the importance functions and roles in different aspects of plants played by *NAC* transcription factors. A special emphasis on the roles of *NAC* transcription factors in context of secondary cell wall deposition and abiotic stress

tolerance will be given. This chapter will also deal with the importance of characterizing the NAC transcription factors in banana.

Chapter II [Material and Methods]

This chapter describes the materials and experimental methodologies used for the studies. Details of bioinformatics analysis, vectors, generation of recombinant vectors, transformation procedures, analysis of transgenic plants including histological observations, biochemical estimations and microscopic analysis, protein overexpression and purification methods and DNA gel shift analysis procedure will be described here.

Chapter III [Studies on *MusaVND1*, *MusaVND2* and *MusaVND3* genes from banana]

This chapter will describe and discuss the characterization of three vascular related *NAC* domain genes from banana by analyzing the overexpression studies, tissue specific regulation of the 5' upstream regulatory region and DNA- protein binding studies.

Three vascular related *NAC* domain genes (*MusaVND1*, *MusaVND2* and *MusaVND3*) from banana were identified by *insilico* analysis and their complete coding sequences were amplified from cDNA of banana tissue. *MusaVND1*, *MusaVND2* and *MusaVND3* encodes a nuclear localized protein as their GFP fusion protein gets localized to nucleus. Transient overexpression of either of *MusaVND1*, *MusaVND2* or *MusaVND3* converts banana embryogenic cells to xylem vessel elements, with a final differentiation frequency of 33.54%, 63.5% and 23.4%. Transgenic banana plants overexpressing either of *MusaVND1*, *MusaVND2* or *MusaVND3* showed transdifferentiation of various types of cells in to xylem vessel elements and ectopic deposition of lignin in cells of various plant organs such as leaf and corm. Tracheary element formation was seen in the cortical region of transgenic corm as well as in epidermal cells and mesophyll cells of leaves. Biochemical analysis indicated significantly higher levels of lignin

and cellulose content in transgenic banana lines overexpressing either of *MusaVND1*, *MusaVND2* or *MusaVND3* than control plants. Transgenic banana harboring either $P_{MusaVND1}$*::GUS*, $P_{MusaVND2}$*::GUS* or $P_{MusaVND3}$*::GUS* showed specific GUS staining in lignified tissues including xylem vessel and tracheids and their GUS activity in different organs was estimated. Purified MusaVND1, MusaVND2 and MusaVND3 proteins in gel shift assay bind to 19-bp secondary-wall NAC binding element (*SNBE*) while it fails to bind mutated *SNBE*. Putative *SNBE* sites in the 5'-upstream regulatory region of important secondary-wall associated genes related to cell-wall modification (*IRX1*/*CesA8*, *IRX3*/*CesA7*,*IRX5*/*CesA4*, *IRX8*, *IRX10* and *IRX12*) and transcriptional regulation (*MYB52*, *MYB85*, *MYB58*/*72*, *MYB46*, and *MYB83*) in banana was identified and mobility of these regulatory regions got retarded by MusaVND1, MusaVND2 and MusaVND3. Transcript level of these important secondary wall associated genes were elevated in transgenic banana overexpressing either *MusaVND1*, *MusaVND2* or *MusaVND3*. Present study suggested promoters with prospective utilization in wall modification in banana and suggest a complex transcriptional regulation of secondary wall deposition in plants.

Chapter IV [Studies on *MusaSNAC1*, a stress associated NAC transcription factor from banana]

Results obtained during characterization of *SNAC1* genes from banana are described in this chapter. Guard cells of the stomata control photosynthesis and transpiration by regulating CO_2 exchange and water loss, thus affecting growth and crop yield. Roles of NAC (NAM, ATAF1/2 and CUC2) protein in regulation of stress-condition has been documented however, their control over stomatal aperture is largely unknown in different plant species. We identified a banana NAC transcription factor, MusaSNAC1 which induced stomatal closure by elevating H_2O_2 content in guard cells during drought-condition imparting durable drought tolerance.

Expression profiling under abiotic stress conditions and analysis of *GUS* (*β-glucuronidase*) activity under *MusaSNAC1* promoter was carried out. Roles of *MusaSNAC1* in drought was confirmed by malondialdehyde estimation, frequency of closed stomata, staining of H_2O_2 in guard cells and relative water content estimation. Purified MusaSNAC1 protein was used in gel shift assay for determination of MusaSNAC1 binding site and its transactivation activity. Overexpression of *MusaSNAC1* in banana resulted in higher number of stomata closure causing reduced water loss and thus elevated drought-tolerance. MusaSNAC1 regulate multiple stress-related genes by binding to core site of NAC-proteins CGT[A/G] in their 5'-upstream region. Results obtained in this study demonstrate a mechanism of drought tolerance through stomatal closure by H_2O_2 generation in guard cells, regulated by NAC-protein in banana. This report suggests a potential role of *NAC* transcription factor for improving the drought tolerance ability in plants.

Chapter V [Studies on *MusaSNAC68*, a stress associated NAC transcription factor from banana]

This chapter describes the results obtained after overexpression of *MusaSNAC68* in transgenic banana. Expression analysis of *NAC68* during stress conditions in banana suggested its positive association to stress conditions. The 5'-proximal region of *MusaNAC68* was isolated and sequence analysis indicated presence of stress related *cis*-elements and *cis*-elements involved in auxin-signaling. Expression of *MusaNAC68* was maximum in roots and positively correlated with application of α-naphthaleneacetic acid. Nuclear localization of *Musa*NAC68 was determined by fusion of green-fluorescent protein with *Musa*NAC68 and transiently overexpressing in banana embryogenic cells. Transgenic lines were marginally taller and displayed more abundant roots than control along with altered expression of auxin-responsive

genes like auxin-responsive factors (*ARFs*) and *IAA/Aux* (Indoleacetic acid-induced protein) genes. Transgenic lines showed better tolerance to stress induced by NaCl and mannitol and produced more shoot biomass. Leaf disc assay showed that transgenic lines retain more chlorophyll and lower malondialdehyde than control under salinity and drought. Transgenic line constitutively overexpressing *MusaNAC68* showed elevated expression of many stress-responsive genes. These data suggest that *MusaNAC68* is a stress responsive gene in banana and is involved in salinity and drought tolerance.

Chapter VI [Conclusion and future prospective]

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

1. *MusaVND1*, *MusaVND2* and *MusaVND3* are efficient regulator of secondary wall development in banana plants and their overexpression induces ectopic secondary wall deposition and transdifferentiation of various cells into xylem vessel elements. However, constitutive expression of these genes resulted in growth retardation and thus reduction in plant biomass. Overexpression of genes under xylem specific promoter will results in a tighter control over their expression and thus will be useful for suitable genetic engineering of plant biomass.
2. Vascular tissue specific activity of banana *VND1*, *VND2* and *VND3* genes was demonstrated by transforming banana with either $P_{MusaVND1}$*::GUS*, $P_{MusaVND2}$*::GUS* or $P_{MusaVND3}$*::GUS* and analyzing the GUS staining. We quantified the activity of $P_{MusaVND1}$, $P_{MusaVND2}$ and $P_{MusaVND3}$ by estimating the GUS activity which indicated differential activity of these regulatory regions in different organs of banana. Such studies are important for generating transgenic plants with tighter control of desired gene expression or for tissue specific expression. A number of *SNBE-like* sites was detected in the 5'upstream regulatory region of multiple secondary wall associated genes suggesting common regulatory mechanism governing these genes. Banana VND1-3 could

specifically bind to *SNBE-like* sequences in a dose dependent manner, and similar binding was observed with 5'upstream regulatory region of *MYB* transcription factors (*AtMYB52-like*, *AtMYB85-like*, *AtMYB58*/*72-like*, *AtMYB46-like* and *AtMYB83-like*) and cell wall modification related genes (*AtIRX1*/*AtCesA8-like*, *AtIRX3-like*, *AtIRX5-like*, *AtIRX8 -like*, *AtIRX10* and *AtIRX 12-like*). Transcript level of these genes were highly elevated due to overexpression of banana *VND1-3* indicating a direct regulation of these genes by banana *VND1-3*. Banana VND1-3 could bind to SNBE like sequences in isolation or as a part of a regulatory region and failed to bind a mutated SNBE sequence, such studies employing a competition assay with a non specific competitor will give better result of DNA binding activity of VND1-3.

3. Study on *MusaSNAC1* suggested its role in controlling drought-response by integrating H_2O_2 induced stomatal closure and simultaneous regulation of multiple stress-responsive genes. Functional analysis of *MusaSNAC1* during drought progression was demonstrated in transgenic banana overexpressing *MusaSNAC1* as well as banana harboring $P_{MusaSNAC1}$*::GUS* and results were supported with histochemical and biochemical analysis, gene expression profiling and protein-DNA binding studies. Present study will not only expand our knowledge about *NAC* transcription factors in drought response but also promote the utilization of *SNAC1* gene for potential crop improvement in future. However, the exact identification and regulation of its target gene on a global basis needs to be carried out by integrating transcriptome sequencing analysis, DNA binding assays and transient transactivation assay of regulatory regions.
4. *MusaNAC68* is rapidly induced by stress conditions. Transgenic banana overexpressing *MusaNAC68* have longer and more abundant roots and altered expression of auxin-responsive genes (*Aux/IAA* and *ARFs*). Role of *MusaNAC68* in conferring salinity and drought tolerance in transgenic banana was demonstrated and elevated transcript level of stress-related genes in

transgenic banana indicated transactivation activity of *Musa*NAC68. Present study also suggests existence of a cross-talk between stress tolerance and, root development and, coordinated regulation of these two aspects by *MusaNAC68*. More studies on regulation of target genes by *MusaNAC68* need to be carried out by transcriptome sequencing analysis and DNA binding activities of MusaNAC68 protein.

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Publications in Refereed Journal:

a. Published

1.Negi S, Tak H, Ganapathi TR (2016) Expression analysis of *MusaNAC68* transcription factor and its functional analysis by overexpression in transgenic banana plants. Plant Cell Tiss Organ Cult. 25:59–70.

2.Negi S, Tak H, Ganapathi TR (2015) Functional characterization of secondary wall deposition regulating transcription factors *MusaVND2* and *MusaVND3* in transgenic banana plants. Protoplasma. DOI:10.1007/s00709-015-0822-5

3. Negi S, Tak H, Ganapathi TR (2015) Cloning and functional characterization of *MusaVND1* using transgenic banana plants. Transgenic Res. 24:571-85

b. Communicated:

1. **Negi S**, Tak H, Ganapathi TR (2017) A banana NAC transcription factor (*MusaSNAC1*) impart drought tolerance by modulating stomatal closure and H_2O_2 content. Submitted to **Plant Molecular Biology.**
2. **Negi S**, Tak H, Ganapathi TR (2017) Native vascular related NAC transcription factors are efficient regulator of multiple classes of secondary wall associated genes in banana. Submitted to **Plant Science.**

Other Publications:

a. Conference/Symposium

1. **Sanjana Negi**, Himanshu Tak, T R Ganapathi 2016. *MusaNAC68* transcription factor positively regulates salinity and drought tolerance in banana. In: 37th annual meeting of Plant tissue Culture Association (PTCA-India) & National Symposium on Plant Biotechnology for Crop Improvement. CSIR-National Botanical Research Institute, Lucknow, India, 25-27 February, 2016. Pp46.

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Chapter 1: General introduction and review of literature

Plants being sessile suffers from a host of envoirmental stress conditions which are broadly classified into abiotic and biotic stress conditions. Abiotic stress conditions such as drought and high salinity reduce the plant growth and reproductivity and in turn crop output. As a counter measures to these stress conditions, plants has evolved numerous dynamic processes at physiological, biochemical and molecular levels including changes in expression of multiple stress related genes to ensure survival and reproductivity under such stress conditions (Pérez-Rodríguez et al. 2010). *NAC* (*NAM*: No apical meristem; *ATAF*: *Arabidopsis* transcription activation factor; *CUC* Cup-shaped cotyledon) transcription factors belong to one of the largest family of plant specific transcription factors with 117 members in *Arabidopsis*, 151 in rice 163 in poplar, 152 in soybean and tobacco, 79 in grapes and 26 members in citrus (Le et al. 2011; Nuruzzaman et al. 2010, 2012; Hu et al. 2010; Rushton et al. 2008). Recent studies on roles of *NAC* transcription factors in plants have suggested their involvement in embryo development (Duval et al., 2002), shoot apical meristem formation (Kim et al. 2007b), secondary walls deposition (Zhong et al. 2010), flower development (Sablowski and Meyerowitz 1998), leaf senescence (Guo et al. 2006), auxin signaling (Guo et al. 2005) and biotic and abiotic stress responses (Olsen et al. 2005a; Tran et al. 2004; Nakashima et al. 2012).

NAC proteins contains a highly conserved N-terminal NAC domain and a variable C-terminal region required for transcriptional activity regulation. NAC domain is divided into five subdomains (A-E) and it contribute towards DNA binding (Subdomain D-E), formation of homodimer or heterdimers and nuclear localization of NAC proteins (Ooka et al. 2003; Olsen et

al. 2005b). The C-terminal divergent region regulates the transcriptional activity determining the nature of NAC proteins as activator or repressor towards target genes. It also contains transmembrane motifs for localization to plasma membrane or endoplasmic reticulum as in case with membrane associated NAC transcription factors (Seo and Park 2010). On the basis of NAC domain sequence similarity, NAC family has been classified into two groups comprising of 18 subgroups (Ooka et al. 2003). Group I is comprised of 14 subgroups (TERN, ONAC022, SENU5, NAP, AtNAC3, ATAF, OsNAC3, NAC2, ANAC011, TIP, OsNAC8, OsNAC7, NAC1, and NAM) while group II is comprised of four subgroups (ANAC001, ONAC003, ONAC001, and ANAC063) suggesting vast diversity of these NAC proteins for different physiological functions (Ooka et al. 2003).

Expression of *NAC* genes is mainly regulated at the level of transcription by multiple cis-elements like stress related cis-elements like *ABRE* (*ABA responsive elements*), *DREs* (*Dehydration responsive elements*) or *AuxRE* (*Auxin responsive element*) among others. Post transcription, the regulation of NAC transcription factor activity is mediated by microRNAs, alternative splicing, or post translational modifications like ubiquitinization and phosphorylation (Nakashima et al. 2012; Puranik et al. 2012).

NAC proteins regulate their target genes by binding to promoter region at certain consensus sequences known as NAC recognition sequence (*NACRS*). *NACRS* contain the core sequence CACG which was identified in the promoter of *Arabidopsis ERD1* gene (Tran et al. 2004). Earlier reports found that the core binding site of NAC protein consists of CGT[A/G] consensus sequence and demonstrated binding of many NAC proteins like ANAC055 (Tran *et al* 2004), TaNAC69 (Xue, 2005), ANAC019, ANAC092 (Olsen *et al*. 2005b), GmNAC11 and GmNAC20 (Hao *et al*. 2011) to this sequence. Some other DNA sequence have also been

reported as the binding site of NAC proteins for example *Arabidopsis* calmodulin-binding NAC, 'CBNAC' could bind to GCTT site (Kim *et al*. 2007a) while NAC factors involved in secondary wall deposition recognize and bind to a 19bp site known as secondary wall NAC binding element (*SNBE*) (T/A)NN(C/T) (T/C/G)TNNNNNNNA(A/C)GN(A/C/T) (Zhong et al. 2010). Surrounding sequences of *NACRS* may provide the binding specificity of certain NAC transcription factors in promoters of different downstream genes which can be broadly grouped into genes with regulatory functions involved in signal transduction or regulation of gene expression and genes encoding for effector proteins involved in functions like protein folding and stabilization, reactive oxygen species detoxification, osmolyte production among others (Puranik et al. 2012).

Drought and salinity stress triggers alterations in multiple responses of plants including gene expression, plant growth, reproduction ability and crop productivity. Multiple NAC genes have been reported to be involved in drought and salinity responses. The first *NAC* gene identified was *RD26/ANAC072* (*RESPONSIVE TO DEHYDRATION 26*) which was involved in ABA dependent stress-response pathway (Fujita *et al*. 2004). Three *Arabidopsis NAC* genes, *ANAC019*, *ANAC055* and *ANAC072* are induced by salinity, drought as well as ABA and could impart drought tolerance in transgenic *Arabidopsis* (Tran *et al*. 2004). Many NAC proteins regulate the formation of the reactive oxygen species (ROS) which are important signaling molecules. *AtNTL4* regulate *Atrboh* genes inducing ROS production and senescence (Lee *et al*. 2012). *SNAC3* from rice was induced in response to drought, salinity, ABA and high temperature and its overexpression increased tolerance to high temperature as well as drought (Fang *et al*. 2015). Overexpression of rice *SNAC3* could increase the expression of ROS-scavenging genes and three ROS-associated enzyme genes were the direct targets of SNAC3 (Fang *et al*. 2015). *AtJUB1*, a

H_2O_2 induced gene delays senescence by lowering intracellular H_2O_2 levels and regulates the *DREB2A* gene by binding to its promoter (Wu *et al*. 2012). A NAC transcription factor from rice, *SNAC1* was induced in guard cells by drought and imparted drought tolerance through increased stomatal closure (Hu *et al*. 2006). Rice *SNAC1* regulates a SRO-protein, *OsSRO1c* which is predominantly expressed in guard cells under stress-conditions (You *et al*. 2013). Overexpression of *OsSRO1c* caused H_2O_2 accumulation in guard cells reducing the number of completely open stomata and thus lowering transpiration induced water loss conditions (You *et al*. 2013). Nearly 167 potential members of NAC family have been identified in banana after a detailed analysis of its genome sequence (Cenci et al. 2014). There is a scarcity of information related to roles of *NAC* transcription factors in banana especially in context of stress tolerance. Six banana *NAC* transcription factors namely *MaNAC1-MaNAC6* were studied for their role in ethylene signaling and *MaNAC1*/*MaNAC2* were indicated for their probable role in fruit ripening via interaction with components of ethylene signaling pathway (Shan et al. 2012). Banana *NAC* transcription factor, *MaNAC5* is involved in disease response and interact with banana *WRKY* gene, *MaWRKY1* and *MaWRKY2* for regulation of *PR* genes expression during disease response (Shan et al. 2015). Another banana *NAC* transcription factor *MaNAC1* is a cold responsive gene and interact with ICE1-CBF signaling pathway for induction of possible cold tolerance in banana fruit (Shan et al. 2014). Because a large number of NAC genes are present in plants and functions of many of them are not known, hence study of their expression pattern under stress conditions may provide identification of NAC factors with putative roles in abiotic and biotic stress conditions. Regulation of members of NAC family along with other stress related genes during abiotic stress conditions ensure establishment of a complex network for regulation of stress conditions. The important roles played by NAC genes in plants during abiotic stress conditions makes them

suitable candidate for generation of transgenic plants with improved drought and high salinity tolerance. Keeping this in view, we have identified two putative NAC genes from banana genome database on the basis of expression profiling and later characterized them by overexpression in transgenic banana plants.

Cell wall is a structural feature of plant cells and is composed of biopolymers like cellulose and provides mechanical support and protection to the plant cell. On the basis of cell type and composition, cell wall has been classified into two types: primary cell wall and secondary cell wall. Primary cell wall is present in all types of plant cells is thin and stretchable compared to secondary cell wall and thus regulates cell shape and size by controlling the cell elongation (Geitmann 2010). Secondary wall also contain lignin in addition to primary wall components which are cellulose, hemicelluloses and pectin and is present in specialized cells like cells of xylem tissue, anther endothecium and in cells of valve margin required for seed dispersal (Barros et al. 2015). Secondary wall is deposited between primary cell wall and cell membrane and provides the cell with rigidity and hydrophobicity in case of xylem cells (Nakano et al. 2015). Evolution of secondary cell wall deposition is a prominent feature which not only provides the mechanical strength required for the vertical growth of plants but also help in the long distance transport of water and minerals. Water transport in plants from roots to other organs occurs through the channels of xylem tissue composed of tracheids and vessel elements. These tracheids develop an array of secondary wall depositions in the form of reticulate, pitted, helical and annular thickenings. Secondary cell wall deposition is regulated and highly coordinated for systematic deposition of multiple components by many genes among which a subgroup of *NAC* transcription factors, *VNDs* (*vascular related NAC transcription factors*) are most important. Secondary cell wall deposition in tracheids is accompanied with programmed

cell death, hence regulation of the activity of secondary cell wall associated genes in specialized cells is of prime importance for proper functioning and homeostasis of plants. In *Arabidopsis*, roles of vascular related NAC domain containing transcription factors (*VND1-VND7*) has been documented showing their imperative roles in secondary wall deposition (Zhou et al. 2014; Yamaguchi et al. 2010). One of the pioneering work showed that *Arabidopsis VND6* and *VND7* are master regulators of secondary wall deposition as their overexpression resulted in transdifferentiation of cells into tracheids (Kubo et al. 2005). However additional work on *VND1-VND5* from *Arabidopsis* has established that these genes also carry the potential to independently trigger the secondary wall deposition and tracheids differentiation (Zhou et al. 2014). Apart from *VND1-VND7*, other members of *NAC* group involved in secondary wall deposition have also been characterized. Secondary wall associated *NAC* domain protein1 (*SND1*) and *NST1/2*, have been shown as an independent master regulator of secondary wall deposition and it functions in differentiation of xylem fibres in *Arabidopsis* (Zhong et al. 2006; Zhong et al. 2007). Another two *NAC* transcription factors, *NST1* and *NST2* have been shown to regulate the secondary wall thickening in anther endothecium (Mitsuda et al. 2005). A common motif in the promoters of secondary wall associated genes for their regulation have been identified and named as *secondary-wall NAC binding element* (*SNBE*)- motif (Zhong et al. 2010; Ohashi-Ito et al. 2010). Further, secondary wall associated transcription factors, *VND6*, *VND7* and *SND1* have been shown to regulate the expression of their downstream target genes by *SNBE*-like motif (Zhong et al. 2010; Ohashi-Ito et al. 2010). The sequence of *SNBE* motif has been worked out as an imperfect palindrome of 19bp with consensus sequence *(T/A)NN(C/T)(T/C/G)TNNNNNNNA(A/C)GN(A/C/T) (A/T)* and has been identified in the 5'upstream regulatory regions of multiple downstream target genes (Zhong et al. 2010).

Arabidopsis SND1 , *VND6*, *VND7*, *NST1* and *NST2* have been shown to regulate the expression of downstream genes like *MYB46*, *MYB83*, *MYB103*, *SND3* and *KNAT7* through such *SNBE*-like motifs in their promoter region (Zhong et al. 2010). Furthermore, *Arabidopsis SND1* , *VND6* and *VND7* also regulate the expression of programmed cell death related gene like *XCP1*(tracheary elements related *cysteine proteases*) through direct binding to *SNBE* motif (Zhong et al. 2010). These finding suggest that *SNBE*-like motifs are common regulatory *cis*-element for direct regulation of secondary wall associated genes.

Thus for understanding the importance of vascular related *NAC* domain transcription factors in regulation of secondary wall deposition in banana, we identified three *VNDs* from banana genome database and later characterized these genes based on overexpression effects in transgenic banana plants, tissue specific activity of the 5'upstream regulatory region and DNA-protein binding studies.

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

1. Expression profiling of two stress related *NAC* transcription factors from banana under different stress treatments.
2. Generation of binary vectors for two stress related *NAC* transcription factors and their utilization for regeneration of transgenic banana plants overexpressing these *NAC* transcription factors.
3. Generation of transgenic banana plants overexpressing three vascular related *NAC* transcription factors.
4. Analysis of transgenic banana plants for either stress tolerance or ectopic secondary wall deposition.

Chapter II : Material and Methods

Plant material and growth conditions

Embryogenic suspension culture of *Musa* cultivar *Rasthali* (AAB) have been established from shoot tips in our laboratory as described earlier (Ganapathi et al. 2001). This embryogenic cell suspension is routinely maintained in our laboratory in M2 medium which is liquid MS medium (pH 5.3) supplemented with 2,4-D (1 mg/l), glutamine (100 mg/l), biotin (1 mg/l), malt extract (100 mg/l) and sucrose (45 g/l) (Cote et al. 1996). Shoots of banana are multiplied on banana shoot multiplication medium comprising of MS medium (pH 5.7) supplemented with benzyl adenine (2mg/l) and adenine sulphate (30mg/l) and sucrose 30gm/l. Individual shoots are rooted on rooting medium (MS medium with NAA (1mg/l) for regenerating complete plantlets. Cloning of stress related NAC transcription factors was carried out from banana cultivar *Karibale Monthan* (ABB) as cultivars with more number of B-genome are considered to be more tolerant than cultivars with A- genome (Robinson and Sauco 2010; Rukundo et al. 2012; Thomas et al. 1998).

Phylogenetic analysis of MusaVND1, MusaVND2 and MusaVND3

Complete coding sequences of poplar *NAC* domain containing genes, *PtVNS01* (BAK14358.1), *PtVNS02* (BAK14359.1) and *PtVNS03* (BAK14360.1) sequences were used for homology searching using tBLASTx search in banana genome database (http://banana-genome.cirad.fr/) and sequence with highest blast score was retrieved. The banana genome loci identifier with highest blast score were designated as *MusaVND1* (GSMUA_Achr8T12100_001), *MusaVND2* (GSMUA_Achr11T03040_001) and *MusaVND3* (GSMUA_Achr11T17510_001) respectively. The selected sequences were then included for BLASTp search in NCBI database for retrieving high scoring sequences of well characterized genes from poplar and *Arabidopsis*. The

phylogenetic analysis was carried out after including sequences of other NAC domain containing transcription factors well characterized for their function in secondary wall development in poplar and *Arabidopsis*. ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with default parameters was then used for aligning different retrieved sequences. A Neighbor joining tree (a bootstrap values with 1000 replicates) was built using MEGA6 software (Kumar et al. 2008). Multiple sequence alignment of *Musa*VND1, *Musa*VND2 and *Musa*VND3 was carried out with characterized homologues from poplar and *Arabidopsis* using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with default parameters. Box shading of conserved amino acid residues was carried out using online available box shade server (http://www.ch.embnet.org/software/BOX_form.html). The homologues used for multiple sequence alignment were identified from NCBI database using BLASTp algorithm.

Construction of *MusaVND1, MusaVND2* and *MusaVND3* overexpression vector

The complete coding sequence of *MusaVND1, MusaVND2* and *MusaVND3* were amplified using cDNA prepared from corm of banana cv. *Rasthali*. Total RNA from banana tissue was isolated using Concert plant RNA reagent (Invitrogen, USA) and further cleaned using RNA binding column of RNeasy plant mini kit (Qiagen, Germany). Total RNA was used for cDNA synthesis with thermoscript AMV-RT (Invitrogen: Cat. No.12236-014) following the manufacturer's protocol. The amplified coding sequences were cloned under the control of *Zea mays* poly ubiquitin promoter in plant overexpression vector *pCAMBIA1301*. For this, the *nos* 3' UTR was first cloned in *Sac*I and *Eco*RI sites of MCS (multiple cloning site) generating *pCAMBIA1301-nos*. Further, in the *pCAMBIA1301-nos*, a three way ligation was performed for cloning the *Zea mays* poly ubiquitin promoter and the amplified coding sequences employing the *Hind*III, *Pst*I and *Kpn*I in the MCS. The cloned coding sequence of *MusaVND1, MusaVND2*

and *MusaVND3* were subsequently sequenced and the sequence information was deposited in NCBI database (KT356872, KP335047 and KP666170 respectively). Further, the generated binary vector (*pCAMBIA1301-ZmUbi-MusaVND1*, *pCAMBIA1301-ZmUbi-MusaVND2* and *pCAMBIA1301-ZmUbi-MusaVND3*) was transformed into the *Agrobacterium tumefaciens* strain *EHA105* employing electroporation.

Amplification of 5' up-stream region of *MusaVND1*, *MusaVND2* and *MusaVND3* and generation of binary vectors

Isolation of genomic DNA was carried out from leaf tissue of banana cv Rasthali according to the protocols given with GenElute Plant Genomic-DNA Miniprep-Kit (Sigma,USA). 5'up-stream region was amplified with 2X PCR master mix (Thermofisher scientific) and using specific primers and purified genomic DNA. Cycling conditions followed was: 98°C(5') , 38-cycles of 98°C(1') , 57°C(1'), 72°C(1.5') and 72°C(20min). 5'up-stream region of *MusaVND1* ($P_{MusaVND1}$) was digested with *Xba*I and *Bsp*HI and ligated with *Xba*I and *Nco*I digested *pCAMBIA1301* generating $P_{MusaVND1}$*::GUS*. Similarly $P_{MusaVND2}$ (5'up-stream region of *MusaVND2*) was digested with *Pst*I and *Bgl*II and ligated upstream of GUS in *pCAMBIA1301* to generate $P_{MusaVND2}$*::GUS*. 5'up-stream region of *MusaVND3* ($P_{MusaVND3}$) was digested with *Bam*HI and *Nco*I, and ligated with *Bam*HI and *Nco*I digested *pCAMBIA1301* generating $P_{MusaVND3}$*::GUS*. All the constructs were sequenced to confirm the integrity of PCR and ligation reaction. Sequences generated are deposited in the NBCI database as MF347605 (for $P_{MusaVND1}$), MF347603 (for $P_{MusaVND2}$) and MF347604 (for $P_{MusaVND3}$).

Transient transformation of banana cv. *Rasthali* embryogenic cells

Transient transformation of binary vector *pCAMBIA1301-ZmUbi-MusaVND1, pCAMBIA1301-ZmUbi-MusaVND2* or *pCAMBIA1301-ZmUbi-MusaVND3* was carried out with modifications

as described earlier (Ganapathi et al. 2001). *Agrobacterium* strain *EHA105* harboring either of the binary vector mentioned above or vector control *pCAMBIA1301* was resuspended in liquid M2 media supplemented with 100µM ACS (acetosyringone) and was further cocultivated with banana embryogenic cells. After cocultivation of 30 minutes, the cells were aspirated onto glass fiber filters and subsequently cultured on M2 media supplemented with 100 µM ACS. The cells were observed daily for formation of xylem vessel elements. After three days of cocultivation in dark at 27 °C the cells were scraped from glass fiber filter and transferred to liquid M2 media supplemented with cefotaxime (400mg/l) and further incubated in dark. The cells were observed for the formation of xylem vessel elements at different time points.

Generation of transgenic banana plants overexpressing *MusaVND1*, *MusaVND2* and *MusaVND3*

Banana genetic transformation was carried out essentially as described earlier (Ganapathi et al. 2001). Around 0.5 ml embryogenic cells were co-cultivated with *Agrobacterium tumefaciens* strain EHA105 for 30 minutes. Further, the cells were aspirated onto glass fiber filters and transferred to semi-solid M2 medium (Cote et al. 1996) for three days in dark at 24^{o}C. After this, the cells along with the filters were transferred to M2 medium supplemented with Cefotaxime (400mg/l) and incubated in light. After three days, the cells were scraped from the filters and cultured on banana embryo induction medium (BEM) supplemented with cefotaxime (400 mg/l) and hygromycin (5 mg/l). A culture of two to three weeks resulted in the development of somatic embryos which were further subcultured for three rounds on BEM for selection. The developing somatic embryos were then transferred onto MS medium supplemented with BA (0.5 mg/l) for development of shoots from the somatic embryos. The shoots were transferred to shoot multiplication medium (Ganapathi et al. 2008) for the generation of multiple shoots. The shoots

were individually isolated from the multiple shoots and cultured on rooting medium (MS medium added with NAA (1mg/l) for regenerating complete plantlets. The rooted plantlets were hardened in the green house.

Subcellular localization of *Musa*VND1, *Musa*VND2 and *Musa*VND3 protein

The complete coding sequence of *MusaVND1* and *MusaVND2* was cloned in frame at the N-terminal end of the *GFP* in the binary vector *pCAMBIA1302* using *Bgl*II and *Spe*I restriction enzymes. Similarly *MusaVND3* coding sequence was cloned in frame with *GFP* in binary vector *pCAMBIA1302* using *Bam*HI and *Spe*I restriction enzymes. The binary vector so generated (*pCAMBIA1302- MusaVND1, pCAMBIA1302-MusaVND2* and *pCAMBIA1302-MusaVND3*) were transformed via electroporation into *Agrobacterium tumefaciens* strain *EHA105*. Further, the transformed *Agrobacterium* was utilized for transiently transforming banana cultivar *Rasthali* embryogenic cells as described above. Three days after transformation and co-cultivation on semi solid M2 medium, the cells were observed for GFP fluorescence employing a fluorescent microscope (Eclipse 80i, Nikon, Japan) for establishing the cellular localization of *Musa*VND1-GFP, *Musa*VND2-GFP and *Musa*VND3-GFP fusion proteins. The position of nucleus in the cells was ascertained using DAPI stain dissolved in phosphate buffer saline (1 $\mu g\ ml^{-1}$).

Histology analysis of transgenic banana plants

Toluidine blue staining was done with 0.1% toluidine blue-O solution in 100mM, pH 7.0 phosphate buffer saline (PBS) for 2 minutes and further rinsed with distilled water. For lignin autofluorescence, the cells and sections were first washed with 100 mM PBS (pH 9.0) and then observed under ultra violet excitation at 365 nm in a fluorescent microscope (Eclipse 80i, Nikon, Japan) and confocal laser-scanning microscope (Carl Zeiss, Germany). Before staining the histological sections were incubated over night in 90% methanol to remove chlorophyll.

Biochemical estimation of lignin and crystalline cellulose

Updegraff method (Updegraff 1969) was followed for estimation of cellulose content of control and transgenic shoots. In short, matrix polysaccharide of the samples was removed by acetic acid-nitric acid solution after which digestion with 67% sulphuric acid was carried out. The released glucose was estimated using anthrone reagent and the absorbance was recorded at 620 nm. Thioglycolic acid reagent method (Lange et al. 1995) with certain modifications was used for lignin estimation. Approximately, 300 mg grounded shoot tissue was resuspended in 1.5 ml methanol and stirred for one hour followed by centrifugation at 12,000 rpm for 10 minutes. The resulting pellet was stirred for 20 minutes sequentially with 2 ml methanol (two times), 1M NaCl, 1% SDS, milli-Q water (two times), ethanol and chloroform/methanol (1:1 v/v). The solution was then centrifuged at 12,000 rpm for 10 minutes. The pellet obtained represents the cell wall and was further dried over night at 60°C in an oven. Further, approximately 10 mg of dried cell wall was treated with 300 µl of thioglycolic acid and 1.2 ml of 2 M HCl followed by heating at 95 °C for four hours. Further, the samples were cooled to room temperature followed by centrifugation at 12,000 rpm for 15 minutes. The resulting pellet containing the lignin-thioglycolic acid complex (LTGA) was washed with milli-Q water (three times). Extraction of LTGA was achieved by resuspending the pellet in one ml of 0.5M NaOH and stirring over night. The supernatant obtained after centrifugation at 12,000 rpm for 10 minutes was stored in a vial. The resulting pellet was washed with 0.5 ml of 0.5 M NaOH. Following centrifugation, the two alkali extracts were mixed together. The extracts were then acidified with 300 µl of concentrated HCl. The LTGA was recovered by centrifugation at 12,000 rpm for 10 minutes after precipitating the LTGA at 4 °C for four hours. The resulting brown color pellet was further

dissolved in 3 ml of 0.5 ml NaOH and the absorbance was recorded at 280 nm after appropriate dilutions.

Molecular analysis of transgenic banana plants overexpressing *MusaVND1*, *MusaVND2* and *MusaVND3*

Genomic DNA from shoots of putative transgenic banana plants and control plants was isolated using GenElute Plant Genomic DNA Miniprep Kit (Sigma, USA). The genomic DNA was further used for PCR amplification of *hygromycin phosphotransferase* (*hpt*) gene (marker gene within the T-DNA) to establish the integration of T-DNA in the genome of putative transgenic shoots. PCR conditions used were 94°C for 3 min for initial denaturation followed by 35 cycles of amplification with each cycle consisting of the following steps: 94°C for 40 s, 56°C for 45 s, and 72 °C for 1 min followed by a final extension of 10 min at 72°C. The PCR product was analyzed on 1% agarose gel. Southern blot analysis was performed as described previously with modifications (Schaewen et al. 1995) to determine the copy number of the T-DNA transferred to transgenic shoots. About 20 µg of *Kpn*I digested genomic DNA was resolved on 1% agarose tris-acetate electrophoresis (TAE; pH 8.0 Tris-acetate buffer). Capillary transfer of digested genomic DNA onto hybond-N nylon membrane (Amersham, cat. no. RPN.203N) was carried out using 10× saline sodium citrate (SSC; 0.045 M trisodium citrate and 0.45 M sodium chloride) buffer. DNA immobilization onto nylon membrane was carried out for 30 minutes at 120 °C. DIG high prime DNA labeling and detection starter kit (Roche, Germany) was employed for DIG labeling of the DNA probe. In short, 1.5 µg of denatured PCR amplified *hpt-II* coding sequence was mixed with 4 µl of DIG high prime supplied with the kit and the labeling of the probe was carried out for 5 hours at 37 °C. DIG Easy Hyb granules supplied with the DIG labeling kit was used for overnight hybridization at 42 °C. Stringency washes under agitation were carried out at

room temperature (using 2X SSC added with 0.1% sodium dodecyl sulfate) and at 62 °C (using 0.5X SSC with 0.1 % SDS). Blocking of the membrane was done using the blocking solution (10X) provided with the above mentioned kit. Hybridization of the DIG-labeled probe was detected using a 1:5,000 dilution of alkaline phosphatase labeled anti-DIG antibody. Further, the hybridization signal was detected by chemiluminescence according to manufacturer's protocol supplied with kit (Roche, Germany). The fold change in the transcript level of *MusaVND1, MusaVND2* and *MusaVND3* in different transgenic lines, due to overexpression was estimated by quantitative real time RT-PCR analysis. Total RNA was isolated as described above and the cDNA prepared was diluted 1:50 with water and further used for real time PCR analysis. The expression of *Musa EF1α* (housekeeping gene) was used for normalization of different Ct values.

Quantitative RT-PCR

Total RNA from leaf tissue of control and different overexpressing plants was isolated using Concert plant RNA reagent (Invitrogen, USA) and further purified by RNA binding column of RNeasy plant mini kit (Qiagen, Germany) during which the traces of genomic DNA contamination was removed by on-column DNAase-digestion (Qiagen, Cat. No.79254) as instructed by manufacturer. Tissue of three independent uniform plants were mixed in equal amount and then utilized for RNA isolation. Integrity of RNA was checked on agarose gel electrophoresis before cDNA synthesis. First strand cDNA was synthesized using thermoscript AMV-RT (Invitrogen: Cat. No.12236-014) as per the protocol supplied with the kit. Quantitative RT-PCR was performed with 2x SYBR Green Jump Start Taq Ready Mix (Sigma, USA) and gene specific primers with 1:50 diluted cDNA. Quantitative RT-PCR was performed on a rotor gene Q platform (Qiagen, Germany) using the conditions of 95°C(4') , 32-cycles of 95°C(15'') ,

57°C(20''), 72°C(20'') followed by a melt curve analysis. Data was normalized against the expression of Banana *EF1α* which has been used as a reference gene. Fold change in transcript level of different genes was calculated by analyzing the data by comparative Ct method ($2^{-\Delta\Delta Ct}$) described earlier (Schmittgen and Livak 2008).

Protein induction and affinity purification of MusaVND1, MusaVND2 and MusaVND3

Complete coding sequence of *MusaVND1*, *MusaVND2* and *MusaVND3* was cloned in *pET28a*-vector in *Nde*I and *Bam*HI sites resulting in fusion of a 6x histidine tag at the N-terminal end of the protein. The recombinant vector was transferred into *E.coli* cells *BL21(DE3)* and the recombinant protein was induced in bacteria growing at 37°C for three hours by 1mM IPTG. Inclusion bodies in the pellet was collected and later solubilized by 0.2% N-lauroylsarcosine for 24hours (Tao et al. 2010). Solubilized fraction was collected by centrifugation (14000 rpm, 30min) which was later used for replacing the N-lauroylsarcosine with , 0.4% triton-X100 and 0.4%CHAPS for 2hours at 4°C. The lysate was then centrifuged at 14000 rpm for 30min to collect the soluble fraction which was then used for affinity purification of recombinant protein using Ni-NTA resin. Ni-NTA resin in column was equilibrated with equilibration buffer (50mM Tris-buffer, pH8 with 250mM NaCl, 10%glycerol) and then loaded with soluble fraction and the flow through was collected. The column was washed with washing buffer (50mM Tris-buffer, pH8 with 250mM NaCl, 50mM imidazole and 10%glycerol) and wash fraction was collected. Elution of the recombinant protein was carried out with 250mM imidazole and different elution fractions were collected. All the fractions were analyzed on 12% SDS-PAGE and the elution containing pure proteins was dialyzed against dialysis buffer (50mM Tris-buffer and 10%glycerol). The recombinant protein was detected by western-blotting using primary antibody against polyhistidine which was later detected by a secondary-antibody conjugated with alkaline-

phosphatase. The color development on PVDF membrane was carried out with NBT/BCIP as suggested by the manufacturer protocol (Sigma USA; 11681451001).

DNA Gel-shift assay (EMSA) of MusaVND1, MusaVND2 and MusaVND3

The 5'upstream regulatory region of different secondary wall related genes was amplified from genomic DNA of banana cv Rasthali using specific primers and PCR conditions 98°C(10') , 35-cycles of 98°C(1') , 55°C(1'), 72°C(30'') and 72°C(10min). Complementary oligonucleotides containing *SNBE* and mutated *SNBE* sites were synthesized by outsourcing. Complementary oligonucleotides was annealed to form ds-DNA by heating at 90°C and then cooling to 37°C in a thermal cycler. Pure protein at different concentration was incubated with DNA substrates with EMSA buffer (10mM Tris-buffer, pH8, 5mM MgCl2 and 10mM KCl). The reaction after 20mintues was resolved on 1.5% agarose-gel and the results were recorded in a gel documentation system.

Stress treatments and expression profiling

Uniform plants of banana cultivar *Karibale Monthan* growing in green house were subjected to high salinity (250mM NaCl), drought (dehydrating on blotting paper), cold (10±2 °C), salicylic acid (2mM), Abscisic acid (100µM ABA) and H_2O_2 (2mM). Drought treatment was given by drying plants on a blotting paper in laminar air flow hood. Cold treatment was carried out at 10±2 °C. At different time points total RNA was isolated using RNeasy plant mini-kit (Qiagen, Germany). On-column DNase (Qiagen, Germany) treatment was performed to remove traces of genomic-DNA contamination. Preparation of first strand cDNA with ThermoScript™ Reverse-Transcriptase (Invitrogen, USA) carried out as per vendor instructions. Expression of banana *EF1α* (housekeeping gene) was used for normalization of C_t-values. Tissues from three

independently treated samples were mixed together during RNA isolation. The real time RT-PCR reactions were performed in triplicates.

Generation of binary vectors for overexpression of *MusaSNAC1* and characterization of 5' upstream regulatory region

Coding sequence and 5'-upstream sequences of *MusaSNAC1* were amplified from banana cv. *Karibale Monthan.* Coding sequence was amplified as follows: 2X PCR master mix (Thermofisher scientific, Catalog number: K0171) was diluted to 1X with water, 5µL cDNA and 20pmols of primers and used in PCR conditions, 94°C(5min), 35 cycles of 95°C(40sec) , 55°C (60sec), 72°C(60sec) and 72°C(10min). Genomic-DNA was isolated from leaves of *Karibale Monthan* using GenElute Plant Genomic-DNA Miniprep-Kit (Sigma,USA) as per manufacturer protocol. 5'-up-stream region of *MusaSNAC1* was amplified using 2X PCR master mix (Thermofisher scientific, Catalog number:K0171) with cycling conditions as follows: 94°C(8min) , 35-cycles of 94°C(50sec) , 55°C(60sec), 72°C(60sec) and 72°C(100min). *pCAMBIA1301* was digested with *Sac*I and *Eco*RI and similarly digested *nos 3'-UTR* was ligated to generate (*pCAMBIA1301-nosT*). *pCAMBIA1301-nosT* was then digested using *Hind*III and *Kpn*I and ligated with *pZmUbi* (promoter of maize polyubiquitin digested with *Hind*III and *Pst*I) and *MusaSNAC1* (digested with *Pst*I and *Kpn*I) in three-way fashion to generate *pCAMBIA1301-pZmUbi- MusaSNAC1-nosT*. 5'-up-stream region of *MusaSNAC1* ($P_{MusaSNAC1}$) was digested with *Pst*I and *BspHI* and ligated upstream of *GUS* in *pCAMBIA1301* digested with *Pst*I and *Nco*I. Sequence generated has been deposited in NCBI data bank as MF347602 (for *MusaSNAC1* coding sequence) and MF347601 (for $P_{MusaSNAC1}$ sequence).

Sequence analysis of MusaSNAC1

A neighbor-joining tree (1000 bootstrap-replicates) after multiple sequence-alignment of MusaSNAC1 obtained by clustal-omega software was built with MEGA6 (Kumar et al. 2008). Box-shading of multiple sequence-alignment was carried out at box-shade server (http://www.ch.embnet.org/software/BOX_form.html). Cis-element analysis in 5'-upstream region was carried out at PlantCARE, JASPAR and PantPan2 database.

Generation and confirmation of transgenic banana lines overexpressing *MusaSNAC1*

Agrobacterium-strain *EHA105* harboring either *pCAMBIA1301-*$P_{MusaSNAC1}$*-GUS* or *pCAMBIA1301-pZmUbi-MusaSNAC1-nosT* was selected on LB-kanamycin (50mg ml^{-1}) plate. Recombinant *Agrobacterium* was induced by acetosyringone and used for genetic transformation of banana cv *Rasthali* as described previously (Ganapathi et al. 2001). Briefly, after co-cultivation of embryogenic cells and *Agrobacterium* for 30min, cells were aspirated and grown on M2-medium for three days (Cote et al. 1996). Banana embryo development medium supplemented with cefotaxime (400mg l^{-1}) and hygromycin (5mg l^{-1}) was used for embryo development and later fully developed embryos was converted into shoots on BA (0.5mg l^{-1}) supplemented medium. Positive GUS stained banana lines growing in green house was used for genomic-DNA isolation with Plant Genomic-DNA Kit (Sigma,USA;G2N350). PCR amplification of *hpt-II* (*hygromycin phosphotransferase*) from genomic-DNA was carried out at 55°C annealing temperature using PCR master mix (Thermofisher). Copy number of T-DNA insertions was detected by Southern-blot analysis. Briefly, genomic-DNA was digested with *Kpn*I for 15hours and then resolved on 0.8% agarose gel. After capillary transfer of DNA onto nylon membrane (Amersham, Catalogue number RPN.203N) with 10x SSC buffer (0.45M NaCl and 0.045M tri- sodium citrate; pH 7.0) , the DNA was cross-linked to membrane by UV-

exposure and then probed with digoxin labeled probed against *hpt-II* coding sequence. Probe preparation, washing and probing of the membrane was carried out as per instructions with DIG labeling kit (Roche, Germany, catalogue number. 11585614910). Hybridization of probe with T-DNA on membrane was allowed for 12hours at 42°C. Stringency washes was carried out with 2X SSC at room temperature and then 0.5X SSC at 65°C. Chemiluminescence signal was observed as per manufacturer's instruction (Roche, Germany). Overexpression in transgenic lines was confirmed by quantitative RT-PCR analysis of *MusaSNAC1* as described above and the data was normalized by the expression of banana *EF1α*. RNA was isolated from three independent plants of each line and quantitative RT-PCR was performed with three replications.

Purification and activity of MusaSNAC1

MusaSNAC1 was ligated under T7-promoter in *pET28a*-vector using *Nde*I and *Bam*HI sites and recombinant vector was transferred to *E.coli BL21 codon-plus. E.coli* was induced by 1mM IPTG when cell density reached approximately 0.6OD. After sonication, inclusion bodies collected by centrifugation were solubilized by 0.2% N-lauroylsarcosine for 24hours (Tao et al. 2010). N-lauroylsarcosine was replaced with 0.4% triton-X100 and 0.4%CHAPS for 2hours. Solubilized MusaSNAC1 protein was purified on Ni-NTA column. Two ml of Nickel resin slurry was washed with 25 bed volume of binding-buffer (50mM Tris-buffer, pH8 with 250mM NaCl, and 10%glycerol) and then soluble fraction was applied. After collection of flow-through, washing was carried out with binding buffer containing 50mM imidazole. Column bound protein was eluted with binding-buffer with 250mM imidazole which was later removed by dialysis. A 12% SDS-PAGE analysis was carried out to check purification profile. Detection of purified MusaSNAC1 by western blot was carried out with anti-polyhistidine antibody linked with

alkaline-phosphatase enzyme and the chromogenic reaction was carried out with NBT/BCIP substrate (Sigma USA; 11681451001).

Gel-shift assay for MusaSNAC1-DNA binding studies

Gel-shift assay was initiated by incubating MusaSNAC1 with DNA substrates in reaction buffer (10mM Tris-buffer, pH8, 5mM MgCl2 and 10mM KCl). The protein-DNA binding was allowed for 20minutes at 25°C, and then analyzed by resolving the reaction on 1.5% agarose-gel stained with ethidium bromide. Different oligonucleotides used was annealed by heating the complementary strands at 90°C and then slowly cooling at room temperature. 5'-upstream region containing CGT[A/G] motif of MusaSNAC1 target genes was PCR amplified from genomic-DNA of banana.

Drought tolerance assay of transgenic banana overexpressing *MusaSNAC1*

Uniform plants of control and transgenic lines of same age was used for drought challenge and recovery assay. Drought was initiated by withdrawing the water for 14 days and the stress-symptoms were recorded. The relative water content (RWC) and MDA content were measured. MDA content was estimated by thiobarbituric-acid method. Homogenized leaf tissue was heated to 95°C (15min) in TBA(0.5%) with TCA(20%). After cooling, absorbance of the supernatant was recorded at 600nm and 532nm. After reducing the reading of 600nm with 532nm reading, the MDA content was calculated by extinction-coefficient of $155 mM^{-1} cm^{-1}$ (Heath and Packer, 1968). Relative water content was estimated in the youngest fully expanded leaves using method described earlier (Gaxiola et al. 2001). H_2O_2 was detected in guard cells by staining the banana leaf with 50µM of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 10min in dark. Fluorescence signal in control and transgenic leaves was detected under similar conditions in a fluorescence enabled microscope (Eclipse 80i, Nikon, Japan). H_2O_2 content of the leaves after

drought stress was estimated as described previously (Loreto and Velikova, 2001). Experiments were repeated at least three times and biochemical estimations were performed with three replications.

Expression of stress related genes in transgenic banana overexpressing *MusaSNAC1*

Many putatively stress-related genes were identified from NCBI and banana genome-database and their expression was quantified by quantitative RT-PCR assay as described above. At least expression of 60 *WRKY*, 90 *CBF/DREB* (dehydration-responsive element-binding proteins), 16 *TIFY* factors , 30 *LEA* and seven genes coding for antioxidant proteins was analyzed. RNA was isolated from three independent plants of each line and quantitative RT-PCR was performed with three replications.

Cloning of *MusaNAC68* in *pCAMBIA1301*

The complete coding sequence of *MusaNAC68* was amplified from leaf cDNA of green house grown banana cv. *Karibale Monthan* using the PCR conditions: 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 55°C for 45 sec and 72°C for 60 seconds followed by final extension of 10 minutes at 72°C. The *nos* 3' UTR was first cloned in *Sac*I and *Eco*RI sites of MCS multiple cloning site (MCS) of vector *pCAMBIA1301* and then the recombinant vector was digested with *Hind*III and *Kpn*I. The coding sequence of *MusaNAC68* (*Pst*I and *Kpn*I digested) and *Zea mays* poly-ubiquitin promoter (*Hind*III and *Pst*I digested) was cloned in a three way fashion in the MCS of the above digested recombinant vector (*pCAMBIA1301-nos*) to generated the *pCAMBIA1301- MusaNAC68*.

Bioinformatics analysis of *Musa*NAC68

The *Musa*NAC68 sequence (Gene bank accession AKI29370) was used for BLASTp search in NCBI database and high scoring sequences of well characterized and annotated genes from

different plant species were retrieved. ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and MEGA5 software (Kumar et al. 2008) were used to construct a Neighbor joining tree (bootstrap values with 1000 replicates). Multiple sequence alignment of *Musa*NAC68 with well annotated NAC proteins from different plant species was carried out using ClustalW2 with default parameters and box shading of the conserved residue was carried out using online available box shade server (http://www.ch.embnet.org/software/BOX_form.html). For identifying *cis*-elements in 5'- upstream region of *MusaNAC68* coding region, the 5' proximal region (Gene bank accession KP861890) was subjected to online available plant *cis*-acting regulatory DNA elements (PLACE) database (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

Subcellular localization of *Musa*NAC68

MusaNAC68 was cloned in frame with *GFP* in the binary vector *pCAMBIA 1302* using the *Bgl*II and *Spe*I restriction sites, so as to generate a translational fusion of *MusaNAC68* and *GFP*. Banana cv *Rasthali* embryogenic cells were transiently transformed by *Agrobacterium tumefaciens* strain *EHA105* harboring recombinant binary vector (*pCAMBIA1302- MusaNAC68*) using the previously established banana transformation protocol (Ganapathi et al. 2001). After five days of transformation, the GFP fluorescence was monitored using a fluorescent microscope (Eclipse 80i, Nikon, Japan) and the nuclei was identified by staining with Hoechst 33258.

Generation of transgenic banana plants overexpressing *MusaNAC68*

Transformation of banana cv *Rasthali* embryogenic cells was carried out with *Agrobacterium tumefaciens EHA 105* harboring *pCAMBIA1301- MusaNAC68* as described earlier (Ganapathi et al. 2001). The cocultivation of *Agrobacterium* and embryogenic cells (0.5 ml settled volume) was carried out for 30 minutes after which the cells were aspirated onto glass fiber filters before

transferring to semisolid M2 medium (Cote et al. 1996). After three days incubation in dark the cells were cultured onto M2 medium supplemented with Cefotaxime (400mg/l) and incubated in light for three days. The selection and growth of transformed banana cells were carried out on banana embryo induction medium (BEM) supplemented with cefotaxime (400 mg/l) and hygromycin (5 mg/l). Well developed embryos were cultured for shoot emergence on MS medium with BA (0.5 mg/l). Multiple shoots of putative transgenic lines on shoot multiplication medium (Ganapathi et al. 2008) were grown individually on rooting medium composed of MS supplemented with NAA (1mg/l) for completed plantlet regeneration.

Confirmation of transgenic plants overexpressing *MusaNAC68*

Genomic DNA of control and putative transgenic banana plants isolated using GenElute Plant Genomic DNA Miniprep Kit (Sigma, USA) was subjected to PCR amplification of *hygromycin phosphotransferase* (*hpt-II*) gene (present within T-DNA region), to confirm the T-DNA integration. PCR conditions were : 94°C for 4 minutes followed by 35 cycles of 94°C for 40 s, 56°C for 45 s, and 72°C for 1 minute followed by 10 min at 72°C. Number of T-DNA integration in genome of different lines was analyzed by Southern blot. The *Kpn*I digested genomic DNA (20 μg) resolved on 0.8 % agarose tris-acetate electrophoresis was transferred to hybond-N nylon membrane (Amersham, cat. no. RPN.203N) using 10X saline sodium citrate (SSC; 0.45M NaCl and 0.045M trisodium citrate, pH 7.0) buffer. Digoxigenin (DIG) labeled probe prepared using denatured coding sequence of *hpt-II* and DIG high prime DNA labeling and detection starter kit (Roche, Germany, cat. no. 11585614910) was hybridized with the membrane overnight at 42 °C. Stringency washes was carried out at room temperature (2X SSC) and at 65 °C (0.5X SSC). Probe hybridization was detected using anti-DIG antibody labeled with alkaline phosphatase and the blot was developed by chemiluminescence as indicated in the kit

protocol (Roche, Germany). For analyzing the overexpression of *MusaNAC68* in different transgenic lines, the cDNA preparation was carried out as described above and the fold change in the transcript level of *MusaNAC68* was determined by quantitative real time RT-PCR. Banana *EF1α* was used as housekeeping gene.

Leaf disc assay

Leaf disc (1 cm diameter) were incubated on either of 250 mM NaCl or 250 mM mannitol in $1/10^{th}$ MS medium with 16/8 hours light and dark establishment at temperature of 27±1° C. The stress treatment was carried out for five days with NaCl and for six days with mannitol. During the period of the treatment, the visual injury to the leaf disc was recorded and, the MDA content and total chlorophyll content were measured. Total chlorophyll estimation was carried out after extraction with 80% acetone (Arnon 1949). The lipid peroxidation (in terms of malondialdehyde) was estimated by thiobarbituric-acid method. Briefly, the homogenized leaf tissue was incubated with 0.5% TBA in 20% TCA (95°C, 15 minutes) and the reaction supernatant was collected by centrifugation (12000 g, 10 minutes) after terminating the reaction on ice. The absorbance at 600nm was subtracted with the absorbance at 532 nm, and the MDA content was estimated with the extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

In vitro* salt and drought assay of transgenic lines overexpressing *MusaNAC68

The *in vitro* salinity and drought tolerance of transgenic lines was carried out by culturing *in vitro* grown shoots of equal age and size onto rooting media (MS media containing 1 mg l^{-1} NAA) supplemented with either 250 mM NaCl or 250 mM mannitol. After subjecting the shoots to stress for 10 days, the recovery of the shoots was monitored by growth on plain rooting medium for 25 days. The weight of plants before maintenance on stress medium and after

culturing on stress free medium was recorded and growth in the form of fresh weight was calculated.

Real time RT-PCR analysis for quantification of gene expression in transgenic banana overexpressing *MusaNAC68*

The expression of different genes was analyzed in control plant and highest overexpressing transgenic line L5. The real time RT-PCR analysis was carried out with 1:50 diluted cDNA and SYBR Green Extract-N-Amp PCR Ready Mix (Sigma) with Rotor-Gene Q platform (Qiagen, Germany).The normalized expression value was derived using $2^{-\Delta Ct}$ (Target gene) and the fold value change of a particular target gene was derived by dividing the $2^{-\Delta Ct}$ value of transgenic lines by $2^{-\Delta Ct}$ value from control plant. The ΔCt is Ct (Target gene) −Ct (*EF1α*) where, *EF1α* is used as housekeeping gene. The fold value change of target gene in control was kept at the value of one.

Chapter III : Studies on *MusaVND1*, *MusaVND2* and *MusaVND3* genes from banana

Introduction

For centuries, plant fibers are being used as renewable resource for energy generation as well as for pulp and paper production. The two principal sclerenchymatous cells of plants are xylem vessels and xylary fibers (Plomion et al. 2001). Biochemical composition of xylem remarkably influences pulp yield, quality and its digestibility (Ona et al. 2001). Xylem consist of two types of water conducting elements, tracheids and vessel elements. Secondary wall depositions in tracheids can be in the form of annular, helical, reticulate or pitted thickening. Secondary wall also contains lignin in addition to primary wall components which are cellulose, hemicelluloses and pectin.

NAC (*NAM*, *ATAF* and *CUC*) family is one of the largest transcription factor family in plants (Aida et al. 1997; Olsen et al. 2005), which includes 110 members in *Arabidopsis* and 140 members in rice, and till now only a few have been identified and characterized for their diverse functions in plants. Secondary wall deposition is regulated by a complex network of genetic factors (Zhong and Ye 2007) including a group of *NAC* domain-containing transcription factors. This group include members like secondary wall associated *NAC* domain protein1 (*SND1*), *NAC* secondary wall thickening promoting factors (*NST1* and *NST2*) and vascular related *NAC* domain factors (*VND6* and *VND7*) which are master regulators of secondary wall deposition. Earlier reports have shown the promoter activity of different vascular related *NAC* domain factors (*VND1-VND7*) in developing xylem vessel elements (Kubo et al. 2005). Transdifferentiation of various kinds of cells into tracheary elements was observed in transgenic plants overexpressing

either of *VND6* and *VND7* (Kubo et al. 2005). The secondary wall deposition in anther endothecium is regulated by *NST1* and *NST2* (Mitsuda et al. 2005) while that in xylem fibers is regulated by a *SND1* (Zhong et al. 2006; Zhong et al. 2007). The downstream targets of such *NAC* domain containing transcription factors are certain *MYB* transcription factors which can activate genes related to secondary wall biosynthesis (Zhong et al. 2008). Transcription factor *MYB46* in *Arabidopsis* is a direct target of *SND1* and can induce the secondary wall deposition (Zhong et al. 2007a). *Arabidopsis* transcription factors *MYB58* and *MYB63*, activates lignin biosynthesis genes at the time of secondary wall formation (Zhou et al. 2009).

Analysis of poplar genes (*PtVNSs*) homologous to vascular development related *NAC* domain transcription factors of *Arabidopsis*, revealed similar secondary wall deposition potential of these poplar genes (Ohtani et al. 2011). The release of banana genome sequence allowed us to identify banana *NAC* domain containing transcription factors homologues in sequence to poplar *PtVNS01-07* genes. The study of poplar vascular related *NAC* domain containing transcription factors has placed *PtVNS01-07* genes among *Arabidopsis VND* group in the phylogenetic tree. Going by this analogy we have named the banana *NAC* domain containing gene homologues to *PtVNS01-07* as *MusaVND1-VND7* respectively. The present study was aimed at characterization of three vascular related NAC domain genes of banana and to investigate whether these banana genes have potential to induce secondary wall development when overexpressed in transgenic banana plants.

Cell wall modification is an emerging field of study as cell wall is a renewable source of energy and pulp production (Ona et al. 2001). Characterization of genetic factors provides a suitable way for cell wall modification, however controlling the expression of such genetic factors is warranted for suitable outcome in this direction as constitute overexpression resulted in

biomass reduction (Yang et al. 2013). Characterization of potential regulatory elements including 5'upstream sequences of xylem specific genetic factors in economically important crops for a tighter control over expression of cell wall regulating genes is essential (Yang et al. 2013; Zhong et al. 2006). Hence, there is a need for characterization of promoter elements with xylem specific activation as these regulatory sequences can be utilized for potential manipulation of secondary cell wall.

Regulation of secondary cell wall depositions and xylem differentiation has been studied in detail in *Arabidopsis*. A group of secondary wall associated *NAC* transcription factors (*SWNs*) in *Arabidopsis*, like *secondary wall associated NAC domain protein1* (*SND1*), *vascular related NAC domain factors* (*VND1-VND7*) and *secondary wall thickening promoting factors* (*NST1* and *NST2*) can cause ectopic secondary wall deposition and thus are key regulators of secondary wall formation in xylem fibers, xylem vessels and anther endothecium (Zhong et al. 2006; Mitsuda et al. 2008; Kubo et al. 2005; Zhou et al. 2014). *Arabidopsis* VND6, VND7 and SND1 directly regulate the expression of many downstream target genes by directly binding to *SNBE* sites in their promoters (Zhong et al. 2010; Ohashi-Ito et al. 2010). In a pioneering study, the consensus binding site of SND1 and other SWNs was identified to be a 19bp imperfect palindrome (*SNBE*) having consensus sequence *(T/A)NN(C/T)(T/C/G)TNNNNNNNA(A/C)GN(A/C/T) (A/T)* which is present in the promoters of multiple downstream target genes thus providing a similar regulatory mode upon these target genes (Zhong et al. 2010). Multiple genes are regulated by *Arabidopsis* SND1 suggesting a complex network leading to activation of effector genes of secondary wall biosynthesis (Zhong et al. 2008). *Arabidopsis MYB46*, *MYB83*, *MYB103* are directly regulated by SND1 during secondary wall deposition (Zhong et al. 2007a). *MYB46* and *MYB83* function redundantly in

secondary wall deposition as their induction caused secondary wall formation in non-parenchymatous cells (Zhong et al. 2007a; McCarthy et al. 2009). *Arabidopsis* SND1 and other SWNs including VND7 regulate *MYB58/72* and *MYB63* which can activate the lignin biosynthesis pathway (Zhou et al. 2009). *Arabidopsis* SND1 also regulate *MYB52* and *MYB85* which are regulators of secondary wall biosynthetic genes leading to secondary wall deposition (Zhong et al. 2008). *Arabidopsis MYB48/59* was induced in xylem during secondary wall formation suggesting its potential role in regulation of secondary wall deposition (Oh et al. 2003). This suggest that *SND1* and other *SWNs* are master regulators regulating downstream *MYB* targets which in turn regulate other *MYB* factors and downstream effector genes of secondary wall deposition. Development of xylem involve secondary wall formation as well as programmed cell death during which tracheary elements specific cysteine proteases (*XCP1* and *XCP2*) are activated (Bollhöner et al. 2012). During secondary wall deposition, *XCP1* and *XCP2* are direct targets of SWNs like *Arabidopsis* VND7 and *Brachypodium* SWN5 (Yamaguchi et al. 2011; Valdivia et al. 2013). *Arabidopsis* VND7 also directly regulate a number of genes involved in cellulose deposition like *irregular xylem* genes like *IRX1*, *IRX2*, *IRX5* and *IRX8* (Yamaguchi et al. 2011). Thus these studies suggest that direct targets of *Arabidopsis* VND6, VND7 and SND1 and their binding towards *SNBE* has been determined which may be common in different plant species because of possible evolutionary conservation in mechanism of secondary cell wall development. However, binding affinity of VND1-VND3 towards SNBE and their direct targets are yet to be ascertained as they have been shown to independently trigger the secondary wall deposition (Zhou et al. 2014; Zhong et al. 2010).

The banana crop can be a sustainable source for biofuel production because of the fact that banana bears fruit only once in its life cycle, leaving behind a remarkably high amount of

unused lignocellulosic biomass (Krishna 1999). Considering that banana can be a potential second-generation biofuel crop, the understanding of regulation of secondary wall deposition in banana is essential. This chapter discusses the characterization of three vascular related *NAC* transcription factors (*MusaVND1, MusaVND2* and *MusaVND3*) and analyzed their probable role in regulating vascular development including secondary wall deposition by utilizing transgenic banana plants. The imperative role of *MusaVND1, MusaVND2* and *MusaVND3* in regulating vascular development was proved by their overexpression in transgenic banana, analyzing the tissue specific expression of their regulatory region and analyzing their transactivation activity on secondary cell wall associated genes in banana.

Results

Phylogenetic analysis of *Musa*VND1, *Musa*VND2 and *Musa*VND3

Phylogenetic analysis indicated that *Musa*VND1, *Musa*VND2 as well as *Musa*VND3 showed high homology with poplar PtVNS01-07 and *Arabidopsis* AtVND1-7 proteins **(Fig. 3.1).** *Musa*VND1 has identities of 64% to PtVNS01, 63% to PtVNS02, 52% to PtVNS03, 50% to PtVNS04, 54% to PtVNS05, 54% to PtVNS06, 52% to PtVNS07, 57% to AtVND1, 62% to AtVND2, 59% to AtVND3, 50% to AtVND4, 51% to AtVND5, 45% to AtVND6 and 48% to AtVND7. *Musa*VND2 has identities of 63% to PtVNS01, 66% to PtVNS02, 50% to PtVNS03, 49% to PtVNS04, 54% to PtVNS05, 55% to PtVNS06, 55% to PtVNS07, 60% to AtVND1, 64% to AtVND2, 60% to AtVND3, 51% to AtVND4, 46% to AtVND5, 48% to AtVND6 and 51% to AtVND7. *Musa*VND3 has identities of 57% to PtVNS01, 57% to PtVNS02, 63% to PtVNS03, 62% to PtVNS04, 60% to PtVNS05, 61% to PtVNS06, 47% to PtVNS07, 55% to AtVND1, 56% to AtVND2, 53% to AtVND3, 57% to AtVND4, 58% to AtVND5, 49% to AtVND6 and 46% to AtVND7. The important function of poplar PtVNS01-07 and *Arabidopsis*

AtVND1-7 proteins has been well documented in earlier reports (Ohtani et al. 2011; Zhou et al. 2014). The sequence of *Musa*VND1, *Musa*VND2 and *Musa*VND3 indicated presence of a highly conserved NAC domain towards the N-terminal end **(Fig. 3.2 and Fig. 3.3)** while the C-terminal region is divergent and is known for regulation of transcriptional activity (Fang et al. 2008).

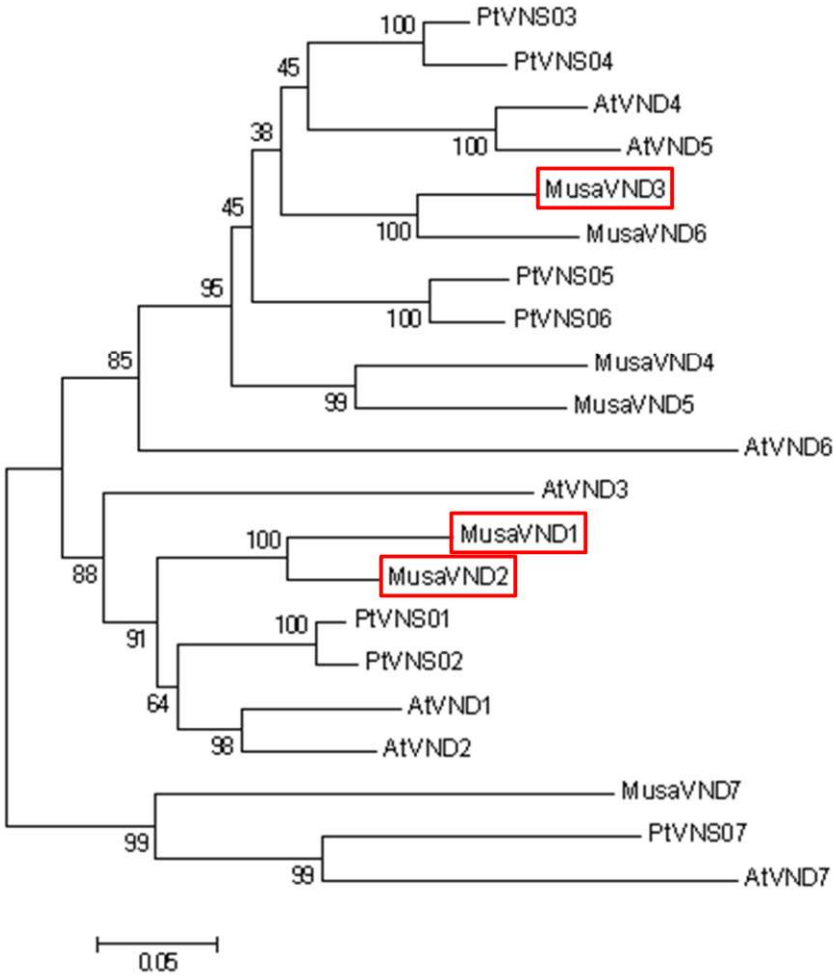

Fig. 3.1 Phylogenetic analysis of *MusaVND1*, *MusaVND2* and *MusaVND3*

The phylogenetic tree (bootstrapped tree with 1000 replicates) was built using annotated vascular related NAC domain protein sequences from poplar and *Arabidopsis*. The amino acid sequences are PtVNSO1 (BAK14358), PtVNSO2 (BAK14359), PtVNS03 (BAK14360), PtVNS04 (BAK14361) , PtVNS05 (BAK14362), PtVNS06 (BAK14363) , PtVNS07 (BAK14364), AtVND1 (At2g18060), AtVND2 (At4g36160), AtVND3 (At5g66300), AtVND4

(At1g12260), AtVND5 (At1g62700), AtVND6 (At5g62380), AtVND7 (At1g71930), *Musa*VND4 (GSMUA_Achr6T36840_001), *Musa*VND5 (GSMUA_Achr7T06640_001), *Musa*VND6 (GSMUA_Achr8T11590_001) and *Musa*VND7 (GSMUA_Achr3T22360_001)

Fig. 3.2 Alignment of MusaVND1 protein with other related protein sequences: PtVNSO1 (BAK14358.1), PtWND5B (ADR00339.1), ThVND1 (XP_007046736.1), AtVND1 (NP_179397.1) and BdSWN1 (NP_001266879.1). Identical residues are black shaded and the highly conserved NAC core domain is underlined in red.

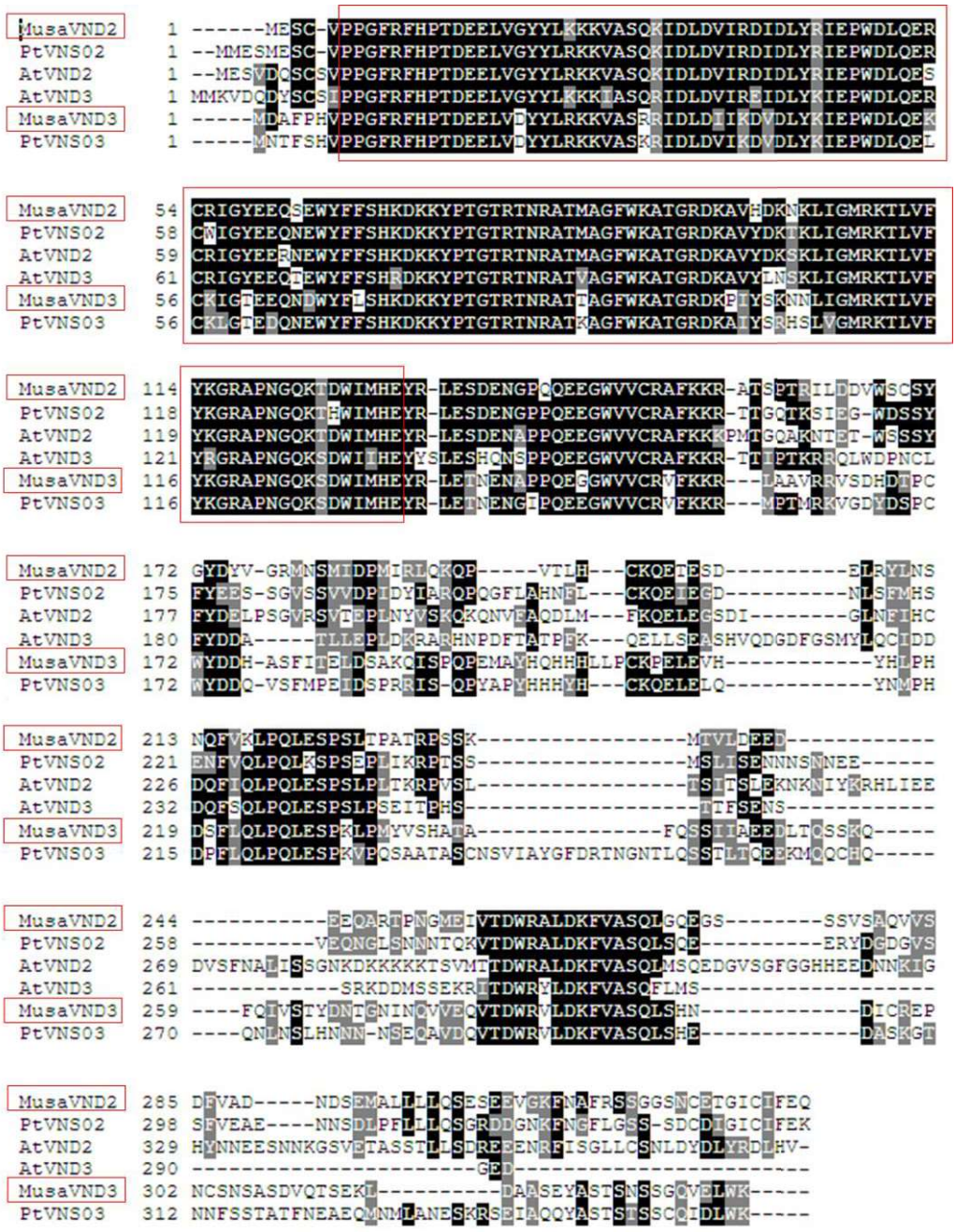

Fig. 3.3 Multiple sequence alignment of *Musa*VND2 and *Musa*VND3. *Musa*VND2 and *Musa*VND3 sequences were aligned with characterized and well annotated sequences of poplar and *Arabidopsis* The amino acid sequences used for alignment are PtVNSO2 (BAK14359), PtVNS03 (BAK14360), AtVND2 (At4g36160) and AtVND3 (At5g66300). Conserved NAC domain is boxed in red. Black shading is done for identical residues.

***In vitro* tracheary element-like cells formation**

Differentiation of tracheary element-like cells was observed after overexpression of either of *MusaVND1, MusaVND2* or *MusaVND3*. These tracheary element-like cells showed characteristic shape of tracheids such as elongated cells and secondary wall deposition (**Fig. 3.4d, 3.4g and 3.4j respectively**). The cells transformed with vector control (*pCAMBIA1301*) did not show any alteration of morphology, indicating that differentiation of tracheary element-like cells is due to overexpression of these transcription factors (**Fig. 3.4a**). Under Confocal microscope, the banana embryogenic cell transformed with vector control (*pCAMBIA1301*) appeared round to oval and devoid of secondary wall fluorescence (**Fig. 3.4b,3.4c**). Images from Confocal laser-scanning microscope (Carl Zeiss, Germany) of these tracheary element-like cells showed the secondary wall deposition. Tracheary element-like cells resulting from overexpression of *MusaVND1, MusaVND2* and *MusaVND3* displayed characteristics of tracheids like wall perforations and annular rings due to secondary wall deposition, observed as lignin autofluorescence (**Fig. 3.4e, 3.4f for VND1, Fig. 3.4h, 3.4i for VND2 and Fig. 3.4k, 3.4l for VND3**). Analysis of tracheary element-like cells differentiation frequency suggested that differentiation started on fourth day and the frequency of these tracheary element-like cells increased from fourth to ninth day. Banana cells transformed with either *MusaVND1*, *MusaVND2* and *MusaVND3*, showed highest differentiation frequency of 33.54%, 63.5% and 23.4% respectively at ninth day after transformation (**Fig. 3.4m, 3.4n**).

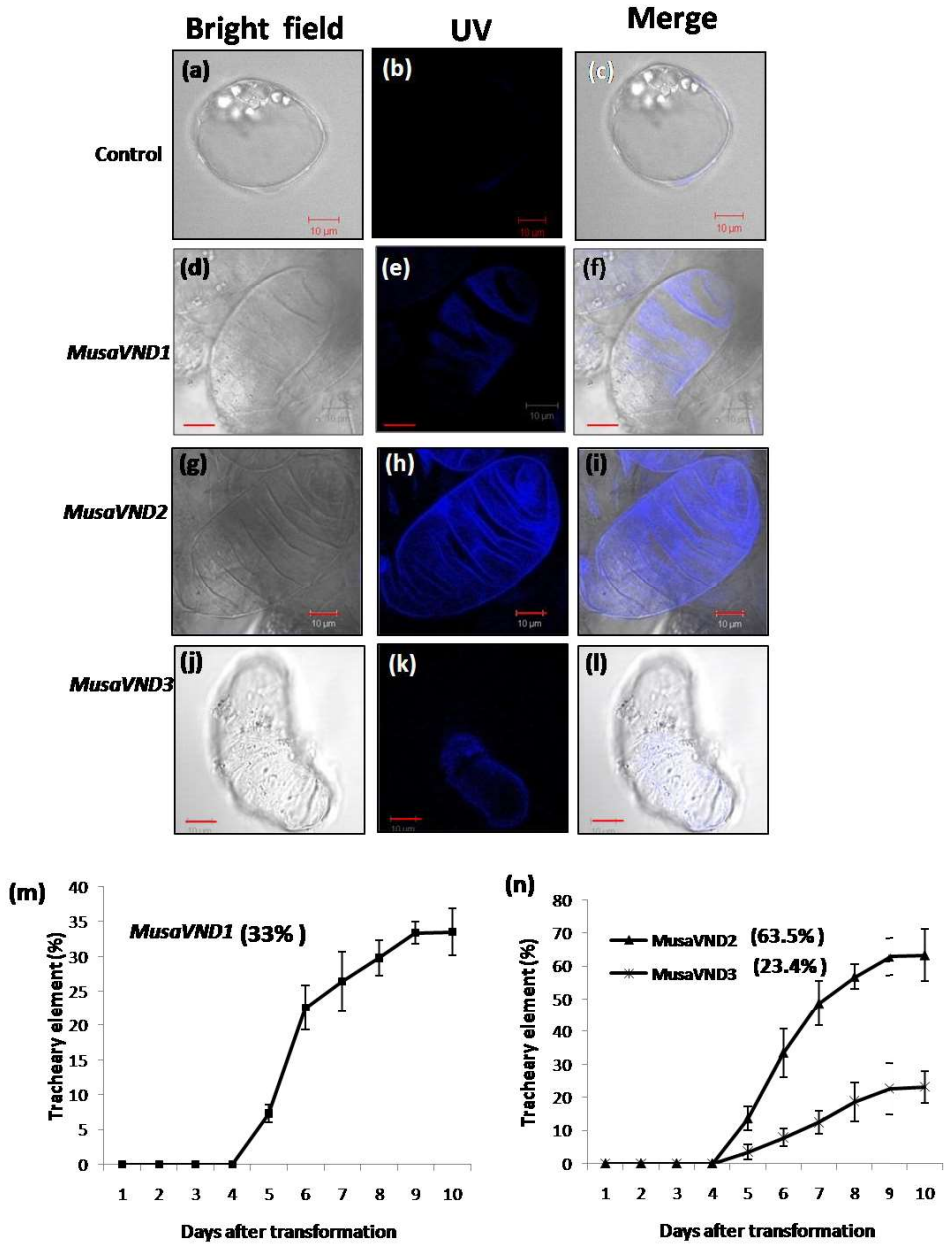

Fig. 3.4 ***In vitro*** **tracheary element-like cells formation**. Tracheary element-like cell formation **was** observed by overexpression of *MusaVND1, MusaVND2* or *MusaVND3*. (a) Banana cells transformed with vector control *pCAMBIA1301*. DIC image of a single control cell. (b) Secondary wall fluorescence from the control cell as observed under Confocal microscope. (c) Merge image of (a) and (b). (d) DIC image of single tracheary element formed after overexpression of *MusaVND1,* observed under Confocal laser scanning microscopy (e) Lignin

autofluorescence of tracheary element observed in (d) exhibiting both wall thickness and wall perforations. (f) Merged image of (d) and (e). (g and j) DIC image of single tracheary element-like cell with characteristic wall perforations obtained after transient overexpression of *MusaVND2* and *MusaVND3*. (h and k) Secondary wall observed with lignin autofluorescence showing wall perforations in tracheary element-like cell induced by overexpression of *MusaVND2* and *MusaVND3* respectively. (i) Merge image of (g) and (h). (l) Merge image of (j) and (k). The bar corresponds to 10 µm. (m) Tracheary element-like cells differentiation rate indicating differentiation increased gradually after transformation.

Subcellular localization of *Musa*VND1, *Musa*VND2 and *Musa*VND3 protein

The translational fusion of *GFP* with either of *MusaVND1, MusaVND2* and *MusaVND3* coding sequence was prepared in vector *pCAMBIA1302* under the control of CaMV35S promoter. To establish the cellular location of *Musa*VND1, *Musa*VND2 and *Musa*VND3 protein, the binary vector *pCAMBIA1302-MusaVND1* or *pCAMBIA1302-MusaVND2* or *pCAMBIA1302-MusaVND3* was transiently expressed in banana cultivar *Rasthali* embryogenic cells and analyzed for GFP fluorescence. The GFP florescence was notably seen in the nucleus of cells transformed with either *pCAMBIA1302-MusaVND1* or *pCAMBIA1302-MusaVND2* or *pCAMBIA1302-MusaVND3* (**Fig. 3.5c, 3.5e, 3.5g respectively**) indicating nuclear location of the proteins. Whereas in vector control (*pCAMBIA1302*) transformed cells the GFP florescence was distributed throughout the entire cell (**Fig. 5a**). These observations proved the nuclear localization of *Musa*VND1, *Musa*VND2 and *Musa*VND3 proteins. DAPI staining was employed to ascertain the nucleus position within these cells (**Fig. 3.5b, 3.5d, 3.5f, 3.5h**).

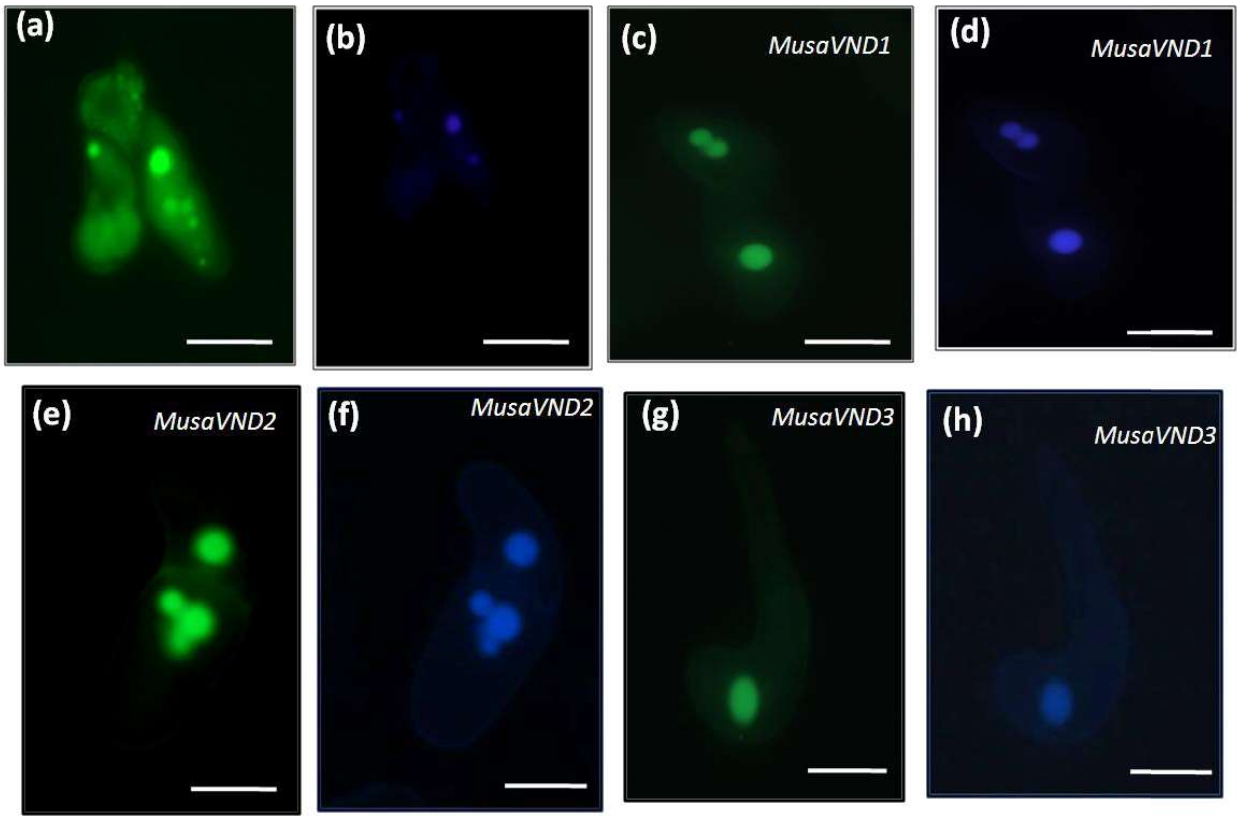

Fig. 3.5 Sub cellular localization of *Musa*VND1, *Musa*VND2 and *Musa*VND3 proteins in banana embryogenic cells. (a) Banana embryogenic cells transformed with vector control *pCAMBIA-1302*. The fluorescence was distributed throughout the cell. (b) DAPI staining of the vector control transformed cells. (c) *Musa*VND1-GFP fusion protein was localized to the nucleus as observed by fluorescence enabled microscopy. (d) The nuclear positions in the banana cells observed in (c) was revealed by DAPI staining. (e) The *Musa*VND2-GFP fusion protein is localized to nucleus. (f) DAPI staining of cells observed in (e). (g) Nuclear localization of *Musa*VND3-GFP fusion protein. (h) The nuclear positions in image (g) of the banana cells was observed after DAPI staining. The bar corresponds to 50 µm.

Regeneration and molecular analysis of transgenic banana plants overexpressing *MusaVND1*

Agrobacterium tumefaciens strain EHA105 containing the *pCAMBIA1301-ZmUbi-MusaVND1* (designed to constitutively express *MusaVND1*) binary vector (**Fig. 3.6a**) was co-cultivated with embryogenic cells of banana cv. *Rasthali* (**Fig. 3.6b**). One month after co-cultivation embryos developed on embryo induction medium supplemented with 5 mg/L hygromycin (**Fig. 3.6c**). The embryos appear white and translucent and further generated secondary embryos (**Fig. 3.6d**). Putative transgenic shoots emerged from embryos when the embryos were sub cultured onto embryo germination medium (**Fig. 3.6e**). The germinating embryos were transferred to banana multiplication medium for the induction of multiple shoots (**Fig. 3.6f).** These putative transgenic shoots were separated and further grown on rooting medium (**Fig. 3.6g**). Full grown rooted plants were hardened using paper cups in a green house (**Fig. 3.6h**). Three transgenic lines were found to be surviving on hygromycin-containing medium. Genomic DNA PCR analysis of control and different *MusaVND1* lines was carried out to confirm the integration of the T-DNA region in the genome of different *MusaVND1* lines. The amplification of the *hpt-II* coding sequence present within the T- DNA region confirms the transgenic nature of *MusaVND1* overexpressing lines. Three transgenic lines were found to be positive for T-DNA integration and were named V1, V2 and V3 respectively (**Fig. 3.6i**). To further confirm the stable integration of the transgene, these three transgenic lines were analyzed by Southern blotting of restricted genomic DNA using a DIG-labeled probe against the *hpt-II* coding sequence. The DIG labeled probe against *hpt-II* coding sequence was hybridized with genomic DNA of these transgenic lines digested with *Kpn*I. As *Kpn*I cuts the T-DNA region of *pCAMBIA1301-ZmUbi-MusaVND1* only once, the number of bands appearing on the autoradiograph is a direct indicator of the

copies of T-DNA transferred to these transgenic lines. Furthermore, the different sized bands detected in various lines proved that these transgenic lines have originated from independent transformation events (**Fig. 3.6j**).

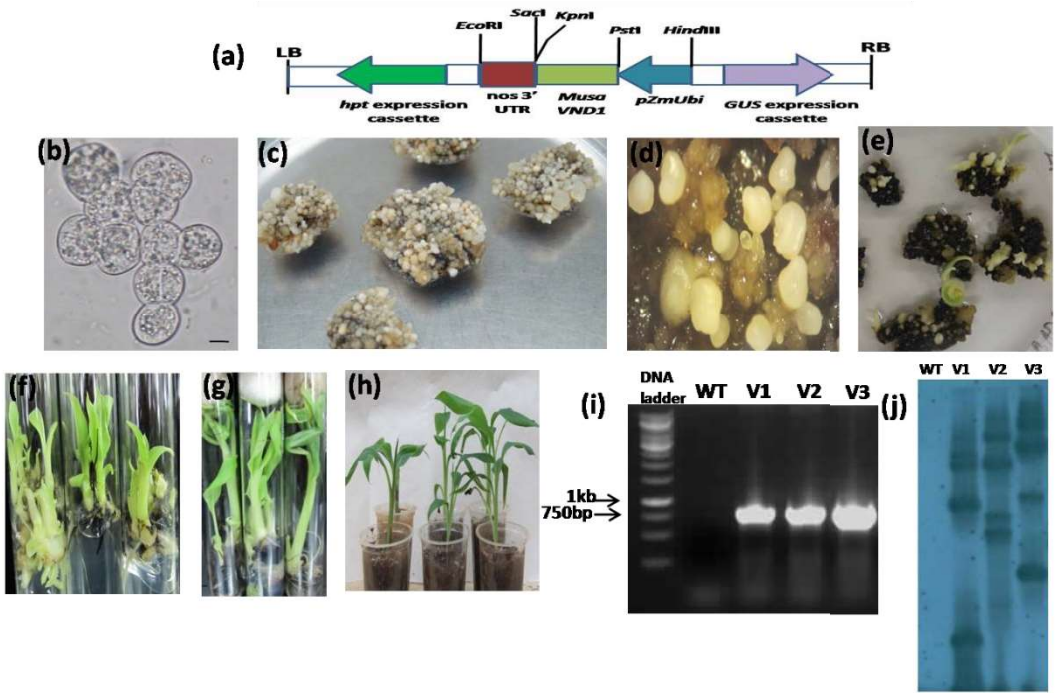

Fig. 3.6 Generation and molecular analysis of transgenic banana plants constitutively overexpressing *MusaVND1* (a) A cartoon representation of the T-DNA region designed to constitutively overexpress *MusaVND1* in transgenic lines. (b) Embryogenic cells of the banana cultivar *Rasthali* used for generating transgenic banana plants. The black bar corresponds to 25 µm. (c) Emergence of embryos on selection medium containing 5 mg/L hygromycin. (d) A close up view of embryos, showing emergence of white translucent secondary embryos. (e) Emergence of putative transgenic shoots on embryo germination medium. (f) Putative transformed multiple shoots in banana multiplication medium. (g) Generation of rooted transgenic banana plants in banana rooting medium containing NAA (1 mg/l). (h) Hardening of transgenic banana plants overexpressing *MusaVND1* in the green house (2 months old). (i) Genomic DNA PCR showing integration of *MusaVND1* coding sequence in different lines of

transgenic banana plants. Amplification of *hygromycin phosphotransferase* gene (788 bp PCR product) from genomic DNA of all the three transgenic lines (V1,V2 and V3) was observed, whereas it was absent in untransformed wild-type plants (WT). (j) Southern blot analysis of transgenic banana lines overexpressing *MusaVND1*. (WT: Wild type plants).

Regeneration of transgenic banana plants over expressing *MusaVND2* and *MusaVND3* and molecular analysis of T-DNA insertion

Binary vectors *pCAMBIA1301-ZmUbi-MusaVND2* (**Fig. 3.7a**) or *pCAMBIA1301-ZmUbi-MusaVND3* (**Fig. 3.8a**) designed to constitutively express *MusaVND2* and *MusaVND3* respectively was used for transformation of embryogenic cells of banana cv. *Rasthali*. One month after co cultivation with *Agrobacterium* strain EHA105 containing either of the binary vector, embryos developed on embryo induction medium supplemented with 5 mg/L hygromycin (**Fig. 3.7b, 3.8b**). The embryos appeared as white globular bodies which subsequently developed secondary embryos (**Fig. 3.7c, 3.8c**). After continued development the globular embryos gets elongated in shape (**Fig. 3.7d, 3.8d**). Further emergence of putative transgenic shoots was observed after transferring the embryos to embryo germination medium (**Fig. 3.7e, 3.8e**). Multiple shoots were generated by transferring the putative transgenic shoots to banana multiplication medium (**Fig. 3.7f, 3.8f**). Individual putative transgenic shoots were separated and grown individually on rooting medium (**Fig. 3.7g, 3.8g**). In both the cases three transgenic lines were found to be surviving on hygromycin-containing medium. Integration of T-DNA region in genome of different transgenic lines was confirmed by genomic DNA PCR analysis. PCR amplification of *hpt-II* coding sequence present in the T-DNA region indicated integration of T-DNA in genome of different transgenic lines. In case of *MusaVND2*, three transgenic lines were found to be positive for T-DNA integration and were named as L2, L4 and L7 respectively (**Fig.**

3.7h). In case of *MusaVND3*, three lines were tested positive for T-DNA insertion and were subsequently named as L1, L3 and L5 respectively (**Fig. 3.8h**). The T-DNA integration in the genome of transgenic lines was reconfirmed by Southern blotting of *Kpn*I restricted genomic DNA by means of a DIG-labeled probe against *hpt-II* coding sequence. T-DNA copy number transferred ranged from one to three was observed in different transgenic lines of *MusaVND2* and *MusaVND3* (**Fig. 3.7i, 3.8i**). The number of bands in Southern blot is a direct indicator of copies of T-DNA transferred to these transgenic lines because *Kpn*I digest the T-DNA region of both *pCAMBIA1301-ZmUbi-MusaVND2* and *pCAMBIA1301-ZmUbi-MusaVND3* only once. Additionally, the dissimilar sized bands detected in different lines established that these transgenic lines were derived from independent transformation events.

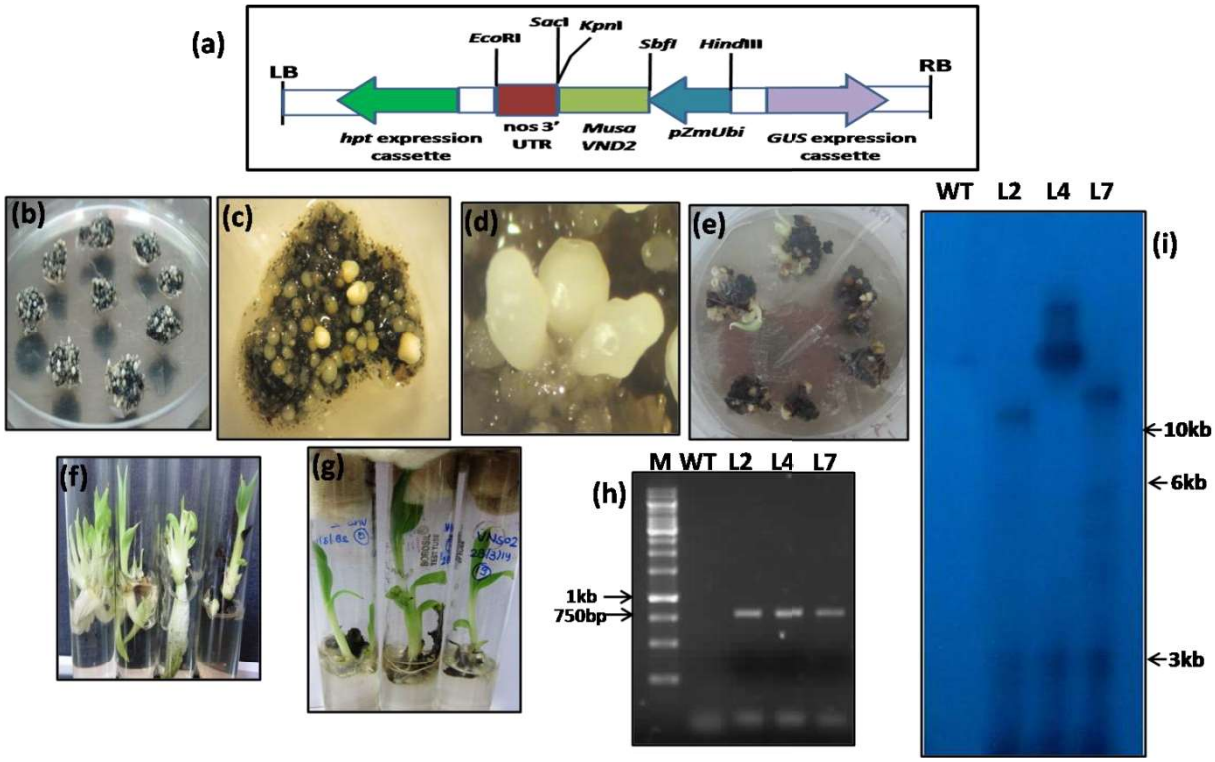

Fig. 3.7 Generation of *MusaVND2* overexpressing transgenic banana plants (a) T-DNA region for constitutive overexpression of *MusaVND2*. (b) Emergence of embryos on medium with selection agent hygromycin (5 mg/L). (c) Close up view of white translucent embryos. (d)

Emerging secondary embryos at torpedo shaped stage. (e) Putative transgenic shoots on embryo germination medium. (f) Multiple shoots of putative transgenic plants in shoot multiplication medium. (g) Rooting in putative transgenic plants on rooting medium. (h) PCR analysis showing integration of T-DNA in genome of different transgenic lines. Transgenic lines (L2, L4 and L7) showed amplification of *hygromycin phosphotransferase* coding sequence (788 bp) which was absent from wild-type plant (WT). (i) Copy number determination of T-DNA insertions in different transgenic lines by Southern blot. The position of marker bands are indicated.

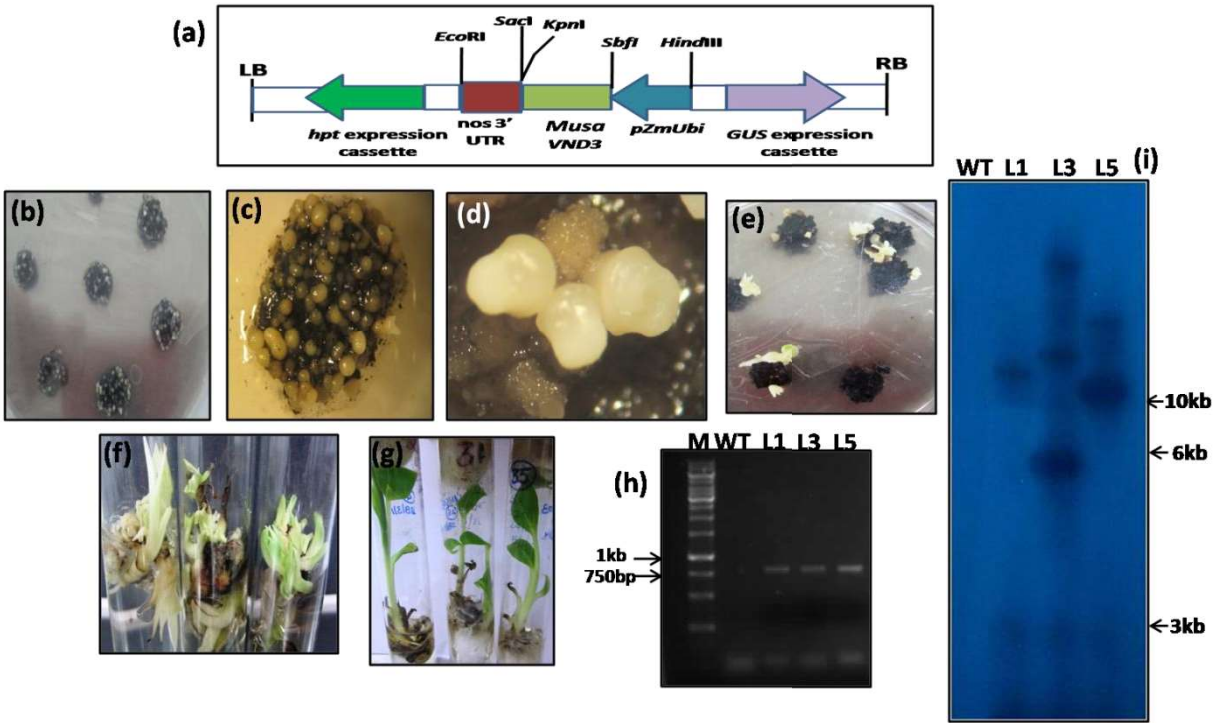

Fig. 3.8 Generation of transgenic banana overexpressing *MusaVND3* and confirmation of T-DNA insertion (a) T-DNA region designed to constitutively over express *MusaVND3*. (b) Embryos emerged on selection medium. (c) White translucent embryos on selection medium. (d) Secondary embryos at torpedo stage. (e) Emergence of shoots on embryo germination medium. (f) Putative transgenic multiple shoots in shoot multiplication medium. (g) Generation of rooted transgenic banana plants. (h) PCR analysis showing insertion of T-DNA carrying *MusaVND3*

coding sequence in genome of different transgenic lines. (i) Southern blot analysis for determination of T-DNA copy number insertion in transgenic banana lines overexpressing *MusaVND3*. Approximate positions of DNA marker are indicated.

Quantitative RT-PCR analysis of overexpression

The transgenic plants overexpressing either MusaVND1 *MusaVND2* or *MusaVND3* were further characterized by quantitative RT-PCR analysis to determine the fold value change in transcript level of MusaVND1 or *MusaVND2* or *MusaVND3* due to overexpression. The fold value change was 9.18 in line V1, 7.65 in line V2 and 13.88 in line V3, relative to control plant (**Fig. 3.9a**). The fold value change was 8.6 in line L2, 10.61 in line L4 and 9.85 in L7 line, relative to control plant (**Fig. 3.9b**). The fold value change in transcript level of *MusaVND3* analyzed by real time RT-PCR analysis was 15.96 in L1, 9.16 in line L3 and 11.31 in Line L5 (**Fig. 3.9c**).

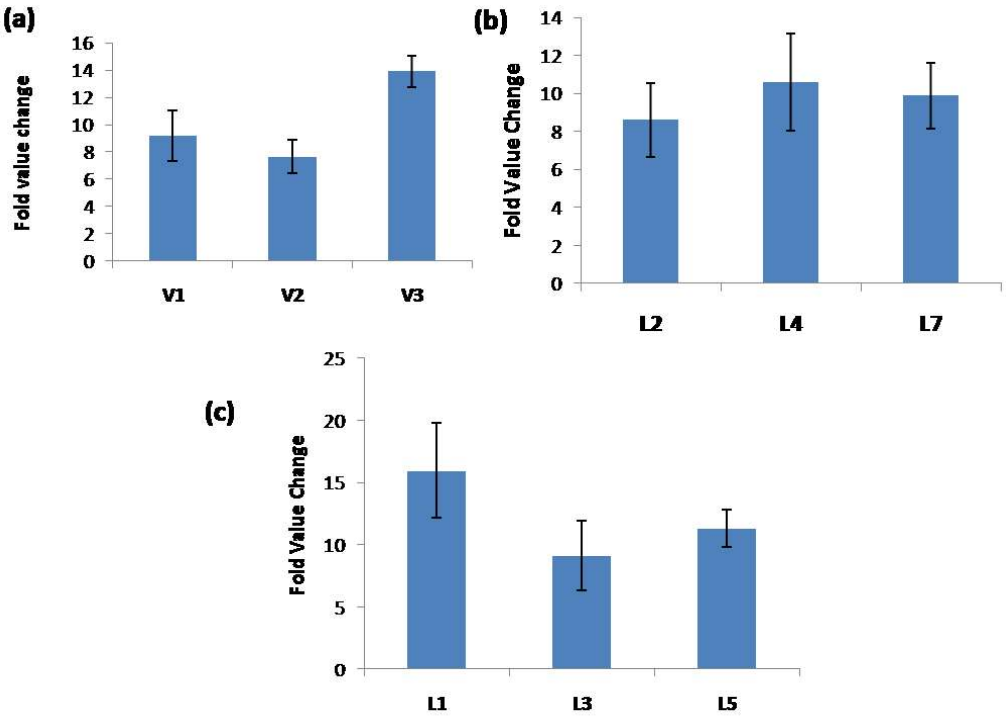

Fig. 3.9 Transgenic lines and Q-PCR analysis of overexpression. (a) Analysis of *MusaVND1* overexpression in different transgenic lines as analyzed by quantitative real time RT-PCR analysis. (b) Analysis of *MusaVND2* overexpression in different transgenic lines by quantitative real time RT-PCR analysis. (c) The over expression of *MusaVND3* in different lines analyzed by quantitative real time RT- PCR. The Ct value of *EF1α* was used to normalize the gene expression values in different transgenic lines. The fold value change of expression in control plants is kept at one. The values are represented as mean ±SE.

Overexpression of *MusaVND1* results in ectopic deposition of lignin and transdifferentiation of various cells into xylem vessel elements

The overexpression of *MusaVND1* causes differentiation of banana cells into tracheary elements, suggesting that the *MusaVND1* might be an important factor regulating secondary wall synthesis in banana plants. To test whether overexpression of *MusaVND1* induces secondary wall synthesis, we generated transgenic banana plants overexpressing *MusaVND1*. Secondary cell wall in leaves of transgenic lines was observed as lignin autofluorescence under a laser scanning Confocal microscope. During visualization under microscope differential interference contrast (DIC) images and lignin autofluorescence images of cells were recorded. Examination of leaf epidermal cells of transgenic lines revealed transdifferentiation of many epidermal cells into tracheary elements. DIC images of these tracheary element-like cells revealed secondary wall deposition in a reticulate pattern (**Fig. 3.10d**). Lignin autofluorescence images of these transgenic leaves exhibit blue color fluorescence in these tracheary element-like cells, indicating deposition of lignin in these reticulate secondary walls (**Fig. 3.10e,f**). Leaf epidermal cells in DIC images of control plants appeared normal (**Fig. 3.10a**) and no lignin autofluorescence was observed from these epidermal cells indicating the presence of only primary wall in these cells

(**Fig. 3.10b,c**). Observation of transverse sections of transgenic lines revealed transdifferentiation of mesophyll cells into tracheary element-like cells with a reticulate pattern of secondary wall deposition (**Fig. 3.10h**). The presence of such lignified secondary walls in normally non sclerenchymatous cells might be the probable reason of leaf growth retardation in transgenic plants overexpressing *MusaVND1*. Leaf mesophyll cells in transverse section from control plants appeared parenchymatous and without deposition of secondary wall (**Fig. 3.10g**). The corm of *MusaVND1* overexpressing transgenic banana plants and of control plants was observed for lignin autofluorescence under UV light illumination. The corm of *MusaVND1* overexpressing transgenic banana plants exhibits lignin autofluorescence in normally non sclerenchymatous cells. The epidermal and cortical cells which are normally non lignified and parenchymatous in control plants exhibit intense lignin autofluorescence in the corm of transgenic lines (**Fig. 3.10j**). The epidermal and cortical cells in the corm section of the control plant do not exhibit any lignin autofluorescence, indicating the absence of secondary wall deposition (**Fig. 3.10i**).

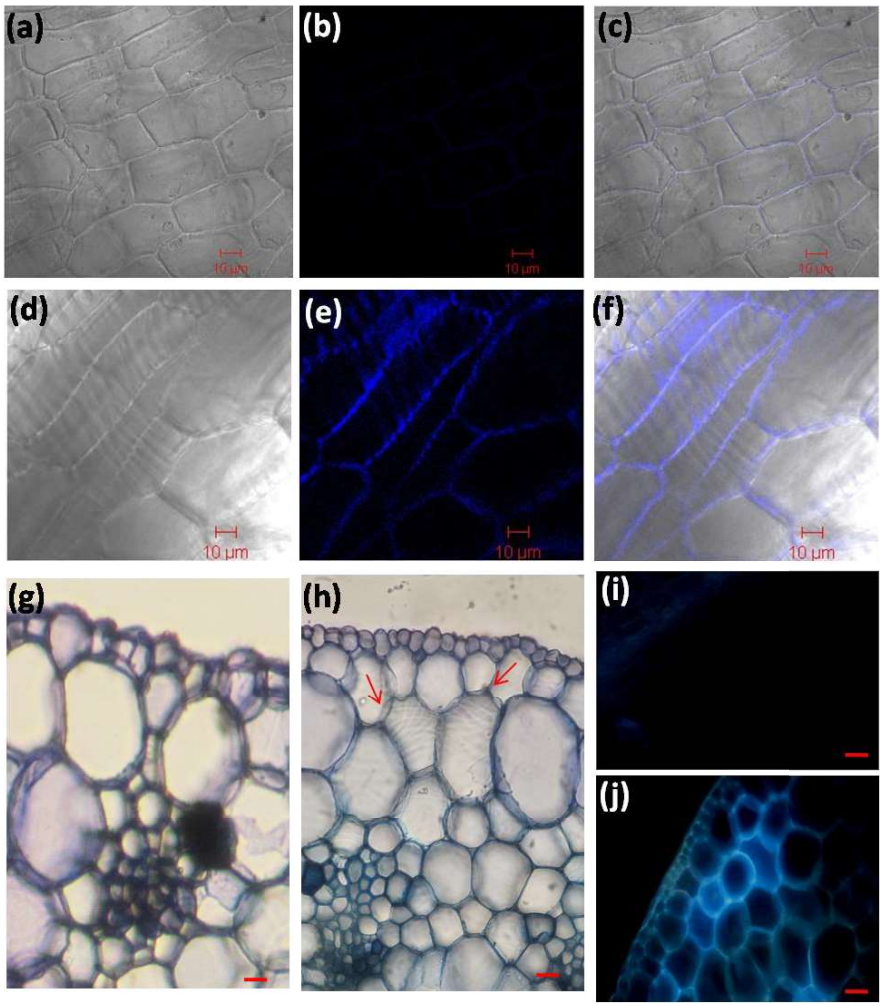

Fig. 3.10 Overexpression of *MusaVND1* causes ectopic deposition of lignified secondary walls in epidermal and mesophyll cells of leaves. Laser scanning Confocal microscopy images for secondary walls and lignin autofluorescence, and differential interference contrast (DIC) and Confocal images were recorded in leaves of transgenic and control plants. (a) DIC image of epidermal cells of control leaves. (b) Lignin autofluorescence of epidermal cells of control leaves. (c) Merged image of image (a) and image (b). (d) DIC image and (e) lignin autofluorescence of epidermal cells of leaf of *MusaVND1* overexpressing plant showing the development of secondary walls in reticulate pattern. (f) Merged image of (d) and (e). (g) Transverse section of leaf of control plant. (h) Transverse section of transgenic leaf showing

transdifferentiation of mesophyll cells into tracheary element-like cells with reticulate thickening (red arrows). (i) Transverse section of corm of control plant observed for lignin autofluorescence under fluorescence microscope. (j) Lignin autofluorescence image of transverse section of transgenic line under fluorescence microscope. The red bar corresponds to 10 µm.

Transgenic plants overexpressing *MusaVND1* have enhanced cellulose and lignin content

Differentiation of tracheary elements in transgenic plants overexpressing *MusaVND1* implied that cell wall biochemical composition may be altered in transgenic plants overexpressing *MusaVND1*. To address this, we analyzed the composition of two major components in the cell wall i.e., lignin and cellulose in cell walls of transgenic and control plants. Biochemical estimation revealed significantly elevated levels of lignin in the pseudostem of transgenic lines compared with control plants (**Fig. 3.11a**). Similarly elevated cellulose levels were detected in cell walls of transgenic lines relative to cellulose levels in control plants (**Fig. 3.11b**).

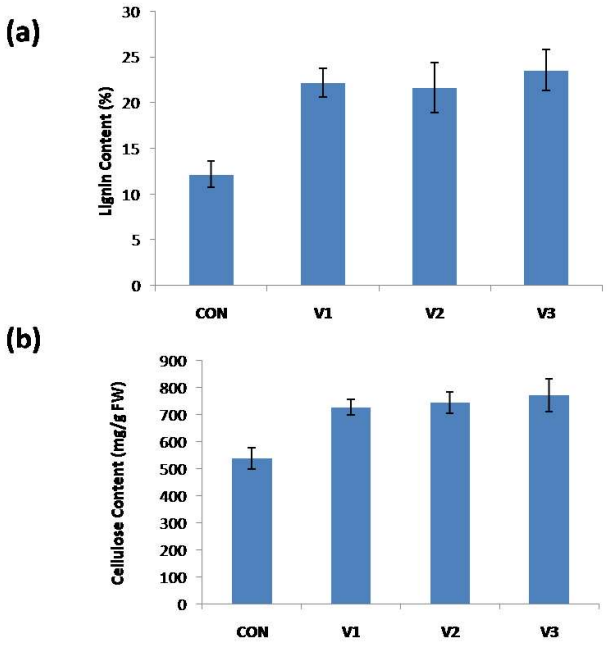

Fig. 3.11 Biochemical estimation of cellulose and lignin in transgenic banana overexpressing *MusaVND1*. (a) Percentage lignin content in cell walls of transgenic lines and

control plants as estimated by thioglycolic acid assay. (b) Cellulose content measured in cell wall of *MusaVND1* overexpressing transgenic lines and wild type plant. (Con: control plants; V1,V2 and V3: different transgenic lines).

Transgenic plants overexpressing either *MusaVND2* or *MusaVND3* showed ectopic deposition of secondary wall and transdifferentiation into tracheary element-like cells

To observe if overexpression of *MusaVND2* or *MusaVND3* can induce ectopic secondary wall deposition, transgenic banana plants overexpressing them were generated. Differential interference contrast (DIC) and lignin autofluorescence images of secondary walls were recorded from leaves of transgenic plants by using laser scanning Confocal microscope. The observation revealed transdifferentiation of many epidermal cells into tracheary element-like cells. DIC images showed secondary wall deposition in reticulate pattern in these tracheary elements like cells from leaves of transgenic lines overexpressing *MusaVND2* or *MusaVND3* (**Fig. 3.12d, 3.12g**). Similarly lignin autofluorescence images from these secondary wall deposition was observed from leaves of transgenic lines overexpressing either of *MusaVND2* or *MusaVND3* (**Fig. 3.12e-f, 3.12h-i**). DIC images of leaf epidermal cells in control plants appeared normal (**Fig. 3.12a**) and without lignin autofluorescence indicating absence of secondary wall in these cells (**Fig. 3.12b,c**). Toluidine blue-O stained leaf transverse sections of transgenic lines overexpressing either of *MusaVND2* or *MusaVND3* revealed transdifferentiation of mesophyll cells into tracheary elements with reticulate pattern of secondary wall deposition, similar to those observed in the transdifferentiated epidermal cells (**Fig. 3.12k, l**). Mesophyll cells from leaf of control plant appeared parenchymatous and devoid of secondary wall depositions (**Fig. 3.12j**). The corm of these different overexpressing transgenic banana plants was similarly analyzed for secondary wall deposition. Transverse sections of corm of transgenic banana plants

overexpressing *MusaVND2* or *MusaVND3* exhibit transdifferentiation of cortical cells with thick deposition of secondary wall (**Fig. 3.12n, 3.12p**). The secondary wall deposition in transgenic plants overexpressing *MusaVND2* was very intense and the secondary wall deposition appeared in the form of band like structure (**Fig. 3.12o**). Observation of corm of control plant showed cortical cells as parenchymatous and non lignified indicating the absence of secondary wall deposition (**Fig. 3.12m**).

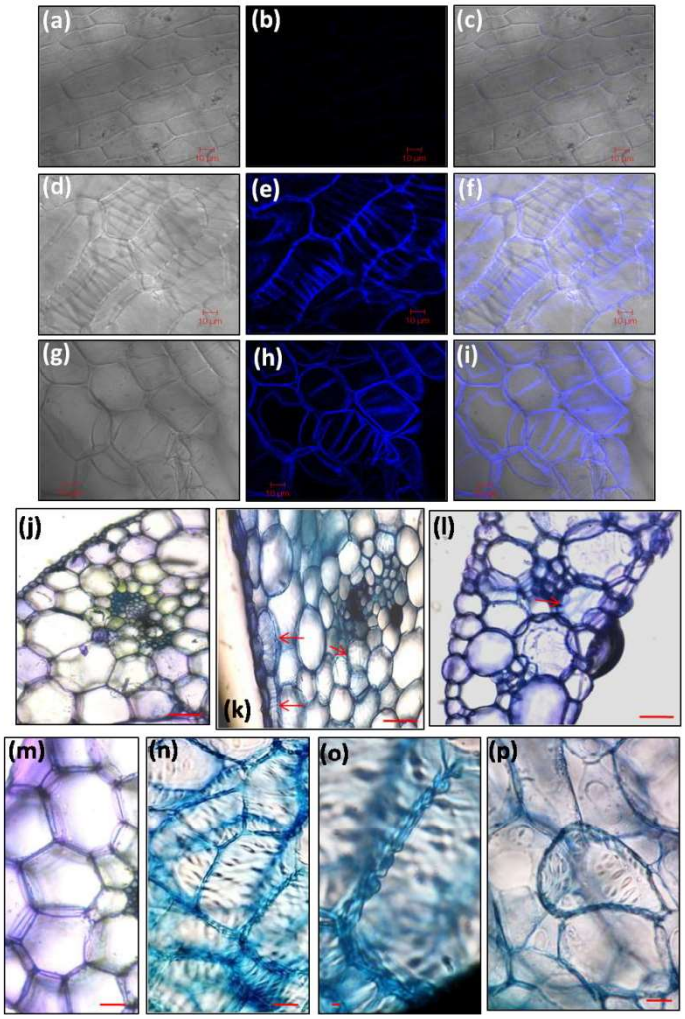

Fig. 3.12 Ectopic secondary wall deposition in transgenic lines overexpressing *MusaVND2* and *MusaVND3*. Differential interference contrast (DIC) and lignin autofluorescence images obtained from leaves of transgenic and control plants. (a) DIC image of leaf epidermal cells of

control plants. (b) Lignin autofluorescence from leaf epidermal cells of control plant. (c) Merge image of image (a) and image (b). (d and g) DIC images and (e and h) lignin autofluorescence images indicating reticulate pattern like secondary wall deposition in leaf epidermal cells of *MusaVND2* and *MusaVND3* overexpressing plant respectively. (f) Merge image of (d) and (e). (i) Merge image of (g) and (h). (j) Transverse section of control leaf. (k and l) Transdifferentiation of mesophyll cells into tracheary elements-like cells with reticulate thickening (red arrows) as observed in leaf transverse section of *MusaVND2* and *MusaVND3* overexpressing plant. (m) Cortical region of corm of control plant showing non sclerenchymatous cells. (n and p) Cortical region of corm of *MusaVND2* and *MusaVND3* overexpressing plant respectively showing secondary wall deposition and transdifferentiation into tracheary elements. (o) A close view of transdifferentiated tracheary element in corm of *MusaVND2* transgenic plant showing secondary wall deposition in the form of band like structure. The red bar corresponds to 10 µm.

Transgenic plants overexpressing *MusaVND2* and *MusaVND3* have enhanced cellulose and lignin content

As the expression of *MusaVND2* and *MusaVND3* caused ectopic secondary wall depositions along with tracheary elements transdifferentiation in the transgenic plants, suggesting that cell wall biochemical composition may be altered in these transgenic plants. We have analyzed the content of cellulose and lignin in cell walls of transgenic and control plants. Elevated levels of lignin in pseudostem of transgenic lines than control plants were observed after biochemical estimation (**Fig. 3.13a, 3.13b**). Similarly, elevated cellulose levels were detected in cell walls of transgenic lines than control plants (**Fig. 3.13c, 3.13d**).

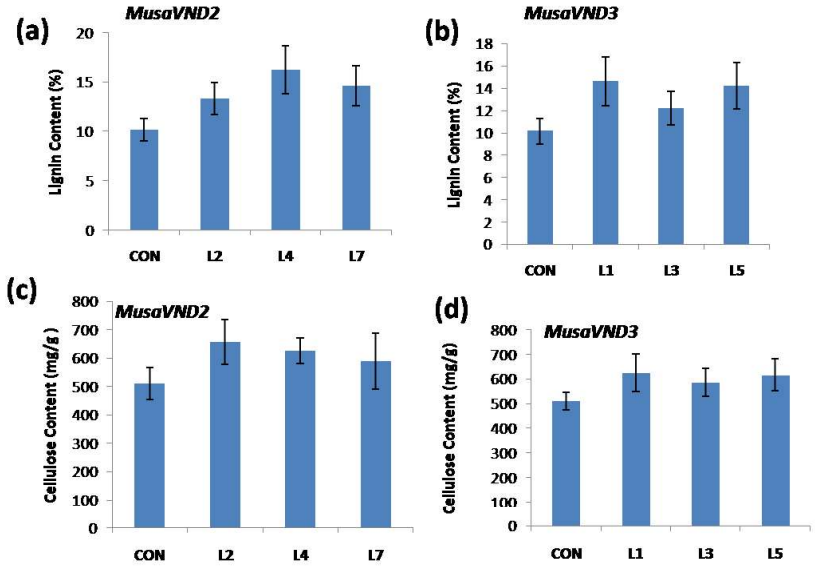

Fig. 3.13 Cellulose and lignin content in cell wall of transgenic banana lines overexpressing *MusaVND2* or *MusaVND3*. (a) Percentage lignin content in cell wall of transgenic lines overexpressing *MusaVND2* and control plants. (b) Lignin content in cell wall of transgenic banana lines overexpressing *MusaVND3*. (c) Cellulose content estimated in cell wall of *MusaVND2* overexpressing transgenic lines and control plant (Con: control plants; L2, L4 and L7: different transgenic lines). (d) Cellulose content analysis after acid hydrolysis in cell wall of *MusaVND3* transgenic plants. (Con: control plants; L1, L3 and L5: different transgenic lines). The experiments were repeated three times and means±SE was plotted.

5'-upstream sequence of *MusaVND1* is active in vascular tissues of banana

To investigate the promoter activity of *MusaVND1*, a 1.35kb 5'-upstream sequence was PCR amplified and cloned upstream of *GUS* in *pCAMBIA1301* and banana plants harboring $P_{MusaVND1}$ *:: GUS* was generated. Various organs of banana were stained by GUS staining buffer to examine the tissue specific activity of $P_{MusaVND1.}$ GUS expression in leaves was observed specifically in longitudinal veins as well as in commissural vascular bundles (**Fig. 3.14a**). Cross section of GUS stained leaf indicate the activity of $P_{MusaVND1}$ to be specific and intense along the

vascular strands and no expression of GUS was detected in epidermal or cortical cells (**Fig. 3.14b,3.14c**). In petiole section, activity of $P_{MusaVND1}$ was detected in vascular bundles present towards center while it was undetectable in vascular bundles along the epidermis (**Fig. 3.14d).** Close up of vascular bundle in petiole show evident GUS expression in vessels and tracheids (**Fig. 3.14e**). In corm the activity of *GUS* under $P_{MusaVND1}$ specifically observed in lignified portion of central stele region and in vascular tissue scattered in the cortex (**Fig. 3.14f**). Activity of $P_{MusaVND1}$ in banana pseudostem composed of overlapping leaf sheaths was observed in vascular bundles scattered all along the section (**Fig. 3.14g**). Moreover, the *GUS* expression under the $P_{MusaVND1}$ increased from central sheath to outer most sheath in pseudostem indicating the developmental expression pattern of *MusaVND1* during vascular tissue development. In roots the expression of $P_{MusaVND1}$ was observed in the central cylinder of the roots , however, the *GUS* expression was evident in the outward protoxylem vessel elements and was absent in central metaxylem elements (**Fig. 3.14h**).

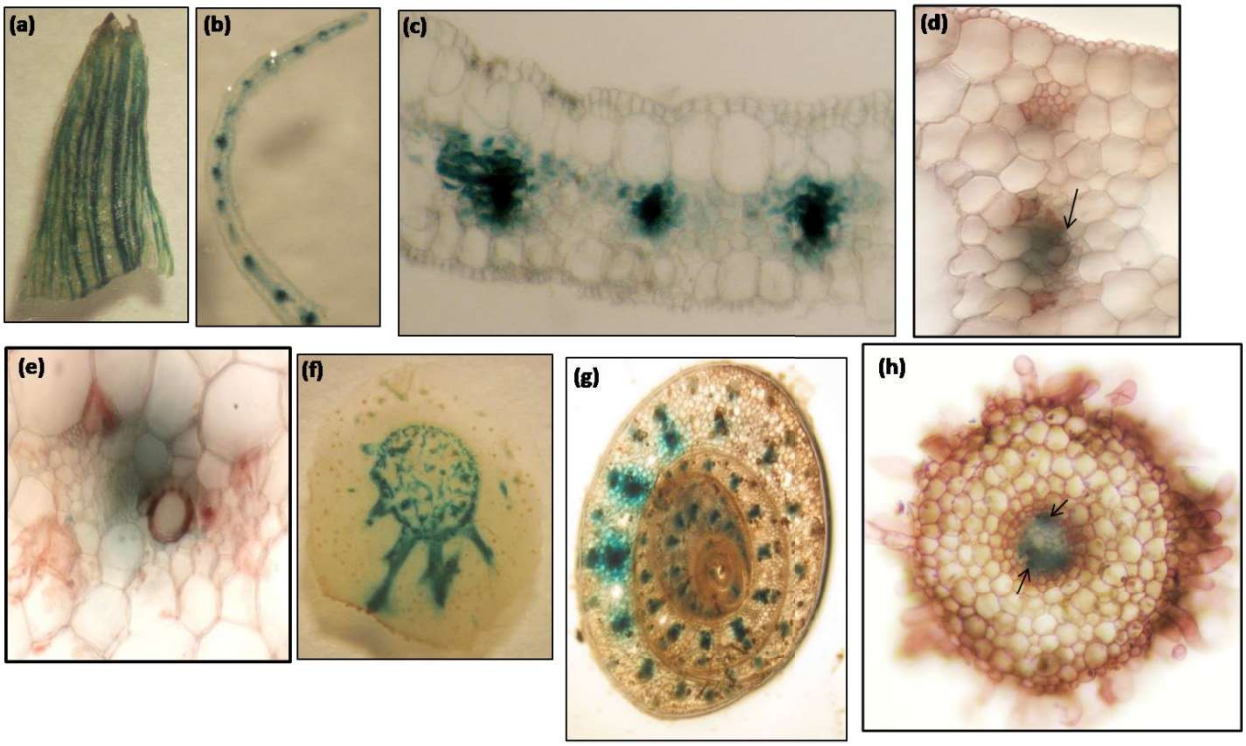

Fig. 3.14 Expression pattern of *MusaVND1* in banana. (a) GUS expression driven by $P_{MusaVND1}$ in banana leaf showing vessel specific activity of $P_{MusaVND1}$. (b) Cross section of *GUS* stained leaf at lower magnification microscopy. (c) High magnification of a leaf section showing intense and specific GUS staining in vascular tissue. (d) Cross section of petiole stained with GUS staining buffer. Arrow indicates the GUS staining in the vascular tissue. (e) Close up of vascular bundle in petiole showing localized GUS staining. (f) GUS staining observed in the corm of transgenic banana. (g) Activity of $P_{MusaVND1}$ in banana pseudostem observed as GUS activity in transformed banana. $P_{MusaVND1}$ *::* *GUS* was mainly active in vascular tissue. (h) Cross section of banana root harboring $P_{MusaVND1}$ *::* *GUS* . Note the GUS activity along the central vascular strand in root indicated by arrow. Safranin was used as counter stain in sections.

Xylem specific activity of 5'-upstream regulatory region of *MusaVND2*

Tissue specific activity of $P_{MusaVND2}$ was analyzed by cloning a 1.2kb 5'-upstream sequence of *MusaVND2* in upstream of *GUS* in *pCAMBIA1301* and generating transgenic banana expressing *GUS* under the control of $P_{MusaVND2}$. In leaf tissue, GUS expression was observed in longitudinal vascular bundles (veins) (**Fig. 3.15a**) which was specifically detected in vessels and surrounding tracheids indicating xylem specific activation of *MusaVND2* (**Fig. 3.15b**). Similar activity of $P_{MusaVND2}$ as GUS staining was observed in vessel and tracheids cells of the petiole (**Fig. 3.15c**). Developmental expression pattern of *MusaVND2* was indicated by *GUS* staining of pseudostem wherein the *GUS* expression get incrementally increased from inner to outer sheath (**Fig. 3.15d**) suggesting an important role of *MusaVND2* in vascular tissue development. In corm, the expression of $P_{MusaVND2}$*::GUS* was observed in central stele region which harbors the vascular strand of the corm (**Fig. 3.15e**). In roots, the expression of *GUS* was strongly detected in all the lignified cells of the central vascular cylinder (**Fig. 3.15f**). In roots the expression of GUS under $P_{MusaVND2}$ was detected in the central metaxylem, peripheral protoxylem and surrounding tracheids all of which showed visible secondary wall thickening (**Fig. 3.15g, 3.15h**).

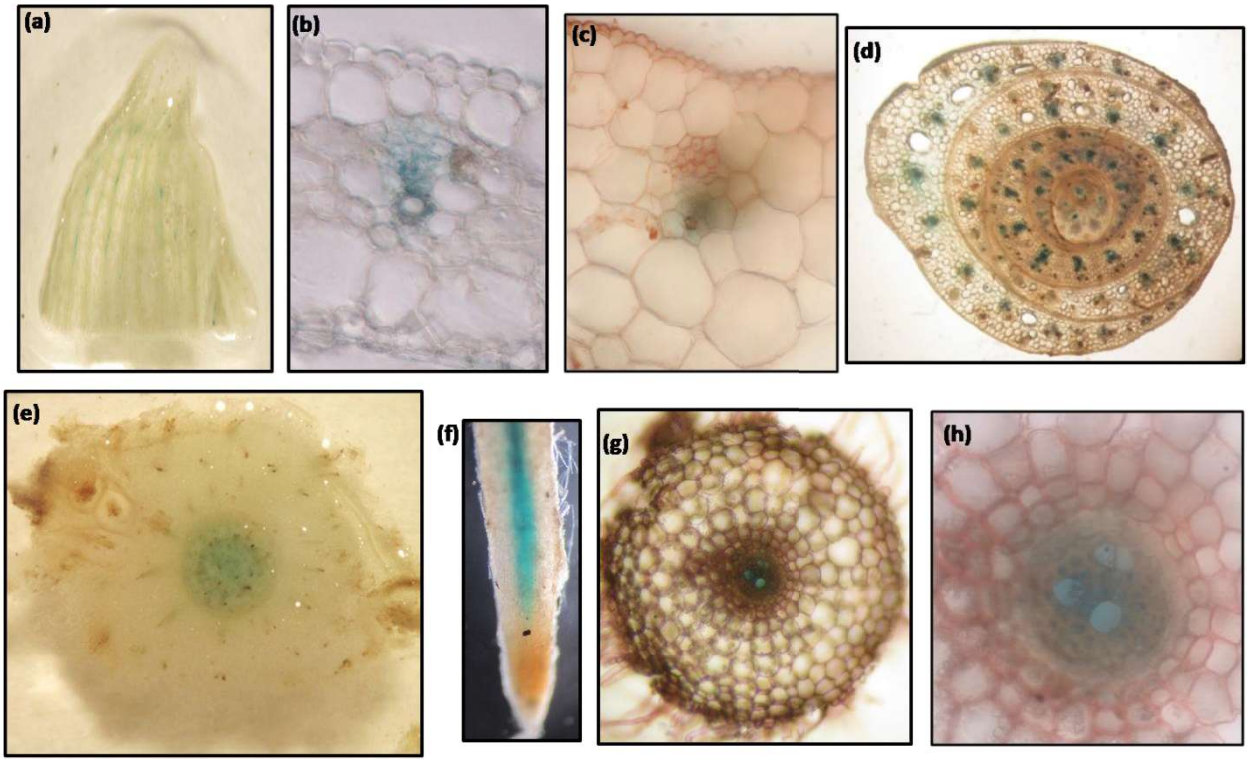

Fig. 3.15 Analysis of transgenic banana harboring $P_{MusaVND2}$ :: *GUS*. (a) Staining of GUS driven by $P_{MusaVND2}$ in veins of banana leaf. (b) Cross section of leaf showing specific GUS expression observed in cells of vascular tissue. (c) Close up of vascular bundle in petiole showing localized GUS staining. (d) Cross section image of GUS buffer stained pseudostem showing vessel specific activity of $P_{MusaVND2}$. (e) Transverse section of corm with GUS expression observed in central vascular region. (f) Root of banana stained with GUS buffer. Note that the *MusaVND2* promoter is specifically active in central vascular region. (g) Root section displaying GUS staining in central vascular region. (h) Transverse section of root at higher magnification showing strong GUS expression in central vascular region. Safranin was used as counter stain in sections.

Analysis of transgenic banana harboring $P_{MusaVND3}$ :: *GUS*

Tissue specific expression of *MusaVND3* was studied by *GUS* staining of transgenic banana harboring $P_{MusaVND3}$ :: *GUS*. For this, a 1.3kb 5'-upstream regulatory region was PCR amplified and cloned upstream of *GUS* and $P_{MusaVND3}$::*GUS* so generated was transferred to banana. In leaves, intense and specific GUS staining was observed in veins comprising of both longitudinal and commissural vascular bundles (**Fig. 3.16a**). Transverse section of leaf indicated very specific GUS staining in vascular bundles including vessels (**Fig. 3.16b**) and surrounding tracheids (**Fig. 3.16c**). In corm, the activity of $P_{MusaVND3}$ was restricted to lignified vascular strands scattered in the central stele and cortex region (**Fig. 3.16d**). In pseudostem, GUS staining was observed in the lignified cells (**Fig. 3.16e**) and a close-up view suggested strong GUS activity in thick walled xylem vessels and proximal cells which is most probably due to diffusion of GUS stain from vessels (**Fig. 3.16f**). Transverse section of petiole showed GUS activity in xylem vessel and nearby tracheids (**Fig. 3.16g**). Transverse section of roots showing GUS staining in central vascular cylinder (**Fig. 3.16h**).However, in roots the GUS expression was observed in the peripheral protoxylem and not in central metaxylem elements and all of which showed visible secondary wall thickening (**Fig. 3.16i**).

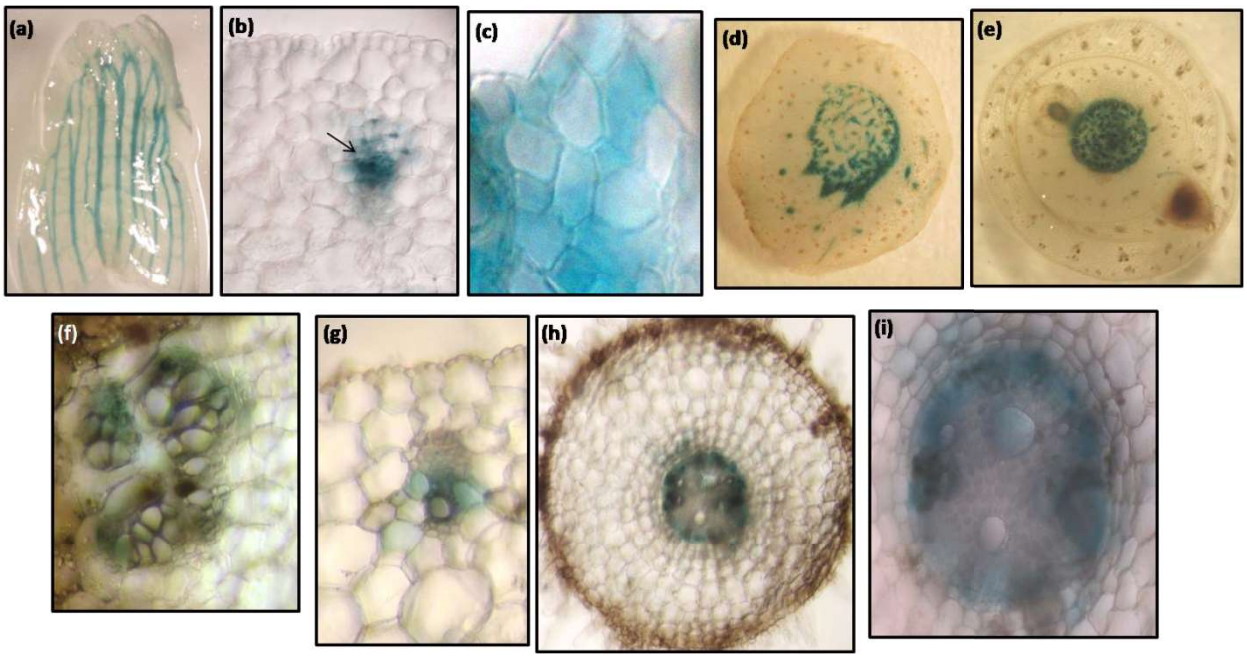

Fig. 3.16 Expression analysis of *MusaVND3* gene. Transgenic banana harboring $P_{MusaVND3}$ ∴ *GUS* was generated and different organs were stained with GUS staining buffer to study the tissue specific activity of $P_{MusaVND3.}$ (a) GUS staining of transgenic banana leaf. Note the strong and specific GUS activity localized in the veins of the leaf. (b) Cross section of the GUS stained leaf. Arrow indicate the vascular bundle showing intense GUS staining. Note the absence of GUS staining in other cells apart cells of vascular tissue. (c) Close up of leaf vascular bundle showing GUS staining in xylem vessels. (d) $P_{MusaVND3}$ activity in the corm of the banana. Note the strong expression in the lignified portion of the corm. (e) GUS staining observed in the pseudostem portion of the banana. (f) Close up of central portion of pseudostem showing strong GUS expression in xylem vessels. (g) Activity of $P_{MusaVND3}$ in petiole cross section. (h) $P_{MusaVND3}$ activity in roots of banana. (i) Close up of central vascular region of root.

GUS activity under the control of $P_{MusaVND1}$, $P_{MusaVND2}$ and $P_{MusaVND3}$

The activity of 5'upstream regulatory regions of banana *VND1-VND3* controlling the *GUS* gene in transgenic banana was estimated. The data obtained suggested that $P_{MusaVND1}$, $P_{MusaVND3}$ and $P_{MusaVND3}$ could drive the expression of GUS in all the organs of banana although at different levels which could be correlated with the level of lignification in different organs of banana. In case of $P_{MusaVND1}$ the highest GUS activity was observed in banana corm while lowest was from leaf tissue (**Fig. 3.17a**). $P_{MusaVND2}$ was most active in corm, followed by pseudostem, leaves and roots (**Fig. 3.17b**). $P_{MusaVND3}$ showed highest activity in terms of GUS activity in pseudostem and corm followed by roots and leaf (**Fig. 3.17c**).

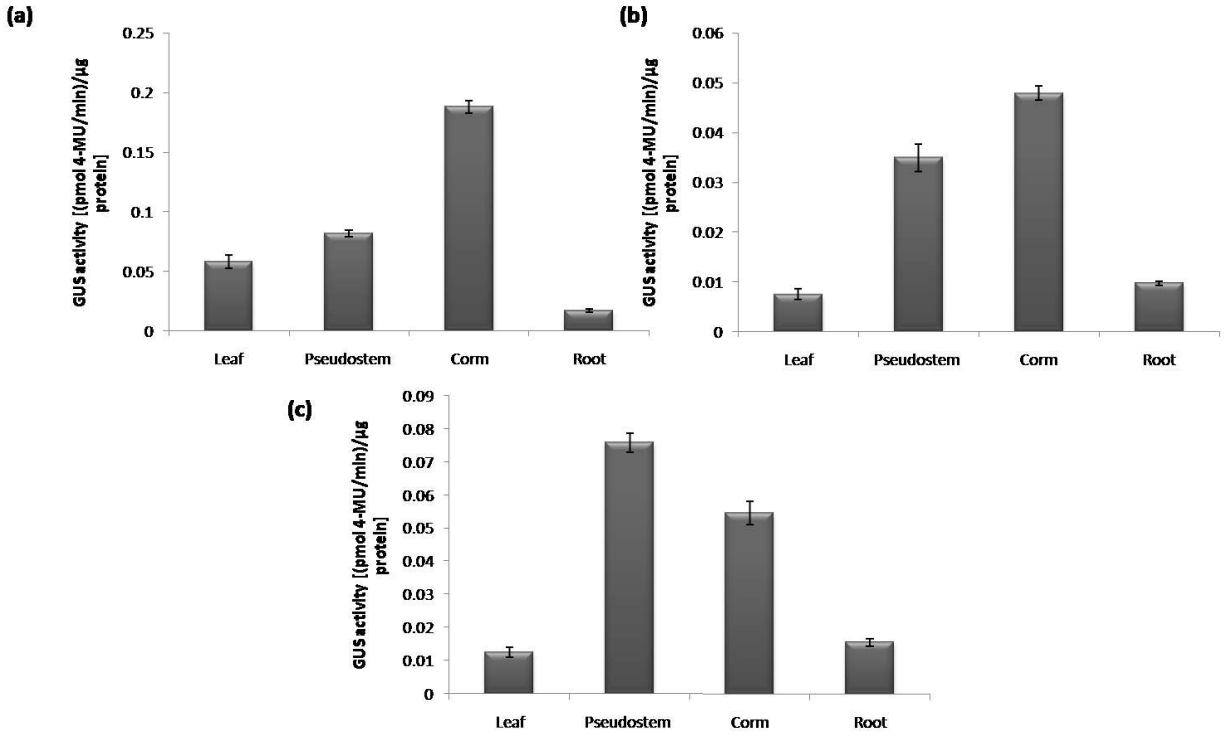

Fig. 3.17 GUS activity in transgenic banana plants harboring $P_{MusaVND1}$ :: *GUS*, $P_{MusaVND2}$ :: *GUS* or $P_{MusaVND3}$ :: *GUS*. (a) GUS activity in different organs of transgenic banana harboring $P_{MusaVND1}$ :: *GUS*. (b) GUS activity in leaf, roots, pseudostem and corm of banana transformed with $P_{MusaVND2}$ :: *GUS*. (c) GUS activity in various tissue of banana harboring $P_{MusaVND3}$ :: *GUS*.

GUS activity was calculated as (pmol 4-MU/min)/µg protein and is represented as mean±SD of three replications.

Purification of banana VND1-3 protein

To investigate the DNA binding activities of banana VND1-VND3, the complete coding sequence of *MusaVND1*, *MusaVND2* and *MusaVND3* was cloned in *pET28a* and induced the protein expression in *E.Coli BL21(DE3)* by 1mM IPTG. The expression of banana VND1-VND3 resulted in inclusion bodies formation which was solubilized by N-lauryl sarcosine for protein purification on Ni-NTA column. Different fractions collected during purification were analyzed in 12% SDS poly acrylamide gel electrophoresis (PAGE) (**Fig. 3.18**). Complete purification of MusaVND1 (**Fig. 3.18a**), MusaVND2 (**Fig. 3.18b**) and MusaVND3 (**Fig. 3.18c**) was obtained and the pure protein in elution fractions was dialyzed for removal of salts and imidazole. The induced banana VND1-VND3 were detected in western blot analysis using an antibody against polyhistidine (**Fig. 3.18d**).

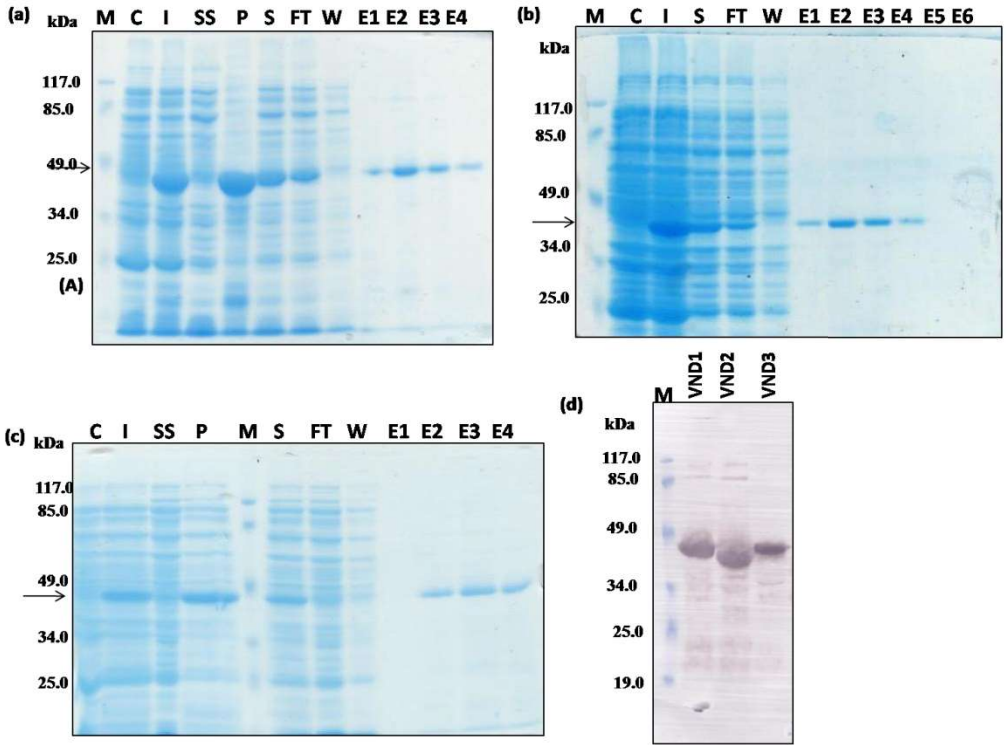

Fig. 3.18 Purification and detection of recombinant protein. (a) Purification profile of MusaVND1 protein in *E.Coli BL21(DE3)* having *pET28a*- MusaVND1 induced by 1mM IPTG. Induced protein (Indicated by an arrow) was purified on Ni-NTA column and analyzed on 12% SDS PAGE. (b) Purification profile of MusaVND2 induced and purified similar to MusaVND1. (c) SDS-PAGE analysis showing purification of MusaVND3 on Ni-NTA column. Induced and purified protein is indicated by arrow. Lane M: Molecular weight marker; C: Control culture; I: induced culture; SS: sonicated supernatant ; P: pellet; S: soluble fraction ; FT: flow through; and W: wash. (d) Western blot analysis to detect the recombinant protein. Different proteins were detected by antibody against polyhistidine.

Banana VND1-VND3 binds to the *SNBE* sites

To analyze whether MusaVND1, MusaVND2 and MusaVND3 bind to *SNBE* sites, complementary oligonucleotides with *SNBE* sites (38bp *SNBE*) were synthesized, annealed and the mobility of ds-DNA in gel in the absence and presence of banana VND1-VND3 was analyzed. Banana VND1-VND3 could retard the mobility of the *SNBE* site containing ds-DNA. The binding of VND1-VND3 with *SNBE*-like site occurred in dose dependent manner as the binding increased with increasing protein content (**Fig. 3.19).**

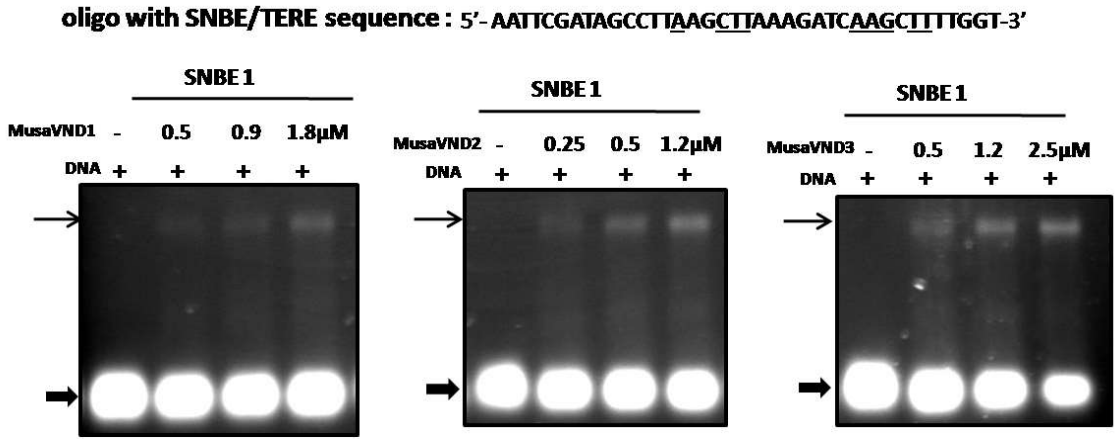

Fig. 3.19 EMSA analysis of MusaVND1, MusaVND2 and MusaVND3 binding to *SNBE* sites. Oligonucleotide with *SNBE* site (38 bp *SNEB*) was employed for EMSA analysis. The consensus residues forming the SNBE site (19bp) are underlined. Binding of MusaVND1, MusaVND2 and MusaVND3 to the *SNBE1* site containing ds-DNA. Amount of protein used in the reaction is indicated on the top of the well. + sign indicate presence of DNA. Protein-DNA complex is indicated by thin arrow while the unbound DNA is shown by a thick arrow. The bound DNA increased with the amount of the protein.

Banana VND1-VND3 binds regulatory sequence of multiple putative secondary wall related genes

Banana *VND1-VND3* could trigger the ectopic secondary wall synthesis in transgenic banana indicating that banana *VND1-VND3* could regulate secondary wall associated genes. Further, banana *VND1-VND3* could bind a 38bp ds-DNA containing *SNBE* site prompting us to analyze the presence of *SNBE* sites in the regulatory regions of secondary wall associated genes in banana. The 5'upstream regulatory regions of *Arabidopsis* homologues of five *MYB* transcription factors with roles in secondary wall deposition regulation (*MYB52*, *MYB85*, *MYB58/72*, *MYB46*, and *MYB83*) and six cell-wall modification genes (*IRX1*/*CesA8*, *IRX3*/*CesA7*,*IRX5*/*CesA4*, *IRX8*, *IRX10* and *IRX12*) was analyzed for presence of putative *SNBE*-like sites (**Table 3.1**). Presence of *SNBE*-like motif indicated possibility of regulation of these genes by banana VND1-VND3 through direct binding to their promoter regions. Our gel shift analysis suggested that mobility of SNBE containing 5'upstream regulatory region of *AtMyb52-like* (227bp region from -634 to -407 and 213 bp region from -40 to -253), *AtMYB85-like* (270 region from -338 to -608), *AtMYB58*/*72-like* (184bp from -490 to -674 and 201bp from -42 to -243), *AtMYB46-like* (218bp from -165 to -383 and 211bp from -649 to -860), *AtMYB83-like* (290bp extending from -282 to -572), *AtIRX1-like* (540bp from -281 to -821), *AtIRX3-like* (219bp from -239 to -458), *AtIRX5-like* (203bp from -656 to -859), *AtIRX8-like* (220bp from -291 to -511), *AtIRX10-like* (206bp from -133 to -339) and *AtIRX12-like* (220bp from -48 t0 -268) was retarded by MusaVND1 (**Fig. 3.20**), MusaVND2 (**Fig. 3.21**) and MusaVND3 (**Fig. 3.22**). All these fragments contain *SNBE*-like sites to which banana VND1-VND3 could bind specifically. Also the retardation of these regulatory regions got increased at increased protein concentration suggesting that banana VND1-VND3 bind efficiently in a dose dependent manner. To investigate whether banana

VND1-VND3 could elevate the expression of these genes from their promoters, quantitative RT-PCR was performed in transgenic banana overexpressing either of *MusaVND1*, *MusaVND2* or *MusaVND3*. Higher transcript level of *AtMyb52-like*, *AtMYB58*/*72-like*, *AtMYB46-like*, *AtMYB83-like*, *AtMYB85-like* transcription factors due to overexpression of *MusaVND1, MusaVND2* and *MusaVND3* in banana suggested that banana *VND1-VND3* are competent regulators of these genes (**Fig. 3.23**). Moreover, banana *VND1-VND3* also elevate the expression of *AtIRX-like* genes suggesting that they trigger the secondary wall deposition by not only increasing regulatory factors like MYB transcription factors but also effector genes involved cell wall biosynthesis (**Fig. 3.23**).These results indicated that banana VND1-VND3 can regulate the secondary wall biosynthesis by triggering a whole cascade of genes involved in the process of secondary wall deposition.

Table 3.1 SNBE-like sequences present in the 5'upstream regulatory region of secondary wall associated genes.

	Gene	**Close homologues in *Arabidopsis***	**Putative SNBE sites detected in 1 kb upstream region**	**Function of *Arabidopsis* homologue in secondary wall deposition**
1	*AtMYB52-like* (GSMUA_Achr9T29090_001)	*AtMYB52* (NM_101658)	-87 TATCTTTTCCTGTAGAGAT -433 AGGCTAACATGAAAAGCCA -594 ATATATCTGATCCAAGAGA	Activator
2	*AtMYB85-like* (GSMUA_Achr4T19880_001)	*AtMYB85* (NM_118394)	-400 CAACGTAGCTTCTACGCAA -444 TGACGTACACCTCAACCTT -604 TTTTCTTTTCTGCAAGGAA	Activator
3	*AtMYB58*/*72-like* (GSMUA_Achr4T27350_001)	*AtMYB58* (XM_002892864) *AtMYB72* (NM_104495)	-181 CTCTCTCGTAGAAAAGAAC -632 GACCGTCTTCTCTAAGATG	Activator
4	*AtMYB46-like* (GSMUA_Achr2T07780_001)	*AtMYB46* (XM_021033783) *AtMYB83* (NM_111685)	-204 TTTCCTGATCGACACGTTC -332 TACCTTGTGAGTGAAGATA -778 TCACGTAGCAATGAAGAAT	Activator
5	*AtMYB83-like* (GSMUA_Achr8T31350_001)	*AtMYB83* (NM_111685)	-387 TACCTTGTGAGTGAAGATA	Activator
6	*AtIRX1*/*AtCesA8-like* (GSMUA_Achr6T31810_001)	*AtIRX1*/*AtCesA8* (NM_117994)	-395 ATTTTTCGGTTCTACGGCT -778 AAACCTGAAAGAAAAGTAT	Cellulose biosynthesis
7	*AtIRX3*/*AtCesA7-like* (GSMUA_Achr7T19410_001)	*AtIRX3*/*AtCesA7* (XM_002873781)	-367 AAGCTTGAAGGAAAAGCTT	Cellulose biosynthesis
8	*AtIRX5* /*AtCesA4-like* (GSMUA_Achr5T06050_001)	*AtCesA1* (XM_021018412) *AtIRX5*/*AtCesA4* (NM_123770)	-366 CGGCCTCGGTCGAACGGAT -411 CCACCTCCATCGGACGCTT -743 TCCATTAAGCACTACGAAT	Cellulose biosynthesis

9	*AtIRX8/ GAUT12-like* (GSMUA_Achr4T33560_001)	*AtIRX8/ GAUT12* (NM_124850)	-400 ATGCTTGGAAAGCAAGTAA -483 CCTCCTTTATTTGAAGTTA -649 TATTTTGGCATTTACGACA -808 AGATGTCCGGTCAAAGACT	Cell wall biosynthesis
10	*AtIRX10/ GUT2-like* (GSMUA_Achr3T18610_001)	*AtIRX10/ GUT2* (XM_021013394)	-248 TCTCTTCAAGCAAAAGTCC -506 TGATTTAAATACAAAGCTC	Cell wall biosynthesis
11	*AtIRX* 12 / *Laccase-4-Like* (GSMUA_Achr6T33600_001)	*AtIRX* 12 / *Laccase*-4 (XM_021029137)	-167 ATCCTTGCTTGCCAAGGTT -216 TTATGTGGGATTTAAGTAA	Cell wall biosynthesis

Table note Conserved residues in *SNEB-like* sites are underlined. Corresponding NCBI accession number or banana genome locus identifier is also indicated.

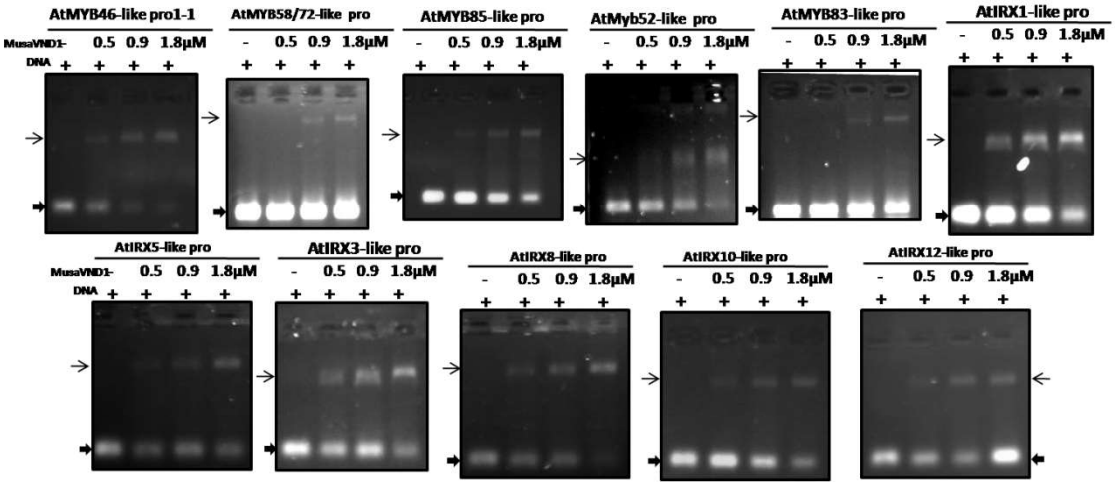

Fig. 3.20 MusaVND1 bind to 5'-upstream region of multiple putative secondary wall related genes. 5'-upstream region of putative secondary wall related was incubated with indicated amount of protein and later resolved on agarose gel. + sign indicate presence of DNA. Protein-DNA complex is indicated by thin arrow while the unbound DNA is shown by a thick arrow. The bound DNA increased with the amount of the protein. Identities of the DNA sequences used are indicated on the top of the respective gels.

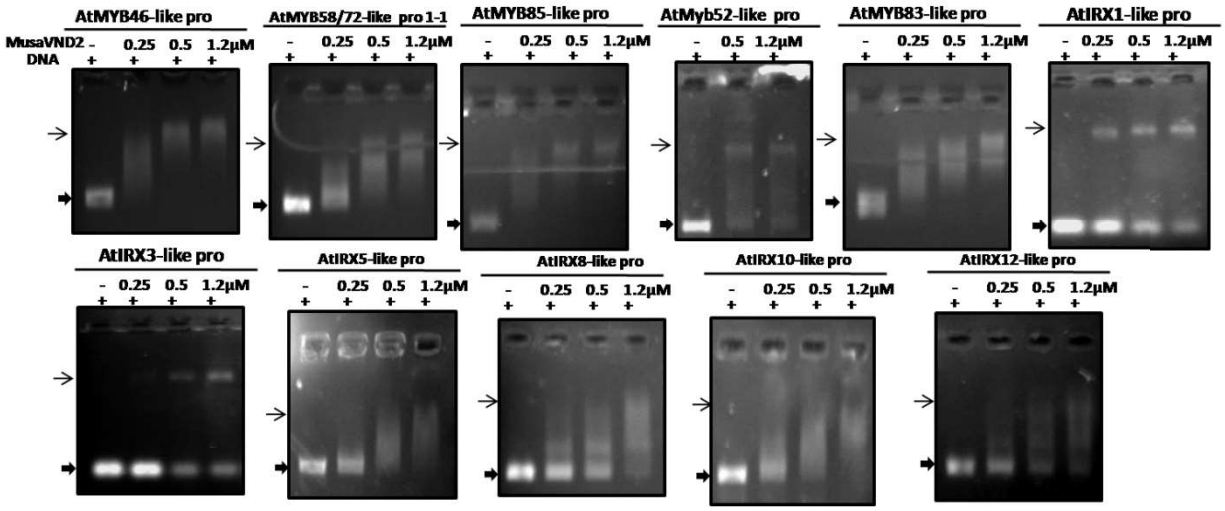

Fig. 3.21 Binding analysis of MusaVND2 with the *SNBE* containing 5'-upstream region of multiple putative secondary wall related genes. Identity of the DNA sequences used are indicated on the top of the respective gels. Indicated amount of protein was incubated with DNA fragments in gel shift assay buffer and later resolved on agarose gel. The bound DNA increased with the amount of the protein. + sign indicate presence of DNA.

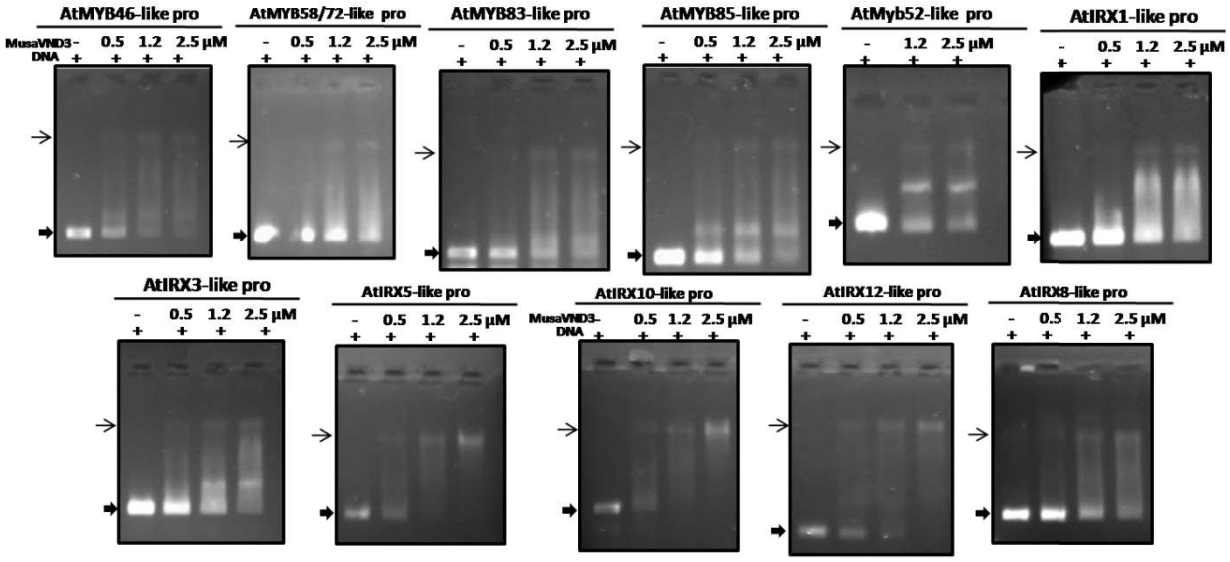

Fig. 3.22 DNA gel shift assay of multiple putative secondary wall related genes with MusaVND3. Different concentrations (0, 0.5, 1.2 and 2.5µM) of MusaVND3 was incubated with different DNA substrates and EMSA reaction was resolved on agarose gel. + sign indicate

presence of DNA. Protein-DNA complex is indicated by thin arrow while the unbound DNA is shown by a thick arrow. The bound DNA increased with the amount of the protein. Identity of the DNA sequences used are indicated on the top of the respective gels.

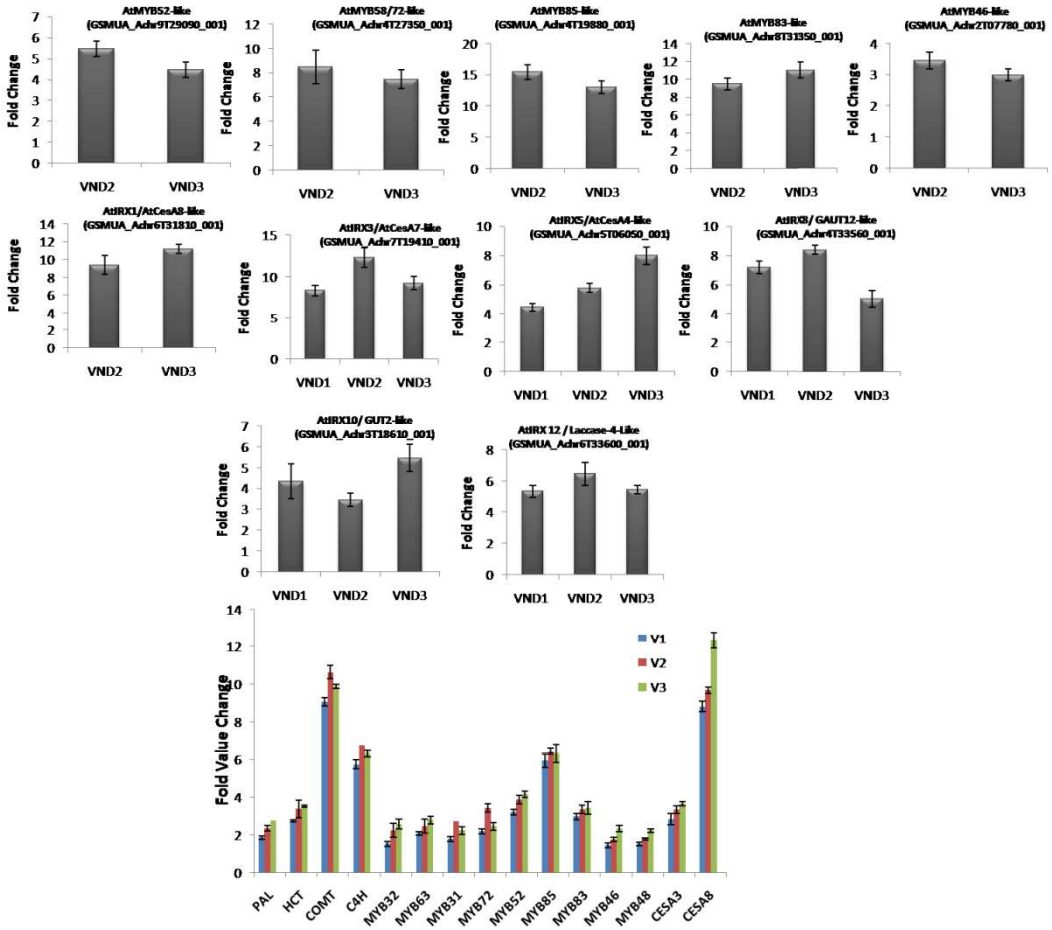

Fig. 3.23 Expression analysis of important secondary wall related genes in transgenic banana overexpressing MusaVND1, MusaVND2 and MusaVND3. Transcript levels of indicated genes were monitored by quantitative real time RT-PCR. Identity and corresponding banana genome locus identifier is indicated on the top of individual graph. Expression of each gene was normalized by expression of banana EF1α and data was represented as fold change relative to control transcript level. mean±SD of three replication is indicated in the bar graph.

Discussion

The lignocellulosic biomass as major renewable energy resource is important as it can be processed into ethanol for liquid fuel production. The production cost of lignocellulosic biomass is very high compared with crude oil prices. The high cost of biomass derived biofuels is due to factors like low sugar content of biomass and difficulties in enzymatic hydrolysis of cell wall (Yang et al. 2013). However, the information about genetic regulations of lignocellulosic biomass deposition is still in infancy. There is a need to analyze potential genetic factors including transcription factors regulating secondary wall deposition as such studies will provide information about coordinated regulation of cell wall deposition. Such studies may offer new avenues for development of lignocellulosic biomass with better desirable characteristic like downstream processing (Yamaguchi and Demura 2010) and increased secondary wall deposition for increasing plant biomass (Yang et al. 2013) and reducing lodging problem (Ma 2009; Li et al. 2009; Ookawa et al. 2010). The desired properties of lignocellulosic biomass can be better modulated by genetic manipulations of such genetic factors regulating secondary wall development because of the fact that these genes hold the ability to change the expression of many other genes. To achieve these aims, characterization of potential genetic factors involved in regulation of secondary wall deposition is indispensable. In banana characterization of genetic factors regulating secondary wall deposition is essential as banana is being considered as a potential second-generation biofuel crop (Clarke et al. 2008). Activation and regulation of both cellulose and lignin biosynthetic pathway is essential for secondary wall deposition. Important functions of secondary wall in plant include formation of water conducting channels and providing mechanical support for vertical growth of plants. A network of genetic factors regulates secondary wall deposition and most important among them are vascular related *NAC*

domain containing transcription factors. Recently the complexity in developmental regulation of xylem cell was proposed as a genetic network comprising of many feed forward loops and it was revealed that the network is perturbed by salt stress (Taylor-Teeples et al. 2015). The regulation of secondary wall deposition and tracheary element development by *VND6* and *VND7* has been emphasized in different plant systems. One of the pioneer work has indicated that *VND6* and *VND7*, respectively induces transdifferentiation into metaxylem and protoxylem like vessel elements and they are suggested to be switches for formation of metaxylem and protoxylem like vessel elements respectively (Kubo et al. 2005). Another study demonstrated that *Arabidopsis VND7* with possible collaboration with *VND2* to *VND5* can regulate differentiation of all types of vessels (Yamaguchi et al. 2008). *Arabidopsis VND6* regulate the tracheary element formation by directly regulating the expression of genes connected with programmed cell death and secondary wall deposition (Ohashi-Ito et al. 2010).

Three vascular related *NAC* domain-containing transcription factors from banana namely *MusaVND1, MusaVND2* and *MusaVND3* were characterized, and evidences for their function in regulating secondary wall deposition are provided. *MusaVND1, Musa*VND2 and *Musa*VND3 are orthologs of poplar secondary wall development related transcription factors, PtVNS01, PtVNS02 and PtVNS03 respectively. Induction of ectopic secondary wall deposition in leaves of transgenic poplar after overexpression of various poplar vessel development related genes revealed that PtVNS01, *PtVNS02* and *PtVNS03* genes are among the important genes involved in secondary wall development (Ohtani et al. 2011). Phylogenetic analysis revealed that *Musa*VND1, *Musa*VND2 and *Musa*VND3 also showed high homology with AtVND1-VND7 transcription factors of *Arabidopsis*, thus indicating a probable similar functions of *Musa*VND1, *Musa*VND2 and *Musa*VND3. A recent study on functional characterization of *AtVND1-VND5*

established the important role of these transcription factors in secondary wall development in *Arabidopsis*. Ectopic secondary wall deposition in transgenic leaves was observed after overexpression of *AtVND1-VND5* while dominant repression of *AtVND3* caused reduced secondary wall thickness in vessels of transgenic plants (Zhou et al. 2014). The expression of *MusaVND1, MusaVND2* was specific in vascular tissue as indicated by GUS staining in banana harboring GUS coding sequence under the control of banana VND1-VND3 promoter. The expression of poplar secondary wall development related transcription factors, *PtVNS01, PtVNS02* and *PtVNS03* showed higher expression in woody tissue like stem and lower in regions like shoot apices and leaves (Ohtani et al. 2011). However, utilization of such genetic factors under constitutive promoter have resulted in biomass reduction and hence, expression of such genetic factors under vascular tissue specific promoter is desirable for a sustainable development of desired lignocellulosic biomass (Yang et al. 2013). The 5'upstream regulatory region ($P_{MusaVND1}$, $P_{MusaVND2}$ and $P_{MusaVND3}$) of three banana *VND* genes was analyzed which showed specific and intense activity in vascular tissue. Banana is being looked upon as a second-generation biofuel crop hence, utilization of these regulatory elements for potential biomass manipulation in near future may be warranted (Clarke et al. 2008). *MusaVND1, MusaVND2* and *MusaVND3* encodes for nuclear localized protein and their transient overexpression results in differentiation of banana embryogenic cells into tracheary element-like cells. One of the earlier reports has utilized a glucocorticoid inducible system for overexpressing *Arabidopsis VND6* and *VND7*, which was able to differentiate BY-2 cells of tobacco into xylem vessel elements (Yamaguchi et al. 2010). Hence, *MusaVND1, MusaVND2* and *MusaVND3* possess similar potential to differentiate banana cells into xylem vessel elements. Transgenic banana plants overexpressing either *MusaVND1, MusaVND2* or *MusaVND3* were generated, and the plants

were analyzed for transdifferentiation, secondary wall deposition and alteration in cell wall components. Transdifferentiation to tracheary element-like cells in leaves and corm of banana overexpressing either *MusaVND1, MusaVND2* or *MusaVND3* indicated that these genes might have important function in regulating secondary wall development in banana. Observation of transdifferentiation in epidermal and mesophyll cells suggests that these genetic factors may be active in only certain kind of cells in banana. However, absence of secondary wall deposition in other cells is perhaps due to causes like deficiency of certain factors or occurrence of some kind of inhibitors in these cells. Measurement of cellulose and lignin content established that transgenic lines over expressing either *MusaVND1, MusaVND2* or *MusaVND3* relative to control plants, had considerably elevated cellulose and lignin content. Elevated cellulose content in transgenic lines indicated that overexpression of these genes is able to modify the expression of cellulose biosynthesis pathway genes along with genes of lignin biosynthesis pathway. These results suggested that banana *VND1-VND3* are efficient regulator of secondary wall development suggesting that these genes can also act as master regulators in monocots similar to dicots. The direct targets of banana VND1-VND3 was analyzed by the binding of banana VND1-VND3 proteins to *SNBE* sites in their target gene. Direct targets of *Arabidopsis* VND6, VND7 and SND1 has been demonstrated by analyzing their binding to *SNBE* sites in the regulatory regions of secondary wall associated genes (Zhong et al. 2010; Ohashi-Ito et al. 2010). Expression pattern of all the different *VND* genes during tracheary element formation (Kubo et al. 2005) and in planta was different suggesting that each *VND* gene might have different function in vascular development (Kubo et al. 2005, Yamaguchi et al. 2008). Banana VND1-VND3 could bind to *SNBE-like* sequences in isolation or as a part of regulatory region of target genes suggesting that banana VND1-VND3 and *Arabidopsis* VND6, VND7 and SND1 share a common recognition

motif in downstream genes. The *SNBE* site has been worked out to be a 19bp imperfect palindrome having consensus sequence *(T/A)NN(C/T) (T/C/G)TNNNNNNNA(A/C)GN(A/C/T) (A/T*) to which different SWNs could bind and regulate their target genes (Zhong et al. 2010). We identified *SNBE-like* sequences in the promoter region of multiple secondary wall associated genes in banana thus indicating that banana VNDs might regulate their downstream genes by these motifs. Some of the direct targets of *Arabidopsis* SND1 and VND7 identified are *MYB46*, *MYB83*, *MYB58*/*72*, *MYB52* and *MYB85* and they have been shown to be involved in regulation of secondary wall deposition (Zhong et al. 2007a; McCarthy et al. 2009; Zhou et al. 2009; Oh et al. 2003). However, distinct set of genes induced by *Arabidopsis* SND1 and VND7 have also been identified suggesting SND1 and VND7 differentially regulate multiple genes which may be due to their binding affinity towards particular *SNBE* site and expression differences of *SND1* and *VND7* in cell (Zhong et al. 2010). We have identified *SNBE-like* sites in the regulatory region of homologues of *MYB46*, *MYB83*, *MYB58*/*72*, *MYB52* and *MYB85* in banana and identified their binding with purified banana VND1-VND3 proteins. Banana VND1-VND3 could bind to *SNBE like* sites containing regulatory region of *MYB46*, *MYB83*, *MYB58*/*72*, *MYB52* and *MYB85* homologues in banana. Further, a number of putative *SNBE-like* sites was detected in 1kb 5'upstream regulatory region of a number of non-transcription factor genes like genes involved in cellulose deposition such as *irregular xylem* genes like *IRX1*, *IRX2*, *IRX5* and *IRX8* and banana VND1-VND3 could retard their mobility in a dose dependent manner in a gel shift assay. Thus banana VND1-VND3 not only regulate downstream regulatory genes but also a number of effector genes in secondary wall synthesis suggesting a possible cooperation with downstream transcription factors to establish an effective transcript concentration. Overexpression of *Arabidopsis MYB46*, *MYB83*, *MYB58*/*72*, *MYB52* and *MYB85* could also

induce secondary wall deposition indicating that these factors may act in cooperatively with upstream *VND* transcription factors (Zhong et al. 2008; Zhong et al. 2007a; McCarthy et al. 2009; Zhou et al. 2009). We could not detect any *SNBE-like* sites in the regulatory regions of lignin biosynthesis related genes in banana suggesting the absence of direct regulation of these genes by *VNDs*. However, overexpression of banana *VND1* resulted in elevation in expression of at least four lignin biosynthesis related genes (*PAL*, *HCT*, *COMT*, and *C4H*) suggesting up regulation of these genes by one of the direct target of the *VND1*. Regulation of lignin biosynthesis genes by *MYB* transcription factors are reported for example poplar *MYB3* and *MYB20* which are orthologos of *Arabidopsis MYB46* and *MYB83* are known to regulate *CCoAOMT* (McCarthy et al. 2010). Many programmed cell death related genes such as *cysteine proteases* (*XCP1* and *XCP2*) and *nucleases* (*ZEN1*) are known to be expressed during tracheary cell differentiation (Bollhöner et al. 2012; Ito and Fukuda 2002). Regulation of *XCP1* and *XCP2* through *SNBE* motifs by *Arabidopsis* VND7 and *Brachypodium* SWN5 has been reported (Yamaguchi et al. 2011; Valdivia et al. 2013).Banana XCP1 contain three *SNBE-like* sequences within 500bp upstream of start codon and banana VND1-VND3 could bind to this regulatory region. Thus banana *VND1*, *VND2* or *VND3* regulate the secondary wall synthesis by inducing directly or indirectly a complete set of genes required for tracheary element differentiation.

Table 3.2 Primers used for cloning and molecular studies on *MusaVND1*, *MusaVND2* and *MusaVND3*

Description	**Forward Primer (5' - 3')**	**Reverse Primer (5' -3')**
Primers for cloning *MusaVND1* in pCAMBIA1301	tctgcagATGGAATCGTGTGTTCCTCCT	tggtaccTCATTGCTCAAATATACAGATGC
Primers for cloning *MusaVND1*coding sequence in pCAMBIA1302	aaAGATCTgGAATCGTGTGTTCCTCCTGGATTC	aaACTAGTTTGCTCAAATATACAGATGCCTTTG
Primers for cloning *MusaVND2* in pCAMBIA1301	tcctgcaggATGGAATCGTGTGTTCCTC	tggtaccTCACTGCTCGAATATACAGATG
Primers for cloning *MusaVND3* in pCAMBIA1301	t*cctgcagg*ATGGATGCATTCCCTCATGT	tggtaccTCATTTCCACAGTTCGACTT
Primers for cloning *MusaVND2* coding sequence in pCAMBIA1302	aaagatctgGAATCGTGTGTTCCTCCAGG	aaactagtCTGCTCGAATATACAGATGCCT
Primers for cloning *MusaVND3* coding sequence in pCAMBIA1302	TTTggatccgGATGCATTCCCTCATGTGCCG	TTTactagtTTTCCACAGTTCGACTTGGCC
Primers for cloning $P_{MusaVND1}$ in pCAMBIA1301	TTTCTAGATCTCTTACTGATCCAGTCAGCG	TTTCATGAGGGTTCCTTTGTCTGCACC
Primers for cloning $P_{MusaVND2}$ in pCAMBIA1301	TTCTGCAGGACCATTCTAGCTAGGGTTC	ttAGATC*G*TaccatCGTCTGCATCGAATTCG
Primers for cloning $P_{MusaVND3}$ in pCAMBIA1301	TTGGATCCCCTTTCCCCGCAATCC	TTCCATGGGTTCTCTAGGCTTTGTAGTCTTTGC
Primers for cloning *MusaVND1* in pET28a	**aaaCATATG**GAATCGTGTGTTCCTCCTG	aaa**GGATCC**TCATTGCTCAAATATACAGATGC
Primers for cloning *MusaVND2* in pET28a	**aaaCATATG**GAATCGTGTGTTCCTCCA	aaa**GGATCC**TCACTGCTCGAATATACAGATG
Primers for cloning *MusaVND3* in pET28a	**aaaCATATG**GATGCATTCCCTCATGTGC	aaa**GGATCC**TCATTTCCACAGTTCGACTT
Hygromycin phosphotransferase (*hpt)* gene	GTCCTGCGGGTAAATAGCTG	ATTTGTGTACGCCCGACAGT
Musa EF1α real time PCR primers	CCGATTGTGCTGTCCTCATT	TTGGCACGAAAGGAATCTTCT

Table 3.3 Lignin biosynthesis pathway genes and the primers used in studies on *MusaVND1*, *MusaVND2* and *MusaVND3*

Gene	Banana genome locus identifier	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
PAL	GSMUA_Achr5T18560_001	CTTGGACTACGGCTTCAAGG	CTTGAGGTTCTCCTCCAGGT
COMT	GSMUA_Achr3T27470_001	CATGATCACCTCCAAACACC	AACGCACTCCACCACTATCA
4CL	GSMUA_Achr1T23450_001	GCGACAAGGAACACCATAGA	CATTCGACCTCACGACAAAC
HCT	GSMUA_Achr6T01940_001	CAGAAGGGCTTATGGGTGAT	TCAGAGCCTCGAGTTCCTC
CAD6	GSMUA_Achr5T11490_001	CGCTGATGATTTCCTCGTTA	CTCCTGCGTCTCCTTCATC
C4H	GSMUA_Achr6T08670_001	AGCAAGATCCTGGTCAACG	GTATGTGGAGGCTGAACTGG
CCoAMT	GSMUA_Achr6T36400_001	GCTCCTCAAACTGATCAACG	CGACCCATGGTTCTTCTCAT
CCR	GSMUA_Achr5T04460_001	GGTGTCTTCCACACTGCTTC	CCTTCCCGTAGCAATACCAG
C3H	GSMUA_Achr4T10110_001	TCAGGGAGGACGAGGTAATC	CAGAGAATTCCGATGATGGAG
F5H	GSMUA_Achr11T17500_001	CAAATCAGCATGGGAGGAC	GAATTAGATGAGAGGAGGTGCAG

Table 3.4 *MYB*, *Ces* and *IRX* genes and the real time PCR primers used in studies on *MusaVND1*, *MusaVND2* and *MusaVND3*

Gene	Banana genome locus identifier	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
AtMYB52-like	GSMUA_Achr9T29090_001	CAGCCAGATCAACAGAAGCC	TTCTCCATTCCTCCTCAGCC
AtMYB85-like	GSMUA_Achr4T19880_001	CAGGAAACCAAGGCCACATC	CCTTCTCGGCAAATTCTCCG
AtMYB58/72-like	GSMUA_Achr4T27350_001	GACACTCACCAAGAAGAGGC	CCTCCATCTCCATTTGCTGC
AtMYB46-like	GSMUA_Achr2T07780_001	CACTTCCTGAGGTTGGGCTA	AGTCTTCCAACTCCCACTCC
AtMYB83-like	GSMUA_Achr8T31350_001	TCCTACTTCCACGAACCACC	TCAAGTCTCCCAACTCCCAC

AtIRX1/AtCesA8-like	GSMUA_Achr6T31810_001	TGCTGGATTTTCTGACGCAC	AGCAGTTGATGGAAGCACAA
AtIRX3/AtCesA7-like	GSMUA_Achr7T19410_001	CCACCACCGTCCTGATCATA	CTGGTATCTGGGCCTTTGGA
AtIRX5 /AtCesA4-like	GSMUA_Achr5T06050_001	AGTCTCCTGATTCCACCGAC	CCCATCGTTGCAGCCTTTT
AtIRX8/ GAUT12-like	GSMUA_Achr4T33560_001	ATGGCATGAATGTCTTCGAT	CTGGGAATGCTATGTCCAAC
AtIRX10/ GUT2-like	GSMUA_Achr3T18610_001	TTCTCACGCTTCTGATCCTC	GGACTTGAGAGCAAGAACCA
AtIRX 12 / ***Laccase-4-Like***	GSMUA_Achr6T33600_001	CAACGAGGACCTCTTCTTCA	CGGTGAAGAGGAGAGAGTGA

Table 3.5 Primer sequences used for gel shift assay studies on *MusaVND1*, *MusaVND2* and *MusaVND3*

Name	**Forward Primer (5' - 3')**	**Reverse Primer (5' -3')**
AtMyb52-like pro 1-1 (--634 to -407)	TCTTTTCAAATGAAGGCACTTT	CCATAACTGGCTTTTCATGTT
AtMyb52-like pro 1-2 (-253 to -40**)**	ACGTACCGTCAATCTGTGGT	ATAACCCCTGCTGAGCTTTC
AtMyb48/59-like pro (488 To -282)	AACATAGCATCAGCTCAATCG	CCAGGTTTATAAGGGTCTCCA
AtMYB85-like pro (-608 to -338**)**	CCCTTTTTCTTTTCTGCAAG	TGGATGATCTCACCTAAATGC
AtMYB58/72-like pro 1-1 (-674 to -490**)**	TGAGACTTGAGGGTGAGGTG	TGCACAACTCAATTCACGAT
AtMYB58/72-like pro 1-2 (-243 to -42**)**	CTTTGTAGCTTGGCGAAAAC	GCTGCCAATAACCATTGAAG
AtMYB46-like pro1-1 (-383 to -165)	CAATCCCCAATCTCTCTCTCT	AGAACCCGGAGAAGGGAATA
AtMYB46-like pro 1-2 (-860 to -649)	TGATATCCAGAATCATACCCACA	GGGGAGCTACGTAAGTACTGG
AtMYB83-like pro	CATGTTCATGATGGCTCAGA	GTGTGTGTGTGCGTGAGAGA

(-572 to -282)		
AtXCP1-like pro (-334 to -7)	AACGCCTTTCATAGCAGTTC	GTTTGTTTTGGCTGGAAAGA
AtIRX1-like pro **(**-821 To -281)	ATCATATTCCACCCACTCGAA	CCGGGTCGGTTCTTGA
AtIRX3-like pro **(**-458 to -239)	TTCCGATCGTATGAAAAGGA	CTCATGCTCACTCAGCCTTC
AtIRX5-like pro **(**-859 to -656)	TAAATGGGCCCAAATAGTCC	CTTCTGCTGTGTCCATCCTC
AtIRX8-like pro **(**-511 to -291)	AGGATCGGGCTTTGGTTACT	CCATGAAGAGTGGTGGTTTG
AtIRX10-like pro **(**-339 to -133)	TCTCTCTCGAAGATGCCAAG	AGTCTCGGGGAGGAGAAAG
AtIRX12-like pro **(**-268 to -48)	ACTCATACAATGTGGGTTCCA	AGCTTGGACTTGCACGAAT
SNBE1	AATTCGATAGCCTTAAGCTTAA AGATCAAGCTTTTGGT	ACCAAAAGCTTGATCTTTAAGCTT AAGGCTATCGAATT

Chapter IV: Studies on *MusaSNAC1*, a stress associated NAC transcription factor from banana

Introduction

Drought negatively affects plant growth and crop yield by reducing the availability of water. Plants evolved an array of mechanisms including molecular, physiological and biochemical responses to counteract the reduced water availability (Ingram and Bartels, 1996). The influx of CO_2 for photosynthesis and transpiration for water loss is regulated by the opening and closing of stomatal aperture formed by guard cells (Hetherington and Woodward, 2003). Stress in plants causes synthesis of abscisic acid (ABA) which induces a variety of effects including stomatal closure (Giraudat et al. 1994). ABA induced stomatal closure in leaves is achieved by influx of Ca^{+2} coupled by solute efflux in guard cells (Blatt and Grabov 1997). H_2O_2 is known to be a second messenger in ABA induced stomatal closure in plants as it resulted in increase in Ca^{+2} content of guard cells by activating Ca^{+2} channels (McAinsh et al. 1996; Pei et al. 2000). ABA induced H_2O_2 generation in guard cells is achieved by activation of NADPH-oxidase through phosphorylation by SnRK2/OST1 (Kwak et al. 2003; Sirichandra et al. 2009). However, other mechanisms regulating H_2O_2 concentration in guard cells impacting the stomatal opening and closing are mostly unknown. Recently a zinc finger transcription factor, *DST* was reported as a negative regulator of stomatal closure acting through reduction of H_2O_2 accumulation in guard cell by modulating genes of H_2O_2 homeostasis (Huang et al. 2009). A NAC transcription factor from rice, *SNAC1* was induced in guard cells by drought and impart drought tolerance through increased stomatal closure (Hu et al. 2006). Rice *SNAC1* regulates a SRO-protein, *OsSRO1c* which is predominantly expressed in guard cells under stress-conditions (You et al. 2013).

Overexpression of *OsSRO1c* caused H_2O_2 accumulation in guard cells reducing the number of completely open stomata and thus lowering transpiration induced water loss conditions (You et al. 2013). Some guard cells specific *MYB* transcription factors have been reported in the past. *Arabidopsis MYB60*, a *R2R3-MYB* gene is specifically expressed in guard cells and *atmyb60-1* plants showed reduced stomatal opening and water loss resulting in less wilting symptoms under drought-conditions. (Cominelli et al. 2005). NAC proteins are plant specific transcription factor family playing important roles in development, secondary cell wall deposition, senescence and stress-responses (Olsen et al. 2005a). The first *NAC* gene identified was *RD26/ANAC072* (*RESPONSIVE TO DEHYDRATION 26*) was involved in ABA dependent stress-response pathway (Fujita et al. 2004). Three *Arabidopsis NAC* genes, *ANAC019*, *ANAC055* and *ANAC072* was induced by salinity, drought as well as ABA and could impart drought tolerance in transgenic *Arabidopsis* (Tran et al. 2004). Many NAC proteins regulate the formation of the reactive oxygen species (ROS) which are important signaling molecules. *AtNTL4* regulate *Atrboh* genes inducing ROS production and senescence (Lee et al. 2012). *SNAC3* from rice was induced in response to drought, salinity, ABA and high temperature and its overexpression increased tolerance to high temperature as well as drought (Fang et al. 2015). Overexpression of rice *SNAC3* could increase the expression of ROS-scavenging genes and three ROS-associated enzyme genes were direct targets of SNAC3 (Fang et al. 2015). *AtJUB1*, a H_2O_2 induced gene delays senescence by lowering intracellular H_2O_2 levels and regulate the *DREB2A* gene by binding to its promoter (Wu et al. 2012).

This chapter discuss the work carried out on a banana *NAC* transcription factor (*MusaSNAC1*) functioning in stomatal closure by increasing guard cells H_2O_2 concentration. *MusaSNAC1* was strongly expressed in guard cells under drought stress and *MusaSNAC1*

overexpressing transgenic banana plants retained more water under drought-conditions than control. Superior drought tolerance of transgenic lines than control was due to increased number of completely closed stomata, reducing the loss of water and thus conserving plant water content. MusaSNAC1 recognize the core binding site of NAC proteins CGT[A/G] in a gel shift assay. MusaSNAC1 was able to retard the mobility of 5'-upstream regions (containing CGT[A/G]) of six stress related genes and transcript level of these stress related genes was elevated in transgenic lines. This work suggested potential of *NAC* transcription factor for improving the drought tolerance ability of plants.

Results

***MusaSNAC1* is stress-responsive gene with expression in guard cells**

MusaSNAC1 was clustered with stress-related NAC transcription factors in a neighbor joining tree (stringency of 1000 bootstrap-replicates) indicating MusaSNAC1 belonged to SNAC subgroup (**Fig. 4.1**). This banana NAC transcription factor was named MusaSNAC1 because of its close similarity with other SNAC1 proteins from other plant species. MusaSNAC1 is a 271 amino acid long protein with conserved N-terminal NAC domain and variable C-terminal region (**Fig. 4.2**). Presence of a WVLCR-motif has been identified in many members of SNAC subgroup (Nuruzzaman et al. 2013) also suggested its association with stress-responsive NAC transcription factors. Transcript levels of *MusaSNAC1* were quantified under different stress-stimuli by quantitative RT-PCR analysis. Expression of *MusaSNAC1* got rapidly elevated during drought, peaking around 12 hours post stress and remained higher till 48hours (**Fig. 4.3a**). Transcripts of *MusaSNAC1* also showed higher expression during high salinity stress (**Fig. 4.3b**) and low temperature (**Fig. 4.3c**) exposure. Exposure of salicylic acid reduced the transcript abundance of *MusaSNAC1*, which remained lower even till 24hours (**Fig. 4.3d**). Spraying and

irrigating banana plants with H_2O_2 strongly reduced the expression of *MusaSNAC1* which remained lower even till 48hours post stress initiation (**Fig. 4.3e)**. Abscisic acid (ABA) application resulted in strong induction of *MusaSNAC1* transcripts, which then returned to normal towards 24 hours post application (**Fig. 4.3f**). The 5'-upstream sequence of this NAC transcription factor was cloned upstream of *GUS* to analyze the tissue nature of this gene. T-DNA containing $P_{MusaSNAC1}$*::GUS* was transferred to banana embryogenic cells and transgenic lines was subjected to stress-conditions (**Fig. 4.3g**). *GUS* expression from $P_{MusaSNAC1}$*::GUS* was detected predominantly in stomata (**Fig. 4.3i**) and close-up analysis indicated higher expression in guard cells (**Fig. 4.3j**). Wild type plants did not show any GUS staining (**Fig. 4.3h**) and microscopic examination of guard cells of control plant (**Fig. 4.3k**) also suggested that expression of *GUS* in lines carrying $P_{MusaSNAC1}$*::GUS* is due to activity of $P_{MusaSNAC1}$.

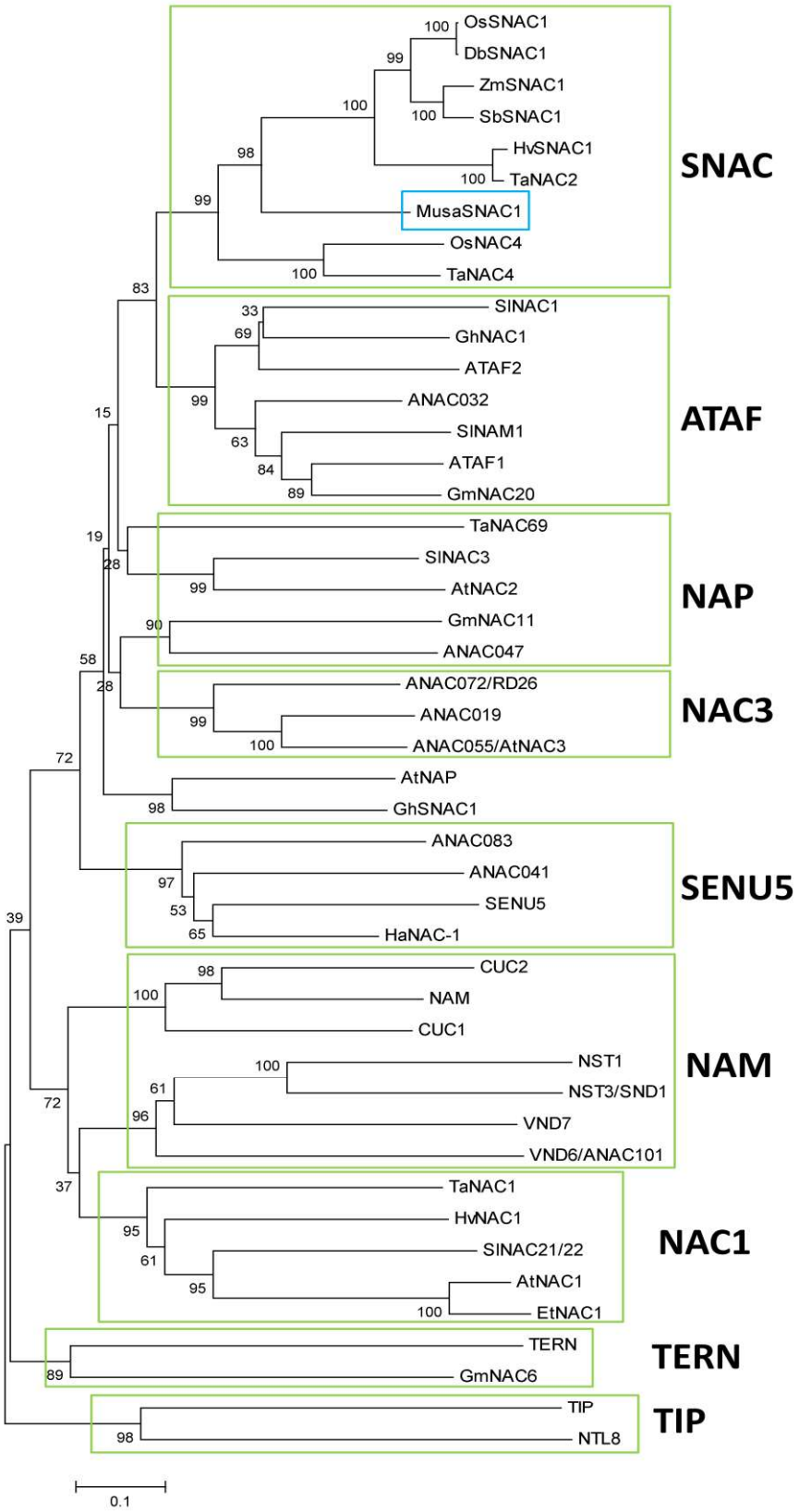
OsSNAC1
DbSNAC1
ZmSNAC1
SbSNAC1
HvSNAC1
TaNAC2
MusaSNAC1
OsNAC4
TaNAC4
SNAC
SlNAC1
GhNAC1
ATAF2
ANAC032
SlNAM1
ATAF1
GmNAC20
ATAF
TaNAC69
SlNAC3
AtNAC2
GmNAC11
ANAC047
NAP
ANAC072/RD26
ANAC019
ANAC055/AtNAC3
NAC3
AtNAP
GhSNAC1
ANAC083
ANAC041
SENU5
HaNAC-1
SENU5
CUC2
NAM
CUC1
NST1
NST3/SND1
VND7
VND6/ANAC101
NAM
TaNAC1
HvNAC1
SlNAC21/22
AtNAC1
EtNAC1
NAC1
TERN
GmNAC6
TERN
TIP
NTL8
TIP
0.1

Fig. 4.1 Phylogenetic relation of MusaSNAC1 with other NAC-proteins. Neighbor-joining-tree with stringency of 1000 bootstrap-replicates showed that MusaSNAC1 has close evolutionary relationship with HvSNAC1 and ZmSNAC1. NAC proteins sequence included in the tree are : OsSNAC1 (ABD52007.1), DbSNAC1 (AIX03021.1), ZmSNAC1 (NP_001123932.1), SbSNAC1 (AGG40203.1), HvSNAC1 (AEG21060.1), TaNAC2 (AAU08786.1), TaNAC4 (AMQ48929.1), OsNAC4 (BAA89798.1), SlNAC1 (NP_001234482.1), GhNAC1 (ACI15341.1), ATAF2 (XP_002873383.1), ANAC032 (XP_002887684.1), SlNAM1 (NP_001234219.1), ATAF1 (NP_171677.1), GmNAC20 (NP_001242756.1), TaNAC69 (AAY44098.1), SlNAC3 (AFD22355.1), AtNAC2 (NP_188170.1), GmNAC11 (ACC66315.1), ANAC047 (NP_187057.2), ANAC072/RD26 (NP_567773.1), ANAC019 (XP_002894409.1), ANAC055/AtNAC3 (NP_188169.1), AtNAP (NP_564966.1), GhSNAC1 (AKA58526.1), ANAC083 (NP_196822.1), ANAC041 (NP_180906.1), SENU5 (CAA99760.1), HaNAC-1 (AAW28153.1), CUC2 (BAA19529.1), NAM (CAA63102.2), CUC1 (BAB20598.1), NST1 (NP_182200.2), NST3/SND1 (NP_174554.1), VND7 (NP_177338.1), VND6/ANAC101 (NP_001318864.1), TaNAC1 (ADG85703.1), HvNAC1 (CAM57979.2), SlNAC21/22 (XP_004243993.1), AtNAC1 (NP_175997.1), EtNAC1 (BAJ33921.1), TERN (BAA78417.1), GmNAC6 (AAY46126.1), TIP (AAF87300.1) and NTL8 (NP_180298.1). Clustering of NAC proteins into different subgroups are indicated on the right side of the tree.

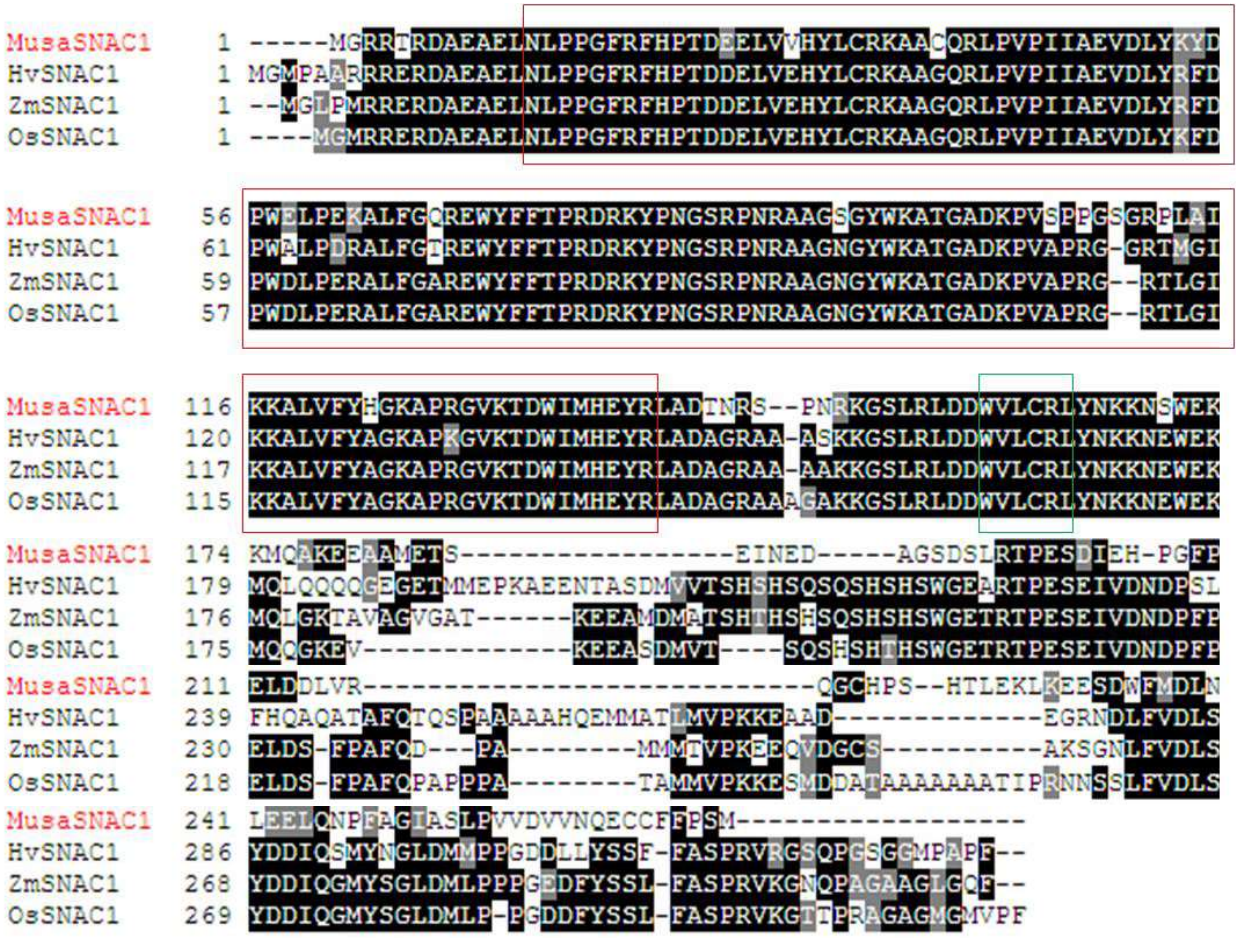

Fig. 4.2 Sequences analysis of MusaSNAC1. (A) Sequence alignment of MusaSNAC1 with other NAC proteins. NAC domain is boxed in red while 'WVLCRL'-motif present in members of SNAC-subgroup is boxed in green. Similar protein residues are shaded in black and position of alignment is indicated by numbers in left.

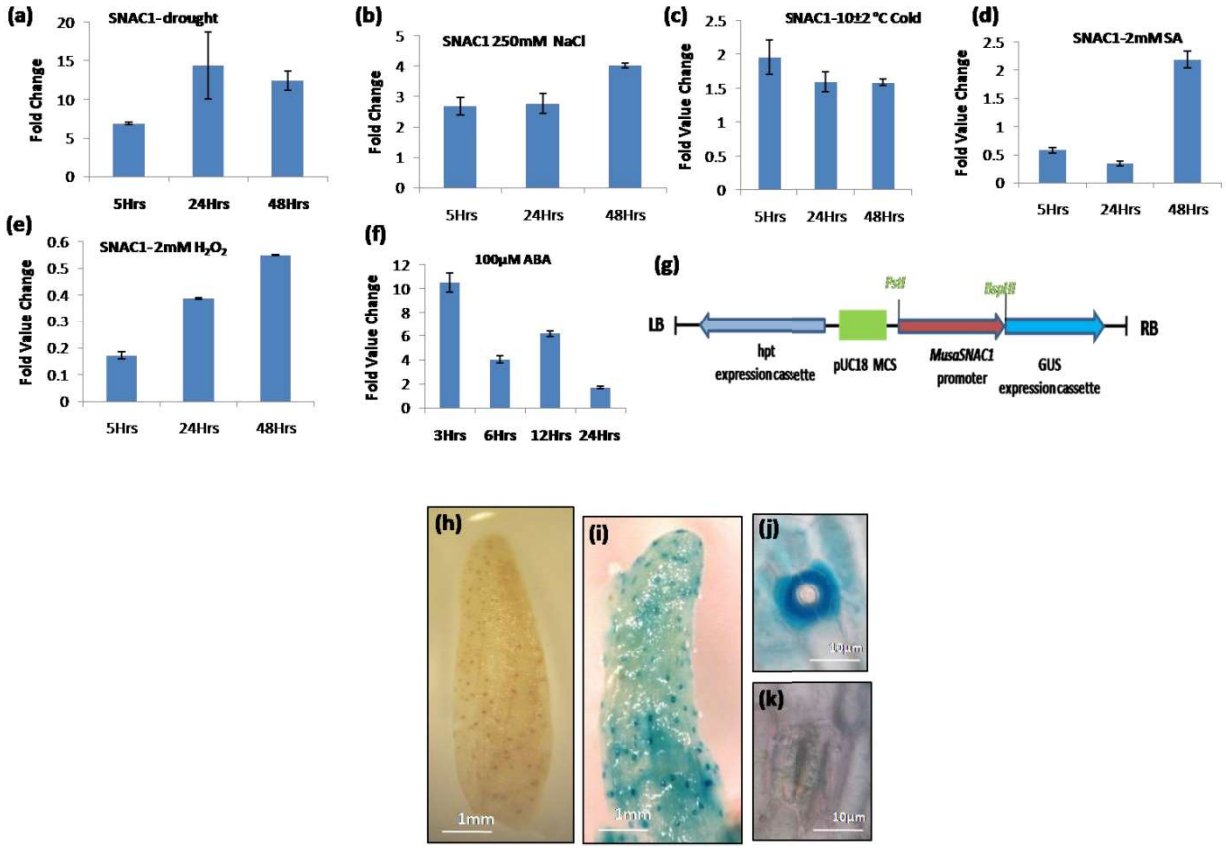

Fig. 4.3 Expression profiling and tissue specific expression of *MusaSNAC1*

Expression analysis of *MusaSNAC1* in banana leaves after stress-treatments. *Musa* cv *Karibale Monthan* was subjected to (a) drought, (b) 250mM NaCl, (c) low-temperature, (d) 2mM Salicylic acid, (e) 2mM H_2O_2 and (f) 100µM ABA. (g) Cartoon representation of T-DNA used for generation of transgenic banana lines containing *GUS* under the control of $P_{MusaSNAC1}$. (h) Histochemical staining of GUS in control banana leaf. (i) Expression of *GUS* in transgenic banana. Note strong expression of *GUS* in stomata. (j) A close-up of stomata showing *GUS* expression in guard-cells of transgenic banana leaf having $P_{MusaSNAC1}$*::GUS*. (k) Close-up of stomata of control banana leaf showing absence of *GUS* staining. Scale bar are indicated in the figure.

Generation and molecular analysis of transgenic banana plants overexpressing *MusaSNAC1*

Strong elevation in expression of *MusaSNAC1* under drought condition suggested its potential role in regulating drought-conditions in banana. To analyze this, we generated transgenic banana overexpressing *MusaSNAC1* after cloning *MusaSNAC1* under *pZmUbi* (maize polyubiquitin promoter) and transforming the T-DNA into banana embryogenic cells (ECS) (**Fig. 4.4a**). Transformation of banana ECS resulted in development of white and opaque somatic embryos (**Fig. 4.4b**) having globular to torpedo shape (**Fig. 4.4c**). Putatively transformed shoots obtained after germination of embryos on shoot elongation medium was multiplied (**Fig. 4.4d**) and rooted (**Fig. 4.4e**) on appropriate medium. Multiple plants of different lines (S1-S4) overexpressing *MusaSNAC1* was hardened in green house for further analysis (**Fig. 4.4f**). T-DNA insertion analyzed by PCR amplification of *hpt-II* (*hygromycin phosphotransferase-II*) sequence from genomic-DNA suggested presence of T-DNA in all the transgenic lines (**Fig. 4.4g**). Southern-blot analysis suggested presence of one T-DNA copy in lines S3, S4 and S5 while two T-DNA copies was detected in line S1 (**Fig. 4.4h**). Presence of different sized bands on blot indicated that each line was obtained as a result of independent transformation event. Transcript levels of *MusaSNAC1* detected by quantitative RT-PCR indicated 8 to 12 fold expression in transgenic lines over control suggesting suitability of these transgenic lines for evaluation of drought related function of *MusaSNAC1* (**Fig. 4.4i**).

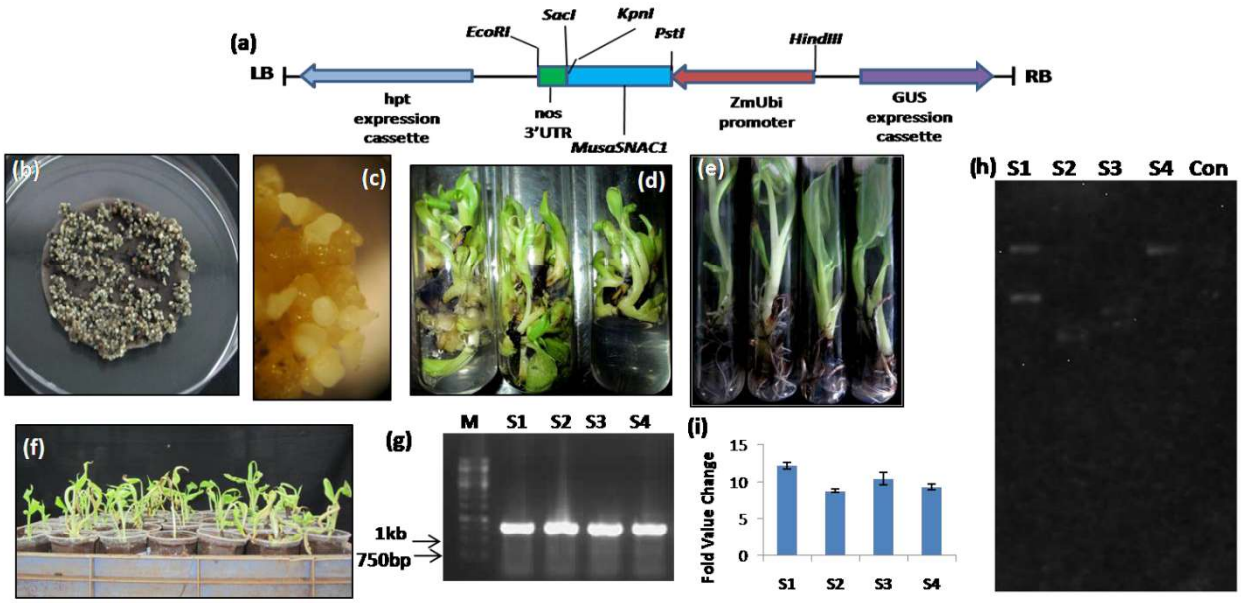

Fig. 4.4 Generation and confirmation of transgenic lines. (a) T-DNA region transferred to transgenic lines contain *MusaSNAC1* under a strong promoter of maize *polyubiquitin* gene for higher-level expression. (b) Somatic embryos developed on occurred on hygromcyin (5mg/l) supplemented medium. (c) Close-up showed different shapes of white and opaque somatic embryos. (d) Embryos germinated into shoots on BAP (2mg/l) containing medium resulted in different transgenic lines. (e) Rooting of different transgenic lines on NAA (1mg/l) medium and hardened plants in green house (f). (g) PCR amplification of *hpt-II* coding sequence from the genomic-DNA of transgenic lines showed successful integration of T-DNA in the genome. M: 1kb ladder, S1-S4: transgenic lines. Size of 750bp and 1kb is indicated by arrows. (i) Quantitative RT-PCR analysis showed many fold transcript level of *MusaSNAC1* in transgenic lines (S1-S4) over control. Data showed was obtained from three biological replicates and represented as mean±SD. (h) Southern-blot analysis indicated stable integration of T-DNA in genome of transgenic lines. Bands on blot indicated copy number of T-DNA in genome of transgenic lines. (S1-S4: transgenic lines; Con: control).

Overexpression of *MusaSNAC1* improves drought tolerance

Four transgenic lines generated showing high level expression of *MusaSNAC1* was chosen for drought exposure and recovery assay under green house conditions. Under control conditions transgenic lines and control plant had similar growth and morphology (**Fig. 4.5a**). This suggest that overexpression of *MusaSNAC1* in banana does not affect its growth and development. Drought was initiated by withdrawing the water supply for 14 days. During 7^{th} and 14^{th}day visual wilting symptoms and biochemical parameters were monitored. Visible drought stress-symptoms like wilting, leaf dropping and yellowing was less pronounced in transgenic lines during 7^{th} and 14^{th}day of drought than control suggesting improved drought-tolerance (**Fig. 4.5b, 4.5c**). Recovery ability of control and transgenic lines monitored by regular watering indicated better recovery of transgenic lines than control (**Fig. 4.5d**). Relative water content analysis suggested that during progression of drought, water loss in transgenic lines was lower than control plants and thus tolerate drought better (**Fig. 4.5e**). MDA (Malondialdehyde) is a marker of stress conditions due to damage caused by reactive oxygen species (ROS) was measured during the period of drought imposition. Transgenic lines displayed lower oxidative damage caused by ROS in terms of lower MDA content than control plants further suggesting the drought protective function of *MusaSNAC1* (**Fig. 4.5f**).

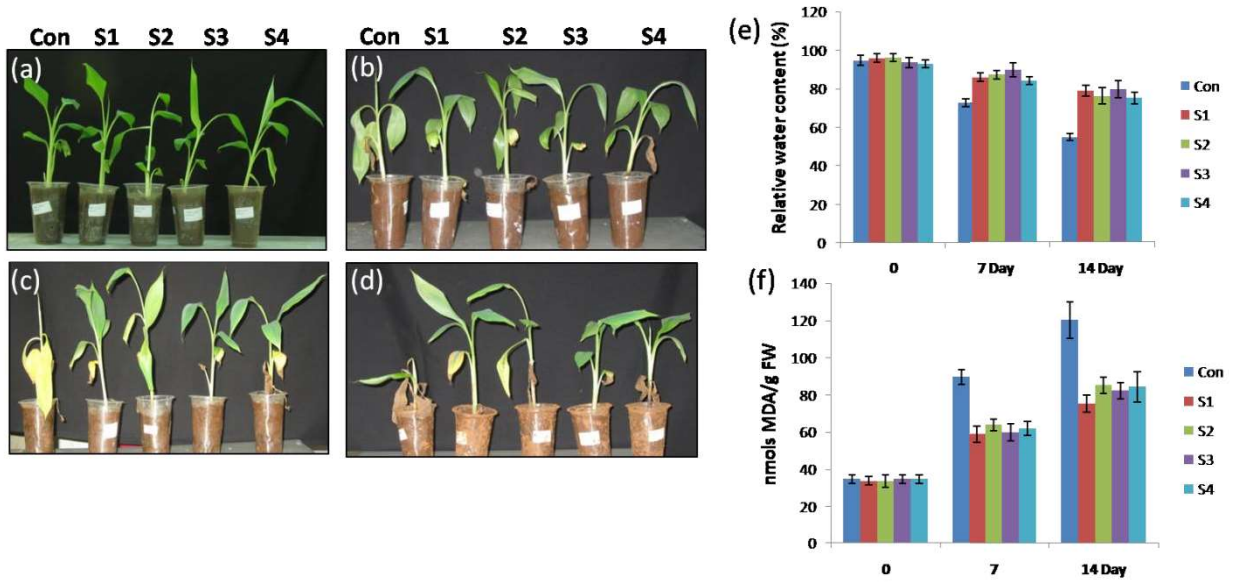

Fig. 4.5 Increased drought-tolerance in transgenic banana lines. (a) Transgenic lines and control plants growing in green house showed similar growth and morphology under completely hydrated condition. (b) Stress-symptoms developed in transgenic lines and control plant after seven days of water withdrawal. (c) Yellowing and wilting in transgenic lines and control plant after 14days of drought. Note the lower stress-symptoms observed in transgenic lines than control. (d) Recovery of drought-stressed transgenic lines and control plant by regular re-watering. Note the better recovery of transgenic lines compared to control. (e-f) Relative water content and MDA content (nanomole of MDA per gram of FW) estimated in transgenic lines and control at start (0 day), and after 7^{th} and 14^{th} day of drought imposition. Data showed was obtained from three biological replicates and represented as mean±SD.

Improved drought tolerance of transgenic lines is due to increased stomatal closure by modulation of H_2O_2 concentration

Expression of *GUS* under $P_{MusaSNAC1}$ got elevated in guard cells under drought-condition and transgenic lines lost water more slowly during drought progression prompting us to analyze the stomatal responses in transgenic lines during drought-stress. Stomatal closing is controlled by

ABA through induction of H_2O_2 production in guard cells (Kwak et al. 2003) and H_2O_2 is an important messenger in stomatal closure in plants (McAinsh et al. 1996; Pei et al. 2000). Higher H_2O_2 accumulation was observed in the guard cells of transgenic lines (**Fig. 4.6b, 4.6d**) than control plants (**Fig. 4.6a, 4.6c**) as suggested by H_2DCFDA staining. Also higher level of H_2O_2 content was observed in transgenic lines than control under drought-condition (**Fig. 4.6e**). As water loss through transpiration is controlled by stomatal aperture, the different levels of stomata in transgenic plants under drought-conditions were observed. Percentage of closed stomata in transgenic lines was approximately 45% (S1), 38% (S2), 42% (S3) and 39% (S4) than 16% in control. Percentage of partially closed stomata did not significantly differ between control and transgenic lines. While the percentage of open stomata was approximately 52% in control and ranged from 20-23% in transgenic lines (**Fig. 4.6f**). These observations suggest that *MusaSNAC1* mediates in drought-tolerance by reducing water loss through increased H_2O_2 induced stomatal closure.

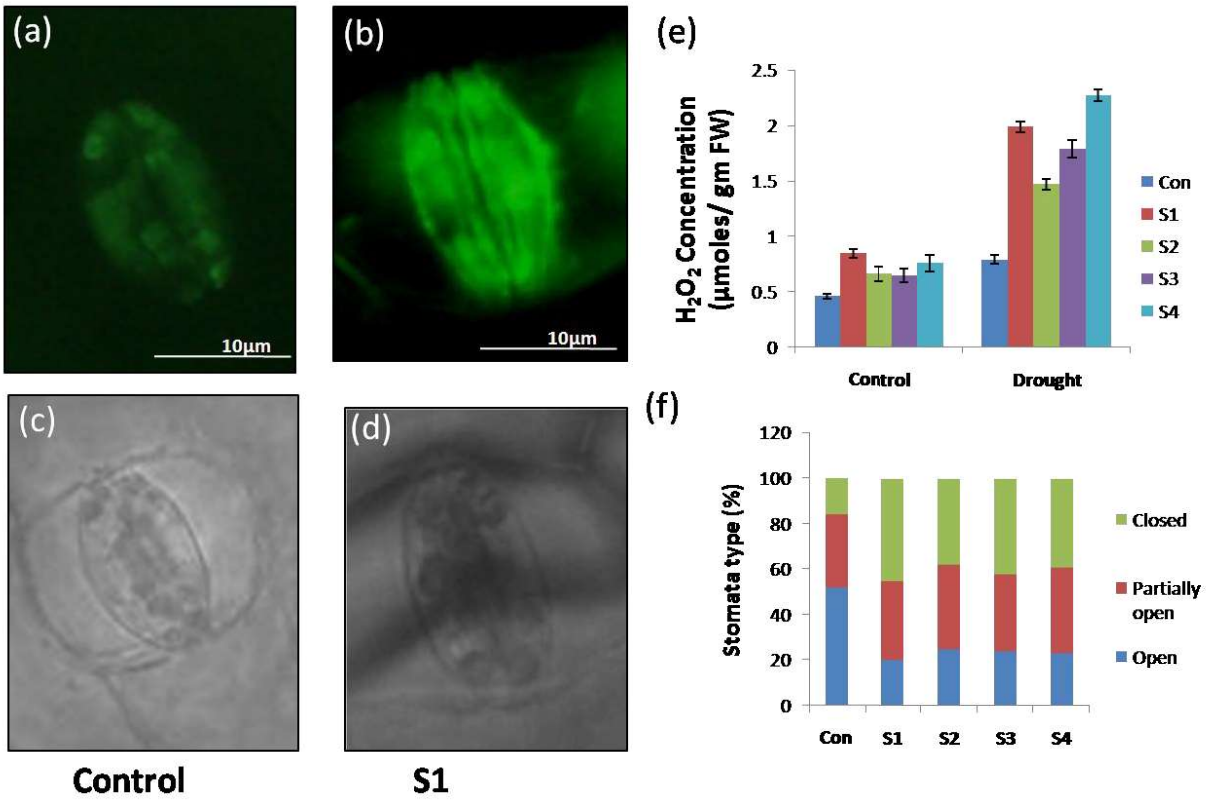

Fig. 4.6 Increased H_2O_2 concentration induced stomata closure in *MusaSNAC1* overexpressing transgenic lines under drought. (a-d) H_2O_2 detection in guard cells by H2DCFDA . Higher fluorescence was detected in guard cells of transgenic line (b) than control (a). (c-d) Corresponding light microscopy image of (a) and (b). Bar correspond to 10µm. (e) H_2O_2 content of control and transgenic lines under control and drought-condition. Note the elevated level of H_2O_2 in transgenic lines than control under drought-condition. (f) Different levels of stomatal opening in control and transgenic lines under drought-conditions. 100 stomata were observed after dehydrating control and transgenic lines.

MusaSNAC1 binds to core binding site of NAC proteins

To analyze the DNA binding ability of MusaSNAC1, MusaSNAC1 protein was purified and gel-shift analysis with different DNA substrate was performed. MusaSNAC1 was induced in *E.Coli BL21-codonplus* by 1mM IPTG after cloning in *pET28a*. The inclusion bodies with induced

MusaSNAC1 was isolated and solubilized by N-lauryl sarcosine and later the solubilized fraction was used for purification of MusaSNAC1 by affinity chromatography. Different fraction collected during purification was analyzed on 12% SDS poly-acrylamide gel-electrophoresis (PAGE) (**Fig. 4.7a**). Purified MusaSNAC1 was detected by anti-polyhistidine antibody in a western-blot (**Fig. 4.7b**). Various DNA motifs for binding of different NAC proteins have been reported in the past. We performed gel-shift analysis with different DNA sequences (DS1-DS6) for analyzing the binding potential of MusaSNAC1. DNA sequences for gel-shift were based on binding site of: DS1- NAC1 and *Arabidopsis* NAM to *CaMV35S* promoter (Duval et al. 2002; Xie et al. 2000), DS2- ANAC019/055/072 to the *ERD1* promoter (Tran et al. 2004), DS3- TaNAC69 (Xue 2005) and DS4- CBNAC (Kim et al. 2007a). Core binding site of NAC proteins was been reported to be CGT[A/G] (Olsen et al. 2005b) and DS5 contain copies of this core site (CGTA and CGTG) while DS6 is the mutated core site (CGCA instead of CGTA and CGCG in place of CGTG) (**Fig. 4.7c**). MusaSNAC1 was able to retard the mobility of all the DNA sequences employed except DS6 and the binding increased with increasing protein amount indicating that MusaSNAC1 binding is specific and occur in dose dependent manner (**Fig. 4.7d**). DS1, DS2 and DS3 contain the core site CGT[A/G] or its reverse complement suggesting that the main binding motif of MusaSNAC1 is CGT[A/G]. Moreover, MusaSNAC1 bind to CGT[A/G] core (DS5) in a dose dependent manner while it failed to bind the mutated core (DS6) even at increasing concentration (**Fig. 4.7d**) suggesting that binding of MusaSNAC1 to CGT[A/G] occurs in specific manner.

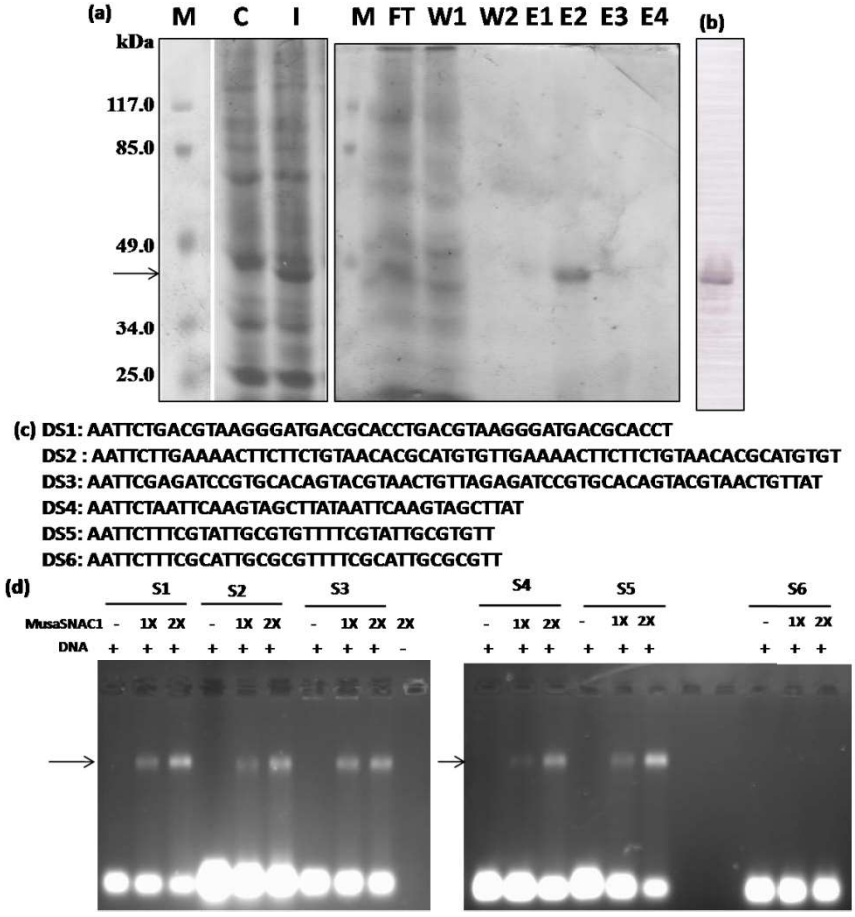

Fig. 4.7 Purification of MusaSNAC1 and DNA binding activity. (a) *E.Coli BL21 codon-plus* transformed with *pET28- MusaSNAC1* was induced by 1mM IPTG (arrow) and induced protein was purified on Ni-NTA column. Different fractions analyzed on 12% SDS-PAGE showed purification of MusaSNAC1 . Lane M: Protein size marker, C: control culture, I: induced culture, FT: flow-through, W1: First wash, E1-E4: Elutions. (b) Detection of purified MusaSNAC1 by western blotting using antibody against 6x-Histidine residues. (c) Sequences of different oligonucleotides (DS1-DS6) used for analyzing the DNA binding activity of MusaSNAC1. DS1-DS5 are DNA containing binding site of different NAC proteins including the core binding site (DS5). (d) MusaSNAC1 bind to all the DNA sequences (DS1-DS5) but

could not bind to the DS6 (mutated form of core site). Arrow indicate the retarded DNA as DNA-protein complex.

MusaSNAC1 regulate expression of multiple stress related genes in banana

MusaSNAC1 transactivate expression of reporter genes in yeast and bind to multiple DNA fragments containing CGT[A/G] motif in gel-shift assay. These result indicated that MusaSNAC1 is a transcriptional factor which possibly regulate target genes by binding to CGT[A/G] elements in their 5'-upstream region. We analyzed the transcript level of multiple putative stress-related genes in transgenic banana overexpressing MusaSNAC1 and identify certain putative direct targets which were unregulated many folds over control value. Sequence analysis of 5'upstream region of these genes indicate presence of MusaSNAC1 binding site CGT[A/G] prompting us to analyze whether MusaSNAC1 can directly bind to these 5'-upstream region. Gel-shift analysis indicated ability of MusaSNAC1 to bind a 216bp upstream DNA fragment (-194 to -410) of GSMUA_AchrUn_randomT10890_001 (putative CBF/DREB factor), a 199bp upstream DNA fragment of FL662828 (putative CBF/DREB factor), a 186bp upstream DNA fragment (-194 to -410) of GSMUA_Achr4G01600_001 (putative WRKY factor), a 194bp upstream DNA fragment (-200 to -394) of GSMUA_Achr6G27090_001 (putative WRKY factor), 207bp upstream DNA fragment (-296 to -503) of GSMUA_Achr6T28760_001 (putative CBF/DREB factor) and 200bp upstream DNA fragment (-286 to -486) of GSMUA_Achr3T13440_001 (putative WRKY factor) (**Fig. 4.8a**). Binding of MusaSNAC1 to these regulatory sequences was specific and efficient as retardation of DNA-protein complex got increased with increased protein concentration. Elevation of these genes was observed in transgenic banana due to overexpression of MusaSNAC1 along with alteration in expression of many other genes. We analyzed expression of multiple members of stress-related

genes (90 CBF/DREB, 60 WRKY, 16 TIFY factors , 30 LEA and seven antioxidant protein coding gene) and expression of genes coding for six putative CBF/DREB, eight putative WRKY, two putative *TIFY* transcription factors and one putative ascorbate peroxidase was altered after overexpression of MusaSNAC1 (**Fig. 4.8b**). Results obtained by gel-shift and quantitative estimation of transcript suggest direct regulation exerted by MusaSNAC1 on these genes by binding to CGT[A/G] motif in their regulatory region.

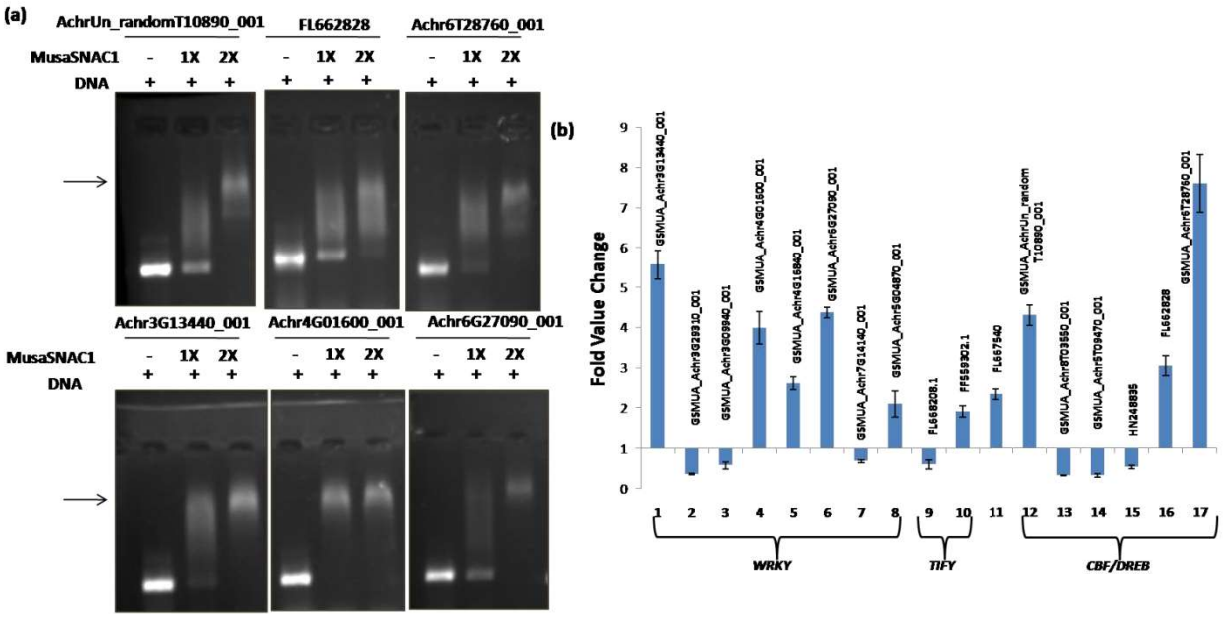

Fig. 4.8 *MusaSNAC1* regulate multiple stress responsive genes in banana. (a) MusaSNAC1 could bind to the region with CGT[A/G] motif (core NAC responsive element) in 5'-upstream region of stress-related genes. DNA-protein complex is indicated by arrow. X indicate the fold of protein used and + or – indicate presence or absence of DNA. (b) Altered transcript levels of stress-related genes in transgenic lines due to overexpression of *MusaSNAC1*. Expression of genes coding for six *CBF/DREBs,* eight *WRKY* and two *TIFY* transcription factors was altered after overexpression of *MusaSNAC1*. The related banana genome locus identifier or NCBI accession number is also indicated. Data was normalized by expression of banana *EF1α* and represented as mean±SD.

Fig. 4.9 Sequence analysis of 5' up-stream region of *MusaSNAC1*. Multiple Dof1 binding sites and cis-elements for stress-responses were detected. Nucleotide position relative to transcription start (+1) is indicated . Transcription start is underlined and start codon is double underlined . Important stress related cis-elements are boxed in red and Dof binding sites are boxed in red. Boxed sequences are: abscisic acid response element (CACGTG; ABRE) at -80 and -329, CGTGAAGGTTCCAG (HSE; heat responsive element) at -434, CCGAAA (LTR; low temperature responsiveness) at -509, TGACCG (MBS; MYB binding site for drought-response) at -180 and -391, GTCGTT (auxin-responsive element) at -921, TGACG (Methyl-jasmonate responsiveness) at -409 and -668, accACGTAaa (NACRS; NAC binding site) at -996, gagCCGACat (AP2/ERF binding site for stress induction) at -362, taGTCGGtgg (AP2/ERF binding site for stress induction) at -290 , cAATCAtgt (bZIP binding site for possible induction in water scarcity) at -700, TAAAG (Dof binding site) at +99, -507 and -680, CTTTA/T (Dof binding site) at -1005, -652, -863 and -944, AAAAG (Dof binding site) at -846 and -851, TAAAAAA (Dof binding site) at -534, GAAAAATT (Dof motif) at -517, CATTTGG (Dof

motif) at -495, CTGTTTC (Dof motif) at -351, CATTAGT (Dof motif) at -280, CTTTGAC (Dof motif) at -183, CGTTGGG (Dof motif) at -160, CTCAAGT (Dof motif) at -27, CAAACGC (Dof motif) at -11, AATAAATTCT (Dof motif) at -936, CAGCATTTTT (Dof motif) at -959, CGTAAAATGA (Dof motif) at -993, CTCTCTTT (Dof motif) at -1009 and TGCTTT (Dof motif) at -383.

Discussion

Worldwide production of banana, an economically important fruit crop is approximately 145 million tonnes per year (FAO,2015). However, yield of banana crop depends on availability of water as it is drought-sensitive crop owing to its morphological features like short root system and permanent green canopy (van Asten et al. 2011; Xu et al. 2014). Banana is a water intensive crop and requires approximately 2000–2500mm rainfall throughout the year (Vanhove et al. 2012) and some studies has also documented the adverse effect of drought as decline in banana production (Surendar et al. 2013; Ravi et al. 2013). In the present study we have characterized a banana stress-related NAC transcription factor, *MusaSNAC1* and showed its utility in improving the drought-tolerance in banana by increasing H_2O_2 induced stomatal closure. Plants lose majority of their water by transpiration through stomata (Xu et al. 2010) and regulation of stomatal closure has been shown to improve plant drought-tolerance (Hu et al. 2006; Huang et al. 2009). Transcript level of *MusaSNAC1* showed positive response to drought, high-salinity, cold as well as ABA application and maximum and early elevation was observed during drought and ABA application. Drought is one of the important stress condition affecting expression of many *NAC* transcription factors including rice *SNAC1* (Hu et al. 2006) and *MusaNAC042* (Tak et al. 2017). Expression of a guard cells regulating zinc finger protein, *DST* in rice was down-regulated under drought and salinity-stress however, *DST* is negative regulator of guard cell

closing (Huang et al. 2009), while *MusaSNAC1* is a positive regulator of stomatal closure. Similar to *MusaSNAC1*, rice *SNAC1* also regulated drought-tolerance by stomatal closure however, rice *SNAC1* and banana *SNAC1* may not have overlapping function as salinity and cold appear to strongly induce rice *SNAC1* and not banana *SNAC1*. Thus rice and banana *SNAC1* may regulate guard cell movement by different pathways. Salicylic acid (SA) is a plant hormone which not only regulates defense-responses as well as has positive roles in senescence (Buchanan-Wollaston et al. 2005). Role of SA in regulation of abiotic stress-conditions is still not fully understood and the response of plant to stress depends on concentration of SA and redox state of cell (Yuan and Lin 2008). SA acts as a negative regulator of *MusaSNAC1* during early hours of exposure suggesting a probable antagonistic cross-talk between *MusaSNAC1* induced stomatal closure and SA dependent signaling pathway. H_2O_2 content in transgenic lines was increased due to *MusaSNAC1* overexpression while transcript level of *MusaSNAC1* was drastically reduced on application of H_2O_2 suggesting a feedback-regulation of H_2O_2 on $P_{MusaSNAC1}$ activity. Rice *SRO1c* which has been identified as a direct target of rice *SNAC1* was elevated by H_2O_2 (You et al. 2013), further indicating that rice *SNAC1* and *MusaSNAC1* do not have overlapping functions and regulate guard cell movement by independent mechanisms. Tissue specific transcription regulation of many stress-associated *NAC* transcription factors has not been documented and this is particularly important in case of *NAC* factors involved in regulation of stress-conditions by modulation of guard cells H_2O_2. $P_{MusaSNAC1}$*::GUS* showed strong expression of *GUS* localized in stomatal guard cells. Expression of *GUS* in guard cells by $P_{MusaSNAC1}$*::GUS* is in line with presence of multiple *Dof1*-elements in 5'-upstream region of *MusaSNAC1* (**Fig. 4.9**). *Dof1*-elements has been attributed to confer guard cells specificity of *AtMYB60* promoter (Cominelli et al. 2011) and *GbSLSP* promoter (Han *et al.* 2013). However,

expression of *MusaSNAC1* was also observed in vascular tissue of leaves and roots suggesting additional regulatory elements conferring activity of $P_{MusaSNAC1}$ in vascular tissues apart from guard cells. Transgenic banana overexpressing *MusaSNAC1* showed improved drought-tolerance observed as lowering of drought-induced wilting and yellowing, higher relative water content, lower MDA content and better recovery of transgenic lines than control. During progression of drought transgenic lines showed higher frequency of closed stomata and lost water slowly coupled with strong activity of $P_{MusaSNAC1}$ in guard cells, indicated a role of *MusaSNAC1* in regulation of guard cells closure. H_2O_2 is an important signaling molecule involved in stomatal closure (McAinsh et al. 1996; Pei et al. 2000) and under drought, transgenic lines overexpressing *MusaSNAC1* had strong accumulation of H_2O_2 in guard cells (detected by H_2DCFDA) suggesting role of *MusaSNAC1* in H_2O_2 mediated guard cells closure during drought. Similar accumulation of H_2O_2 in guard cells was observed in rice *dst* mutant (Huang et al. 2009) and transgenic rice overexpressing rice *SRO1c* (regulated by *SNAC1*) (You et al. 2013). However, apart from closure of stomata, H_2O_2 can also act as an important signaling molecule which can reinforce stress-tolerance by regulating ROS metabolism and altering expression of stress-related genes (Hossain et al. 2015). MusaSNAC1 mediated H_2O_2 induced guard cells closure and being a transcription factor increased the expression of stress-protective genes which can counteract the negative effect of elevated H_2O_2 content apart from supporting the *MusaSNAC1* promoted drought-tolerance observed in transgenic banana lines. This was corroborated by the observation that transgenic plants overexpressing rice *SNAC1* showed significantly higher oxidative stress-tolerance than control plant (Hu et al. 2006) while its downstream target Os*SRO1c* (You et al. 2013) increased oxidative stress sensitivity of overexpressing transgenic plants.

In case of NAC proteins CGT[A/G] has been reported as consensus binding site and multiple NAC protein like ANAC055 (Tran et al. 2004), TaNAC69 (Xue 2005), ANAC019, ANAC092 (Olsen et al. 2005b), GmNAC11 and GmNAC20 (Hao et al. 2011) could bind to CGT[A/G]. MusaSNAC1 could bind to DNA molecules containing direct or complement repeat of CGT[A/G] (DS1-3) similar to other NAC proteins reported in earlier reports (Tran et al. 2004; Xue 2005; Xie et al. 2000) as well as multiple repeats of core site (CGTA and CGTG in DS5). MusaSNAC1 could not bind mutated core (DS6) suggesting a specific interaction with CGT[A/G]. However, MusaSNAC1 could also bind to a DNA sequence containing GCTT site (DS4) which has been reported as binding site of *Arabidopsis* CBNAC (Kim et al. 2007a) suggesting other possible binding motifs of MusaSNAC1 apart from CGT[A/G]. Multiple NAC transcription factors have been reported to induce *CBF/DREBs* (*C-repeat-binding proteins/ dehydration-responsive element-binding proteins*) factors apart from other important stress-related genes. A stress-responsive banana *NAC* transcription factor, *MusaNAC042* was involved in salinity and drought stress-tolerance and increased transcript level of at-least 9 *CBF/DREBs* in transgenic banana (Tak et al. 2017). *Arabidopsis JUNGBRUNNEN1*, a *NAC* transcription factor regulated H_2O_2 level and *DREB2A* by directly binding to its *JUB1* motif in the promoter region (Wu et al. 2012). MusaSNAC1 could bind to 5'-upstream region containing CGT[A/G] site of at least six putative stress-related genes in a gel-shift assay. Furthermore, transcript level of these genes (along with other putative stress-responsive genes) was altered in transgenic banana overexpressing *MusaSNAC1* suggesting that *MusaSNAC1* could regulate expression of these six genes in banana. These results suggest that *MusaSNAC1* is a probable upstream regulator of these genes and they are probably co-regulated during drought-stress in banana.

Table 4.1 Primers used for molecular studies in *MusaSNAC1*

Gene	Forward Primer (5' - 3')	Reverse Primer (5' -3')
Primers for cloning *MusaSNAC1* in pCAMBIA1301	aactgcagATGGGGAGGAGGACGAGAG	aaggtaccTCACATAGATGGGAAGAAGCAGC
MusaSNAC1 real time PCR primers	GTCGGAGATCAACGAGGAC	GATTCTGCAACTCCTCCAGA
Hygromycin phosphotransferase (*hpt)* gene	GTCCTGCGGGTAAATAGCTG	ATTTGTGTACGCCCGACAGT
Musa EF1α real time PCR primers	CCGATTGTGCTGTCCTCATT	TTGGCACGAAAGGAATCTTCT
Primers for cloning of $P_{MusaSNAC1}$ upstream of GUS in pCAMBIA1301	TTctgcagCTCATCTCATCTCATCTCTCTTTAGG	TTTCATGACGCTCCTATGTCTCCTTCTTC
Primers for cloning *MusaSNAC1* in pET28a	aaaCATATGGGGAGGAGGACGAGAGAC	aaaGGATCCTCACATAGATGGGAAGAAGC

Table 4.2 Primer sequences used to study expression of putative stress-related genes up-regulated in transgenic banana overexpressing *MusaSNAC1*

Type	NCBI accession number/ banana genome identifier	Forward Primer (5' - 3')	Reverse Primer (5' -3')
WRKY	GSMUA_Achr3G13440_001	GATCGGCTTGGTTGGATCT	GAGGTGTACTTCGCCGTGAT
WRKY	GSMUA_Achr3G29310_001	GGAAGGAGTGGAAGACGCTAA	AGAAGATGCTGTGGTTGAAGC
WRKY	GSMUA_Achr3G09940_001	AGCCTCTGTCCACCAGAAAG	GGAGTCTGCACCACCACTCT
WRKY	GSMUA_Achr4G01600_001	CAAACCTTGCCATAGCTGGA	AGCTGAGGTCCTAGAGGAAGC
WRKY	GSMUA_Achr4G16840_001	GTGGATGAGCAAAACGAACC	ACCATCTTCCGTAACGCTGT
WRKY	GSMUA_Achr6G27090_001	GTAGTCGCAGGAGCAAGACC	TCCCTCTCCCTCTTCTCTCC
WRKY	GSMUA_Achr7G14140_001	CCTTCTCCTTCCCTTCGACT	AATGATCTCGGTGAGGTCAGA
WRKY	GSMUA_Achr5G04870_001	TTGACCCTCGACTTCACGA	GAGGAGAGCGGAGGCTTG
TIFY	FL668208	AAGCGGAAGAGGAAGAGGAAT	ATTATGGCCTGTGCTTTTTCA
TIFY	FF559302	ACCAACACTGAGCAGCAAACT	CTTTACCCGCCAACAACATAA

L-ascorbate peroxidase 6, chloroplastic	FL667540	TCAAGGAGCTGCTCAAGACC	TAGCACTGGCCAACTGGAAC
CBF/DREB	GSMUA_AchrUn_randomT10890_001	AGCATGGGTGAATCGGTTAG	TGGCTTCACACCTATGTTGC
CBF/DREB	GSMUA_Achr8T03550_001	AGTAGCCAGAGCAGCACCAT	GAAGAGTCGGAGTCGCTTTG
CBF/DREB	GSMUA_Achr5T09470_001	TCCAAGATCCAGGCCATCT	CGAGCACTTCCCAGATCAAT
CBF/DREB	HN248835	ATTACCGAGGGGTGAGGAAG	TGCGGGAAGTTGGTCTTG
CBF/DREB	FL662828	ACGGGTCGAAGAAGAAGAAGA	ACTGCAGGTCCATCAGATTCA
CBF/DREB	GSMUA_Achr6T28760_001	TTCGGAAAGTCGGACACAG	CTTTGAGCAATTCTGCGTGT

Table 4.3 Primer sequences used in gel shift assay of *MusaSNAC1*

Name	**Forward Primer (5' - 3')**	**Reverse Primer (5' -3')**
GSMUA_AchrUn_randomT10890_001 (-194 to -410)	CGCAGTCTTTGATTCTCGAT	CCCGTCCTTTTATTTCGATT
FL662828	CATCTCTTGCCCAAACCTAA	CGATTCGAGTTAGGGTGTTT
GSMUA_Achr4G01600_001 (-194 to -410)	AGCTCGAAGCTGACCAATC	CGGAATGAAGCTACCAAAAA
GSMUA_Achr6G27090_001 (-200 to -394)	GGAGATCGGAGGGATGTTAG	GTCGGTGGCAGAGACAAG
GSMUA_Achr6T28760_001 (-296 to -503)	ACACCACCAGTTGTGGACCT	TCATCTTCTCTGTTGTTGGAGTTC
GSMUA_Achr3T13440_001 (-286 to -486)	CGCCCACATTCCCAATATAC	GTTCTTGGTGGACATGCTTTG
S1	aattcTGACGTAAGGGATGACGCACCTGACGTAAGGGATGACGCACCt	CtagaGGTGCGTCATCCCTTACGTCAGGTGCGTCATCCCTTACGTCAg
S2	aattcTTGAAAACTTCTTCTGTAACACGCATGTGTTGAAAACTTCTTCTGTAACACGCATGTGt	ctagaCACATGCGTGTTACAGAAGAAGTTTTCAACACATGCGTGTTACAGAAGAAGTTTTCAAg
S3	aattcGAGATCCGTGCACAGTACGTAACTGTTAGAGATCCGTGCACAGTACGTAACTGTTAt	ctagaTAACAGTTACGTACTGTGCACGGATCTCTAACAGTTACGTACTGTGCACGGATCTCg

S4	aattcTAATTCAAGTAGCTTATAA TTCAAGTAGCTTAt	ctagaTAAGCTACTTGAATTATAAG CTACTTGAATTAg
S5	AattcTTTCGTATTGCGTGTTTTC GTATTGCGTGTt	ctagaACACGCAATACGAAAACAC GCAATACGAAAg
S6	AattcTTTCGCATTGCGCGTTTTC GCATTGCGCGTt	ctagaACGCGCAATGCGAAAACGC GCAATGCGAAAg

Chapter V: Studies on *MusaSNAC68*, a stress associated NAC transcription factor from banana

Introduction

Productivity of plants is threatened by stress-conditions like high-salinity and drought. One of plant specific transcription factor family, which is explored for involvement in stress-responses is of NAC (NAM, ATAF and CUC) proteins (Fujita et al. 2004; Nakashima et al. 2007). *NAC* transcription factor was first identified in *Arabidopsis* as a gene responsive to dehydration and named as *RESPONSIVE-TO-DEHYDRATION* 26 (*RD26*) (Yamaguchi-Shinozaki et al. 1992). *NAC* factors have roles in physiological processes and development including shoot apical meristem formation (Souer et al. 1996), secondary-wall thickening (Mitsuda et al. 2005), lateral root formation (Guo et al. 2005), embryogenesis (Aida et al. 1999), leaf-senescence (Lee et al. 2012), auxin-signaling (Xie et al. 2000) and fruit ripening (Shan et al. 2012). *Arabidopsis NAC1* (Xie et al. 2000) and *NAC2* (He et al. 2005) are involved in auxin-signaling and root development. Nearly 110 in *Arabidopsis* (Olsen et al. 2005a) and 151 members of *NAC* family in rice (Nuruzzaman et al. 2010) have been identified. *Arabidopsis NAC* factors *ANAC019*, *ANAC055* and *ANAC072* are induced by drought and salinity and overexpression of these factors improve drought tolerance (Tran et al. 2004). *Arabidopsis NAC* gene *ATAF1* is induced by drought and high-salinity and its overexpression improve drought tolerance (Wu et al. 2009). Transgenic rice overexpressing stress-responsive gene *SNAC1*, showed significantly enhanced drought tolerance and up-regulation of multiple stress-related genes (Hu et al. 2006). Rice

overexpressing *NAC5* have enlarged root and improved drought tolerance along with higher grain yield (Jeong et al. 2013).

Recent years has seen an increase in number of research studies aimed at elucidating roles of the physiological, molecular and genetic factors involved in stress responses and developmental pathways in banana. Recently transgenic banana plants with superior tolerance to drought and salinity has been generated by overexpression of banana *aquaporin* genes *MusaPIP1;2* and *MusaPIP2;6* (Sreedharan et al. 2013, 2015), peanut *PR-10* gene, *AhSIPR10* (Rustagi et al. 2015), banana *SAP1* gene (Sreedharan et al. 2012), *MusaWRKY71* (Shekhawat and Ganapathi 2013), *MusaDHN-1* (Shekhawat et al. 2011) and *MusabZIP53* (Shekhawat and Ganapathi 2014). There is a dearth of information related to roles of *NAC* transcription factors in banana especially in the context of stress tolerance. Six banana *NAC* transcription factors namely *MaNAC1-MaNAC6* were studied for their role in ethylene signaling and *MaNAC1*/*MaNAC2* were indicated for their probable role in fruit ripening via interaction with components of ethylene signaling pathway (Shan et al. 2012). Banana *NAC* transcription factor, *MaNAC5* is involved in disease response and interact with banana *WRKY* gene, *MaWRKY1* and *MaWRKY2* for regulation of *PR* genes expression during disease response (Shan et al. 2015). Another banana *NAC* transcription factor *MaNAC1* is a cold responsive gene and interact with ICE1-CBF signaling pathway for induction of possible cold tolerance in banana fruit (Shan et al. 2014).

In this chapter, we discuss the characterization of a banana *NAC* transcription factor (*MusaNAC68*) with dual role in root development and stress tolerance. *MusaNAC68* is induced by stress conditions and auxin, α-naphthaleneacetic acid. *Musa*NAC68-GFP (green fluorescent protein) overexpression indicated nuclear localization of *Musa*NAC68. Overexpression of *MusaNAC68* result in marginally taller plants with greater root abundance than control.

Transgenic plants displayed alteration in expression of auxin-responsive factors (*ARFs*) and *IAA/Aux* (Indoleacetic acid-induced protein) along with stress-related genes. The transgenic lines displayed better drought and salt tolerance ability, indicating that *MusaNAC68* play an important role in abiotic stress-responses and thus *MusaNAC68* can be a potential target for engineering stress tolerance in banana.

Results

Sequence analysis of *MusaNAC68*

MusaNAC68 coding sequence was amplified from banana cultivar *Karibale Monthan* and the sequence (KR078315) was submitted in NCBI database. Open reading frame of *MusaNAC68* have 897 bp coding for polypeptide of 298 amino acids with predicted molecular weight of 34.1 kDa and theoretical pI of 5.91. The sequence was named as *Musa*NAC68 because of high similarity with NAC68 sequences of other plant species. *Musa*NAC68 showed high homology with other NAC proteins from different plant species. *Musa*NAC68 has identities of 65% to PdNAC68 (XP_008792531), 70% to EgNAC1 (ABB72842), 61% to VvNAC68 (XP_002283807), 55% to ZmNAC1 (ADK25055), 59% to BdNAC67 (XP_003557366), 53% to SbSNAC1 (AGG40203), 57% to TaNAC2 (AAU08786), 55% to OsNAC1 (ABD52007), 54% to ZmSNAC1 (AEY78612), 59% to SbNAC1 (AIA57772), 57% to ObNAC67 (XP_006650786), 57% to HvSNAC1 (AEG21060), 54% to OsSNAC1 (ACX71077), 56% to SiNAC68 (XP_004970362), 55% to OsNAC4 (BAA89798), 58% to TaNAC67 (AHB32901), 55% to ZmNAC67 (XP_008644394), 58% to TaNAC4 (ADD10666), 59% to ZmNAC5 (NP_001146648) and 56% to BdNAC68 (XP_003563852) (**Fig. 5.1**). Wheat *NAC* transcription factor, *TaNAC67* is induced in response to drought, salinity, cold and ABA application and transgenic lines displayed enhanced tolerance to drought, high salinity and cold stresses along with elevated expression of

multiple stress responsive genes (Mao et al. 2014). *TaNAC2*, a wheat *NAC* transcription factor is induced in multiple stress conditions and transgenic *Arabidopsis* lines overexpressing wheat *NAC2* showed increased tolerance to multiple abiotic stress conditions (Mao et al. 2012). Maize stress-responsive *NAC* transcription factor (induced by cold, high-salinity and drought), *ZmSNAC1* is a transcriptional activator and provide drought tolerance in transgenic *Arabidopsis* plants (Lu et al. 2012). *Hordeum vulgare* stress induced transcription factor, *HvSNAC1* imparts high drought tolerance in transgenic barley resulting in significantly improved yield (Al Abdallat et al. 2014). *SbSNAC1* is a stress responsive *NAC* transcription factor from sorghum with maximum expression in roots and has ability to confer better drought tolerance in transgenic *Arabidopsis* (Lu et al. 2013). N-terminal end of *Musa*NAC68 has a conserved NAC domain while C-terminal domain is divergent. *Musa*NAC68 contain a WVLCR motif outside highly conserved NAC-domain (**Fig. 5.2**). WVLCR motif was identified as a motif among members of SNAC (stress-responsive NAC) group (Nuruzzaman et al. 2013).

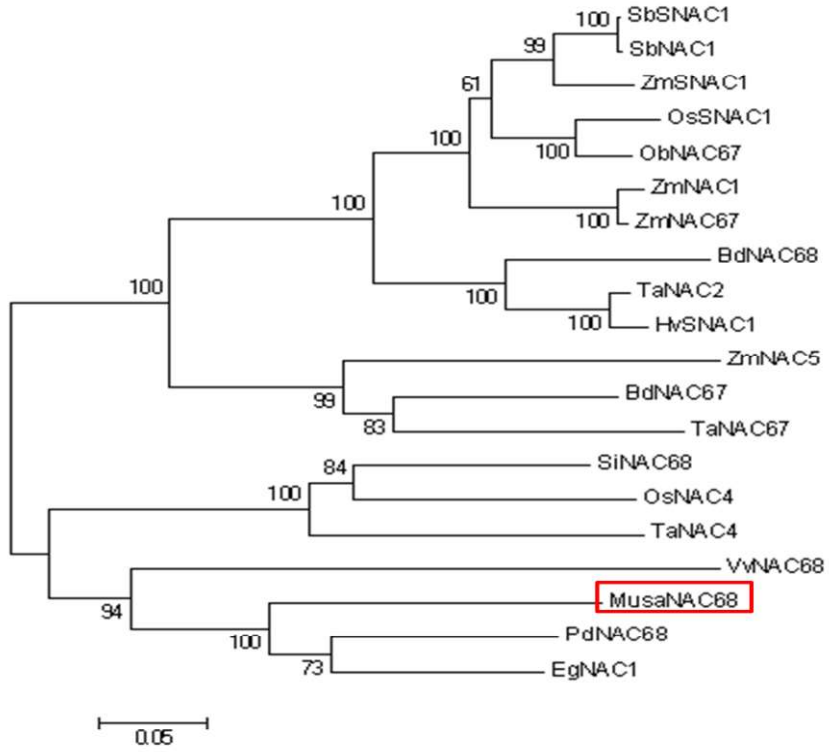

Fig. 5.1 Phylogenetic analysis of *Musa*NAC68 protein and other closely related NAC domain containing proteins from different plant species. *Sorghum bicolor* SbSNAC1 (AGG40203), *Sorghum bicolor* SbNAC1 (AIA57772), *Zea mays* ZmSNAC1 (AEY78612), *Oryza sativa* OsSNAC1 (ACX71077), *Oryza brachyantha* ObNAC67 (XP_006650786), *Zea mays* ZmNAC1 (ADK25055), *Zea mays* ZmNAC67 (XP_008644394), *Brachypodium distachyon* BdNAC68 (XP_003563852), *Triticum aestivum* TaNAC2 (AAU08786), *Hordeum vulgare* HvSNAC1 (AEG21060), *Zea mays* ZmNAC5 (NP_001146648), *Brachypodium distachyon* BdNAC67 (XP_003557366), *Triticum aestivum* TaNAC67 (AHB32901), *Setaria italica* SiNAC68 (XP_004970362), *Oryza sativa* OsNAC4 (BAA89798), *Triticum aestivum* TaNAC4 (ADD10666), *Vitis vinifera* VvNAC68 (XP_002283807), *Phoenix dactylifera* PdNAC68 (XP_008792531) and *Elaeis guineensis* EgNAC1 (ABB72842). The bootstrapped tree was build with 1000 replicate.

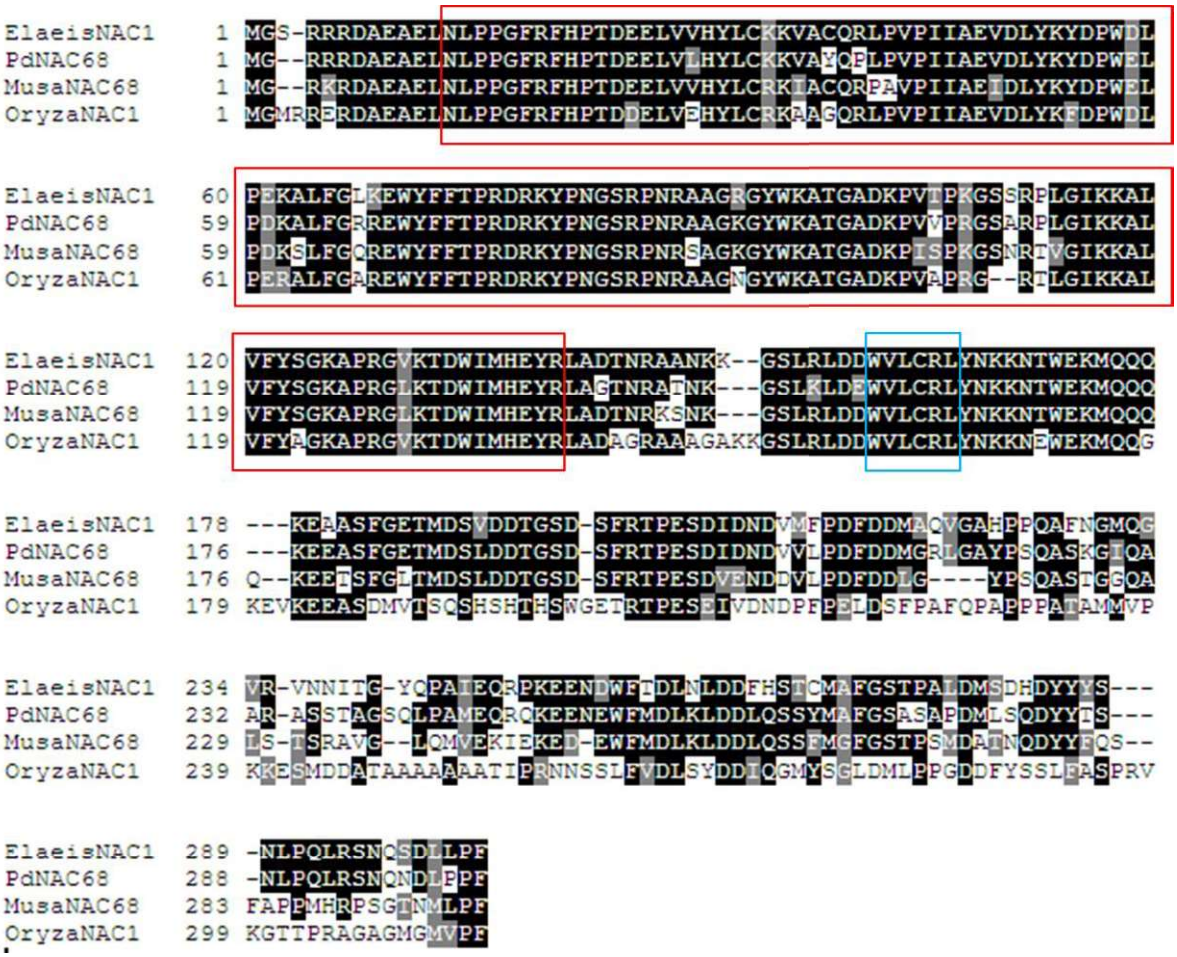

Fig. 5.2 Multiple sequence alignment of *Musa*NAC68. *Musa*NAC68 protein sequence was aligned with sequences from *Elaeis guineensis* NAC1 (ABB72842.1), *Phoenix dactylifera* PdNAC68 (XP_008792531) and *Oryza sativa* NAC1 (ABD52007.1). Identical residues are black shaded and the highly conserved NAC core domain is red boxed. The conserved WVLCR motif present among members of SNAC group is boxed in blue.

Expression analysis of *MusaNAC68*

MusaNAC68 was induced during drought, high salinity, cold and upon exposure to methyl-jasmonate, ABA and H_2O_2. *MusaNAC68* is induced in both leaves and roots. During cold exposure, expression of *MusaNAC68* was maximum at 24h and get reduced but remained higher

than control even up to 48h post exposure (**Fig. 5.3a**). In roots, expression of *MusaNAC68* during cold was maximum at 5h and reduced to uninduced level around 48h (**Fig. 5.4a**). Drought result in maximum expression of *MusaNAC68* at 5h in leaves (**Fig. 5.3b**) and 24h in roots (**Fig. 5.4b**), which reduced but remained higher than control level even at 48h. During high salinity, expression of *MusaNAC68* in leaves peak around 5h and gradually reduced but remain higher than control (**Fig. 5.3c**). In roots, expression of *MusaNAC68* during high salinity gradually increases and remain high even after 48h post exposure (**Fig. 5.4c**). In leaves, ABA as well as methyl-jasmonate induces *MusaNAC68*, and expression peak around 5h post treatment and it gradually returned to uninduced level (**Fig. 5.3d, 5.3e**). In roots, *MusaNAC68* expression after ABA and methyl-jasmonate treatment was maximum at 5h and then it slightly reduced in case of ABA treatment (**Fig. 5.4d**) while it returned to control level in case of methyl-jasmonate (**Fig. 5.4e**). H_2O_2 did not change expression of *MusaNAC68* noticeably until 5h after which significant elevation was observed at 24h and it remained higher even up to 48h in both leaves (**Fig. 5.3f**) and roots (**Fig. 5.4f**).

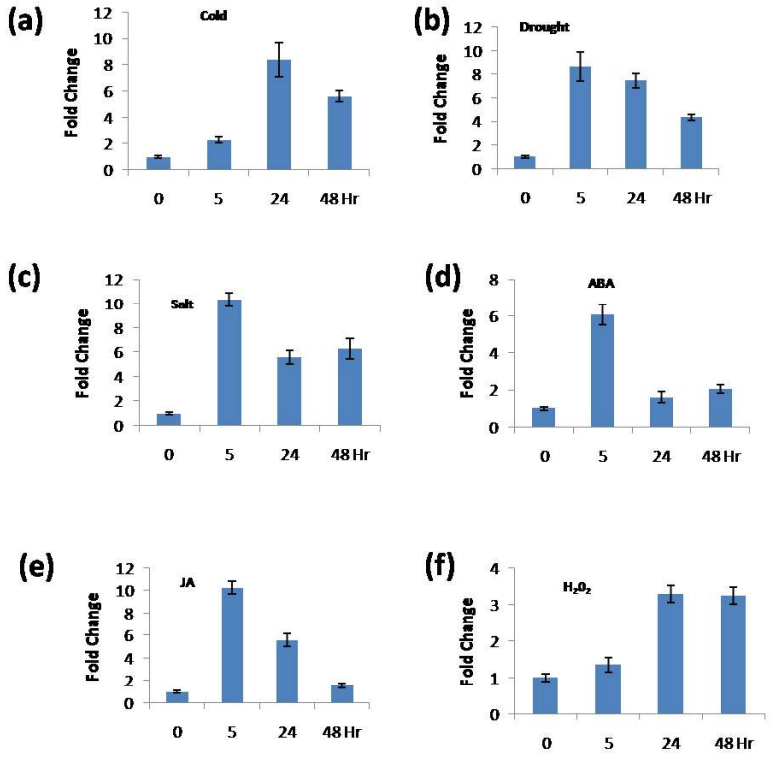

Fig. 5.3 Expression analysis of *MusaNAC68* in leaves. Expression profiles in leaves after exposure to (a) cold, (b) drought, (c) 250mM NaCl , (d) 100µM ABA (e) 200µM MeJA and (f) 2mM H_2O_2. Values are mean±SE.

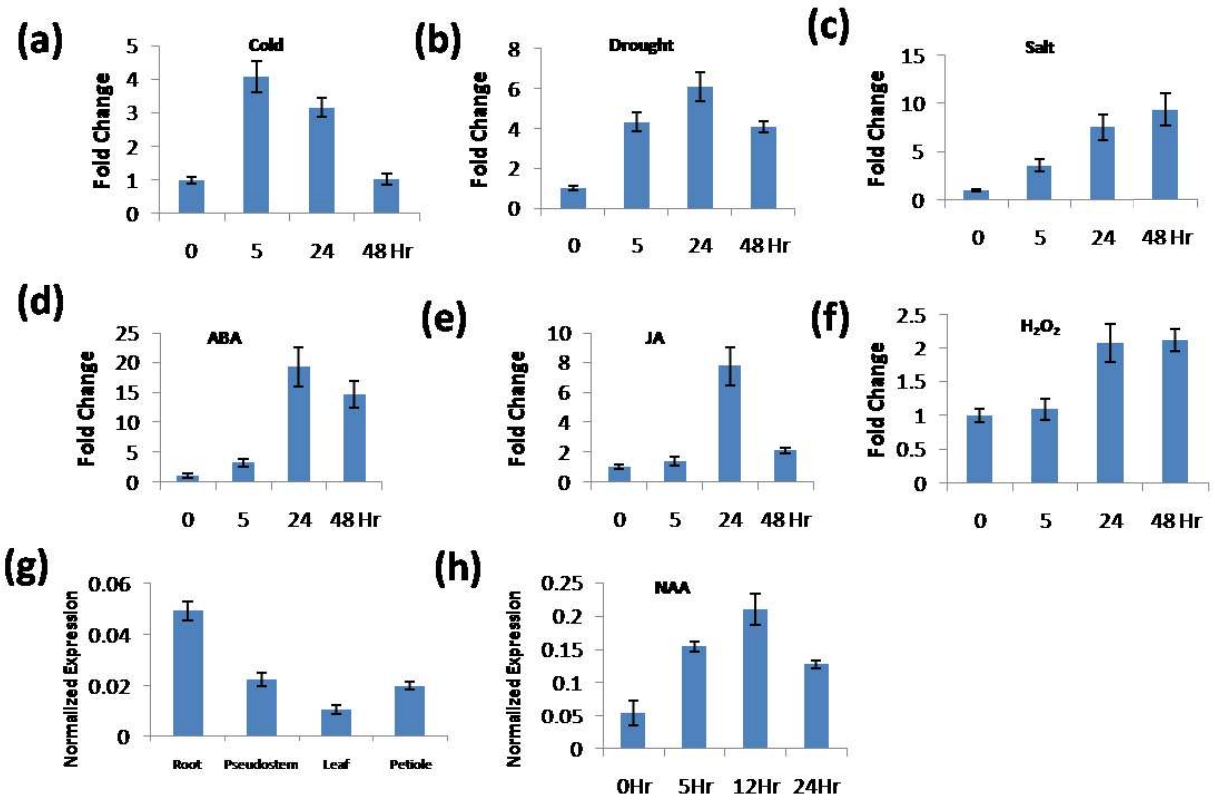

Fig. 5.4 Expression profiles of *MusaNAC68* in roots. Expression after exposure to (a) cold, (b) drought, (c) 250mM NaCl, (d) 100µM ABA (e) 200µM MeJA and (f) 2mM H_2O_2. (g) Expression of *MusaNAC68* in different parts of banana. (h) Induction of *MusaNAC68* in response to auxin. *In vitro* shoots were cultured in medium with 5µM NAA. RNA was isolated from roots at time points indicated in graph.

Promoter analysis for detection of putative *cis*-elements

Induction of *MusaNAC68* in stress conditions suggest probable presence of stress related *cis*-elements in promoter region (Genebank accession KP861890). Drought stress-related *cis*-elements identified are ACGTATERD1 (ACGT), MYB1AT (AAACCA) and MYB2AT (TAACTG). CBFHV (GTCGGC) is *cis*-elements involved in cold stress and was also identified in the 5'-proximal region of *MusaNAC68*. Important *cis*-elements like MYCCONSENSUSAT (CAGATG) for drought as well as cold stress are identified. Presence of ABA related *cis*-elements like ABRELATERD1 (ACGTG), DPBFCOREDCDC3 (ACACGTG) and MYB2CONSENSUSAT (CAACGG) in promoter region indicates responsiveness of *MusaNAC68* towards abscisic-acid application. *Cis*-element like GCCCORE (GCCGCC) for jasmonate-responsiveness, GT1-consensus (GGAAAT) and ELRECOREPCRP1 (TTGACC) for salicylic-acid responsiveness and GT1GSSCAM4 motif (GAAAAA) for salt stress were also detected in promoter region (**Fig. 5.5**).

Fig. 5.5 *Cis*-element analysis in the 5′ upstream region (KP861890) of *MusaNAC68*. The probable TATA box and translation initiation codon are double underlined in black. Important *cis*-elements like ACGTATERD1 (ACGT), MYB1AT (AAACCA), MYB2AT (TAACTG), CBFHV (GTCGGC), MYCCONSENSUSAT (CAGATG), ABRELATERD1 (ACGTG), DPBFCOREDCDC3 (ACACGTG), MYB2CONSENSUSAT (CAACGG), GCCCORE (GCCGCC), GT1-consensus (GGAAAT), ELRECOREPCRP1 (TTGACC), GT1GSSCAM4 motif (GAAAAA), ARFAT (TGTCTC) and CATATGGMSAUR (CATATG) are boxed in red.

Subcellular localization

*Musa*NAC68 subcellular localization was determined by transiently overexpressing *Musa*NAC68-GFP in banana embryogenic cells. Vector control (*pCAMBIA1302*) transformed cells indicate distribution of GFP fluorescence throughout cells (**Fig. 5.6a**) while in cells

overexpressing *Musa*NAC68-GFP, fluorescence was prominent in nucleus (**Fig. 5.6b**) indicating nuclear localization of *Musa*NAC68-GFP. Positions of nuclei was detected by Hoechst-33258 staining (**Fig. 5.6c, 5.6d**).

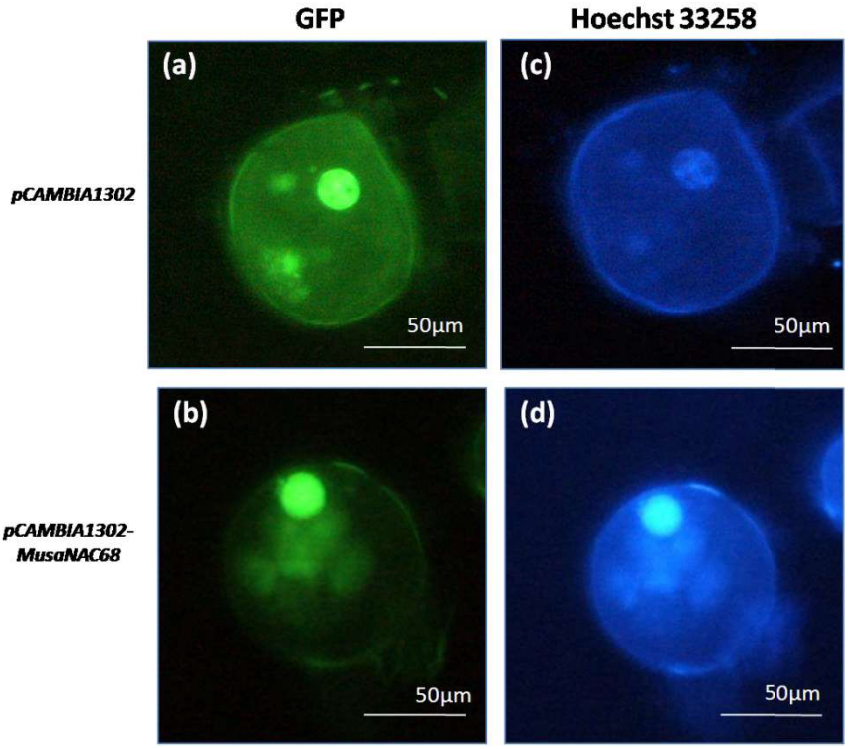

Fig. 5.6 Nuclear localization of *Musa*NAC68. (a) GFP fluorescence observed in banana cell transformed with pCAMBIA-1302. (b) The nuclear localization of GFP fluorescence in *pCAMBIA1302-MusaNAC68* transformed cell indicating nuclear localization of *Musa*NAC68. (c and d) The nuclear position in banana cell (a and b respectively) observed after Hoechst 33258 staining.

Regeneration and confirmation of transgenic plants

To generate transgenic plants, *MusaNAC68* coding sequence was cloned under control of maize polyubiquitin promoter in binary vector *pCAMBIA1301* (**Fig. 5.7a**). *Agrobacterium* mediated transformation of banana cultivar *Rasthali* resulted in emergence of globular-embryos (**Fig. 5.7b**) which further regenerate secondary-embryos (**Fig. 5.7c**) on embryo-induction medium supplemented with strong selection agent hygromycin. On embryo-germination medium conversion of well developed embryos into putative transgenic shoots occurred (**Fig. 5.7d**), which were subsequently converted into multiple shoots on shoot multiplication medium (**Fig. 5.7e**). Rooted plants from multiple shoots generated on rooting-medium (**Fig. 5.7f**) were hardened in green house. Four transgenic lines surviving on selection medium hardened in the green house were taller than control plants (**Fig. 5.7g**). Transgenic nature of lines was screened by PCR analysis, where amplification of *hpt-II* (Hygromycin phosphotransferase) from genomic-DNA indicated successful integration of T-DNA. Four transgenic lines found to be positive for T-DNA integration were named as L2, L5, L8 and L11 respectively (**Fig. 5.7h**). Stable integration of T-DNA in genome of transgenic lines was confirmed by Southern blot using a DIG-labeled probe against *hpt-II*. T-DNA copies from two to four were present in genome of transgenic lines (**Fig. 5.7j**). Overexpression of *MusaNAC68* due to T-DNA copies was analyzed as fold value change by real time RT-PCR. Fold value change in transcript level of *MusaNAC68* was 9.4 in line L2, 12.7 in line L5, 6.6 in L8 to 4.5 in L11 line, relative to control (**Fig. 5.7i**).

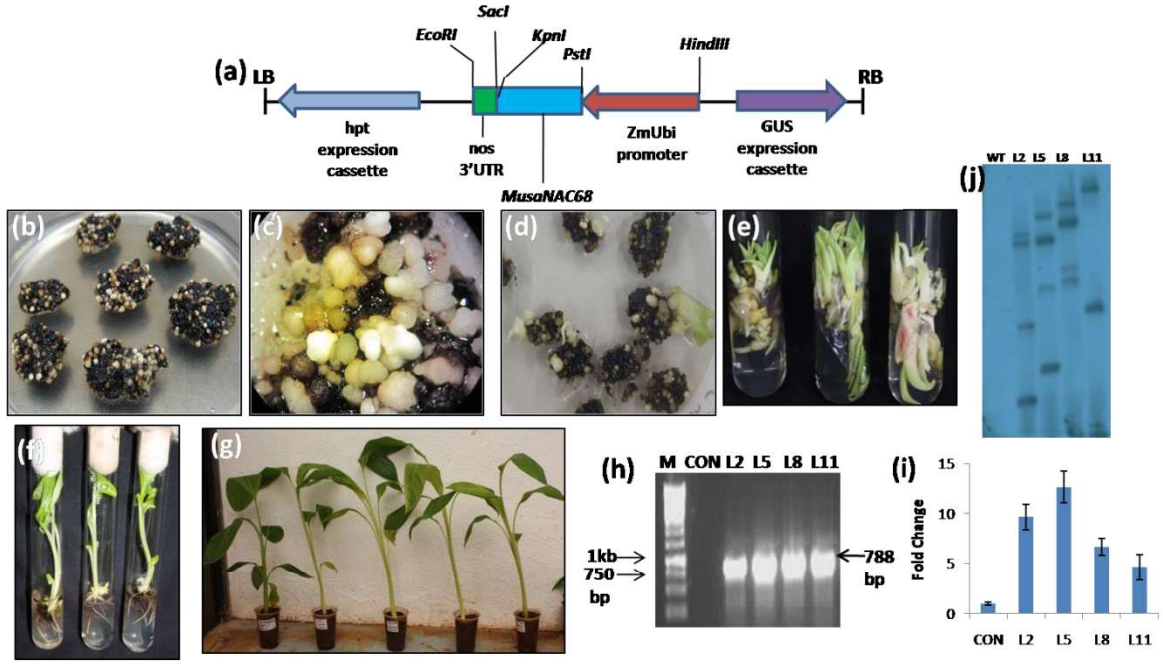

Fig. 5.7 Generation of transgenic banana plants constitutively over expressing *MusaNAC68*. (a) T-DNA region of binary vector used to generate transgenic banana lines overexpressing *MusaNAC68.* (b) Embryos emerging from banana embryogenic cells on selection medium containing 5 mg/L hygromycin. (c) A close up of white and translucent embryos on selection medium. (d) Putatively transformed shoots emerging on selection medium. (e) Generation of multiple shoots from shoots emerging on selection medium. (f) Rooted banana plants obtained from multiple shoots. (g) Transgenic lines and control plants hardened in green house. (h) PCR analysis of genomic DNA showing integration of T-DNA in different transgenic banana lines. The amplification of *hpt-II* (788 bp) present between left and right border of T-DNA indicated successful integration of T-DNA in the genome of different transgenic lines (L2, L5, L8 and L11). (i) The overexpression of *MusaNAC68* in different lines was analyzed by quantitative real time RT- PCR. The values are represented as mean ±SE. (j) Copy number of T-DNA insertion in the genome of different transgenic lines was analyzed by Southern blot.

Transgenic lines have more abundance of roots

Roots of transgenic lines are more abundant and dense than control indicating the probable role of *MusaNAC68* in root development in banana (**Fig. 5.8a**). Transgenic lines were found to have considerably higher root biomass when measured in terms of fresh weight (**Fig. 5.8b**) and dry weight (**Fig. 5.8c**). Maximum expression of *MusaNAC68* in roots compared to other organs support role of *MusaNAC68* in root development (**Fig. 5.4g**). Many reports link root development with auxin-signaling (Halliday et al. 2009; Fu and Harberd 2003; Overvoorde et al. 2010) suggesting that altered root phenotype of transgenic lines may be due to change in auxin-mediated pathways. NAA (α-naphthaleneacetic acid) increase transcript level of *MusaNAC68* in roots indicating that *MusaNAC68* has probable involvement in auxin-mediated root development (**Fig. 5.4h**). Role of *MusaNAC68* in auxin-signaling was further probed by analyzing expression of *Aux/IAA* and *ARF* (Auxin-response factor) genes. Transcript abundance of *ARF6*, *8* and *19* were remarkable while expression of *ARF2* was slightly down regulated in line L5 (**Fig. 5.8e**). Elevation of *Aux/IAA* was lower than increase in expression of *ARF* genes (**Fig. 5.8d**). These findings suggest a probable role of *MusaNAC68* in regulating root development in banana plants by altering auxin-signaling pathway.

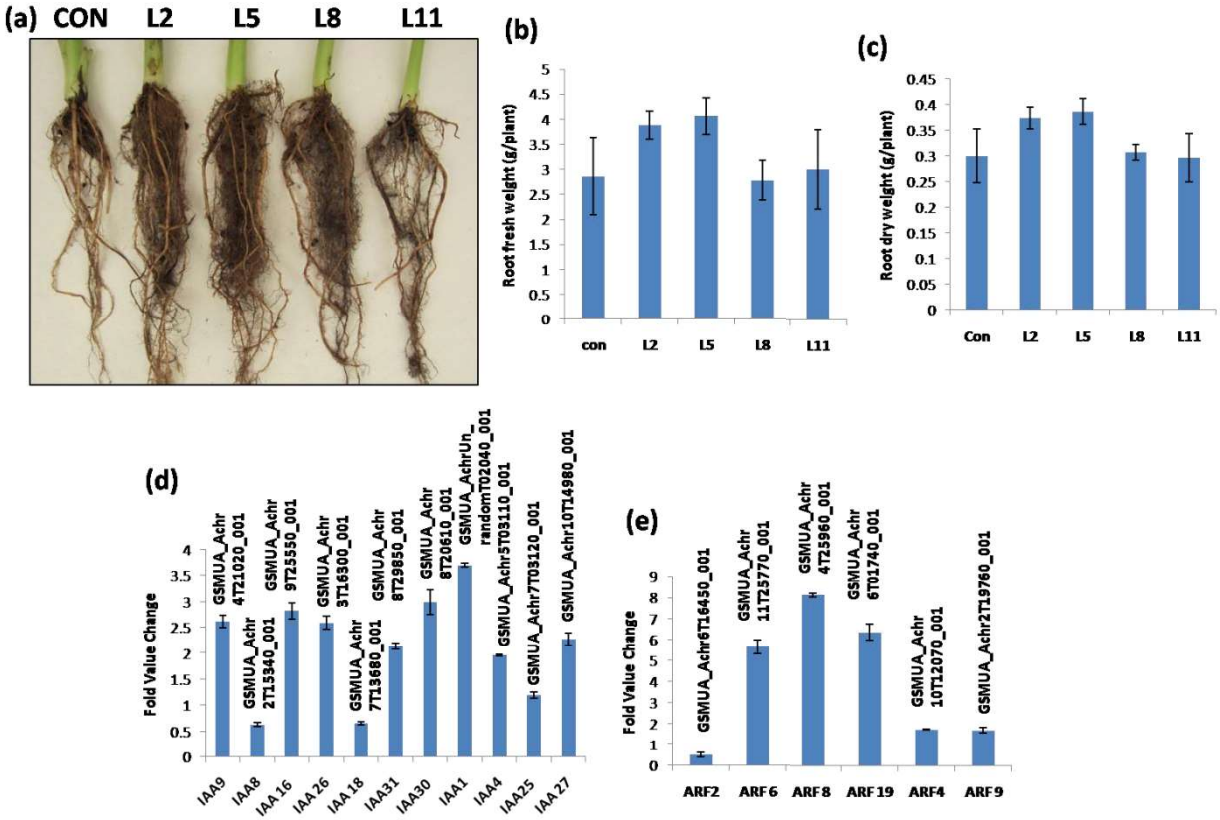

Fig. 5.8 Analysis of root and expression of auxin-responsive genes (a) Root abundance of transgenic lines and control plant (2 months old). (b) Root biomass of control and transgenic lines in term of fresh weight (gm/ plant). (c) Root biomass of control and transgenic lines in terms of dry weight (gm/ plant). (d) Expression of *Aux/IAA* genes in line L5 and control. (e) Quantitative PCR of *ARF* genes in line L5 and control. The fold change in control is 1.Values are mean±SE. (CON=Control; L2, L5, L8 and L1=transgenic lines). Banana genome database locus identifier/NCBI accession number is shown.

Leaf disc analysis

Superior drought and salinity tolerance of transgenic leaf discs was observed as less severe injury to discs incubated in 250mM NaCl or 250mM mannitol. Amount of bleaching in transgenic discs was remarkably lower than control after five days of treatment with 250mM NaCl (**Fig. 5.9a**) and six days of treatment with 250mM mannitol (**Fig. 5.9b**). NaCl and mannitol induced decrease in

chlorophyll content was lower in leaf discs of transgenic plants compared to control (**Fig. 5.9c, 5.9e**). Biochemical estimations indicated remarkably lower MDA content in transgenic discs than control discs on NaCl (**Fig. 5.9d**) or mannitol (**Fig. 5.9f**) indicating superior drought and salinity tolerance of transgenic lines than control plants.

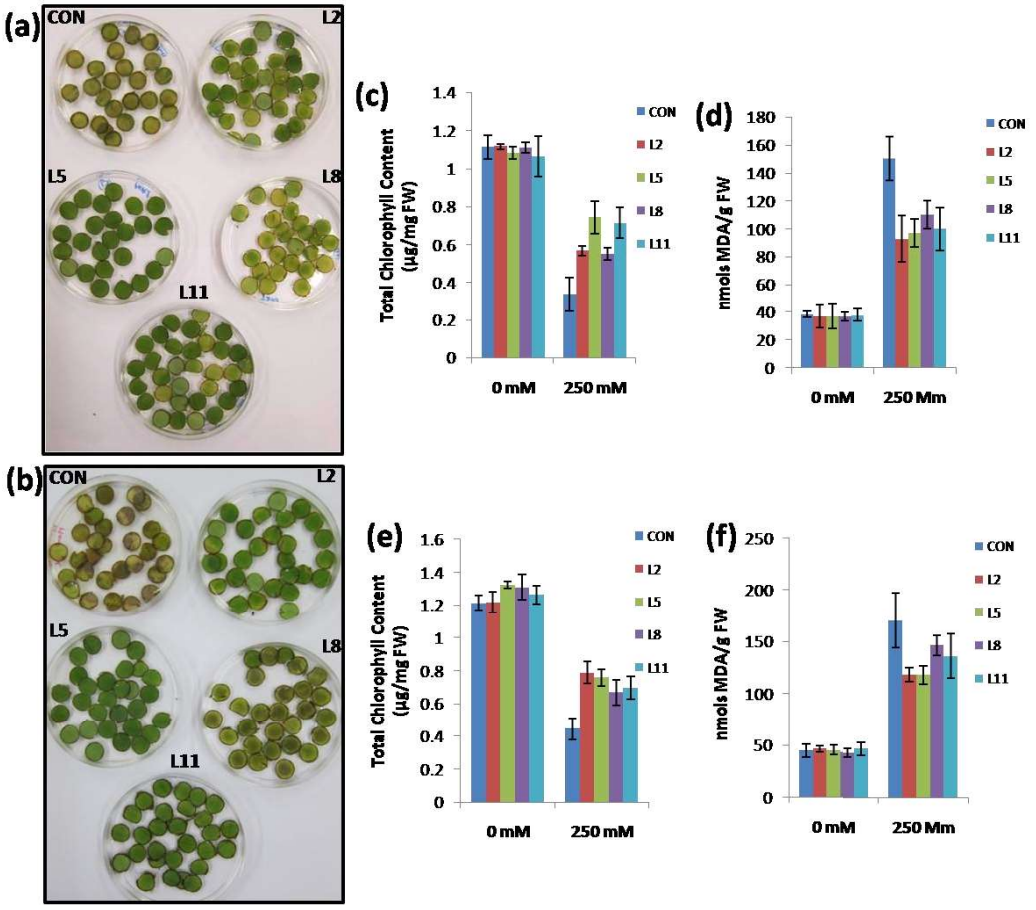

Fig. 5.9 Leaf disc assay. (a) Assay for salinity tolerance in transgenic lines. Visual injury as bleaching is less sever in transgenic lines than control in 250mM NaCl solution. Control leaf discs bleached profoundly in same solution. (b) Leaf disc assay for drought tolerance in transgenic lines. Transgenic leaf discs were more resistant to bleaching than control disc in 250mM mannitol. (c) Chlorophyll (µg/mg FW) content in leaf discs after exposure to 250mM NaCl. (d) MDA (nmols/g FW) content of leaf discs after NaCl exposure. (e) Chlorophyll (µg/mg FW) content of leaf discs subjected to 250mM mannitol. (f) MDA (nmols/g FW) levels in leaf discs of

different lines after mannitol exposure. MDA levels were lower in leaf discs of transgenic lines indicating tolerance toward dehydration induced oxidative damage. Values are mean±SE. (CON=Control; L2, L5, L8 and L11= Transgenic lines).

***In vitro* salinity and drought assay**

We further studied superior stress tolerance of transgenic lines utilizing whole plants. *In vitro* grown plants were subjected to NaCl and mannitol induced stress for ten days (**Fig. 5.10a**) and recovery was estimated after culturing in plain rooting medium for 25 days. Transgenic plants stressed on 250mM mannitol supplemented medium and later recovered on plain rooting medium, displayed remarkably better growth (**Fig. 5.10e**) and significantly longer shoot length than control (**Fig. 5.10b, 5.10d**). Similarly, recovery of transgenic lines stressed to 250mM NaCl was better than control. Superior performance of transgenic lines in salinity stress was evident as shoot length (**Fig. 5.10c, 5.10f**) and fresh weight growth (**Fig. 5.10g**) was better than control.

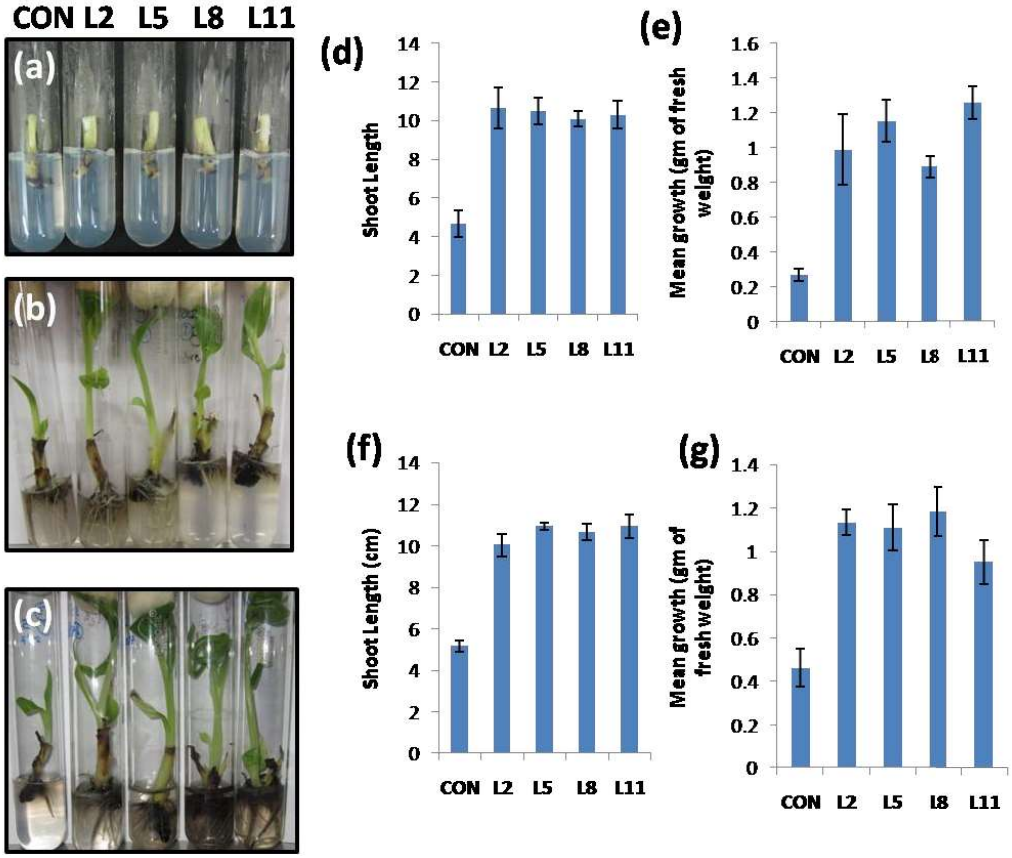

Fig. 5.10 ***In vitro*** **salinity and drought tolerance assay**. (a) Control and transgenic lines on medium with either 250mM NaCl or 250mM mannitol. (b) Recovery of transgenic lines and control stressed by 250mM mannitol. (c) Recovery of transgenic lines and control stressed by 250mM NaCl. (d) Transgenic lines gain better shoot length on rooting-medium after treatment with 250mM mannitol. (e) Fresh weight (gm) gained during recovery was more in transgenic lines than control subjected to 250mM mannitol. (f) Shoot length of NaCl stressed transgenic lines and control on rooting-medium after 25 days. (g) Fresh weight growth (gm) of NaCl stressed transgenic lines and control after 25 days on plain rooting-medium. Values are mean±SE. (CON=Control; L2, L5, L8 and L11= Transgenic lines).

Real time RT-PCR analysis of stress-related genes in transgenic banana

To better understand enhanced stress tolerance of transgenic lines, expression of abiotic stress-related genes like *DREB/CBF* (dehydration-responsive element-binding proteins/ C-repeat-binding proteins), *LEA* (late embryogenesis abundant) and *TIFY* transcription factors was analyzed by real time RT-PCR. Expression of genes coding for 2DREB, 4LEA and 5TIFY transcription factors were significantly up-regulated in transgenic line L5 than control (**Fig. 5.11**).

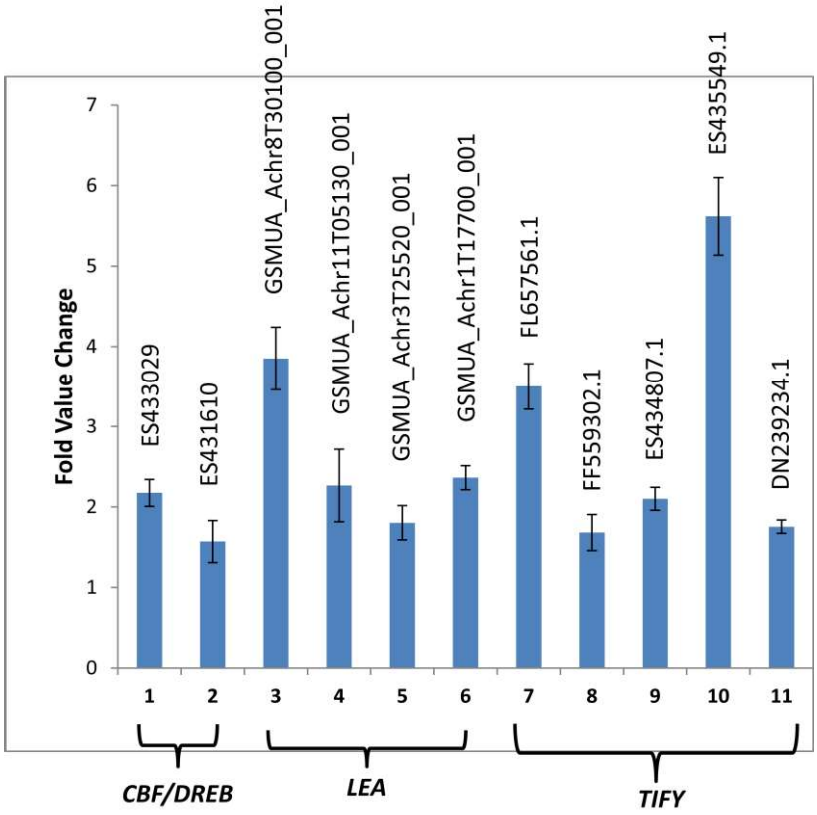

Fig. 5.11 Real time PCR of stress-related genes. Among the genes analyzed, the expression of 2*DREBs*, 4*LEA* and 5*TIFY* factor genes were significantly elevated in transgenic line L5. Values are mean±SE.

Discussion

We have investigated functions of a stress-responsive transcription factor, *NAC68* from banana. *Musa*NAC68 contain characteristic N-terminal NAC domain and is nuclear localized as seen by overexpression of *Musa*NAC68::GFP in banana cells. Transcript of *MusaNAC68* was remarkably altered in conditions like drought, high salinity, cold and application of ABA, H_2O_2 and jasmonic-acid. Multiple stress-related *cis*-acting elements were identified in 5′-proximal region of *MusaNAC68*. *Cis*-element like GT1GSSCAM4 present in 5′-proximal region of *MusaNAC68* has been indicated for salt induced expression of *SCaM-4* promoter in soybean (Park et al. 2004). *Cis*-regulatory elements like MYB1AT (AAACCA), MYB2AT (TAACTG) etc in 5′-proximal region correlate with induction of *MusaNAC68* in drought. *MYB2* factor of *Arabidopsis* is induced by drought and regulate many drought stress related genes by binding to their promoter (Urao et al. 1993). *Cis*-elements like MYB2CONSENSUSAT (CAACGG) and ABRELATERD1 (ACGTG) in 5′-proximal region is probable reason for induction of *MusaNAC68* with ABA. *ABREs* (ABA-responsive elements) are *cis*-element in promoter of drought and ABA inducible gene, *RD29* (Nakashima et al. 2006). *Arabidopsis PDF1*.2, a marker for jasmonate-dependent defense responses contain GCC-core linked with jasmonate-responsive expression (Brown et al. 2003). GCCCORE (GCCGCC) in 5′-proximal region can be correlated with the jasmonate-responsive expression of *MusaNAC68*.

Banana varieties with AAB or ABB genome are considered to be more abiotic stress tolerant relative to varieties with AAA genome constitution indicating that superior tolerance ability of such varieties might be attributed to B-genome (Robinson and Sauco 2010; Rukundo et al. 2012; Thomas et al. 1998). The cloning and characterization of *MusaNAC68* was carried out in banana cv. *Karibale Monthan* which posses ABB genome and thus is considered to be more

strong with respect to abiotic stress tolerance. A recent report emphasized on screening of banana biodiversity for drought tolerant varieties indicated that among varieties with different genome constitutions, banana variety with ABB genome was more tolerant and further proteomics approach showed altered expression of multiple genes in this tolerant variety (ABB) (Vanhove et al. 2012).

Four transgenic lines overexpressing *MusaNAC68* were regenerated. For Southern blot, DNA was restricted with *Kpn*I which digest T-DNA only once; hence number of T-DNA in genome of transgenic lines can be correlated with number of bands in autoradiograph. Difference in size of bands detected in Southern blot proved that transgenic lines originated from independent transformation events. However, copy number of T-DNA could not be correlated with fold value change in *MusaNAC68* transcript in transgenic lines. Variation in expression of T-DNA in lines might be due to "position effect" wherein different copies of T-DNA might have integrated into regions of genome with variation in transcriptional activity. Our study had shown that *MusaNAC68* overexpressing transgenic plants are marginally taller than control. Auxin is known to promote shoot elongation. *Arabidopsis* overexpressing *NAC1* were bigger than control, with thick stem and large leaves and showed significantly higher fresh weight than control plants (Xie et al. 2000). Poplar overexpressing *Pinus sylvestris glutamine synthetase* (*GS1a*) showed high levels of indole acetic-acid (IAA) and improved growth with taller phenotype (Jing et al. 2004; Man et al. 2011). Overexpression of *MusaNAC68* in banana increased root abundance and altered expression of auxin-responsive genes. Transgenic line with maximum *MusaNAC68* overexpression (L5) displayed higher transcript level of many *Aux/IAA* (Indoleacetic acid-induced protein) and *ARF* (Auxin-responsive factors). *Aux/IAA* are induced by auxin and mutants with gain-of-function showed altered stem elongation and root development. Current information

about *Aux/IAA* indicated that they might both activate and inhibit auxin responses (Reed 2001). *MusaNAC68* overexpressing banana line L5 showed elevated transcript of *ARF6*, *8*, and *19* and reduced expression of *ARF2*. *ARF6* and *ARF8* are transcriptional activators in auxin-signaling (Tiwari et al. 2003) and *Arabidopsis arf6* and *arf8* single-mutants have delayed stem elongation and floral growth (Nagpal et al. 2005). Mango (*Mangifera indica*) *ARF2* inhibits root growth in *Arabidopsis* suggesting *ARF2* negatively regulate root development (Wu et al. 2011). *Arabidopsis ARF19* is a transcriptional activator and *Arabidopsis* double-mutant lacking *ARF7* and *ARF19* showed considerable reduction in lateral and adventitious roots (Wilmoth et al. 2005). *Arabidopsis* overexpressing *NAC1* have more abundant roots and greater number of lateral roots along with alteration in expression of auxin-responsive genes *AIR3* and *DBP* (Xie et al. 2000). Induction of *MusaNAC68* after α-naphthaleneacetic acid application and presence of two auxin-responsive *cis*-elements ARFAT (TGTCTC) and CATATGGMSAUR (CATATG) in 5'-proximal region, suggested probable role of *MusaNAC68* in regulation of root development by auxin-signaling. ARFAT (TGTCTC) is an auxin-response element (*AuxREs*) in promoter of auxin-responsive genes (Goda et al. 2004). SMALL AUXIN UP RNA (*SAUR*) are auxin-responsive genes and promoter of *SAUR15A* gene contain CATATGGMSAUR (CATATG) *cis*-regulatory element involved in auxin-responsiveness (Xu et al. 1997). Reports of multiple *NAC* transcription factors with dual function of regulating root development and abiotic stress tolerance has emerged in recent years. Three rice *NAC* factors, *OsNAC5*,*OsNAC9* and *OsNAC10* are involved in root development and drought tolerance (Jeong et al. 2010, 2013; Redillas et al. 2012). *SNAC1* of rice increase root biomass with longer root system in transgenic cotton, resulting in better drought tolerance (Liu et al. 2014). Banana plants overexpressing *MusaNAC68* showed significantly increased tolerance to high salinity and drought. Reports indicated that overexpression of

transcription factors like rice *NAC6* and *DREB1* lead to growth retardation (Ito et al. 2006; Nakashima et al. 2007). Such growth retardation which can finally result in reduction of crop yield, was not observed in banana overexpressing *MusaNAC68*, suggesting that *MusaNAC68* can be potential gene for stress engineering in field grown banana plants.

In vitro stress tolerance assay along with detached leaf assay or leaf disc assay are frequently used in our laboratory to assess the tolerance capacity of different transgenic banana plants (Shekhawat and Ganapathi 2013, 2014; Shekhawat et al. 2011; Sreedharan et al. 2012). Many studies has employed *in vitro* assay to assess the stress tolerance ability of plants wherein the tolerance has been measured in terms of shoot growth and rooting capacity (Bidabadi et al. 2012; Hamrouni et al. 2008). In an earlier study, an *in vitro* assay to analyze the stress tolerance ability of banana has been carried out and drought tolerance and sensitivity of different banana varieties was studied in terms of parameters like gain of weight and total number of new leaves suggesting the suitability of *in vitro* assay to analyze the stress tolerance in plants (Rukundo et al. 2012). One of the recent report has analyzed drought tolerance ability in banana varieties using *in vitro* assay and suggested that such assay have advantage of being rapid as majority of banana are kept as *in vitro* cultures along with factors like possibility to analyze vast number of plants in short time and ability to control the experimental conditions (Vanhove et al. 2012).

Significant elevated expression was observed for at least 2*DREB/CBF*, 4*LEA* and 5*TIFY* genes in line L5. *DREBs* are important transcription factors involved in regulation of stress related genes and play critical role in regulating abiotic stress (Lata and Prasad 2011). *LEA* genes are induced in abiotic stress conditions and their accumulation in embryos is correlated with drought tolerance ability (Liu et al. 2013). *TIFY* belong to one of most vital transcription factor family with important role in jasmonate signaling and stress tolerance (Wu et al. 2015). Increased

expression of stress-related genes in transgenic banana overexpressing *MusaNAC68* indicated direct and indirect transactivation activity of *Musa*NAC68.

Table 5.1 Primers used for cloning and molecular studies involving *MusaSNAC68*

		Forward Primer (5' - 3')	Reverse Primer (5' -3')
1	Primers for cloning *MusaNAC68* in pCAMBIA1301	AATTCTGCAGATGGGAAGGA AGAGAGATGCTG	ACGTGGTACCTCAGAATGGTAACAT GTTGGTG
2	Primers for cloning *MusaNAC68* coding sequence in pCAMBIA1302	aaaAgatctg ggaaggaagagagatgctgag	AAAactagtgaatggtaacatgttggtgccgc
3	Hygromycin phosphotransferase (*hpt)* gene	GTCCTGCGGGTAAATAGCTG	ATTTGTGTACGCCCGACAGT
4.	*Musa EF1α* real time PCR primers	CCGATTGTGCTGTCCTCATT	TTGGCACGAAAGGAATCTTCT
5.	Primers for amplification of *MusaNAC68* 5' proximal region	TAAAAAAAACTCTCCAAGTA CAACG	GCCTCAGCATCTCTCTTC

Table 5.2 Auxin responsive genes and their qPCR primers used in *MusaSNAC68* study

Name	Banana genome locus identifier	Forward Primer (5' - 3')	Reverse Primer (5' -3')
ARF2	GSMUA_Achr6T16450_001	ACTGAAAGGTTG GTTGGTGC	TGTTGGGAGACACTAGTGCA
ARF4	GSMUA_Achr10T12070_001	TTTGAATTTGAGG GTGCGCT	CGGAATTGTCAGGGTCAGGA
ARF9	GSMUA_Achr2T19760_001	GCATTCACCACA GGACAGTC	GGTCATCGCCAACTAGCATC
ARF6	GSMUA_Achr11T25770_001	AATTTTGTGGCTT CCTCGGG	CCAGCCTGATCTCAAAGGGT
ARF8	GSMUA_Achr4T25960_001	AAGTCAGGATCG GTTGGGAG	CCCTGTTTTCCCATTTTCAGC
ARF19	GSMUA_Achr6T01740_001	CCGGACCTGGCTT TCAATTC	CGTCACCAACAAGCAGAACA
IAA9	GSMUA_Achr4T21020_001	ACTGTGGCTCTCA AGGAATGA	TTGCACTTCTCCATTGCTCTT
IAA8	GSMUA_Achr2T15340_001	CCTCAGCACTCGA GAAGATGT	CTCTTGCAGGAGTTGGTGAA C
IAA16	GSMUA_Achr9T25550_001	AGGGGGTTCGCT GAGACTAT	CCTTGCTTCCCTTCTCAGAGT
IAA26	GSMUA_Achr3T16300_001	CTGCTCCTGTTGT TGGTTGG	AAGAAGGCCAAGGAAGAGC T

IAA18	GSMUA_Achr7T13680_001	AGAACACGACTG CAACACAC	CTCGGAACAGTTCATCGACG
IAA31	GSMUA_Achr8T29850_001	GCTAGGACTTGG CCTCTCAA	TCAGGGCATATGATCGTCGT
IAA30	GSMUA_Achr8T20610_001	ACCGTCGACCTA AAGCTTCA	CGCCATCCATGCTAACCTTC
IAA1	GSMUA_AchrUn_randomT02040_0 01	TTCTGCTTTTGAC AGGGCAC	CCAACAAGCATCCAGTCACC
IAA4	GSMUA_Achr5T03110_001	TCAAGGACACCG AGCTAAGG	GAGCTCCTTGTAACCCCTGT
IAA25	GSMUA_Achr7T03120_001	AGGCTTGGCATGT CACTAGT	TGCCTCTGAGCTCATCGTAG
IAA27	GSMUA_Achr10T14980_001	AACAAGGGCTAG GGTGTCTC	CACCAACAAGCATCCAGTCC

Table 5.3 *TIFY* transcription factors and real time primers used in *MusaSNAC68* study

NCBI accession number	**Forward Primer (5' - 3')**	**Reverse Primer (5' -3')**
FL663717	TGACCAAACTCAGAACAACGA	ATCAGATCCTTGGCTTTCTCG
FL665822	GGATCTCTTTCCCCACAAGTC	GATGGGTGGAAAAGCTGAGAT
FL668208	AAGCGGAAGAGGAAGAGGAAT	ATTATGGCCTGTGCTTTTTCA
FF559078	CACTTGCAAGGGATGTTCAAT	CGTTTGCAATCGTCATTATGTT
ES437332	CCATCTTTTATTGCGGAAAGG	CATGAGGAACCGATGAAGAGA
FF558898	ATCCTGTTTCGGTTGTTGATG	GGTGCCATGAAACTTGAAGAA
ES433722	TCCGTGAAAAGCGTAAAGAAA	GCATTGCTGGTGTCATCTTTT
FL651123	AGTCCTCCCAATTGACGATCT	ATATCAGATGCATTGGCTTGG
ES435111	CGAAGGAACGATTCTGTAACG	CTCCTCTCGTATGGACCTGCT
ES431249	CACTGTGGCATCAACTCAAAA	ATTAGCATGTGGCAGCAGTTC
FF562392	GGCAACATATCTGAACCGAAA	CGTTTGGAACCATGCAACTAT
FL657561	CAGTCCATCTCTCCCTCAATG	AGCCACAGCAGCAGTGTTATT
FF559302	ACCAACACTGAGCAGCAAACT	CTTTACCCGCCAACAACATAA
ES434807	TCCATGGATCTGTTTCCTCAG	GGCGGAGAACCTAAACTTCTG
ES435549	TGGAACACATCGAAGTCCTTT	TTGCGAGACAGAGGTATTGCT
DN239234	TGAAGACTCGACGGTAACCTG	TACAAAACCAACCCAGTCAGC

Table 5.4 *DREB* genes and their real time primers used in *MusaSNAC68* study

NCBI accession number	**Forward Primer (5' - 3')**	**Reverse Primer (5' -3')**
ES432749	AAGCAGCACGGTGGAGTC	GTCAGACGGTGGTGGTTCAT

ES436780	GACACCGCGCTCTTCTACC	GAACCGTTTCTGATCTCCTTCC
ES431610	GGCTAAGACCAACTTCCCAATC	AAGCAGCGCCTAGGAACAAG
ES435265	GGCCGCGGAGATAAGAGA	GCGTAGTCGCTGGGGTTC
ES437095	GAGCGATCATCTCTGACTTCG	TTCCTCGGGTCTCTTATCTCC
ES432514	CCAGTAGTCAGAGCAGCACCT	GACTTCGCGTCGACAAAGA
FL662828	ACGGGTCGAAGAAGAAGAAGA	ACTGCAGGTCCATCAGATTCA
ES435892	CAGTTCACAGAAACAGGGGAAG	TCCCATGATGGATACTTGTGC
ES432610	GCCGGATTCGGTTCCATA	TGCAGCCTTCGTTGAAGC
FF558748	CTTCCGAAACTAGCTGCATCA	AATTCATCCCAGCCACCAC
ES436488	GCTCCGGCTACTGCTTCC	GCGGGCCGTATCTCCTTC
ES432382	GCTGCCCCTCAATGAAAAC	GGGCACAGTTGCAGATGAA
HN260232	TACGCTGCGGAGATACGG	GAGGACTCCACCGTGCTG
HN240556	CGGCCTTCGACTACACCTT	AATCGCCAAACTCCACCTC
HN253812	GGTTTGATGGATCTGCTGCTA	GATGATGCAGTTGCTGCTGT
HN261004	CGGCGGAGATAAGGGACT	CCTCCTCCTTAACGAGTGCTT
FL659594	AGGCCCCTTCCTCCTGAT	ATCAGCGCCCTCCAGTTTA
FF559848	CCACAAGAGATGCTCAATGTCA	TTGGGGGTTTTAACCTCACTTT
ES433029	GAGGAGAAGGAAGGAGATCAGC	TGAGATCTGGATGGCTTGTTTC
FF560353	GGAGGTGTGAGGAGGGATTC	CCACCCTCATCTTCTAATTCCAC
ES433424	CAGGGATAAAACCAGCTCCAC	CCCAGCAGCACTTTCCTTAGT
FF561178	ATCAGGAGATTTTAGTGGATCTGC	ATAGGCTCTTGCAGCCTCTTC
FF561371	TCAAATGTAGCCAAAGCACCT	GAAAGAGTTGCTTCCCTGGTC
FF560746	GGCTTCTTGGAAGAGAAGGTG	TGTCTGAGGGTTATCGTGTCC
FF561432	CGCCCACAAGAAAGTCTCAA	ACAAAGGAGTTGCTTCCCTGA
FF559386	AGCATTCCGACCATCCAAAT	TGATCTCAGGGGTTTTGTCCT
FF560823	GAGTTCGATGACGAGGAGGAG	ATGGACGTTGGCGGATTC
HN248835	ATTACCGAGGGGTGAGGAAG	TGCGGGAAGTTGGTCTTG
HA107944	GATCCTGACGCCACTGATTC	GCCTGACTCCCTTAAACTTGG
HN250386	ATGCAAGTCAGGCAAAAGGA	TTATGGCCACGAATCCTACG
HN248085	ATGATGATCCGGACACTTCC	CCACATCTGGTCGGGTTC
ES431669	GGAGCTCTAGCTTCGGGAGA	CCTGCTGCTGCTGTTGAGA

Table 5.5 *LEA* genes and their real time primers used in *MusaSNAC68* study

Banana genome locus identifier	**Forward Primer (5' - 3')**	**Reverse Primer (5' -3')**
GSMUA_Achr10T04300_001	CAATAGCAAACCTCCACC TGA	CCCTTCCCCTGATCATATCC
GSMUA_Achr4T19200_001	GGAGAAGGTGAAGGACAT GG	GACTGGTAAGGGGAACACG A
GSMUA_Achr7T15580_001	CGAGACTGTCGTCCCTGGT	GCTCTCCTCCCGACTCCTC
GSMUA_Achr10T21680_001	GCAATAGAGGCAGCCAAG AG	ACGCCAATGGTTTCCAGTA G
GSMUA_Achr1T16570_001	AGTCCGGCATGGACAAGA C	CCCTCGACAGGGTAGAATC C
GSMUA_Achr5T09440_001	CATCTGCGAGATCTCCTAC ACC	GAGAGGGGGATGGTGAAGT C

GSMUA_Achr6T20420_001	TGGTTAACCGGAGGGGATA	TTGCTTGTGGGAGAGAGTCA
GSMUA_Achr6T34630_001	AGATGCCCGGAGCACTAAT	ATGCAGGAATAGGTGCCAGA
GSMUA_Achr9T26430_001	TTCCCTGACTGGTTGGAGAT	CGCCAGATAGGGTAAACCAA
GSMUA_Achr10T12880_001	GGGCTATCACTTGCTCACATT	CAAGCCTGCAATTACCATCA
GSMUA_Achr8T30100_001	CTGGAGCCTAAAGCTACAAAGG	TGTTGAGCTTCTTCAGCCTTC
GSMUA_Achr11T05130_001	CGCAAAAGGCTAATCTGGAG	TTGTAGCGAGCGGCAATAGT
GSMUA_Achr3T25520_001	ACAGGGGTAAATACCGTGCTG	GGACGCATCTCCAGGTTTG
GSMUA_Achr1T17700_001	CAAGTTCTTTGGGCTGTCG	CTCATCTTGCTGGTGCAGTC

Chapter 6: Conclusion and future prospective

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

1. *MusaVND1*, *MusaVND2* and *MusaVND3* are efficient regulators of secondary wall development in banana plants and their overexpression induces ectopic secondary wall deposition and transdifferentiation of various cells into xylem vessel elements. However, constitutive expression of these genes resulted in growth retardation and thus reduction in plant biomass. Overexpression of genes under xylem specific promoter may result in a tighter control over their expression and thus could be useful for suitable genetic engineering for increased plant biomass development.
2. Vascular tissue specific activity of banana *VND1*, *VND2* and *VND3* genes was demonstrated by transforming banana with either $P_{MusaVND1}$*::GUS*, $P_{MusaVND2}$*::GUS* or $P_{MusaVND3}$*::GUS* and analyzing the GUS staining. We quantified the activity of $P_{MusaVND1}$, $P_{MusaVND2}$ and $P_{MusaVND3}$ by estimating the GUS activity which indicated differential activity of these regulatory regions in different organs of banana. Such studies are important for generating transgenic plants with stringent control of desired gene expression or for tissue specific expression. A number of *SNBE-like* sites was detected in the 5'upstream regulatory region of multiple secondary wall associated genes suggesting common regulatory mechanism governing these genes. Banana VND1-3 could specifically bind to *SNBE-like* sequences in a dose dependent manner, and similar binding was observed with 5'upstream regulatory region of *MYB* transcription factors (*AtMYB52-like*, *AtMYB85-like*, *AtMYB58*/*72-like*, *AtMYB46-like* and *AtMYB83-like*) and cell wall

modification related genes (*AtIRX1*/*AtCesA8-like*, *AtIRX3-like*, *AtIRX5-like*, *AtIRX8 -like*, *AtIRX10* and *AtIRX 12-like*). Transcript level of these genes were highly elevated due to overexpression of banana *VND1-3* indicating a direct regulation of these genes by banana *VND1-3*. Banana VND1-3 could bind to SNBE like sequences in isolation or as a part of a regulatory region and failed to bind a mutated SNBE sequence, such studies employing a competition assay with a non specific competitor will give better result of DNA binding activity of VND1-3.

3. Study on *MusaSNAC1* suggested its role in controlling drought-response by integrating H_2O_2 induced stomatal closure and simultaneous regulation of multiple stress-responsive genes. Functional analysis of *MusaSNAC1* during drought progression was demonstrated in transgenic banana overexpressing *MusaSNAC1* as well as banana harboring $P_{MusaSNAC1}$*::GUS* and results were supported with histochemical and biochemical analysis, gene expression profiling and protein-DNA binding studies. Present study will not only expand our knowledge about *NAC* transcription factors in drought response but also promote the utilization of *SNAC1* gene for crop improvement programmes. However, the exact identification and regulation of its target gene on a global basis needs to be carried out by integrating transcriptome sequencing analysis, DNA binding assays and transient transactivation assay of regulatory regions.
4. *MusaNAC68* is rapidly induced by stress conditions. Transgenic banana overexpressing *MusaNAC68* exhibited longer and more abundant roots and altered expression of auxin-responsive genes (*Aux/IAA* and *ARFs*). Role of *MusaNAC68* in conferring salinity and drought tolerance in transgenic banana was demonstrated and elevated transcript level of stress-related genes in transgenic banana indicated transactivation activity of

*Musa*NAC68. Present study also suggests existence of a cross-talk between stress tolerance and, root development and, coordinated regulation of these two aspects by *MusaNAC68*. More studies on regulation of target genes by *MusaNAC68* need to be carried out by transcriptome sequencing analysis and DNA binding activities of MusaNAC68 protein.

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