Cloning and characterization of three abiotic stress related genes (*Dehydrin, WRKY and bZIP*) and their overexpression in banana

By

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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.

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List of Publications arising from the thesis

Journals

1. "Cloning and characterization of a novel stress-responsive WRKY transcription factor gene (*MusaWRKY71*) from *Musa* spp. cv. *Karibale Monthan* (ABB group) using transformed banana cells", **Shekhawat UKS**, Ganapathi TR, Srinivas L, **2011**, **Molecular Biology Reports** *38*, 4023-4035.

2. "*MusaDHN-1*, a novel multiple stress-inducible SK_3-type dehydrin gene, contributes affirmatively to drought- and salt-stress tolerance in banana" **Shekhawat UKS**, Ganapathi TR, Srinivas L , **2011**, **Planta** *234*, 915-932.

3. *"MusaWRKY71* overexpression in banana plants leads to altered abiotic and biotic stress responses", **Shekhawat UKS**, Ganapathi TR, **2013**, **PLoS One**, *8*, e75506.

4. "Transgenic banana plants overexpressing *MusabZIP53* display severe growth retardation with enhanced sucrose and polyphenol oxidase activity"**,** **Shekhawat UKS**, Ganapathi TR, **2014**, **Plant Cell, Tissue and Organ Culture**, *116*, 387-402.

Conferences

1. "Overexpression of a native SK_3-type dehydrin enhances tolerance to drought and salt stress in banana", **Shekhawat UKS**, Srinivas L, Ganapathi TR, **2011,** National Symposium on Recent advances in plant tissue culture and biotechnological researches in India at M.N. Institute of Applied Sciences, Bikaner, 4-6 Feb.

2. "Characterization of *MusaWRKY71*, a multiple stress inducible WRKY gene using transgenic banana plants", **Shekhawat UKS,** Ganapathi TR, 2012, DAE BRNS Life Science symposium on "Trends in Plant, Agriculture and Food Sciences" at BARC, Mumbai, 17-19 Dec.

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1. "Studies on *MusaWRKY71*, a multiple stress inducible transcription factor gene from banana which is involved in diverse stress responses", **Shekhawat UKS,** Ganapathi TR, **2013**, BARC Newsletter, *Oct 2013*.

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CONTENTS

SYNOPSIS

Preamble

Plant growth and development are adversely affected by different abiotic and biotic stress factors. In order to survive these stresses, plants have evolved the capacity to sense and react to these diverse external signals by means of specialized physiological and biochemical strategies. Upon stress perception, plants trigger a cascade of cellular events involving several parallel transduction pathways that eventually modulate the level of specific transcription factors resulting finally in the up or down regulation of genes coding for synthesis of effector proteins and/ or metabolites which participate in stress tolerance. Detailed investigations into the functions of each of these transcription factors and effector proteins are required in order to understand and modulate stress response pathways in important crop plants. Banana (*Musa spp.*) is one of the most important food and fruit crops in the world and is widely grown in many of the tropical countries. India is the highest producer of bananas in the world with a total production of 31.89 million metric tones, which is approx. 34.5% of total production of top 20 banana growing countries (http://www.fao.org). Like for any other important crop plant, the yield and productivity of banana is limited by the several abiotic stress factors to which the banana plant is continuously exposed. Among these, cold temperatures, drought and high salt conditions are most important in context of banana. Banana is a very cold sensitive plant, with its growth being severely curtailed in ambient temperatures of less than 8°C (Santos et al., 2009). For this reason, banana cultivation is mostly concentrated in the tropical regions of the world which do not experience severe cold

temperatures at any time of the year. Further, it is a water intensive plant owing to its shallow roots and a permanent green canopy. Due to this reason banana cultivation needs an abundant and steady supply of water and any level of drought imparts a severe effect on its yield and productivity (van Asten et al., 2011). Additionally, banana plant is known to be highly salt sensitive also and it has been reported previously that high soluble salt contents in the soil cause accelerated collapse of banana root system (Gauggel et al., 2005). Thus, there is an urgent need to study the genes involved in abiotic stress responses in banana plant and to further device suitable strategies to effectively mitigate the effects of abiotic stress through the development of abiotic stress tolerant transgenic banana lines.

Chapter I [General Introduction]

This is an introductory chapter that describes the literature related to the important abiotic stress related genes widely studied in plants. Since this study has been concentrated on genes coding for dehydrins, WRKY transcription factors and bZIP transcription factors, the roles and functions of these genes in plants have been dealt with in detail.

Dehydrins are highly hydrophilic proteins involved in playing key adaptive roles in response to abiotic stress conditions having dehydration as a common component (Hara, 2010). Dehydrins, belonging to a broader class of late embryogenesis abundant (LEA) proteins, have been extensively studied in various plants owing to their affirmative association with abiotic stress tolerance. Several studies have shown induction of dehydrins in response to different abiotic stress conditions signifying their role in ameliorating the cellular effects of these stresses

(Richard et al., 2000; Bae et al., 2009). *Arabidopsis thaliana* overexpressing a wheat dehydrin demonstrated enhanced tolerance to salt and drought stress (Brini et al., 2007). Several reports describe improved freezing tolerance in plants overexpressing specific dehydrin proteins (Hara et al., 2003; Puhakainen et al., 2004). WRKY transcription factors are specifically involved in the transcriptional reprogramming following incidence of abiotic or biotic stress on plants (Rushton et al., 2010). Several reports published recently indicate that overexpression of select specific *WRKY* genes in transgenic plants can lead to significantly improved stress tolerance in plants. *VvWRKY1* overexpression in grapes activated the expression of jasmonic acid pathway-related genes and improved the tolerance to the downy mildew (Marchive et al., 2013). Overexpression of *ZmWRKY33* in *Arabidopsis* induced *RD29A* known to be involved in stress-signaling and enhanced salt stress tolerance in the transgenic plants (Li et al., 2013). bZIP transcription factors have been implicated in several important cellular processes and hence investigations into the roles played by various members of *bZIP* gene family in plants is important. Many of these bZIP transcription factors have been in the past associated with abiotic stress responses wherein their overexpression has resulted in improved abiotic stress tolerance in the transgenic plants. *ThbZIP1* gene from *Tamarix hispida* enhanced the salt stress tolerance in transgenic tobacco plants (Wang et al., 2010). Transgenic *Arabidopsis* plants overexpressing three soybean *bZIP* genes displayed better tolerance to salt and freezing stress as compared to respective controls (Liao et al., 2008). Transgenic tobacco plants overexpressing a wheat *bZIP* gene *Wlip19* showed significantly improved freezing stress tolerance (Kobayashi et al., 2008). *Arabidopsis bZIP* gene

ABF3 improved abiotic stress tolerance in rice without stunting the growth of transgenic rice plants (Oh et al., 2005).

Chapter II [Studies on *MusaDHN-1*, an SK_3-type dehydrin gene from banana]

Cellular mechanisms involved in responding to environmental stress conditions which generate reduced water potential typically include production of an array of different proteins to help the cells tide over unfavorable growth conditions. Specific hydrophilic proteins known as LEA proteins are the major proteins expressed in response to the water-deficit conditions. Group 2 LEA proteins, also called as dehydrins, are by far the most well studied putative dehydration protective proteins in higher plants. Dehydrins are characteristically rich in glycine and charged polar amino acids, whereas they lack cysteine and tryptophan. Dehydrins have conserved lysine-rich motifs called K-segments (EKKGIMDKIKEKLPG or its derivatives) which probably form amphiphilic α-helices. These α-helices interact with intracellular molecules, chiefly proteins and membranes by hydrophobic interactions thereby inhibiting their coagulation in water-stressed conditions. Other distinctive features of dehydrins include consensus motifs called Y-segment (T/VDEYGNP) and S-segment (a track of serine residues). A novel banana SK_3-type dehydrin gene, *MusaDHN-1* was identified from banana EST database (Genbank accession no. ES436956). The coding sequence of *MusaDHN-1* consisted of 711 nucleotides encoding a protein of 236 amino acids. MusaDHN-1 protein is classified as a SK_3-type dehydrin as it possesses a single tract of characteristic serine residues, the S-segment in the N-terminal half and three lysine-rich K-segments. Expression profiling in native banana plants (cv. *Karibale Monthan*) demonstrated that *MusaDHN-*

1 transcript was induced in leaves and roots by drought, salinity, cold, oxidative and heavy metal stress as well as by treatment with signaling molecules like abscisic acid, ethylene and methyl jasmonate.

To explain stress inducibility of *MusaDHN-1* and to get clues for its possible functions in banana, a 1071-bp fragment present upstream of *MusaDHN-1* coding sequence was amplified from banana cv. *Karibale Monthan* genomic DNA by employing TAIL-PCR. Several putative cis-acting elements demonstrated to be integral to stress-responsive gene regulation pathways were identified in the promoter region of *MusaDHN-1* using online PlantCARE and PLACE software. Promoter characterisation carried out by making a *MusaDHN-1* promoter::GUS fusion construct and its transformation in tobacco plants reconfirmed the abiotic stress inducibility of *MusaDHN-1*. To further characterize its functions in banana plants, banana embryogenic cells were transformed with an expression cassette designed to constitutively overexpress *MusaDHN-1* in the transgenic plants. Transgenic nature of these plants was confirmed by genomic DNA PCR and Southern blotting and overexpression of *MusaDHN-1* was confirmed by real-time quantitative RT-PCR. Transgenic banana plants constitutively overexpressing *MusaDHN-1* were phenotypically normal and displayed improved tolerance to drought and salt stress treatments in both *in vitro* and *ex vitro* assays. Better relative water content, enhanced accumulation of proline and reduced malondialdehyde levels in drought and salt stressed *MusaDHN-1* overexpressing plants indicated their superior performance in stressed conditions.

Chapter III [Studies on *MusaWRKY71,* a WRKY transcription factor gene from banana]

Plant stress responses are generally controlled by a network of specialized genes through intricate regulation by specific transcription factors. Among these transcription factors, members of WRKY family have been well known for their central roles in regulating the plant response to stress. WRKY transcription factors comprise one of the largest families of transcription factors in plants and are involved in a variety of physiological processes most prominent among them being biotic and abiotic stress responses. WRKY proteins possess a specific 60 residues long DNA binding domain which is named as the WRKY domain after the almost invariant WRKY amino acid sequence present towards the N-terminus. Most members of WRKY family have been implicated in transcriptional reprogramming linked with the plant immune responses where they serve as central components of the innate immune system. Additionally, specific WRKY proteins have been demonstrated to be involved in cold, heat, drought and salt tolerance thereby indicating that the WRKY web of signalling encompasses both biotic stress and abiotic stress responses.

We identified a novel *WRKY* gene, *MusaWRKY71*, using in silico approaches from an abiotic stress-related EST library maintained at NCBI. Since this EST was incomplete at the 3' end, 3' RACE was employed to decipher the full sequence of *MusaWRKY71* transcript. The 1299-bp long cDNA of *MusaWRKY71*, comprising of a 76 nucleotide long 5'UTR, 843 nucleotide coding sequence and a 380 nucleotide 3' UTR, encodes a protein with 280 amino acids and contains a characteristic WRKY domain in the C-terminal half. Among the characterized proteins, MusaWRKY71

showed the best homology with WRKY71 proteins of *Oryza sativa, Zea mays* and *Triticum aestivum* and hence by analogy this banana *WRKY* gene was designated as *MusaWRKY71*. Although MusaWRKY71 shares good similarity with other monocot WRKY proteins the substantial size difference makes it a unique member of the WRKY family in higher plants. The 918-bp long 5' proximal promoter region determined using TAIL-PCR has many putative cis-acting elements and transcription factor binding motifs. Subcellular localization assay of MusaWRKY71 performed using a MusaWRKY71::GFP fusion protein construct confirmed its nuclear targeting in transformed banana suspension cells. *MusaWRKY71* expression in banana plantlets was up-regulated manifold by cold, dehydration, salt, ABA, H_2O_2, ethylene, salicylic acid and methyl jasmonate treatment indicating its involvement in response to a variety of stress conditions in banana. When *MusaWRKY71* was constitutively overexpressed in transgenic banana plants, the resulting transgenic plants were phenotypically normal. Stable integration and overexpression of *MusaWRKY71* in these transgenic banana plants was proved by Southern blot analysis and quantitative real-time RT-PCR respectively. Transgenic banana plants overexpressing *MusaWRKY71* displayed enhanced tolerance towards oxidative and salt stress as indicated by better photosynthesis efficiency (Fv/Fm) and lower membrane damage of the assayed leaves. Further, differential regulation of putative downstream genes of MusaWRKY71 was investigated by real-time RT-PCR expression analysis using the transgenic plants. Out of a total of 122 genes belonging to *WRKY*, *pathogenesis-related (PR) protein genes*, *non-expressor of pathogenesis-related genes 1* (*NPR1*)

and *chitinase* families analyzed, 10 genes showed significant differential regulation in *MusaWRKY71* overexpressing lines.

Chapter IV [Studies on *MusabZIP53,* a bZIP transcription factor gene from banana]

Plant hormone abscisic acid (ABA) plays an especially vital role in the signalling processes associated with abiotic stress in plants. Generally, when a plant encounters adverse environment conditions, ABA levels increase and this leads to a chain of signal transduction events wherein multiple pathways are modulated to finally reorient the internal milieu of a plant cell such that it now has enhanced capacity to overcome the stress period. Most genes which are observed to respond to drought, high salt and cold stress treatments are also up-regulated by exogenous ABA application. Majority of such genes have a ABA-responsive element [(C/T)ACGTGGC] in their proximal promoter regions. Modulation of gene expression through ABA-responsive elements is predominantly regulated by basic leucine zipper proteins (bZIPs) transcription factors. bZIPs constitute a large family of transcription factor proteins having a highly conserved bZIP domain composed of two invariate structural features: a basic domain (rich in basic amino acids) required for sequence specific DNA binding, and the leucine zipper domain which contains at least 3 - 4 repeats of leucine at every 7^{th} position (heptad leucine repeat) that mediates dimerisation through hydrophobic bonding.

We identified a *bZIP* gene, *MusabZIP53*, from banana EST database (Genbank accession no. DN239778) based on its differential abundance in contrasting sets of abiotic stress tissue derived EST sequences. Full length coding sequence of

MusabZIP53 consisted of 435 nucleotides coding for a 144 amino acid protein. A 493 bp long 5' UTR and a 225 nucleotide long 3' UTR of *MusabZIP53* gene were also identified. Expression profiling in native banana plants proved that *MusabZIP53* transcript was strongly up-regulated by cold and drought stress and by ABA treatment in both leaf and root tissues. Cellular localization of MusabZIP53 protein investigated via a MusabZIP53::GUS fusion protein construct confirmed its nuclear targeting. *MusabZIP53* was overexpressed in transgenic banana plants using a constitutive promoter derived from *Zea mays* polyubiquitin gene. Transgenic banana plants constitutively overexpressing *MusabZIP53* displayed growth retardation from early stages of transformation/ regeneration protocol. The embryos were slow to emerge from the transgenic tissues and they were significantly lower in numbers as compared to the equivalent controls. Further, multiple shoot induction and rooting of these putatively transgenic tissues was also slow. Subsequently, the hardened plants were observed to be severely dwarfed as compared to controls. The root growth was also severely stunted. The leaves of these transgenic plants showed a very typical morphology in that they were significantly thicker than the control leaves of the same length and were arranged to form a stiff rosette on top of the plant. Genomic DNA PCR and Southern blotting was performed to confirm the transgenic nature of these plants. Further, the overexpression of *MusabZIP53* in transgenic banana plants was established by northern blotting. Genes belonging to several families known to be involved in abiotic stress perception and mitigation were found to be differentially regulated in these transgenic plants. These included genes coding for dehydration response element binding proteins, LEA proteins, anti-oxidant enzymes, aquaporins,

polyphenol oxidases (PPOs), Aux/IAA proteins and proteins involved in amino acid metabolism. In total, 25 genes out of a total of 189 tested were found to be differentially regulated in *MusabZIP53* overexpressing plants.

Plants overexpressing close homologs of *MusabZIP53* have been reported to have elevated sucrose levels in their leaves. Further, these homologs have also been reported to possess a highly conserved untranslated ORF (uORF) in their long 5' UTRs. These studies have postulated that these *bZIP* genes possessing this uORF are involved in sucrose sensing such that their translation is slowed down if the amount of cellular sucrose increases and vice versa. In our studies also, we observed the levels of sucrose were higher by approx. 40 % in the transgenic leaves as compared to the equivalent controls. Further, to prove that sucrose indeed affects the translation of *MusabZIP53* gene, we used banana embryogenic suspension culture cells growing in modified MS medium with 4.5 % (w/v) sucrose. When these cells were starved of sucrose for 24 hrs the transcript levels of two genes, which were up-regulated in untreated *MusabZIP53* overexpressing leaves, were induced to significant levels indicating sucrose starvation indeed leads to elevated translation levels of MusabZIP53 protein in banana cells thereby inducing the expression of the above two genes. All four banana PPO sequences detected in EST and transcriptome databases were found to be up-regulated in *MusabZIP53* overexpressing plants. Since all the four expressed PPO coding genes were up-regulated in the transgenic *MusabZIP53* overexpressing plants, we wanted to investigate whether the *MusabZIP53* gene exerts a coordinated control of the total PPO activity in banana and whether these PPO genes are in any way related to abiotic stress response pathways in banana plant.

Hence, PPO coding gene transcript levels were determined in cold and ABA stressed banana leaves. All the four PPO coding transcripts demonstrated to be up-regulated in untreated *MusabZIP53* overexpressing plants were found to be induced in ABA and cold treated banana leaves also. To validate the correlation between elevated transcript levels and increased protein activity, total PPO activity was determined in the leaves of four transgenic plants and in the untransformed controls and also in cold and ABA treated untransformed banana plants. Approx. 20 - 25 % higher PPO activity was noticed in all the four transgenic lines and stress treated leaves indicating that higher PPO coding transcript levels lead to increased protein activity.

Chapter V [Conclusions and future work]

The major conclusions of the present thesis are summarised as below

1. Constitutive overexpression of a specific effector protein like dehydrin imparts multiple abiotic stress tolerance in banana plants without compromising on any important vital growth parameter. This study was the first to report characterization of a banana gene using transgenic banana plants. Further, *MusaDHN-1* dehydrin gene characterized in this study can potentially be used to develop abiotic stress tolerant lines in other important crop plants.

2. *MusaWRKY71* is an important member of the *WRKY* gene family in banana and it possess the capacity to cross regulate other *WRKY* gene members as well as other genes involved in biotic stress signalling pathways. Since overexpression of this gene provides the transgenic plants with an improved oxidative and salt stress tolerance, it may well be a significant link in the

interface between abiotic and biotic stress pathways in banana. Further studies involving microarray experiments based on the newly uncovered banana genome sequence are warranted in future to fully establish the multi faceted roles played by *MusaWRKY71* gene in banana stress responses.

3. *MusabZIP53* gene plays important roles in multiple cellular networks involved in abiotic stress related signalling in banana. Further, it also contributes to the maintenance of sucrose homeostasis in banana plant through a linked uORF present in its 5'UTR. Also, our findings pertaining to the coordinated induction of all four expressed PPO coding genes by *MusabZIP53* gene (in effect controlling the total cellular PPO activity) assign a hitherto unknown role for *bZIP* genes in plants.

4. Among the three genes characterised as part of this study, the *MusaDHN-1*dehydrin gene is the most suited to develop abiotic stress tolerant banana plants. Being an effector protein in contrast to the regulatory nature of MusaWRKY71 and MusabZIP53 transcription factor proteins, MusaDHN-1 works as a molecular shield and directly protects the various cellular macromolecules from the effects of dehydration. As is evident from our results, *MusaWRKY71* and *MusabZIP53* transcription factor genes are involved in multiple regulatory pathways in banana plant and hence constitutive overexpression of these genes might not be the most appropriate way to develop stress tolerant banana plants.

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List of Figures

List of Tables

Chapter 1

General Introduction

Modern-day plants have evolved from simple autotrophic organisms in response to continuous abiotic and biotic environmental changes. Among the abiotic factors that have influenced the overall progression of plant evolution, water availability, soil salinity and prolonged incidence of low temperatures are the most important. These 50also represent the major impediments for agriculture worldwide because these adverse environmental factors prevent plants from realizing their full genetic yield potential. In order to survive these challenges, plants have evolved the capacity to sense and react to these diverse external signals by means of specialized physiological and biochemical strategies. Upon stress perception, plants trigger a cascade of cellular events involving several parallel transduction pathways that eventually modulate the level of specific transcription factors resulting finally in the up- or down-regulation of genes coding for synthesis of effector proteins and/ or metabolites which participate in stress tolerance. Among the abiotic factors, water stress in its broadest context encompasses both drought and salt stress and also low temperature stress. Because cell signaling controls plant responses to stress stimuli and eventual adaptation to these adverse conditions, it is not an exaggeration to observe that water stress signaling has played a dominant part in shaping the flora on earth.

Banana is among the most important fruit crops in the world and it also fulfills a huge food security role for millions of people across the world, mainly in the African continent. India is the largest producer of banana with approx. 31 million metric tones comprising about 30 % of world production. Bananas are especially sensitive towards any sort of water limitation and it has been reported that they rarely attain their full genetic yield potential due to limitations imposed by water availability

[1]. A shallow root system coupled with permanently green canopy comprising of big leaves and numerous stomata translates into huge water requirements for banana plant. [2, 3] have reported that banana requires upto 550 mm of water per month. Water limitation in banana plant is reflected in the form of reduced stomatal conductance and leaf size and increased leaf senescence. This heightened drought sensitivity of cultivated banana varieties became evident when banana production in Tamilnadu declined in 2001-2004 due to severe drought conditions [1]. Similarly, other abiotic stress conditions like high salinity and low temperatures also affect the realization of full genetic yield potential of banana by indirectly limiting the physiological water availability.

In response to conditions which create water limitations, land plants have developed special mechanisms to prevent loss of intracellular hydration. In banana, a Brazilian cultivar known as 'Cachaco' is considered to be highly drought tolerant [4]. Among the genes which are induced in water limiting conditions in this cultivar (as demonstrated by their differential occurrence in contrasting EST datasets; http://www.ncbi.nlm.nih.gov/nucest/?term=cachaco), we have identified a late embryogenesis abundant (LEA) protein coding gene, a WRKY transcription factor protein coding gene and a bZIP transcription factor protein coding gene for detailed stidies in the present study.

High accumulation of LEA proteins has been accepted as the most common mechanism evolved to overcome water stress [5, 6]. LEA proteins described first in cotton, were originally reported to be abundantly expressed during last stages of embryo development. Dehydrins comprise a distinct biochemical group of LEA

proteins called as group 2 LEA proteins. These proteins are expressed at high levels during late embryogenesis or they can be induced in vegetative tissues by different stress stimuli which cause cellular dehydration (like drought, salinity, cold, heat etc). Dehydrins are omnipresent in higher plants and are also reported to be present in other photosynthetic organisms including ferns, mosses, algae and cyanobacteria [7]. Dehydrins vary greatly in size from 9-200 kDa. Study of different dehydrins from varied plants have led to the identification of conserved motifs, the most conserved among them being known as K-segment (named so due to the predominance of lysine amino acid residues). Dehydrins can also possess tyrosine rich Y-segments and serine rich S-segments. In aqueous solutions, dehydrin molecules are dominantly present in the random coil conformation (i.e. they form a majority of hydrogen bonds with neighboring water molecules and least hydrogen bonds between different amino acid residues). Due to this, dehydrins appear unstructured with high proportions of hydrophilic amino acid residues and have the ability to change their conformation in response to changes in their microenvironment. Under reduced hydration, the above described K-segments assume α-helical conformation (similar to amphipathic α-helices) wherein negatively charged amino acids are placed on one side of the helix, hydrophobic amino acid are on the opposite side of the helix and basic amino acids lie on the polar-non polar interface. The amphipathic α-helices of these dehydrins interact with partly dehydrated surfaces of other proteins and biomembranes. The binding of dehydrins to the partly dehydrated surface of proteins and lipids protects them from further loss of water envelope (which can lead to their denaturation). These interactions between partly dehydrated surfaces of dehydrin proteins and other

vital biomolecules form the basis of dehydration protective functions of dehydrins [8]. Several transgenic studies performed over the last decade have revealed positive effect of dehydrin gene expression on plant abiotic stress tolerance. Transgenic *Physcomitrella patens* moss in which the only dehydrin gene present was disrupted, had an impaired ability to recover after salt and osmotic stress [9]. Transgenic *Arabidopsis* plants overexpressing *RcDHN5* (an SK_2 type dehydrin) sourced from frost-tolerant *Rhododendron catawbiense*, showed improved frost tolerance [10]. Expression of *CuCOR19* derived from *Citrus unshiu* in tobacco mitochondria resulted in reduced lipid peroxidation [11]. The expression of wheat *DHN-5* in *Arabidopsis* led to an increase in salt and osmotic stress tolerance [12]. Further, ectopic expression of BjDHN2 and BjDHN3 proteins from *Brassica juncea* in transgenic tobacco plants resulted in improved tolerance to heavy-metal (Cd^{2+} and Zn^{2+}) stress [13]. The transgenic plants demonstrated reduced electrolyte leakage and malondialdehyde generation, indicating that BjDHN2 and BjDHN3 probably enhanced tolerance to heavy metals by reducing lipid peroxidation thereby protecting cellular membranes. The fact that dehydrins are known to be induced in response to multiple abiotic stress stimuli coupled with the above said studies showing positive correlation between dehydrin expression and stress tolerance prompted us to investigate the role of dehydrins in banana abiotic stress response pathways. According, an SK_3-type dehydrin gene was identified from banana EST database and later characterized by overexpressing it in transgenic banana plants cv. Rasthali. Rasthali (AAB 'Silk' group) is a highly prized banana cultivar preferred for its unique fruit aroma and golden yellow colour

Detailed studies performed after elucidation of first genomes of plant species like *Arabidopsis* and rice have revealed that plants devote a large proportion of their genome capacity to transcription, with the *Arabidopsis* and rice genomes respectively coding for more than 2100 and 2300 transcription factors [14]. Most of these transcription factor genes belong to large gene families, some of whom are plant-specific. WRKY transcription factor family is one such plant specific family of regulatory proteins. Apart from biotic and abiotic stress responses, WRKY transcription factors have been shown to be involved in several important developmental and physiological processes like embryogenesis, seed coat and trichome development, leaf senescence and plant hormone signaling [15]. The WRKY protein family is named after the highly conserved ~ 60 amino acid long WRKY domain, which has a conserved amino acid motif WRKYGQK at N-termini and a unique zinc-finger-like motif at C-termini. Both these motifs contribute to the high binding affinity of WRKY transcription factors to their cognate consensus cis-acting element, the W box (TTGACT/C). WRKY proteins are known to function as important components in the complex signaling networks activated during plant biotic stress responses. Compared with the research progress in roles of WRKY proteins in biotic stresses, considerably less information is available to elucidate the functions of WRKY proteins in abiotic stress responses. Several studies have demonstrated that specific WRKY genes are strongly and rapidly induced in response to multiple abiotic stress stimuli such as cold, drought or salinity indicating their possible regulatory role in these signaling pathways [16, 17]. In one such earliest study, a *WRKY* gene derived from *Larrea tridentata* (a xerophyte) was shown act as an activator of ABA signaling

[18]. In rice, OsWRKY24 and OsWRKY45 were shown to act as repressors of an ABA-inducible promoter, and OsWRKY72 and OsWRKY77 were demonstrated to be activators of the same promoter [19]. Heat shock inducible *HSP101* promoter driven overexpression of *OsWRKY11* in rice improved heat and drought stress tolerance [20]. Likewise, overexpression of *OsWRKY45* led to enhanced salt and drought tolerance as well as increased disease resistance [21]. Similarly, in *Arabidopsis*, overexpression of either *AtWRKY25* or *AtWRKY33* improved salt stress tolerance [22]. These studies amply illustrated that WRKY factors form integral part of the signaling processes involved in transcriptional reprogramming when plants are exposed to any sort of abiotic or environmental stress. While taking part in plant stress responses, WRKY proteins act both as repressors as well as activators of other genes. Furthermore, there is increasing evidence that a single WRKY transcription factor might be involved in regulating several plant processes. They interact with a diverse array of protein partners, including MAP kinases, 14-3-3 proteins, calmodulin, histone deacetylases, resistance proteins and other WRKY transcription factors. Also, *WRKY* genes show extensive autoregulation and cross-regulation of other members of the *WRKY* family which facilitates transcriptional responses in a complicated web with built-in redundancy [15]. Strong experimental evidence showing that WRKY transcription factors are involved in a host of plant processes including abiotic stress tolerance prompted us to investigate the role of *WRKY* genes in banana abiotic stress response pathways. Accordingly, a *WRKY* gene was identified from banana EST database and later characterized by overexpression in transgenic banana plants.

Another transcription factor family of significance in context of plant abiotic stress tolerance is the bZIP transcription factor family. The bZIP family is among the largest transcription factor families in higher plants and members of this family regulate multiple plant processes involved in plant growth and development and responses to environmental factors. This family is characterized by a basic region mediating sequence-specific DNA-binding and a leucine zipper region required for dimerization. According to DNA binding specificities and sequence homology in the bZIP domains, these bZIP proteins have been classified into 13 groups (A, B, C, D, E, F, G, H, I, J, K, L and S) [23]. Group A bZIP transcription factors in *Arabidopsis*, like ABI5, bind to the ABA responsive elements and function in ABA signaling. These group A members are mainly involved in plant responses to dehydration and salt stress [24]. Group S members like AtbZIP1 act in transcriptional activation of hypoosmolarity inducible proline dehydrogenase and specifically activate the G-box containing ASN1 promoter in a protein kinase KIN10 mediated energy/ stress signaling cascade in *Arabidopsis*. Apart from ABA, expression of *bZIP* genes is commonly regulated by methyl jasmonate as well as various abiotic and biotic stress treatments, including salt, cold, dehydration, methyl viologen and pathogen invasion. By analogy, this indicates that bZIP proteins have important roles in plant abiotic stress tolerance. Constitutive expression of maize *ABP9*, which encodes a bZIP transcription factor, in transgenic *Arabidopsis* plants leads to improved tolerance to multiple stresses [25]. Overexpression of *SlAREB* (a member of the ABF/AREB subfamily in *Solanum lycopersicum*) improves the survival of transgenic tobacco plants under drought stress conditions [26]. Also, constitutive expression of *ThbZIP1*

in tobacco was shown to enhance the activity of peroxidase and superoxide dismutase [27]. Apart from that, several other reports show that plants expressing ectopic bZIP proteins demonstrate higher tolerance towards salt stress [28], freezing stress [28], drought stress and heat shock [29]. Also, some *bZIP* genes have roles in signaling pathways involved in mediating endoplasmic reticulum stress responses arising out of unfolded protein response in plants [30].

Thus keeping in mind the importance of bZIP family of proteins in diverse plant functions, we identified a putative *bZIP* gene from banana EST database and constitutively overexpressed the same in transgenic banana plants to characterize its functions.

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

1. Amplification of gene transcripts coding for a dehydrin, a WRKY transcription factor and a bZIP transcription factor from banana tissue derived cDNA.
2. Expression profiling of the three selected gene transcripts in banana tissues exposed to different stress treatments.
3. Overexpression of full length coding sequences of the three selected gene transcripts using a strong constitutive promoter in transgenic banana plants.
4. Analysis of the characteristics of the resultant transgenics in context with abiotic stress tolerance in banana.

Chapter 2

Studies on *MusaDHN-1*, a SK_3-type dehydrin gene from banana

2.1 Introduction

Environmental stresses including drought, high salinity and cold induce significant physiological and molecular alterations in crop plants leading to considerable reduction in their growth and productivity [31, 32]. Cellular mechanisms involved in responding to these environmental conditions, which generate reduced water potential, typically include production of an array of different proteins to help the cells tide over unfavorable growth conditions [33]. Specific hydrophilic proteins known as Late Embryogenesis Abundant (LEA) proteins are the major proteins expressed in response to the water-deficit conditions [34]. Group 2 LEA proteins, also called as dehydrins, are by far the most well studied putative dehydration protective proteins in higher plants [35, 36]. Dehydrins are characteristically rich in glycine and charged polar amino acids, whereas they lack cysteine and tryptophan. Dehydrins have conserved lysine-rich motifs called K-segments (EKKGIMDKIKEKLPG or its derivatives) which probably form an amphiphilic helix. Other distinctive features of dehydrins include consensus motifs called Y-segments (T/VDEYGNP) and S-segments (a track of serine residues). In fact, the number and order of the K-, S- and Y- motifs in the protein sequence defines the different dehydrin subclasses: Y_nSK_n, Y_nK_n, SK_n, K_n and K_nS. Although the way dehydrins function in plants is still an open question, several postulates suggesting their role in protecting cellular lipid membranes against peroxidation, in providing cryoprotective function to freezing sensitive enzymes, in binding to lipid vesicles containing acidic phospholipids or in directly regulating gene function by DNA binding have been proposed [37, 38]. Since the expression of dehydrins is significantly induced by abiotic stresses such as

drought, cold and high salinity, it has been postulated that a positive correlation exists between dehydrin expression and abiotic stress tolerance in plants.

We have identified a novel SK_3-type dehydrin (*MusaDHN-1*) from a banana leaf cDNA library (Genbank accession no. ES436956). Banana, which is the world's largest fruit crop, is a water intensive plant owing to its shallow roots and a permanent green canopy [1]. Due to this reason banana cultivation needs an abundant and steady supply of water and any level of drought imparts a severe effect on its yield and productivity [39, 40]. Global water scarcity leading from adverse weather changes has necessitated development of drought tolerating varieties of important food security crops like banana. Further, development of cultivars tolerant to drought and other stress conditions in banana is difficult using conventional methods as most of the cultivated bananas are triploids and hence sterile. Genetic transformation with genes like dehydrins, which have enabled other crop plants to tide over water and salt stress conditions [12], is one of the viable strategies for obtaining banana plants displaying enhanced survival in drought and salinity conditions. *MusaDHN-1*, identified as part of this study, was found to be significantly upregulated in response to different abiotic stress conditions like drought, cold and high salinity in a robust and hardy cultivar of banana (cv. *Karibale Monthan*, ABB group). Banana cultivars possessing more number of B genomes have, in general, been considered to be more tolerant to various types of environmental stresses [41, 42] and hence the studied up-regulation of the *MusaDHN-1* in this cultivar in response to various abiotic stresses led us to postulate that constitutive overexpression of this dehydrin in any of the elite edible cultivars of banana may result in improved abiotic stress tolerance thereby

increasing their productivity in environmentally stressed conditions. In this chapter, we describe the cloning and overexpression of *MusaDHN-1* in an elite edible banana cultivar cv. *Rasthali* (AAB group) along with detailed assays for improved drought and salt stress tolerance.

2.2 Materials and methods

2.2.1 Primers

The primer sequences used in this study are shown in Table 2.1

2.2.2 Amplification and sequence analysis of *MusaDHN-1*

Total RNA extracted from young banana cv. *Karibale Monthan* leaves using Concert Plant RNA Reagent (Invitrogen, USA) was cleaned up and subsequently treated with DNase using RNeasy Plant Mini Kit (Qiagen, Germany). This RNA (approx. 5 µg) was then used to make first strand cDNA using Oligo $(dT)_{12–18}$ primer (Invitrogen, USA) and AccuScript Reverse Transcriptase (Stratagene, USA) according to manufacturer's instructions. Full length coding sequence of *MusaDHN-1* was amplified from this cDNA using Pfu Ultra AD DNA Polymerase (Stratagene, USA) as per suppliers protocol. The PCR product obtained was subsequently sequenced. The online ExPASy translate tool (http://au.expasy.org/tools/dna.html) was used to translate the *MusaDHN-1* cDNA sequence. ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to align the predicted MusaDHN-1 protein sequence with its closest homologues. The K- and S- segments of MusaDHN-1 protein were identified using ExPASy prosite server (http://au.expasy.org/prosite/). MEGA 5 software was used to construct a tree showing phylogenetic relationships for MusaDHN-1 protein.

2.2.3 Determination of copy number of *MusaDHN-1* in banana genome

Genomic DNA was isolated from young banana cv. *Karibale Monthan* leaves using GenElute Plant Genomic DNA Miniprep Kit (Sigma, USA). Twenty micrograms genomic DNA was digested overnight with *Eco*RI, *Bam*HI and *Pst*I restriction enzymes. Following enzyme deactivation the completely digested genomic DNA was purified using High Pure PCR Product Purification Kit (Roche Applied Science, Germany) and separated overnight at a field strength of 1.25 V/cm in a 0.9% (w/v) agarose TAE gel. DNA was transferred to a nylon membrane by capillary method using 20X SSC. Restricted DNA was subsequently immobilized on the membrane by baking at 120°C for 30 min. The membrane was then exposed to DIG-labeled probes generated using *MusaDHN-1* 5'UTR as a template. Chemiluminescent detection of hybridization signals was carried out using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany) according to manufacturer's instructions.

2.2.4 Expression profiling of *MusaDHN-1* in different stress conditions using real time PCR

In vitro rooted plantlets of banana cv. *Karibale Monthan* were hardened in greenhouse for 2 months. Uniformly sized plants at similar growth stage were treated with 100 µM ABA, 200 µM methyl jasmonate, 5 mM ethephon, 10 µM methyl viologen, 250 mM NaCl and 100 µM $CuSO_4$. Cold treatment was exerted by exposing the plants to 10 ± 2°C in a growth chamber in a 16 h light/8 h dark regime. For drought stress, plantlets were washed thoroughly to eliminate soil and then left to dry on filter papers in the greenhouse. Plants exposed only to sterile water were taken

as experimental controls. Leaf and root tissues collected from the various treated plants at specific time points were immediately frozen in liquid nitrogen and stored at -80°C. Total RNA isolation was performed as described above. The first-strand cDNA synthesis was performed using 5 µg total RNA, Oligo $(dT)_{12–18}$ primer and ThermoScript Reverse Transcriptase (Invitrogen, USA) according to manufacturer's instructions. For each treatment samples from three independent plants were pooled prior to RNA isolation to ensure reproducibility of results. Quantitative real time RT-PCR reactions were performed in triplicates with SYBR Green Extract-N-Amp PCR ReadyMix (Sigma, USA). Banana *actin* gene (GenBank accession no. EF672732) was also amplified along with *MusaDHN-1* gene to allow gene-expression normalization and subsequent quantification. All real time quantitative RT-PCR reactions were carried out using Corbett Rotor Gene 3000 platform (Corbett Life Science, Australia). C_t values obtained were analyzed using REST-MCS software [43] to arrive at relative expression level values for *MusaDHN-1* gene in response to various stress treatments. cDNA samples were established to be DNA-free by performing 40 cycles of amplification with banana *actin* primers.

2.2.5 Isolation, sequence analysis and characterization of 5' proximal promoter region of *MusaDHN-1*

The 5' proximal promoter region of *MusaDHN-1* was deduced by TAIL-PCR [44]. Nine arbitrary primers were tested with three *MusaDHN-1* sequence specific sequential nested primers to amplify the 5' proximal promoter region. After analyzing the results of secondary and the tertiary reactions of TAIL-PCR, an aptly sized band was eluted and subsequently sequenced. *MusaDHN-1* 5' proximal promoter sequence

was subjected to in silico analysis for motif search using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) databases. Further, this promoter sequence was cloned in place of CaMV 35S promoter upstream of β-glucuronidase gene in pCAMBIA-1301 binary vector (Genbank accession no. AF234297). This newly constructed binary vector (p*MusaDHN-1*-1301) was mobilized into *Agrobacterium tumefaciens* strain EHA 105 [45] by electroporation and subsequently used to transform tobacco (*Nicotiana tabacum* L. cv. *Havana 425*) leaf discs as described [46]. Transgenic rooted tobacco plantlets selected on 20 mg l^{-1} hygromycin were treated with 300 mM NaCl for 5 hours or dehydrated for 14 hours before GUS histochemical staining was performed as described by [47]. GUS stained plantlets were photographed after the chlorophyll was removed by treatment with 70% (v/v) ethanol.

2.2.6 Construction of binary vector for overexpression of *MusaDHN-1*

Expression cassette for overexpression of *MusaDHN-1* in transgenic banana plants was assembled in the multiple cloning site of pCAMBIA-1301 binary vector. Firstly, the nos 3' UTR amplified from pBI121 binary vector, the *Zea mays* polyubiquitin promoter amplified from *Zea mays* genomic DNA and the *MusaDHN-1* coding sequence amplified from the banana cv. *Karibale Monthan* leaf cDNA were all individually cloned in Fermentas plasmid pTZ57R/t. Subsequently, nos 3' UTR was cloned in the multiple cloning site of pCAMBIA-1301 using *Sac*I and *Eco*RI restriction enzymes. Finally, modified pCAMBIA-1301 binary vector (containing nos 3' UTR in its multiple cloning site) digested with *Hin*dIII and *Kpn*I restriction

enzymes, *Zea mays* polyubiquitin promoter (released using *Hin*dIII and *Pst*I) and *MusaDHN-1* coding sequence (released using *Pst*I and *Kpn*I) were ligated directionally in a three-way ligation reaction to form a new binary vector denoted as *MusaDHN-1*-1301. This vector, containing the expression cassette [pZmUbi-*MusaDHN-1*-nos], was sequenced to verify the sequence of *MusaDHN-1* coding sequence. *MusaDHN-1*-1301 binary vector was then mobilized into *Agrobacterium tumefaciens* strain EHA 105 by electroporation and later used to transform banana embryogenic cells. Unmodified pCAMBIA-1301 binary vector was also similarly mobilized into *Agrobacterium* and used to transform banana embryogenic cells.

2.2.7 *Agrobacterium*-mediated transformation of banana embryogenic cells and generation of transgenic banana plants

A single *Agrobacterium* colony was grown in liquid YENB medium (0.75% w/v yeast extract with 0.8% w/v nutrient broth) supplemented with 50 mg l^{-1} kanamycin overnight at 27°C with an orbital shaking of 180 rpm. *Agrobacteria* from the densely grown culture were resuspended in the same medium with kanamycin at an $OD_{600\ nm}$ of 0.1 in the morning and grown under the same conditions until an $OD_{600\ nm}$ of 0.6 - 0.8 was achieved. The suspension was subsequently centrifuged at 6,500*g* for 10 min and the pellet obtained was resuspended in M2 medium [48] added with 100 µM ACS at a final $OD_{600\ nm}$ of ~ 0.2. The bacterial suspension so obtained was used for cocultivation with banana cv. *Rasthali* embryogenic cell suspension cultures as essentially described [49]. Seven days post-subculture cells (0.5 ml PCV) sieved through an 85-µm sieve were cocultivated with *Agrobacterium* for 30 minutes and subsequently aspirated onto glass filter discs which were transferred on to semi solid

M2 medium supplemented with 100 µM ACS. The plates were maintained in dark for three days at 23 ± 2°C. Then, the filters along with the cells were transferred to fresh semi solid M2 medium added with cefotaxime (400 mg l^{-1}). After further three days, the cells were transferred to banana embryo induction medium added with cefotaxime (400 mg l^{-1}) and hygromycin (5 mg l^{-1}). Embryo development was noticed after three to four weeks and the selection was continued for three rounds by subculturing the developing embryos on to fresh medium of the same composition. The well developed embryos were transferred to MS medium supplemented with BAP (0.5 mg l^{-1}) for shoot emergence. The germinating embryos were transferred to banana multiplication medium for multiple shoot induction [50]. The single shoots were isolated from the multiple shoots and rooted on MS medium supplemented with NAA (1 mg l^{-1}). The rooted plantlets were hardened in the greenhouse.

2.2.8 Molecular analysis of transgenic banana plants

Six transgenic banana lines, out of a total of twenty four lines generated, were chosen for further molecular and biochemical analysis. Genomic DNA isolated (as described in subsection 2.2.3) from these six transgenic banana lines was used as template in PCR reactions designed to amplify *hygromycin phosphotransferase* gene present within the T-DNA borders of the binary vector used to obtain these plants. Genomic DNA isolated from untransformed banana plant served as a control in these PCR reactions. Further, the confirmation of transgenic nature of these lines and T-DNA copy number determination in each of these lines was performed by Southern blot analysis (performed as described in subsection 2.2.3) using a DIG-labeled probe against *hygromycin phosphotransferase* gene.

Total RNA isolation from young leaf of these transgenics and the subsequent first strand cDNA synthesis using ThermoScript Reverse Transcriptase were performed as described in subsection 2.2.4. The quantitation of the quantum of overexpression of *MusaDHN-1* in these transgenic lines was deduced by real time RT-PCR analysis performed as described for expression profiling of *MusaDHN-1* gene in native banana plant in response to various stress conditions. cDNA obtained from untransformed banana plants served as a control in these RT-PCR reactions.

2.2.9 Assay for drought tolerance

The assay for improved drought tolerance of the *MusaDHN-1* overexpressing lines was performed in a two way manner which utilized both the in vitro maintained multiple shoot cultures of the transgenic lines and the hardened transgenic plants growing in the green house. Uniform individual shoots approximately 2 cm in length and 7 mm in thickness (derived from multiple shoot cultures of the confirmed transgenic lines) were cultured for 10 days on rooting media added separately with two dehydrating agents namely mannitol (at 50 and 100 mM) and PEG (MW 8000) (at 2 % and 5% w/v). The shoots so treated were subsequently transferred to plain rooting media and grown for further 20-25 days under 16 hr /8 hr light dark regime.

To check how the transgenic hardened plants behave during water scarcity, the greenhouse growing plants were deprived of water for a period of 15 days. Subsequently, the plants were recovered with regular watering.

Relative water content (RWC) in the transgenic plants during the course of the drought stress was determined in separate experiments after a period of 10 and 14 days from the start of drought stress. Leaves from each plant were detached and their

fresh weights were recorded immediately. The turgid weight of the leaves was recorded after floating the leaves in deionized water at 4°C overnight. Subsequently, the dry weight of the leaves was determined after drying them in a hot air oven for two days at 60°C. RWC was calculated using the following equation: RWC = (fresh weight – dry weight)/ (turgid weight – dry weight) X 100.

Malondialdehyde (MDA) content of the hardened plants exposed to drought stress for 7 and 14 days was determined by the method described by [51] with modifications. 500 mg leaf tissue was crushed under liquid N_2 and homogenized with 12 ml of 80:20 (v:v) ethanol:methanol mixture added with 0.01% (w/v) butylated hydroxytoluene. The extract was immediately centrifuged at 8000 g for 5 min at 4°C. The supernatant obtained was diluted 1:10 in 80:20 (v:v) ethanol:methanol mixture added with 0.01% (w/v) butylated hydroxytoluene. 1 ml of this diluted supernatant was then added to a tube with 1 ml of either (i) (-) thiobarbituric acid (TBA) solution comprised of 20.0% (w/v) trichloroacetic acid or (ii) (+) TBA solution containing the above added with 0.65% (w/v) TBA. Samples were mixed vigorously and heated in a dry bath for 20 min, cooled in an ice bath, and subsequently centrifuged at 8000 g for 10 min. Absorbances were recorded in a spectrophotometer at 440 nm, 532 nm, and 600 nm. MDA equivalents were calculated as described in [51].

All in vitro and ex vitro assays were performed in triplicates. Subsequently, representative plants for each assay were photographed. Leaves derived from three independent plants for each line were used in all biochemical assays. In vitro shoots and hardened plants derived from unmodified pCAMBIA-1301 transformed banana lines were used as controls in all the above assays.

2.2.10 Assay for salt tolerance

Similar to the assay for improved drought tolerance, the assay for improved salt stress tolerance was also done in a two way manner utilizing both in vitro maintained multiple shoot cultures of the transgenic lines and the hardened transgenic plants growing in the green house. Herein, the approx. 2 cm long uniform shoots were exposed to different concentrations of NaCl (at 50 and 100 mM) in rooting medium for 10 days and then in plain rooting medium for 20- 25 days before recording the data.

Survival of the hardened plants under salt stress was ascertained by irrigating the plants with 25 ml 250 mM NaCl every alternate day for 10 days followed by recovery by regular irrigation with water.

Proline content of the hardened plants exposed to salt stress (applied as above) was determined by the method described by [52] with modifications. Briefly, 500 mg leaf tissue was homogenized in 10 ml 3 % (w/v) sulfosalicylic acid. The homogenate was heated in a water bath at 100°C for 15 min and then cooled under running water. Subsequent to centrifugation at 10000g for 2 min, 2 ml supernatant was added to 2 ml acid-ninhydrin reagent and 2 ml of glacial acetic acid. This mixture was heated at 100°C for 45 min in a water bath followed by termination of reaction in an ice bath. The reaction mixture was extracted with 4 ml toluene by vigorous shaking. The color intensity of the toluene was subsequently read at 520 nm to determine the amount of free proline in the tissues.

MDA content of the hardened plants exposed to salt stress (applied as above) was also determined as explained above in subsection 2.2.9.

All the in vitro, ex vitro and biochemical analyses were done in triplicates as described for drought tolerance assays. Controls similar to those used in drought tolerance assays were used here.

2.3 Results

2.3.1 Isolation and sequence analysis of *MusaDHN-1*

A novel *dehydrin* gene was identified from a banana leaf cDNA library (EST Genbank accession no. ES436956) using in silico approaches. As this was the first dehydrin gene to be identified in banana, it was named *MusaDHN-1*. The coding sequence of *MusaDHN-1* consisted of 711 nucleotides encoding a protein of 236 amino acids (Figure 2.1). The MusaDHN-1 protein has a predicted molecular weight of 26.21 kDa and a theoretical pI of 5.02. MusaDHN-1 protein is classified as a SK_3-type dehydrin as it possesses a single tract of characteristic serine residues, the S-segment in the N-terminal half and three lysine-rich K-segments (Figure 2.1), their sequences being slightly divergent from that of other similar plant dehydrins. MusaDHN-1 protein displayed the best homology with two other monocot dehydrins namely *Oryza sativa* SK_3-type dehydrin (Genbank accession no. ABS44866) and *Triticum aestivum* cold acclimation protein WCOR410c (Genbank accession no. AAB18202). In spite of the fact that MusaDHN-1 protein is considerably shorter in size as compared to its close homologues from other monocots, the sequence and position of the single S-segment and three K-segments in MusaDHN-1 protein matches broadly with that of similar segments in these related dehydrins indicating close evolutionary relationship between these proteins (Figure 2.2 and 2.3).

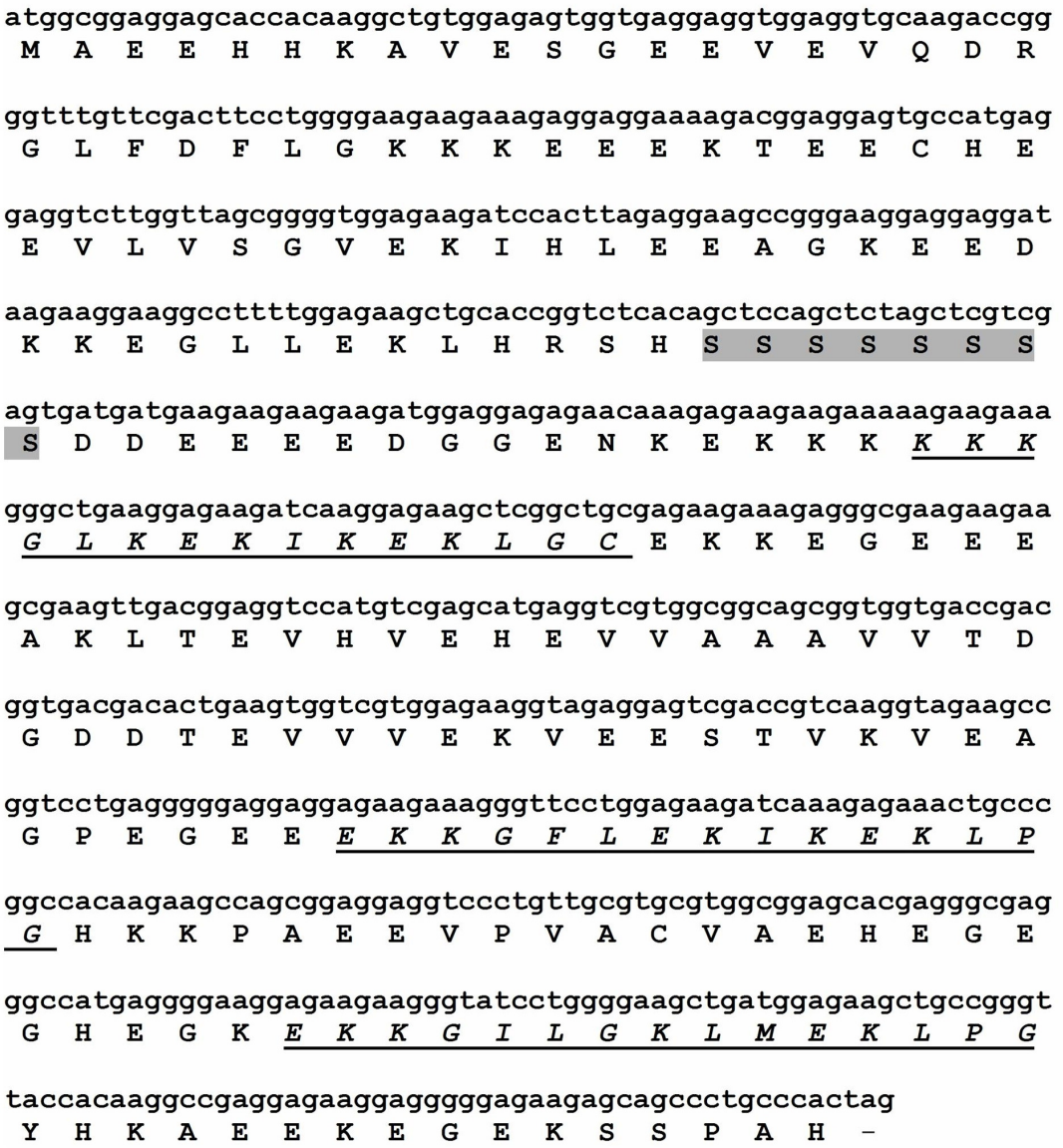


Figure 2.1 Sequence analysis of *MusaDHN-1* cDNA derived from banana cv. *Karibale Monthan*. Nucleotide sequence of *MusaDHN-1* cDNA together with its predicted amino acid sequence. The conserved S-segment having eight serine residues is boxed. The three K-segments are underlined.

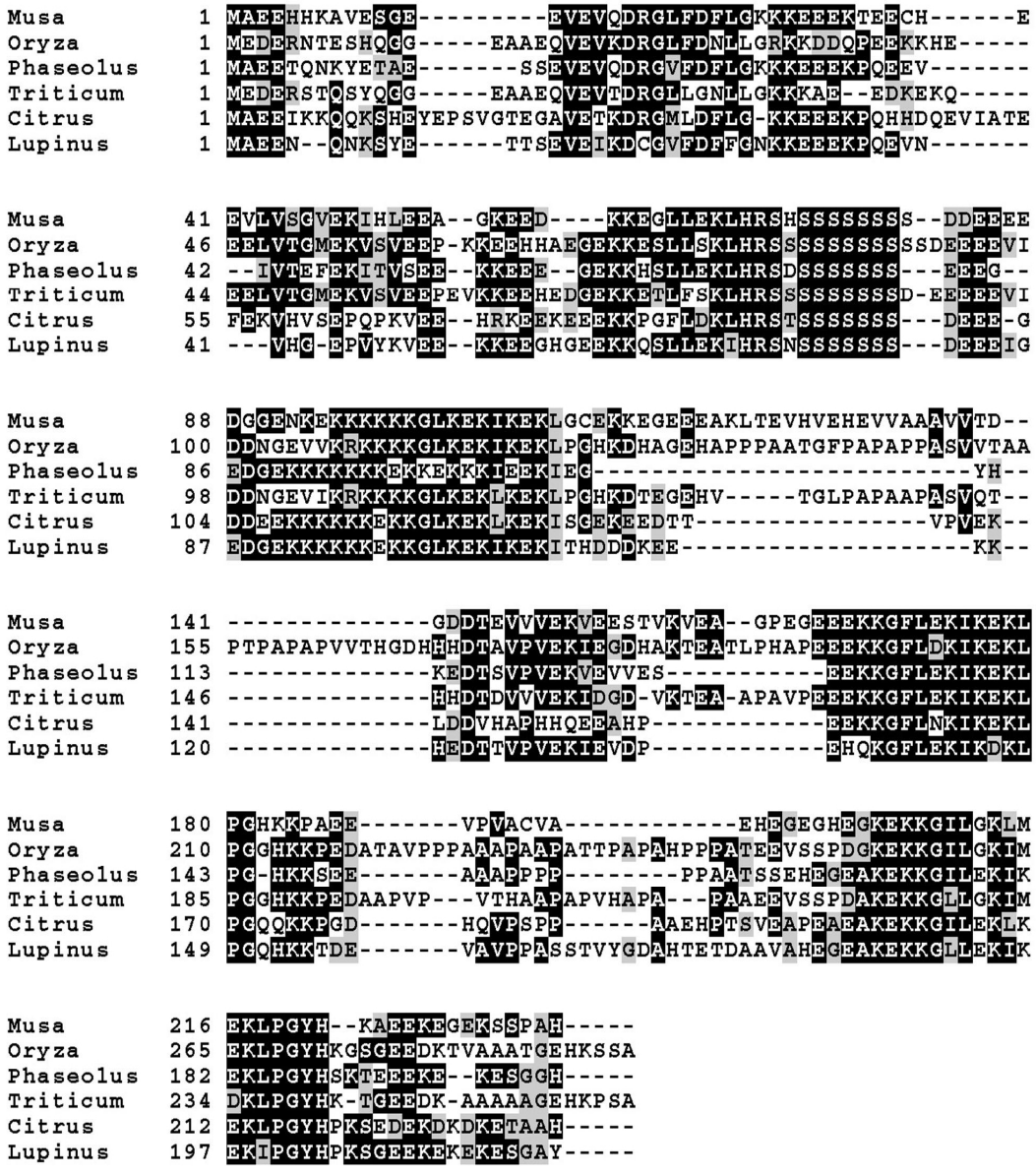


Figure 2.2 Alignment of MusaDHN-1 protein sequence derived from cv. *Karibale Monthan* with closely related sequences. Alignment of MusaDHN-1 protein sequence with sequences from *Oryza sativa* (ABS44866)**,** *Phaseolus vulgaris* (AAB00554), *Triticum aestivum* (AAB18202), *Citrus x paradisi* (AAN78125) and *Lupinus albus* (AAT06600).

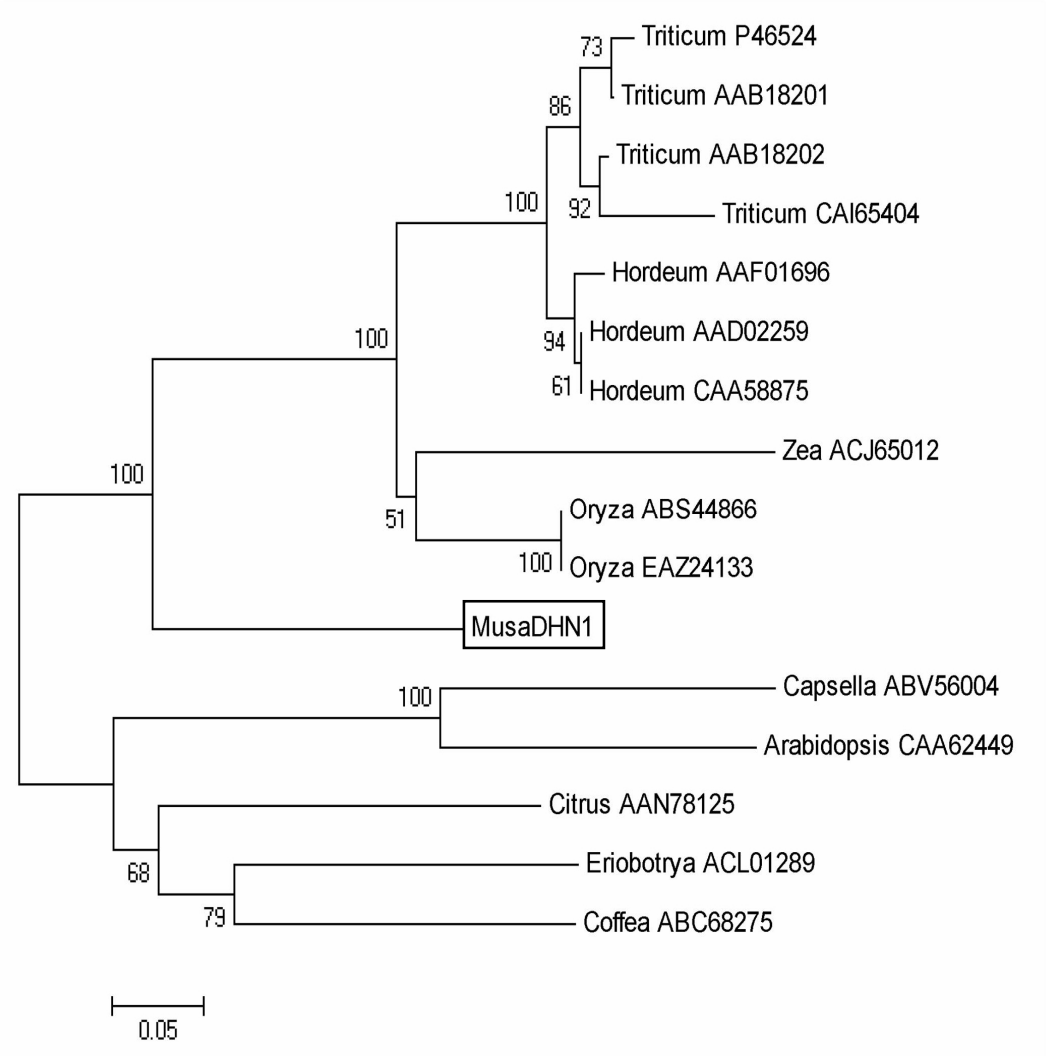


Figure 2.3 Phylogenetic relationship of MusaDHN-1 protein sequence derived from cv. *Karibale Monthan* with other closely related dehydrin proteins. The accession numbers of the protein sequences used for building up the phylogenic tree are given alongside. The bootstrapped tree with 1,000 replicates was constructed using ClustalW2 and MEGA 5 tools.

2.3.2 Copy number determination of *MusaDHN-1* in banana genome

Southern blot analysis of banana cv. *Karibale Monthan* genomic DNA digested with *Bam*HI, *Eco*RI and *Pst*I restriction endonucleases and performed using DIG-labeled probe against *MusaDHN-1* 5' UTR indicated that *MusaDHN-1* occurs as a low copy number gene in banana genome (Figure 2.4).

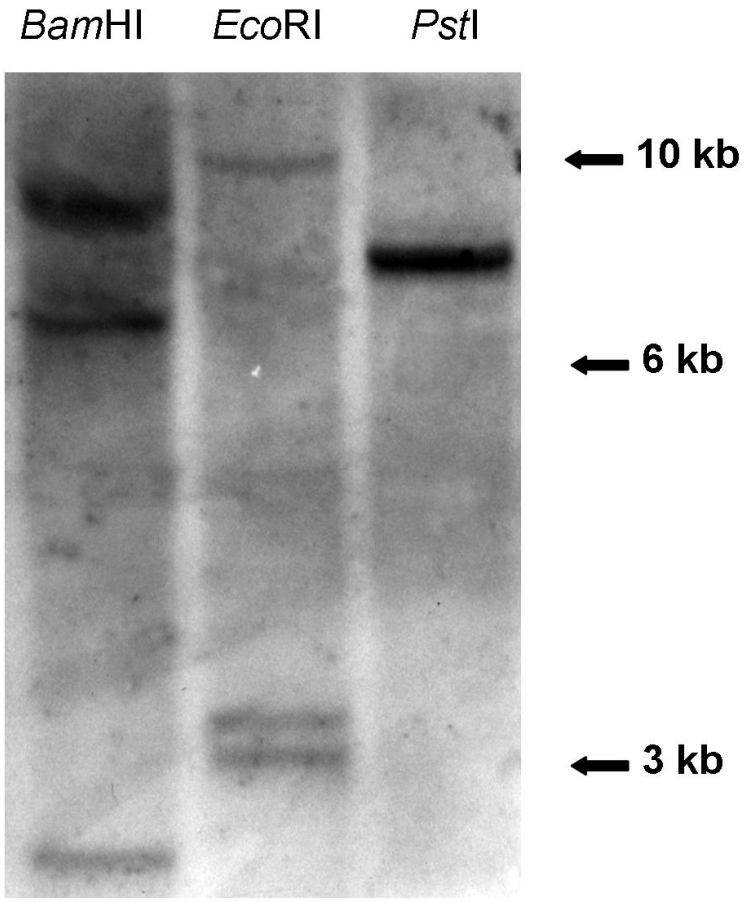
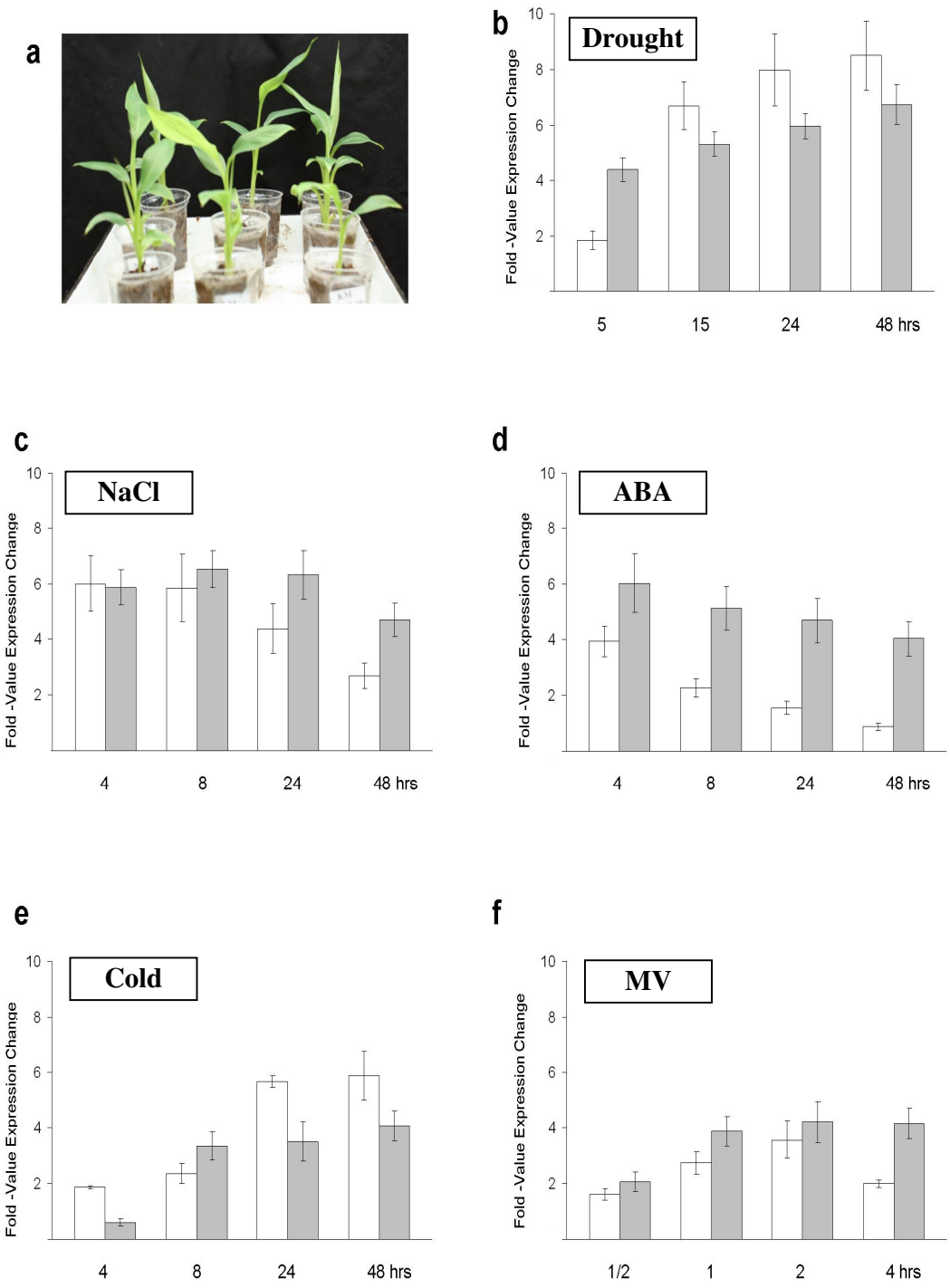


Figure 2.4 Southern blot analysis of *MusaDHN-1* gene from banana cv. *Karibale Monthan*. Genomic DNA isolated from young leaves was restricted with *Bam*HI, *Eco*RI and *Pst*I before being filter hybridized with DIG-labeled probes against *MusaDHN-1* 5' UTR sequence. Approximate positions of the DNA marker bands are indicated.

2.3.3 Expression profiles of *MusaDHN-1* under different stress treatments

As expression levels of several previously studied dehydrins are induced in response to different abiotic stresses, the expression pattern of *MusaDHN-1* was studied after exposing banana plants to various stress conditions for different periods of time. Real time quantitative RT-PCR performed using cDNA derived from stressed and non-stressed leaves and roots of banana cv. *Karibale Monthan* plantlets demonstrated the inducibility of *MusaDHN-1* expression in response to salt, dehydration and cold stress as well as ABA, methyl viologen, $CuSO_4$, ethylene and methyl jasmonate treatments (Figure 2.5 a-i). Salt stress lead to immediate induction of *MusaDHN-1* expression which then slowly tapered down through 48 hours of treatment. Drought treatment caused slow induction of *MusaDHN-1* expression which continued to rise even after 48 hrs post initiation into dry conditions. Cold treatment resulted in a *MusaDHN-1* expression pattern broadly similar to that under drought conditions. ABA treatment induced *MusaDHN-1* expression within 4 hours after which it slowly reverted back to near its control levels. Expression level of *MusaDHN-1* in response to methyl viologen, which is potent inducer of oxidative stress in plants, reached a peak within 2 hours of application following which it slowly came down to near its un-induced levels. Ethylene application induced and kept the expression levels of *MusaDHN-1* at considerably high levels till around 24 hours after treatment. Methyl jasmonate was another agent which induced *MusaDHN-1* expression quickly within 4 hours. Application of heavy metal stress in the form of $CuSO_4$ application also induced the expression of *MusaDHN-1* considerably. The expression pattern of *MusaDHN-1* in plants exposed to different stress conditions was found to be broadly similar in leaves

and roots. Also, treatments where only the roots were exposed to different abiotic stress treatments showed expression patterns similar to the treatments where both leaf and roots were treated. The unambiguous inducibility of *MusaDHN-1* in response to such varied stress treatments indicates a specific and important role for this dehydrin in abiotic stress responses of banana plant.



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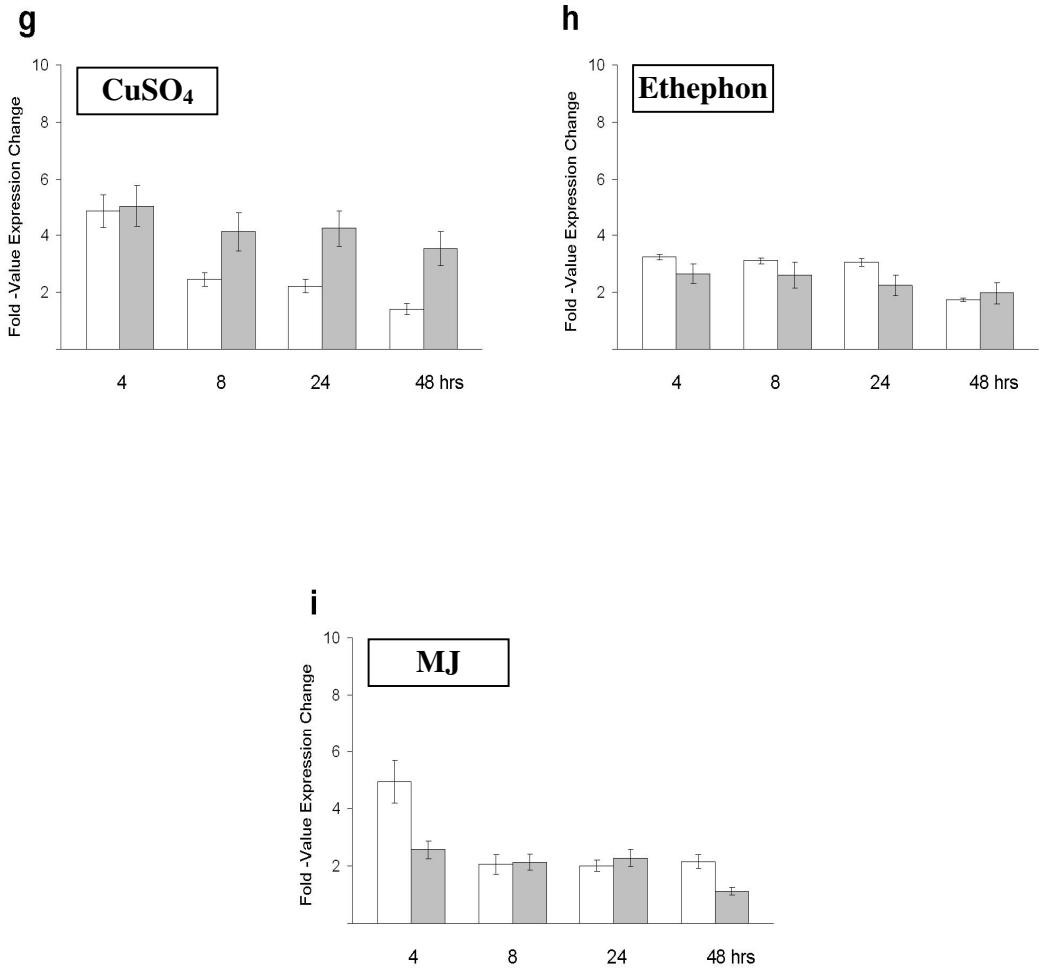


Figure 2.5 Expression profiles of *MusaDHN-1* in banana cv. *Karibale Monthan* leaves (white bars) and roots (grey bars) in response to different abiotic stresses as determined by quantitative real time RT-PCR. (a) Two months old greenhouse hardened plants used for various abiotic stress treatments. Time-course study of *MusaDHN-1* induction after exposure to drought (b), 250 mM NaCl (c), 100 µM ABA (d), Cold [8 ± 2°C] (e), 10 µM methyl viologen (f), 100 µM $CuSO_4$ (g), 5 mM ethephon (h), 200 µM methyl jasmonate (i). The x-axis represents the expression level of *MusaDHN-1* in control conditions. Values are mean ± SE.

2.3.4 Detection of putative cis-acting elements in the 5' proximal region of *MusaDHN-1*

To further explain the expression pattern of *MusaDHN-1* obtained as above and to get clues for its possible functions in banana, a 1071-bp fragment present upstream of *MusaDHN-1* coding sequence was amplified from banana cv. *Karibale Monthan* genomic DNA by employing TAIL-PCR. Several putative cis-acting elements demonstrated to be integral to stress-responsive gene regulation pathways were identified in the promoter region of *MusaDHN-1* using online PlantCARE and PLACE software (Figure 2.6 a). Prominent among them were ABRE (C/TACGTG), LTRE/DRE/C repeats (C/TCGAC) and CGTCA-motif (for methyl jasmonate responsiveness). Although a classical GCC box (TAAGAGCCGCC) responsible for ethylene inducibilty was not present in the 1071-bp proximal promoter region, the CACGTG (G-box like motif) present here has been shown to take part in ethylene-induced expression of pathogenesis-related genes [53]. The GCC box may be present upstream of the 1071-bp cloned promoter. Importantly, this 1071-bp *MusaDHN-1* promoter region, when tagged upstream of *β-glucuronidase* gene, replicated its expression pattern in response to salt and drought stress in transgenic tobacco plants transformed with this *MusaDHN-1* promoter- β-glucuronidase fusion construct (Figure 2.6 b, c, d and e).

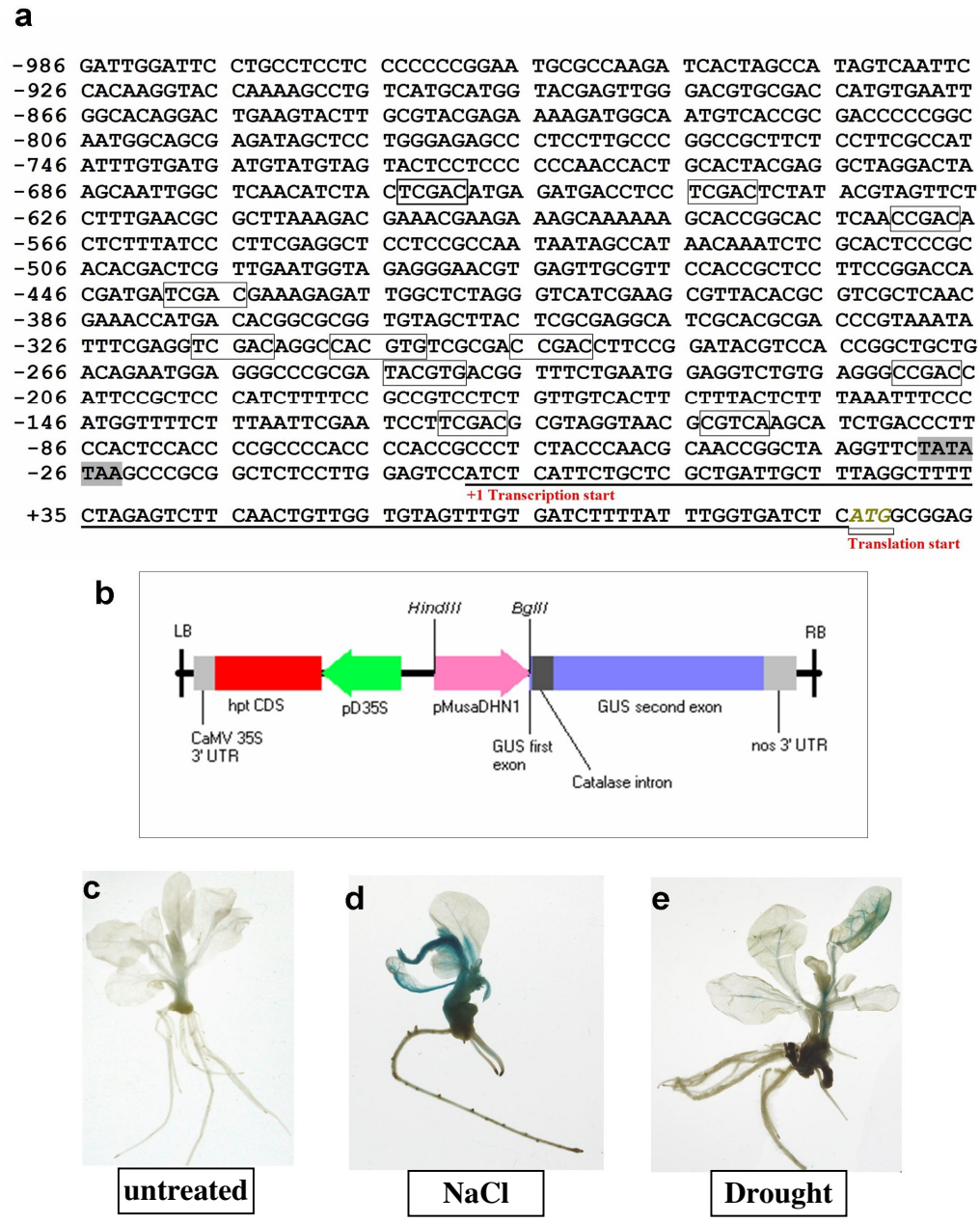


Figure 2.6 Characterization of 5' proximal promoter region of *MusaDHN-1* gene (derived from cv. *Karibale Monthan*). (a) 1071-bp nucleotide sequence of the 5' upstream region of *MusaDHN-1*. The putative transcription and translation start sites are marked. The underlined region is the predicted 5' UTR region of the *MusaDHN-1* mRNA. Probable TATA-box is shaded. ABRE (C/TACGTG), LTRE/DRE/C repeats (C/TCGAC) and CGTCA-motif (CGTCA) are boxed. (b) T-DNA region of binary vector p*MusaDHN-1*-1301 designed to characterize *MusaDHN-1* promoter. Expression pattern of β-glucuronidase driven by *MusaDHN-1* promoter in transgenic tobacco plants: untreated (c) and exposed to 300 mM NaCl for 5 h (d) and drought for 14 h (e).

2.3.5 Generation and analysis of transgenic banana plants

Banana cv. *Rasthali* embryogenic cells were transformed with an expression cassette designed to constitutively overexpress *MusaDHN-1* in the transgenic plants (Figure 2.7 a and b). Whitish embryos developed on the banana embryo induction medium supplemented with hygromycin (5 mg l^{-1}) three weeks after transformation (Figure 2.7 c). Secondary embryos developed from these primary embryos upon subculturing on the fresh medium of same composition. Subsequent to three cycles of subculture, the embryos were allowed to germinate on embryo germination medium (Figure 2.7 d). The germinating embryos were transferred to banana multiplication medium for multiple shoot induction (Figure 2.7 e). These plantlets were rooted further on MS medium added with NAA (Figure 2.7 f). Here, the transgenic plantlets developed rapidly to produce a profuse root system. Subsequently, the transgenic plants were hardened in a contained greenhouse (Figure 2.7 g). Six transgenic lines were selected from a total of twenty four lines based on their vigorous growth in hygromycin containing medium and intensity of GUS staining in the leaf tissue. These two parameters putatively indicated that the T-DNA(s) containing the GUS, 'pZmUbi-*MusaDHN-1*-nos' and the hygromycin phosphotransferase expression cassettes were inserted in these lines in transcriptionally active euchromatin regions of the genome.

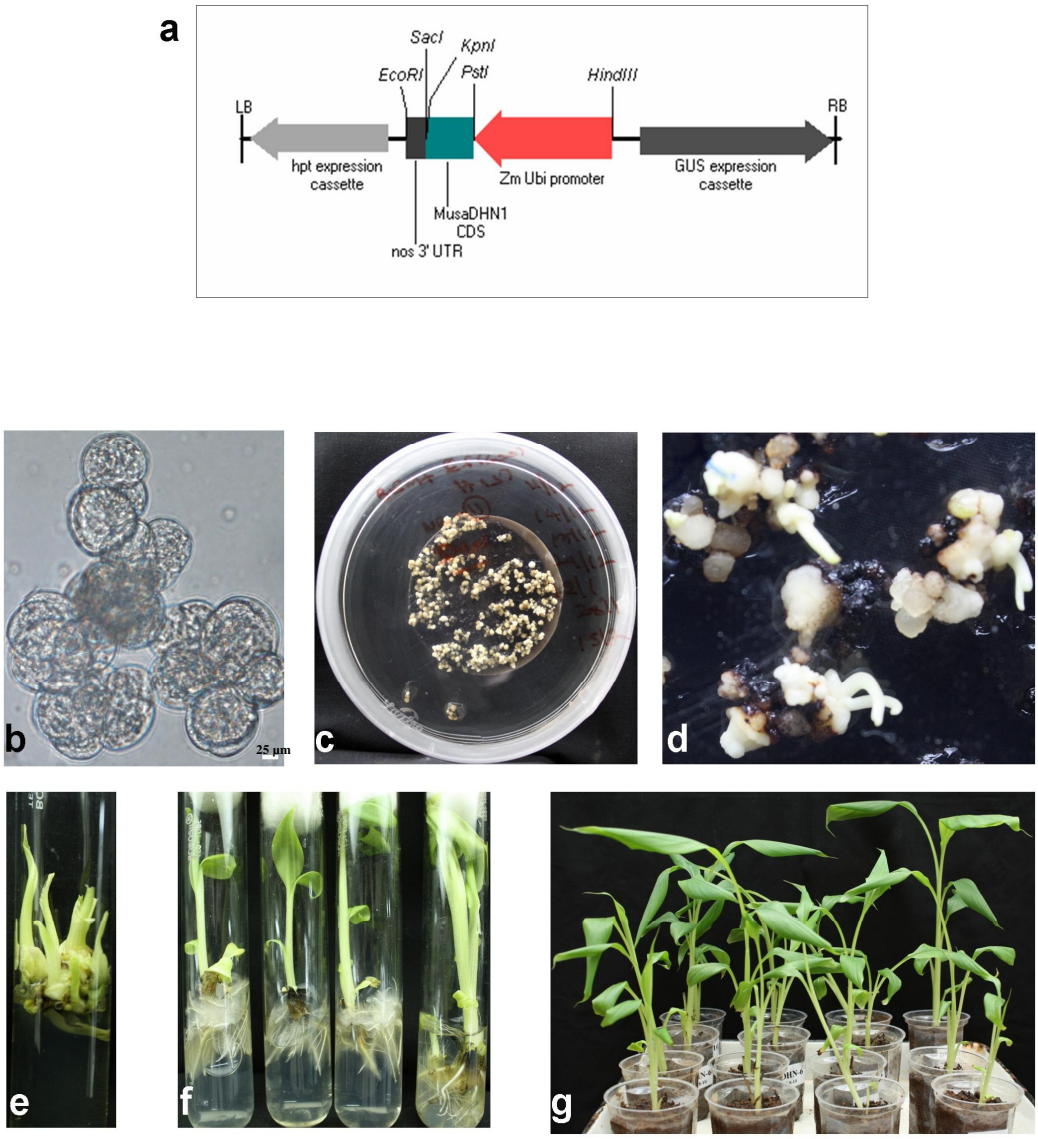


Figure 2.7 Generation of transgenic banana cv. *Rasthali* plants overexpressing *MusaDHN-1*. (a) T-DNA region of binary vector *MusaDHN-1*-1301 designed to constitutively overexpress *MusaDHN-1* in transgenic banana plants. (b) Densely cytoplasmic embryogenic cells of banana cv. *Rasthali*. White bar corresponds to 25 µm (c) Transformed embryos on embryo induction medium. (d) Germinating embryos on embryo germination medium. (e) Transgenic multiple shoots on multiple shoot induction medium. (f) Transgenic rooted plantlets on rooting medium. (g) Transgenic hardened plants in greenhouse (2-months old).

PCR analysis of these six selected *MusaDHN-1* overexpressing lines clearly showed the amplification of a single 788-bp fragment derived from *hygromycin phosphotransferase* coding sequence while being absent in untransformed control plants (Figure 2.8 a). To further confirm the stable integration of the transgenes, all these six transgenic lines were also successfully characterized by Southern blotting of restricted genomic DNA using a DIG-labeled probe against *hygromycin phosphotransferase* gene (Figure 2.8 b). Restriction enzyme *Kpn*I was used to digest the genomic DNAs as it cuts the T-DNA of the overexpression vector only once and hence the number of bands appearing on the autoradiographs can be directly correlated with the copy number of the T-DNAs transferred to banana genome in these transgenic lines. T-DNA copy numbers ranging from 1 to 4 were determined in the six transgenic lines analyzed. Further, the different sizes of the bands detected in different lines proved that these six transgenic lines have originated from independent transformation events.

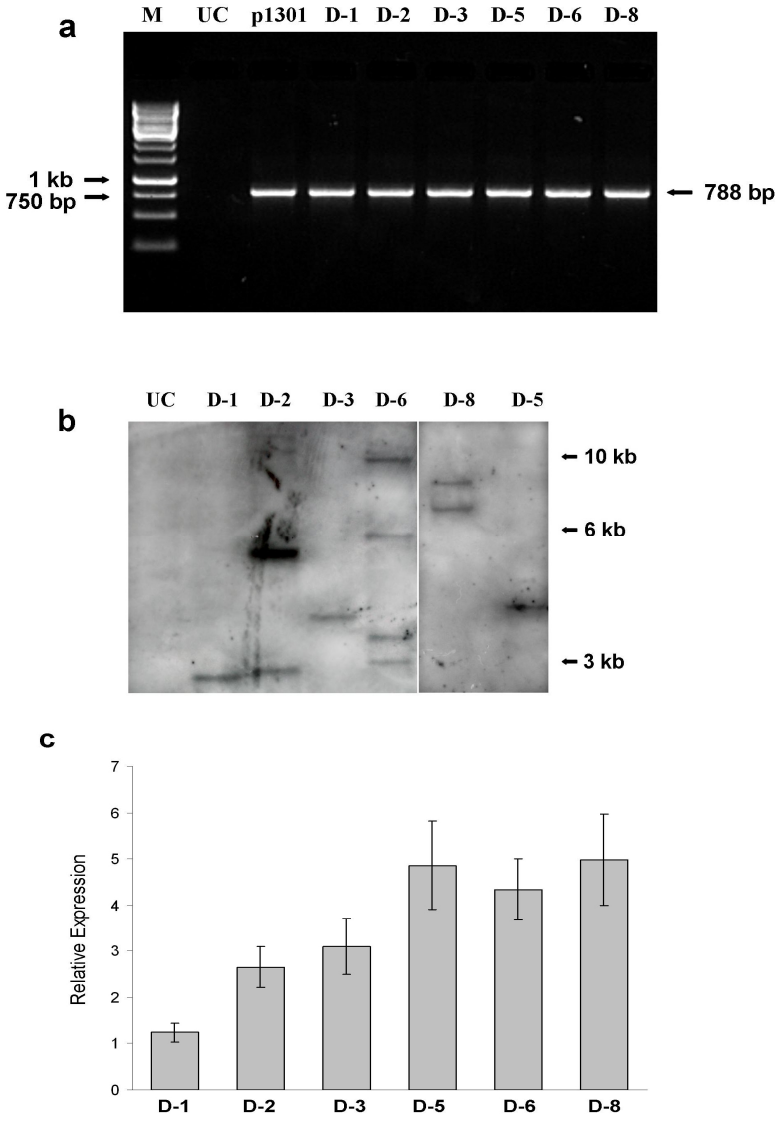


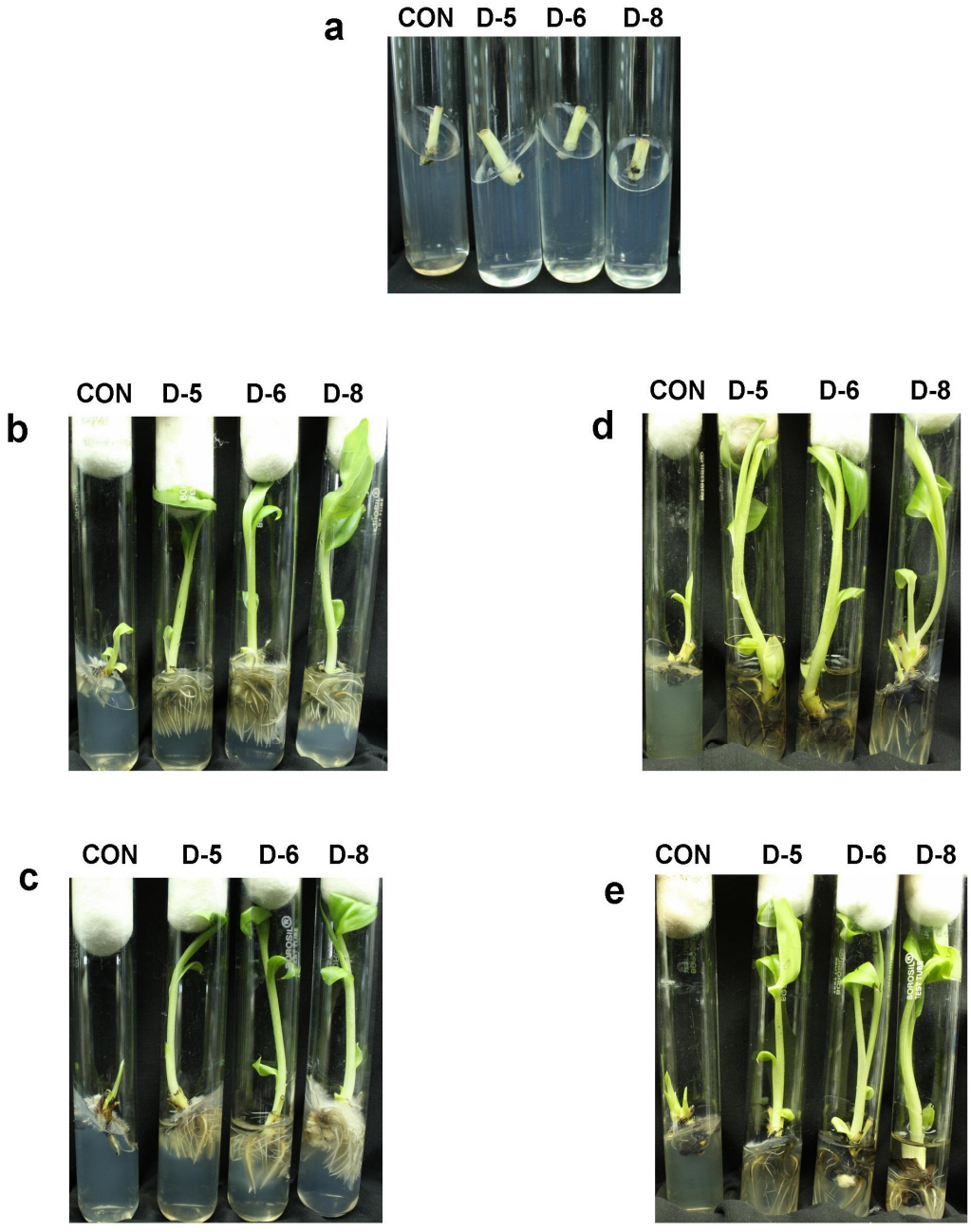
Figure 2.8 Molecular analysis of transgenic banana plants overexpressing *MusaDHN-1* gene. (a) PCR analysis using genomic DNA isolated from control as well as transgenic plants as template. A 788-bp amplification product derived from *hygromycin phosphotransferase* gene was noticed in pCAMBIA-1301 transformed plant (p1301) and all *MusaDHN-1* overexpressing lines (D-1, D-2, D-3, D-5, D-6 and D-8) whereas it was absent in untransformed banana plants (UC). (b) Southern blot analysis of *MusaDHN-1* overexpressing banana plants. Genomic DNA isolated from young leaves was restricted with *Kpn*I before being filter hybridized with DIG-labeled probes against *hygromycin phosphotransferase* gene sequence. Approximate positions of the DNA marker bands are indicated. (c) Quantum of *MusaDHN-1* overexpression in transgenic banana lines determined using real time quantitative RT-PCR. The expression level of native *MusaDHN-1* in transgenic plants is assumed to be 1. Values are mean ± SE.

Real time PCR analysis performed using cDNA obtained from the six selected *MusaDHN-1* overexpressing lines showed that the exact quantum of overexpression varied greatly between the different lines with the 2 Log expression ratios between the untransformed plants and the *MusaDHN-1* overexpressing lines falling in the range of 1.2 to 4.9 (Figure 2.8 c). Further, no direct correlation was found between the level of overexpression and the T-DNA copy number determined using Southern blotting. This apparent discrepancy probably arose due to the so called "position effects", whereby the integration into the actively transcribing regions results in better transgene expression as compared to that from other less active genomic regions. The three transgenic lines (D-5, D-6 and D-8) showing good overexpression of *MusaDHN-1* were selected for further analysis.

2.3.6 Increased tolerance of transgenic plants to drought and salt stress treatments

The phenotype, growth and development, both in vitro and ex vitro, of transgenic banana plants overexpressing *MusaDHN-1* were identical to that of control plants (those transformed with unmodified pCAMBIA-1301 vector or the untransformed controls). Owing to the inability to obtain transgenic seeds in a vegetatively propagated crop like banana, we chose to use small transgenic shoots of uniform length and thickness derived from in vitro maintained multiple shoot cultures as our explants for various stress treatments. These multiple shoot cultures were derived from single transgenic embryos and hence were clonal in nature (Figure 2.9 a). Stress treatments were given as additives in rooting media in which these transgenic shoots were grown. Further, as described by [54] roots are generally the first and the most

sensitive plant organ to respond to abiotic stress conditions. Hence, allowing the in vitro generated shoots to develop roots in the presence of stress-inducing components like PEG, mannitol and NaCl gives the best indication of their improved stress tolerance. When individual shoots derived from control, D-5, D-6 and D-8 transgenic lines were put for rooting in presence of PEG (MW 8000) at 2 and 5% (w/v) or mannitol at 50 and 100 mM or NaCl at 50 and 100 mM, the transgenic lines responded in a significantly better way in the recovery medium (without stress inducing agents) by first generating profuse adventitious roots followed by rapid elongation of leaves and leaf stalks. The control shoots displayed stunted root and shoot growth under same treatments (Figure 2.9 b, c, d and e and Figure 2.10 a and b). PEG and mannitol are widely used as drought stress mimics in plant systems [55] and hence the ability of the single shoots to tolerate and grow robustly in their presence proves the increased tolerance of transgenic lines towards drought stress. Similarly, application of salt stress using NaCl is a standard procedure followed to demonstrate improved tolerance to salinity in plants. Vigorous growth of *MusaDHN-1* overexpressing shoots (similar to the growth of control shoots in plain rooting media) was observed once the shoots were transferred to plain rooting media after treatment in media supplemented with NaCl.



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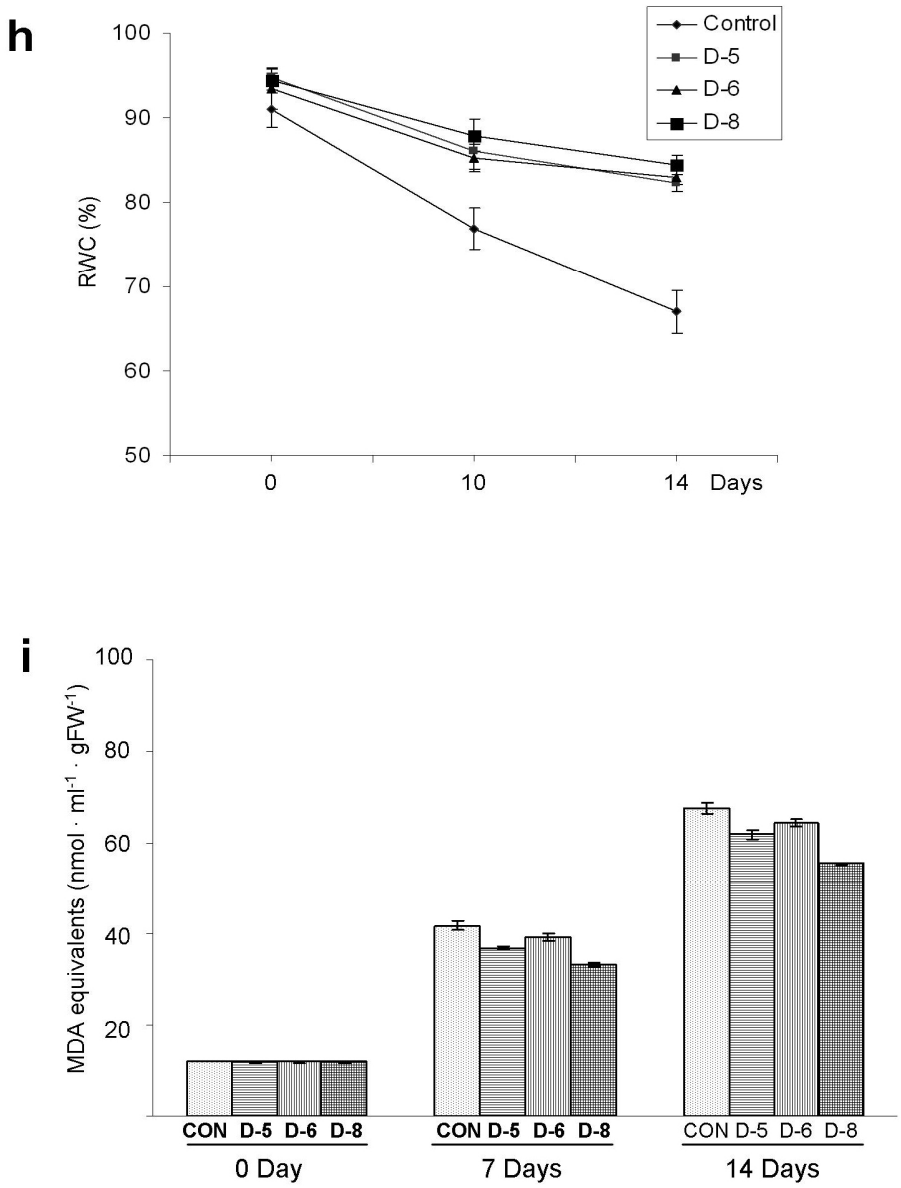


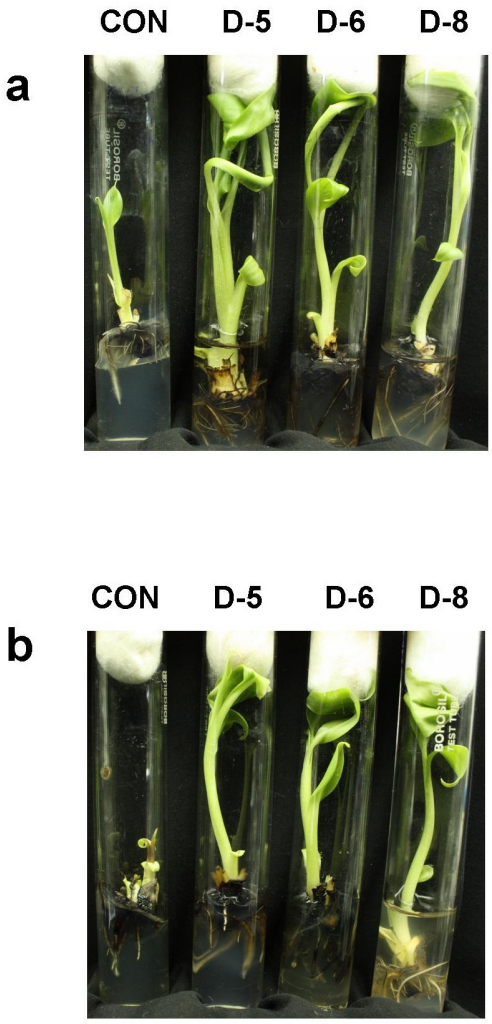
Figure 2.9 Assay for drought tolerance in *MusaDHN-1* overexpressing transgenic banana plants. (a) Single in vitro maintained shoots used as explants for all in vitro tolerance assays. (b) and (c) drought tolerance assay performed by supplementing rooting medium with 2% and 5% (w/v) PEG (MW 8000) respectively. (d) and (e) drought tolerance assay performed by supplementing rooting medium with 50 mM and 100 mM mannitol respectively. Compare the growth of control explants with those in figure 2.7f (f) and (g) *MusaDHN-1* overexpressing lines along with control 15 and 30 days respectively after initiation of drought stress recovery. (h) RWC in *MusaDHN-1* overexpressing lines and control plant 10 and 14 days after the start of drought stress. (i) MDA equivalents in *MusaDHN-1* overexpressing lines and control plant 7 and 14 days after the start of drought stress.

Additionally, the fact that small single shoots could tolerate non-optimal stress conditions indicated that full grown transgenic plants of these lines with well developed shoot and root systems will be able to tolerate much stronger stress conditions. To put to test this hypothesis, 2-3 months old transgenic hardened plants were put through standard drought and salinity stress application procedures. *MusaDHN-1* overexpressing greenhouse plants stressed by withholding water for 15 days followed by regular watering were able to fully recover from the drought treatment whereas the control plants got completely dried and could not recover (Figure 2.9. f and g). Similarly, upon irrigation with 250 mM NaCl the control plants started to show symptoms of chlorosis within 10 days from the end of treatment. The yellowing of leaves continued and became more prominent at 20 days from the end of salt treatment. Contrastingly, the overexpressing lines remained green and grew normally after the end of salt treatment (Figure 2.10 c and d).

Biochemical and physiological changes in the control and the overexpressing lines were monitored by evaluating parameters like RWC and membrane damage in drought stressed plants and proline accumulation and membrane damage in salt stressed plants. RWC in control plants reduced from 90.95% in unstressed plants to approx 76.87% and 66.97% at 10 and 14 days from the start of drought treatment. In contrast, in the *MusaDHN-1* overexpressing lines this reduction was 86.05% (D-5), 85.23% (D-6), 87.94% (D-8) and 82.27% (D-5), 82.91% (D-6), 84.44% (D-8) at 10 and 14 days from the start of drought treatment from an original RWC of 94.75% (D-5), 93.47% (D-6), 94.46% (D-8) in unstressed leaves. This result indicated that *MusaDHN-1* overexpressing lines maintained higher water content in drought

conditions as compared to equivalent controls and hence were less likely to be affected by water scarcity in their immediate environment (Figure 2.9 h).

Proline is considered to be one of the most important low molecular weight osmolytes which gets accumulated in salt stressed plants. Analysis of the proline contents in leaves of salt stressed lines showed that all the three *MusaDHN-1* overexpressing lines accumulated this amino acid at higher levels as compared to control plants. The proline content of the unstressed *MusaDHN-1* overexpressing and control plants differered significantly. Proline content increased in all the lines subjected to stress for upto 14 days. The D-8 transgenic line showed the maximum accumulation of proline in stressed leaves after 14 days of salt stress which was 6.85 times that of unstressed control. Proline content in control plant was significantly lesser under same salt stress conditions thereby indicating superior tolerance to salt stress in *MusaDHN-1* overexpressing plants (Figure 2.10 e). Further, these finding confirmed our earlier observations of substantially better growth and rooting of *MusaDHN-1* overexpressing shoots in medium supplemented with NaCl.



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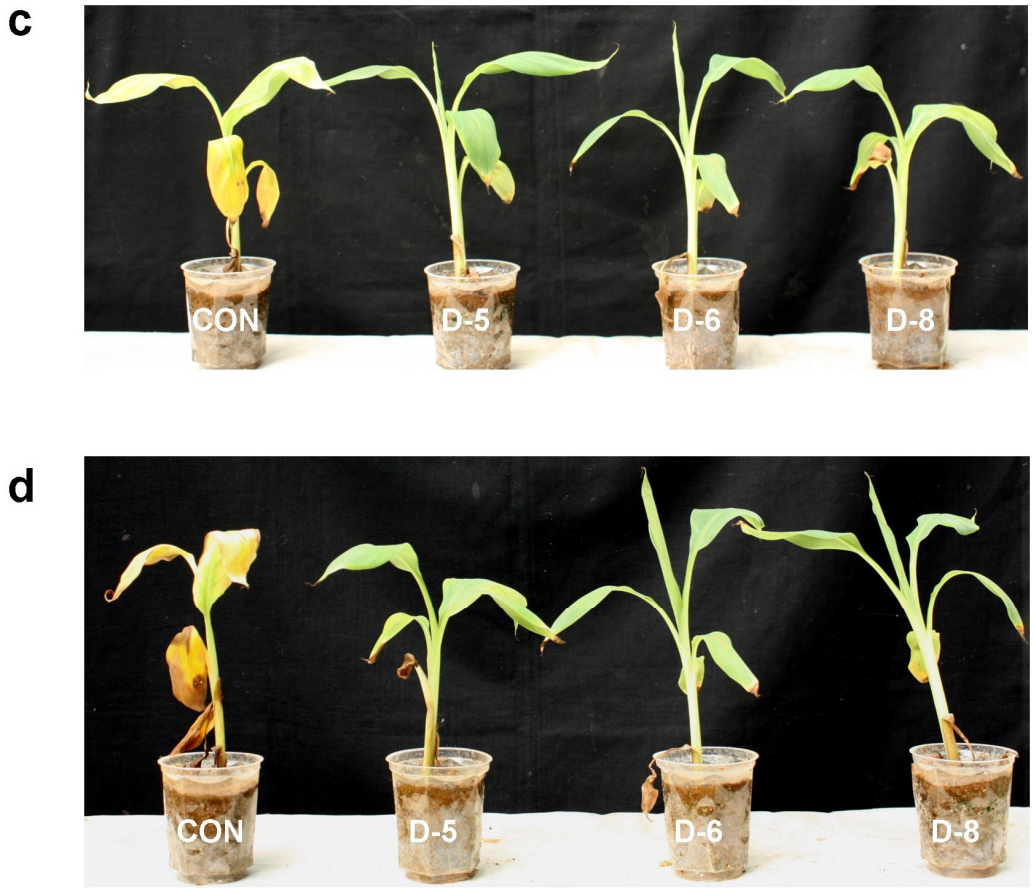

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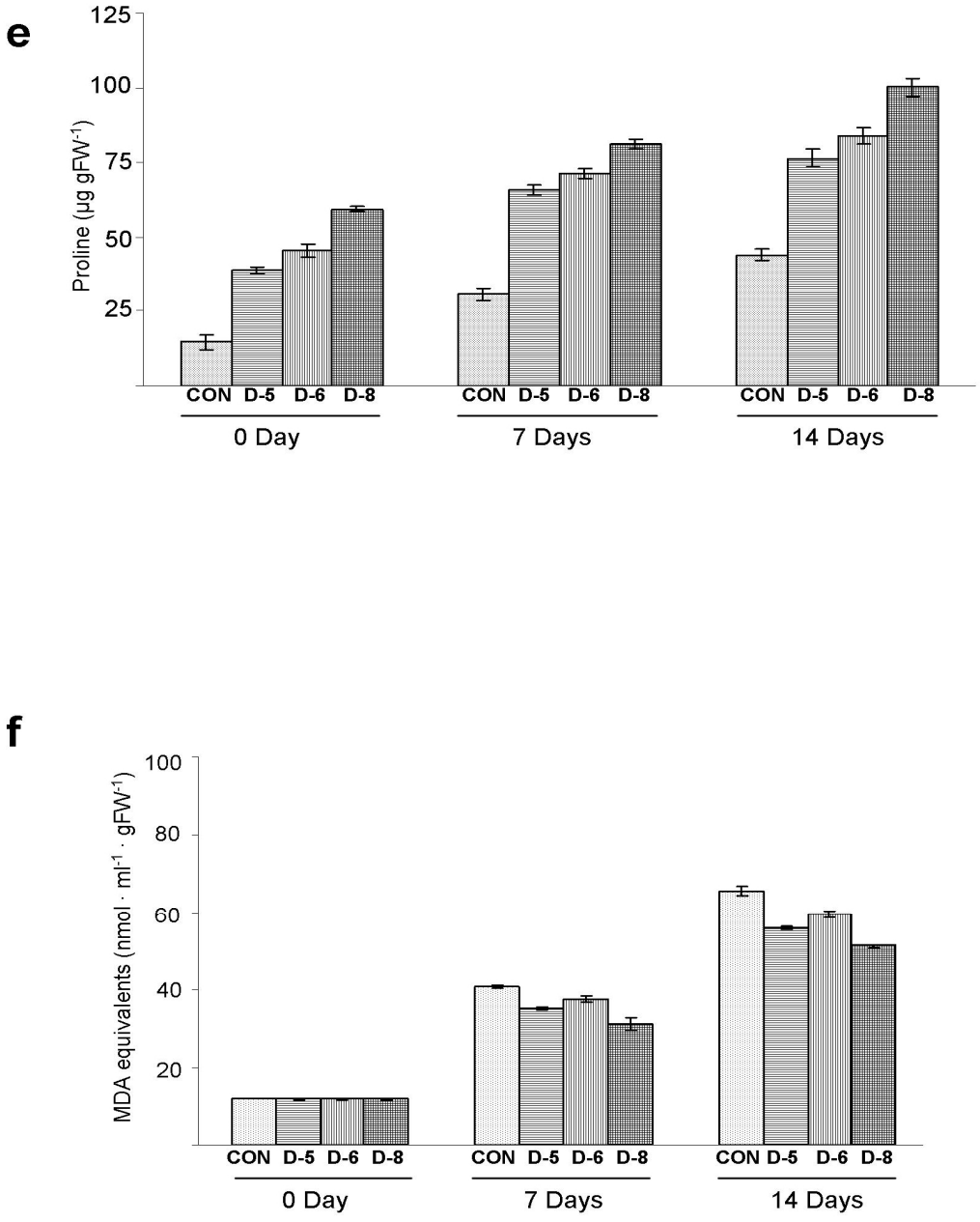

Figure 2.10 Assay for salt stress tolerance in *MusaDHN-1* overexpressing transgenic banana plants. (a) and (b) salt stress tolerance assays performed by supplementing rooting medium with 50 mM and 100 mM NaCl respectively. Compare the growth of control explants with those in figure 2.7f (c) and (d) *MusaDHN-1* overexpressing lines along with control 10 and 20 days respectively after initiation of salt stress recovery. (e) proline content in *MusaDHN-1* overexpressing lines and control plant 7 and 14 days after the start of salt stress. (f) MDA equivalents in *MusaDHN-1* overexpressing lines and control plant 7 and 14 days after the start of salt stress.

Free radical formation and subsequent lipid peroxidation was measured by analysing the MDA levels in both control and *MusaDHN-1* overexpressing transgenic lines in unstressed and salt or drought stressed plants. No significant difference was observed between the control and *MusaDHN-1* overexpressing lines under optimal growing conditions. MDA content increased in all the plants upon application of drought or salt stress. However, this increase was significantly more pronounced in the control plants under stress as compared to *MusaDHN-1* overexpressing lines. The amount of MDA increased only 5.2 (D-5), 5.45 (D-6) and 4.7 (D-8) folds in the *MusaDHN-1* overexpressing lines as against 5.65 fold in control plants at 14 days from the start of drought stress (Figure 2.9 i). Similarly, the amount of MDA increased only 4.72 (D-5), 5.04 (D-6) and 4.34 (D-8) folds in the *MusaDHN-1* overexpressing lines as against 5.5 fold in control plants at 14 days from the start of salt stress (Figure 2.10 f). These results indicate that constitutively higher expression of *MusaDHN-1* increases the protective antioxidative capacity in these plants under drought and salt stress conditions thereby reducing the free radical-induced damage to the cellular membranes of transgenic banana plants.

2.4 Discussion

Dehydrins, belonging to a broader class of LEA proteins, have been extensively studied in various plants owing to their affirmative association with abiotic stress tolerance. Several studies have shown induction of expression of dehydrins in response to different abiotic stress conditions signifying their role in ameliorating the cellular effects of these stresses [56, 57]. Most of these studies have concentrated on a select few model plants like *Arabidopsis* [58] or on major cereal crops such as rice

[59] or wheat [60] with plants like banana with huge economic importance being largely left out. Banana is among the most important food crops in the world and India ranks first in its production. Banana and plantain constitute key staple food crops of African continent where they fulfill a threefold role of providing nutrition, income and food security [40]. Research in banana has mostly been limited to studying the mechanisms underlying ripening and associated processes [61]. Only a few studies have been undertaken for studying banana or plaintain abiotic stress responses [42, 62].

The present study undertook cloning and characterization of a novel banana dehydrin by generating transgenic banana plants overexpressing this dehydrin. This SK_3-type dehydrin was found to be inducible in response to several abiotic stress treatments including drought, salinity, cold, metal toxicity, oxidative stress and application of ABA, methyl jasmonate and ethylene. Further, the presence of different stress-related cis-acting elements in the 5' proximal promoter region of this dehydrin gene reconfirmed its inducibilty in response to varied abiotic stress conditions. In fact, the 1071-bp promoter region of *MusaDHN-1* determined by TAIL-PCR was found to be sufficient to drive inducible expression of β-glucuronidase under salt and drought conditions in transgenic tobacco plants.

Structurally, dehydrins have been documented to be rich in amphiphilic α-helices and random coils. These α-helices are proposed to interact with intracellular molecules, chiefly proteins and membranes by hydrophobic interactions thereby inhibiting their coagulation in water-stressed conditions. Additionally, water-binding activity of the random coils could ensure maintenance of adequate water in

dehydrated cells [36]. These functions in part explain the inducibility of *MusaDHN-1* in conditions generating reduced water potential such as drought, salinity, and low temperatures. Further, signaling molecules like ABA, methyl jasmonate and ethylene serve as the link between the actual reception of stress by different receptors and the subsequent modulation of gene expression [63]. Heavy metal ions exert oxidative stress in plants by mediating the generation of reactive oxygen species which cause lipid peroxidation and enzyme deactivation. Dehydrins have been proposed to mitigate this stress by binding to heavy metal ions thereby reducing there concentration and also by directly scavenging the free radicals thus preventing any damage to cellular membranes [64]. Recently, it has been proposed that dehydrins could possibly be involved in pathogen response in *Arabidopsis* by modulation of some jasmonic acid - responsive genes [65]. This study noted that wheat dehydrin *DHN-5* overexpression in *Arabidopsis* down regulated three jasmonate-ZIM domain proteins which act as negative regulators of jasmonate signaling. As jasmonic acid is known to act as an important signaling molecule in defence responses against insects and pathogens, the study postulates that wheat *DHN-5* modulates these defense responses in *Arabidopsis* by causing down-regulaltion of some jasmonate-ZIM domain encoding genes.

To further examine and characterize the role of this multiple-stress inducible dehydrin, *MusaDHN-1* was overexpressed in transgenic banana plants using constitutively active *Zea mays* polyubiquitin promoter together with its intron containing 5' UTR region. *Zea mays* polyubiquitin promoter and 5' UTR region were used here to drive the constitutive expression of *MusaDHN-1* as they have been

reported in the past to give the best expression levels in transgenic banana plants [66]. Embryogenic suspension culture cells of banana cv. *Rasthali* were the explants of choice here for genetic transformation as the transgenic plants obtained using these cells are least likely to be chimeric and hence guarantee the most stable expression of transgenes introduced into their genome [67]. Further, *Rasthali* is considered to be a highly prized banana cultivar preferred for its unique fruit aroma and golden yellow color.

A number of studies have successfully demonstrated that the overexpression of one or multiple dehydrins results in enhancement of abiotic stress tolerance in plants. *Arabidopsis thaliana* overexpressing a wheat dehydrin demonstrated enhanced tolerance to salt and drought stress [12]. Several reports describe improved freezing tolerance in plants overexpressing specific dehydrin proteins [11, 68]. However, most of these studies presented evidence of improved abiotic stress tolerance in model plants like *Arabidopsis* or tobacco. Further, the best possible way to elucidate the functions of a protein is its overexpression in the native parent plant enabling analysis of the effects of its interactions with various other macromolecules present in the native system. We therefore chose transgenic banana plants to overexpress this dehydrin. Characterization of these transgenic plants showed that constitutive overexpression of *MusaDHN-1* resulted in plants with improved salt and drought tolerance as compared to equivalent controls. By using *Zea mays* polyubiquitin promoter and 5' UTR region we were able to achieve expression levels of *MusaDHN-1* which were comparable to the maximum induced levels (post application of salt or drought stress) of the same gene in untransformed banana plants. Maintenance of

these higher expression levels of *MusaDHN-1* at a constitutive and systemic level in transgenic banana plants resulted in enhanced abiotic stress tolerance.

We found the transgenic dehydrin overproducing plants to be phenotypically similar to the untransformed control plants with no identifiable morphological and developmental alterations noticed. Although, earlier studies document some level of growth retardation in transgenic plants constitutively expressing single or multiple genes associated with stress tolerance [69], dehydrin expression in transgenic plants does not impede normal developmental pathway of plants. The *MusaDHN-1* transgenic lines displayed enhanced tolerance to high concentrations of NaCl. These plants performed better than controls in both in vitro and ex vitro assays conducted by respectively using small single shoots and greenhouse maintained plants. The extent of chlorosis symptoms in greenhouse plants irrigated with NaCl were far lesser and delayed as compared to that in controls. Interestingly, no apparent variation was noticed between the in vitro shoots derived from the three *MusaDHN-1* overexpressing lines and the controls when they were grown on rooting medium containing NaCl, mannitol and PEG (MW 8000). But, after they were transferred to plain rooting media, the *MusaDHN-1* overexpressing lines generated profuse adventitious roots much earlier than controls where there was little or no development of roots indicating that the damage done by high salinity and drought conditions was much less in *MusaDHN-1* overexpressing lines as compared to controls. A significantly lesser increase in endogenous MDA levels in transgenic plants stressed similarly also indicates the same. Similar behaviour of dehydrin overproducing plants during recovery from stress application has been documented before [9, 12]. Our

observation only reconfirms the importance of studying plant responses during recovery after stress treatment. Further, non-permanent nature of environmental stress conditions also necessitates such studies.

Higher RWC noticed during drought conditions in *MusaDHN-1* overexpressing lines as compared to controls correlates well with the acknowledged role of dehydrins in binding to water molecules owing to their peculiar secondary structures. Water-binding by dehydrins slows the rate of water loss from leaves and hence the dehydrin overproducing plants are better able to retain water during drought conditions.

Accumulation of proline in leaves of transgenic lines was further studied to investigate the mechanism of salt tolerance. Increase in free proline content and its role as an osmoprotectant in abiotic stress conditions has been well studied in the past [70]. Proline acts by helping to maintain the intracellular osmotic balance thereby preserving the structures of various enzymes and other sub-cellular constituents. Its accumulation also increases cellular osmolarity thereby providing the turgor pressure needed for cell expansion and growth in stressed environment. Elevated endogenous proline concentrations noticed in salt stressed *MusaDHN-1* overexpressing lines agrees well with several such reports wherein proline accumulation through different strategies has been well correlated with increased salinity tolerance. In fact, the accumulation of proline in transgenic lines was greater than that in the controls even under standard growth conditions. Similar phenomenon has been observed by [12] where overexpression of a wheat dehydrin *DHN-5* led to increased proline biosynthesis in unstressed plants. A detailed microarray analysis in their case [65]

failed to detect any up regulation of proline biosynthetic enzymes. However, they have suggested that wheat *DHN-5* due to its chaperone like activity improves the stability of proline biosynthetic enzymes and therefore the proline synthesis is enhanced. Further, dehydrins have been proposed to directly interact with compatible solutes like proline to serve as structural stabilizers of macromolecules under conditions of water deficit [71].

In conclusion, the study described here identified a novel dehydrin gene *MusaDHN-1* which is inducible in response to a variety of abiotic stress conditions and application of various signaling molecules. This study is the first one to generate transgenic lines showing improved drought and salt stress tolerance in an economically important fruit crop banana. Further, this study also describes for the first time characterization of a banana gene using transgenic banana plants. *MusaDHN-1* dehydrin gene characterized in this study can potentially be used to develop abiotic stress tolerant lines in other important crop plants also.

Table 2.1 Primers used for studies on *MusaDHN-1*

Amplicon / Description	Primer Sequence (5'to 3')
MusaDHN-1 full length CDS	CTGCAGATGGCGGAGGAGCACCACAAGG
	GGTACCGTTCCTAGTGGGCAGGGCTGCT CTT
MusaDHN-1 sequential nested primers for TAIL-PCR	CGGCTTCCTCTAAGTGGATCTTC
	GCACTCCTCCGTCTTTTCCTC
	CTTTCTTCTTCCCCAGGAAGTC
MusaDHN-1 promoter	TGCAAGCTTGCGCCAAGATCACTAGCCA TAG
	CTCAGATCTACCATGAGATCACCAAATA AAAGATCACAAAC
MusaDHN-1 partial CDS	ACAAGGCTGTGGAGAGTGGT
	TCATGCTCGACATGGACCT
Banana *actin* partial CDS	ATGGCTGACGGCGAGGATA
	GGGAAAGAACAGCCTGAATTGC
nos 3' UTR	CTACCGAGCTCGAATTTCCCCGATCGTT
	CGGCCAGTGAATTCCCGATCTAGTAACA
Zea mays polyubiquitin promoter	AATTAAGCTTCCGGTCGTGCCCCTCTCTA
	AGCTCTGCAGAAGTAACACCAAACAACA GG
Hygromycin phosphotransferase partial CDS	GTCCTGCGGGTAAATAGCTG
	ATTTGTGTACGCCCGACAGT
Arbitrary degenerate primers for TAIL-PCR	NTCGASTWTSGWGTT
	NGTCGASWGANAWGAA
	WGTGNAGWANCANAGA
	AGWGNAGWANCAWAGG
	TGWGNAGSANCASAGA
	STTGNTASTNCTNTGC
	GTNCGASWCANAWGTT
	WGCNAGTNAGWANAAG
	AWGCANGNCWGANATA

Chapter 3

Studies on *MusaWRKY71*, a WRKY transcription factor gene from banana

3.1 Introduction

Plant growth and productivity are adversely affected by various abiotic and biotic stresses [72]. To overcome these challenges, plants have developed the ability to perceive and respond to these diverse external signals using specialized physiological and biochemical strategies [73]. Plant stress responses are generally controlled by a network of specialized genes through intricate regulation by specific transcription factors [74]. Among these transcription factors, members of ERF [75], bZIP [76], MYB [77], NAC [78] and WRKY families [15] have been well known for their central roles in regulating the plant responses to stress. A variety of specific methodologies like large-scale single pass sequencing of cDNA clones to generate ESTs have been utilized for studying modulation of gene expression in response to various abiotic and biotic stresses. ESTs obtained from a specific cDNA pool provide a quantitative method to measure specific transcripts within a cDNA library and represent a powerful tool for gene discovery, gene expression, gene mapping and the generation of gene profiles [79]. Recently, [80] have demonstrated the importance of moderately abundant ESTs in genomic studies and have shown that comparative analysis of stressed and non-stressed tissue derived EST data sets can lead to reasonable prediction about differential expression of moderately abundant ESTs. We have utilized a unique EST (GenBank accession no. DN239172) from a cDNA library constructed from *Musa acuminata* ssp. *burmannicoides* var. *Calcutta 4* (AA group) leaves subjected to low temperature stress [81]. This unique EST which corresponds to a novel *WRKY* transcription factor gene is specifically represented in a single copy in the low temperature stressed leaf cDNA library while being absent in other banana

cDNA libraries including that derived from high temperature stressed leaves and dehydration tolerant cultivar "Cachaco" (http://www.musagenomics.org/research/est_and_cos.html).

WRKY transcription factors comprise one of the largest families of transcription factors in plants and are involved in a variety of physiological processes, most prominent among them being biotic and abiotic stress responses. WRKY proteins possess a specific 60 residues long DNA binding domain which is named as the WRKY domain after the almost invariant WRKY amino acid sequence present towards the N-terminus. Apart from this WRKY signature sequence, WRKY proteins also possess an atypical zinc-finger structure at the C-terminus of WRKY domain which is either $Cx_{4-5}Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$. The number of WRKY proteins present in different organisms is highly variable with unicellular green alga *Chlamydomonas reinhardtii* having a single WRKY transcription factor, the moss *Physcomitrella patens* having 37, *Arabidopsis* with 74 and soybean with almost 200. Most members of WRKY family have been implicated in transcriptional reprogramming linked with the plant immune responses where they serve as central components of the innate immune system [82]. Additionally, specific WRKY proteins have been demonstrated to be involved in cold, heat, drought and salt tolerance thereby indicating that the WRKY web of signaling encompasses both biotic stress and abiotic stress responses [83]. Further, WRKY transcription factors are also involved in regulating the complex hormonal crosstalk during embryogenesis [84] and seed germination in different plants [85].

In this chapter, we have characterized the first *WRKY* gene from banana (*MusaWRKY71*) in detail using transgenic banana plants and established its involvement in diverse stress responses. Further, differential expression of several putative *MusaWRKY71* target genes involved in biotic stress response pathways has also been studied.

3.2 Material and methods

3.2.1 Plant materials and growth conditions

In vitro plantlets of banana cv. *Karibale Monthan* maintained at 25 ± 2ºC in a 16 hr light/8 hr dark regime were hardened in a greenhouse for 3 months. Uniform size plantlets at 6 or 7 leaf stage were used in all the experiments.

3.2.2 Primers

Primers used in the current study are listed in Table 3.1.

3.2.3 3'-rapid amplification of cDNA ends

In order to obtain the full length cDNA sequence of the EST with GenBank accession no. DN239172, 3'-RACE was performed using 5'/3' RACE Kit (Roche Applied Science, Germany) according to manufacturers instructions. Total RNA was extracted from 100 mg young leaves after exposing them to 10 ± 2ºC for 24 hrs using Concert Plant RNA Reagent (Invitrogen, USA) followed by RNA clean up and on column DNase digestion using RNeasy Plant Mini Kit (Qiagen, Germany). The PCR products were analyzed on a 1.5 % agarose gel and subsequently sequenced.

3.2.4 DNA and protein sequence analysis of the *MusaWRKY71* gene

Amplification of full-length genomic sequence of *MusaWRKY71* gene was performed using the sequence information obtained from 3'-RACE. Genomic sequence for

MusaWRKY71 gene so amplified was then sequenced. The full length cDNA sequence for *MusaWRKY71* gene was amplified from cold stressed leaf cDNA (isolated for 3'-RACE) using Pfu Ultra AD DNA Polymerase (Stratagene, USA) according to manufacturers instructions. This cDNA was also subsequently sequenced. The sequence and position of the three introns was ascertained by aligning the genomic and transcribed sequence of *MusaWRKY71* gene using the online ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). ClustalW2 was also used to align MusaWRKY71 amino acid sequence with its close homologues. MEGA 5 software was used to construct a phylogenetic tree for MusaWRKY71.

3.2.5 Cellular localization of MusaWRKY71 protein

MusaWRKY71 gene cDNA was cloned in-frame at the N-terminal end of GFP in the binary vector pCAMBIA-1302 (GenBank accession no. AF234298) using *Nco*I and *Spe*I restriction enzymes. *MusaWRKY71* gene coding sequence without native stop codon was amplified from cold stressed leaf cDNA using Pfu Ultra AD DNA Polymerase. The resulting binary vector (p1302-*MusaWRKY71*) was mobilized into *Agrobacterium tumefaciens* strain EHA105 by electroporation. This *Agrobacterium* culture was then used to transform banana suspension culture cells (cv. *Rasthali*) at an OD_{600nm} of 0.1 as described previously [49]. Five days post *Agrobacterium* infection when the transient expression of the GFP reporter is at its peak in the transformed cells, localization of the fusion protein was ascertained using a fluorescence microscope (Eclipse 80i, Nikon). To confirm the nuclear localization of the GFP fusion protein, cells were stained in dark for 10 min in a staining solution containing

10 mM tris, 1 mM EDTA (pH 8), 0.005% (v/v) triton X-100 [86] and 1μg ml^{-1} Hoechst 33258 nucleus specific stain. *MusaWRKY71*::GFP fusion protein was visualized using GFP filter set (excitation 460–500 nm, emission 515–550 nm) whereas Hoechst 33258 was seen using DAPI filter set (excitation 340-380nm, emission 435-485 nm). Online prediction of subcellular localization was done using WoLF PSORT prediction software (http://psort.nibb.ac.jp).

3.2.6 Determination of copy number of the *MusaWRKY71* gene by Southern blot analysis

A minimum of 20 μg genomic DNA (isolated as described before) was digested overnight with *Eco*RI and *Xho*I restriction enzymes. The completely digested genomic DNA was purified using High Pure PCR Product Purification Kit (Roche Applied Science, Germany) and separated overnight on a 0.9% [w/v] agarose TAE gel. DNA transfer to nylon membrane was performed overnight by capillary method using 20X SSC. Restricted DNA was immobilized on the membrane by baking at 120°C for 30 min. The membrane was probed with a DIG-labeled probe of *MusaWRKY71* gene. Chemiluminescent detection of hybridization signals was performed according to the DIG application manual for filter hybridization (Roche Diagnostics GmbH, Germany).

3.2.7 Expression profiling of *MusaWRKY71* under different stress stimuli using semi-quantitative RT-PCR

Healthy banana cv. *Karibale Monthan* plantlets growing in the green house were subjected to various treatments. The plantlets were treated with 2 mM SA, 100 μM ABA, 200 μM MeJA, 5 mM ethephon, 2 mM H_2O_2 and 200 mM NaCl. Cold and

heat treatments were carried out at 10 ± 2°C and 45 ± 2°C respectively in a growth chamber in a 16 hr light/ 8 hr dark regime whereas for dehydration stress the plantlets were left to dry in a laminar air flow under a 16 hr light/ 8 hr dark regime. Plantlets treated only with sterile water were taken as control. Leaf and root tissues harvested from different stressed plants at different points of time were frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from each sample as mentioned before and the first-strand cDNA was synthesized using ~ 5 µg total RNA and Oligo dT_{12-18} primer and Thermoscript Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions. PCR was performed with diluted cDNAs (1:20, 1:50 and 1:100 dilutions of cDNAs obtained using cold treated plants) using Taq DNA Polymerase under the following conditions: 94°C for 5 min followed by the 26, 28, 30 or 32 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min 30 sec. The amplified products were electrophoresed on 1.5% (w/v) agarose gel. Analysis of this gel led to determination of appropriate PCR cycle number to effectively avoid the saturation of DNA amplification during the PCR. Subsequently, PCR was repeated thrice with the appropriate cycle number (30) with all the cDNA samples. Densitometric quantification using raw volume of ethidium bromide stained bands was done using the Genetools program with G:BOX EF gel documentation system (Syngene, UK). Expression level of *MusaWRKY71* gene under different stress stimuli was normalized (independently for each stress treatment for both leaves and roots) against banana *actin* gene (GenBank accession number EF672732). cDNA samples were confirmed to be free of genomic DNA contamination by performing 40 cycles of amplification of banana *actin* gene.

3.2.8 Isolation and sequence analysis of the *MusaWRKY71* gene 5' flanking region

The 5' upstream region of *MusaWRKY71* gene was determined using TAIL-PCR [44]. A total of nine arbitrary primers were tested with three *MusaWRKY71* specific sequential nested primers for amplifying the 5' upstream region. An appropriately sized band was eluted after analyzing the results of secondary and tertiary reactions of the TAIL-PCR and subsequently sequenced. The sequence analysis and motif search for the *MusaWRKY71* gene 5' proximal region was performed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) databases.

3.2.9 Generation of *MusaWRKY71* overexpressing banana plants

The full length cDNA for *MusaWRKY71* gene amplified by Pfu Ultra AD DNA Polymerase was cloned in place of β-glucuronidase coding sequence in pCAMBIA-1301 binary vector using restriction sites for *Nco*I and *Pml*I. The new binary vector was appropriately sequenced to confirm proper insertion of *MusaWRKY71* cDNA. This vector (p1301-*MusaWRKY71*) was then electroporated into *Agrobacterium tumefaciens* strain EHA105 and a culture derived from this *Agrobacterium* was then cocultivated with 0.5 ml packed cell volume of banana embryogenic cells. These cells were then aspirated onto glass fibre filters and cultured on to solidified M2 medium added with 100 µM ACS. After three days of incubation in dark at 25 ± 1^{o}C, the cells (along with the filters) were transferred to fresh M2 medium added with cefotaxime (400 mg l^{-1}). Three days later the cells were removed from the filters and transferred onto banana embryo induction medium supplemented with cefotaxime (400 mg l^{-1})

and hygromycin (5 mg l^{-1}). Somatic embryos developed in 3 to 4 weeks were then cultured on the same medium for three subculture routines of three weeks duration on hygromycin selection medium. Developed embryos were then transferred to MS medium supplemented with BAP (0.5 mg l^{-1}) for germination. Rapidly developing embryos were subsequently transferred to banana multiplication medium to multiply each putatively transformed line. Individual shoots isolated from the multiple shoot cultures were transferred to MS medium added with NAA (1 mg l^{-1}) for rooting. These tissue culture generated plantlets were then hardened in the greenhouse and used for all the analysis.

3.2.10 Molecular analysis of the transgenic plants

Genomic DNA isolated from seven putatively transformed banana lines actively growing on hygromycin containing medium using GenElute Genomic DNA miniprep kit (Sigma, USA) was used as template in PCR with primers specific for *hygromycin phosphotransferase* gene present within the T-DNA borders of p1301-*MusaWRKY71* binary vector. Genomic DNA isolated from untransformed banana plant served as negative control in these PCR. Four lines were then randomly chosen from the lines which showed bands specific for hygromycin phosphotransferase gene. These four lines (W2, W3, W8 and W22) were then analyzed by Southern blot analysis (performed using DIG-labeled probes specific for *hygromycin phosphotransferase* gene) for estimation of T-DNA copy numbers in these lines. Chemiluminescence was detected on the nylon filters using a chemiluminescence enabled gel-documentation system. The exact quantum of overexpression of *MusaWRKY71* in these four selected transgenic lines was examined by real time quantitative RT-PCR analysis. Total RNA

extraction from young leaves of transgenic plants and first strand cDNA synthesis were done as described before. Samples derived from three independent plants of each line were pooled prior to RNA isolation to ensure reproducibility of results. cDNA derived from untransformed banana plants were used as controls in these RT-PCR performed using SYBR Green Extract-N-Amp PCR ReadyMix (Sigma, USA). Banana *EF 1α gene* [87] was amplified together with *MusaWRKY71* gene to allow gene-expression normalization and subsequent quantification. All real-time quantitative RT-PCR reactions were performed using Qiagen make Rotor-Gene Q platform. Ct values obtained from Rotor-Gene Q software were analyzed using REST-MCS software [43] to arrive at relative expression level values for *MusaWRKY71* gene in different overexpression lines.

3.2.11 Detached leaf stress tolerance assays for *MusaWRKY71* overexpressing lines

Detached leaves derived from 2-3 months old transgenic hardened *MusaWRKY71* overexpressing lines were used in assays for improved oxidative and salt stress tolerance. Leaves were excised along with their petioles and their cut ends were dipped in 1/10 MS medium added individually with methyl viologen (10µM) or NaCl (350 mM) for 7 days in 16 hour light/ 8 hour dark regime. Relatively higher levels of methyl viologen and NaCl were used in these assays to reduce the assay time as otherwise severe fungal contamination was noted in the flasks. Detached leaves so treated with methyl viologen or NaCl were then analysed for membrane damage by estimating their malondialdehyde (MDA) content. Further, continuous excitation plant efficiency analyzer (Hansatech Instruments make Model no. Handy-Pea) was

used to estimate photosynthetic efficiency (Fv/Fm) in all detached leaf samples. Leaves derived from hardened greenhouse maintained plants of untransformed banana cv. *Rasthali* were used as controls in all the above assays. All the stress assays were conducted in triplicates and after their completion representative samples for each assay were photographed.

3.2.12 Differential expression of putative *MusaWRKY71* target genes in *MusaWRKY71* overexpressing lines

To investigate the effect of *MusaWRKY71* overexpression on expression of other *WRKY* gene family members in banana, cDNA isolated from leaves of *MusaWRKY71* overexpressing banana lines was utilized in real time quantitative RT-PCR together with primers specific for 67 annotated *WRKY* genes from banana genome project [88]. cDNA isolated from untransformed control plants was used as control in these reactions. REST-MCS software was used to arrive at relative expression values [43] and primers specific to banana *EF1α* gene were used for normalization of expression values. Similarly, primers specific to 20 *PR protein* genes, 26 *chitinase* genes and 9 *NPR1* genes were also tested to determine their differential expression in *MusaWRKY71* overexpressing lines.

3.3 Results

3.3.1 Cloning and sequence analysis of *MusaWRKY71*

Partial sequence of the unique EST derived from a cold stressed EST library of banana leaves (DN239172) was extended towards the 3' end using 3'RACE. The full-length coding sequence of *MusaWRKY71* cDNA consisted of 843 nucleotides. A 380 nucleotide long 3' UTR was also deduced from the 3'RACE sequence. Also, a 76

nucleotide long 5' UTR has been predicted based on the sequence of 5' end of the EST DN239172 and the analysis of 5' proximal sequence (obtained using TAIL-PCR) by TSSP program hosted at www.softberry.ru. When primers flanking the *MusaWRKY71* cDNA were used to amplify the full-length gene sequence from the genomic DNA of banana cv. *Karibale Monthan*, a fragment of 1073 bp was obtained. Comparative alignment analysis of the genomic sequence and the cDNA sequence of *MusaWRKY71* gene indicated the presence of three introns (84 bp, 68 bp and 78 bp in length respectively) in *MusaWRKY71* gene, all of which were present in the N-terminal half of the *MusaWRKY71* gene. All three introns displayed distinctive characteristics of plant introns like being relatively AT rich (AT content 60.7% for intron 1, 58.8% for intron 2, 56.4% for intron 3) and having conserved 5'-GT and AG-3' splicing sites (Figure 3.1)

+1

TCTCAGACCTGTTATCATTCATAGAAGCACAGCTTTATTAGTTAGTCACCACCTTACCTTGCCTCCTACT
CTAATTATGGGGTCAAGTTGGTTGGATTGCCCCTCTCTTAATCTCGACCTCAACGTCGGCTTGCTCCCG
TTGCCTGTCGATTCTCCGGTGAGTGCAATCGGTCTTCCATCTTCTTTCTGAACAGTTTTTGATTCATTTTGTTGATTAGTCTGT
TCTTGATGGGTTGTGCAGAAAGCTGTTTCGGCCGTGGAATCCAAGCATGTCGACAAGAAGGTGTCGATCAA
AGAAGAGAAAGCTGTATCGATTAGCTGCTGAGTTTATATATGTTACGGAGTTCTGATGATGTGCCACCTTCTGTCTCTTAGA
TTAAGGCTCTTGAGGCGGAGCTCGTTCTGGCAACCGAGGAGAACAAGAAGCTGAGTGAAGTGCTCGC
GGCCACGATCGCCAGCTACAGCGCGGTTCGGAAACAGCTGATCGAGCAGATGAACACCCCTCCCCCCG
AAGGCGGGTCTCGATCTAACTCGCCGCCGGGAAAGAGGAAGAGCGAGAGCCTCGATGCTAATATGGA
GAGCACGTCGAGCGAAGGCTCCTGCAAGCGAGTTAGAGACGACTGTAAACCCAAGGTCTGGAAGCTC
CATGTCCGCTCCGACCCGTCTGATACAAGCCTCGTAGGTTCCACAACCTTTCTTTCTTCTTTCTTACATGTTCCTG
GATCGAAGCCGACATCTTCTTCGGGTCTTTAGGTCGTGAGAGATGGGTATCAATGGAGGAAATATGGGCAGAAG
GTGACCCGAGACAATCCATGCCCAAGAGCTTACTTCAGATGCTCCTTCGCCCCTTCTTGCCCAGTTAAG
AAGAAGGTGCAGAGGAGCGCGGAGGACACGTCGATCTTAGTGGCGACGTACGAAGGCGAGCACAAC
CATGACCTGCGTTCTCGGCCTGGAGCTCCCAGTCTGCGTCCCAATACTGCAGCACCTGACCTCAAGTCA
TCAGGATCGCAACCGGAGATGGAGTCGCAGGAGTTCCAGCGCAGTTTGGTGGAGCATATGGCTTTCTC
GTTGTCGGAAGATCCAGCCTTCAAGGCTGCACTGGCCACCGCCATCTCCGGGAAAATGCTTCCCCTAC
GAACTAGCTAACTCTCTGCTCCTCGAGATGACCGAGGTGATGCCAAAAGTTTGGTTTCTGTTCTTATTA
GTTGAAGTAGACGATCCACGAATCCACAGACTGAGTTTGATTCGGAAGAAGAAGTTCCGAGTTGAAC
ACACCGATCGATCGATCCCGAACGAAACTTTCTCTCGGAACAAAGGTCAAAAACACGTGGTGTGGACT
TCTCGGTCAACGGTGTCAGCCAAGGAAGTTTCGCAGTCGCTTATCAGGACGATGTCGTTGTAAATGCT
TTAAAAATTGTGTCGGTATGATAATGGACATCCAGCCATTGATTTGTGTATGATGAATACGAATGCGA
ATACAAATGTGATACGCAATGGATATTAGTTAGTTAAAAAAAAAAAAAAAA

Figure 3.1 Deduced nucleotide sequence of *MusaWRKY71* mRNA derived from cv. *Karibale Monthan*. Transcription start site has been indicated by +1. Translation initiation and termination codons have been boxed. Arrowheads denote the junction between exon and intron. Introns are colored red and shown in smaller font. 5' and 3' UTR's have been underlined.

The *MusaWRKY71* cDNA encodes a protein of 280 amino acid residues with a predicted molecular weight of 30.69 kDa and a theoretical pI of 8.80. WRKY transcription factors have been categorized into three groups based on the number of WRKY domains they contain (two domains in Group I proteins and one in the others) and the features of their zinc finger domain. Based on the primary amino acid sequence of the single WRKY domain that MusaWRKY71 possesses, it has been assigned to group IIa of WRKY transcription factors. Among the characterized proteins, MusaWRKY71 showed the best homology with WRKY71 proteins of *Oryza sativa, Zea mays* and *Triticum aestivum* and hence by analogy the present banana *WRKY* gene was designated as *MusaWRKY71* (Figure 3.2 and 3.3). *MusaWRKY71* possesses both monopartite (PPGKRKSESL comprising 103-112 amino acids) and bipartite nuclear localization signals (RSNSPPGKRKSESLDANMESTSSEGSCKRVRDD comprising 99-131 amino acids). In addition, two nine-amino-acids transactivation domains (PKAVSAVES comprising 27-35 amino acids and SLVEHMAFS comprising 247-255 amino acids) have also been identified in *MusaWRKY71*. As is evident from Figure 3.3, *MusaWRKY71* differs substantially in the N- and C-terminal flanking regions of WRKY domain from its closest known homologues. Also, among the three introns the position of only the third intron has been found to be conserved among its closest relatives. All these evidences point out to the fact that *MusaWRKY71* represents a novel member of *WRKY* gene family.

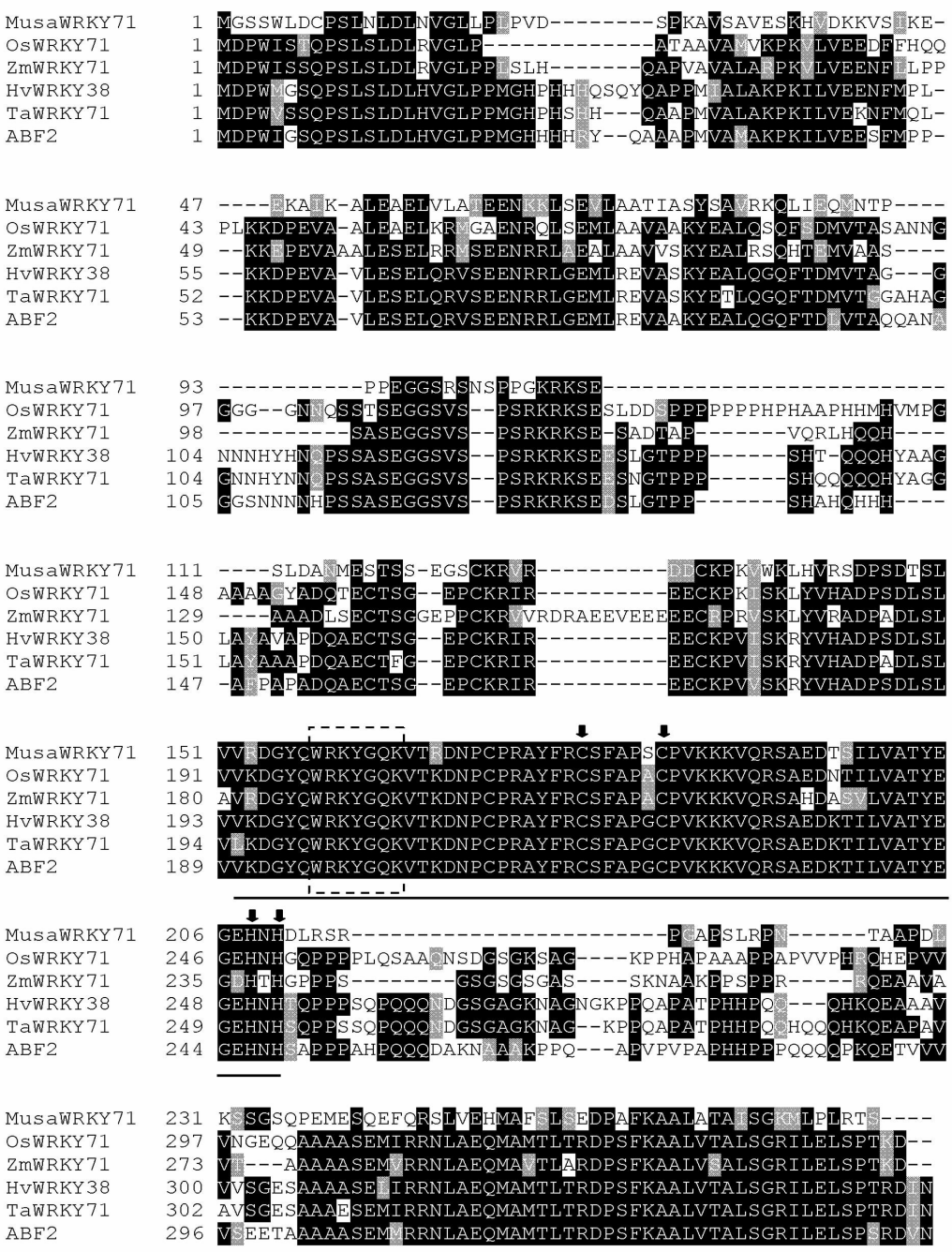

Figure 3.2 Alignment of MusaWRKY71 protein sequence derived from cv. *Karibale Monthan* with closely related protein sequences. Sequences from *Oryza sativa* (AAT84158), *Zea mays* (ACG28515), *Hordeum vulgare* (AAS48544), *Triticum aestivum* (ABN43177) and *Avena sativa* (CAA88331) have been aligned with MusaWRKY71 protein sequence. The WRKY domain has been underlined and the fully conserved WRKYGQK core sequence is boxed. The cysteine and histidine amino acid residues of the putative zinc finger motif have been marked by arrowheads.

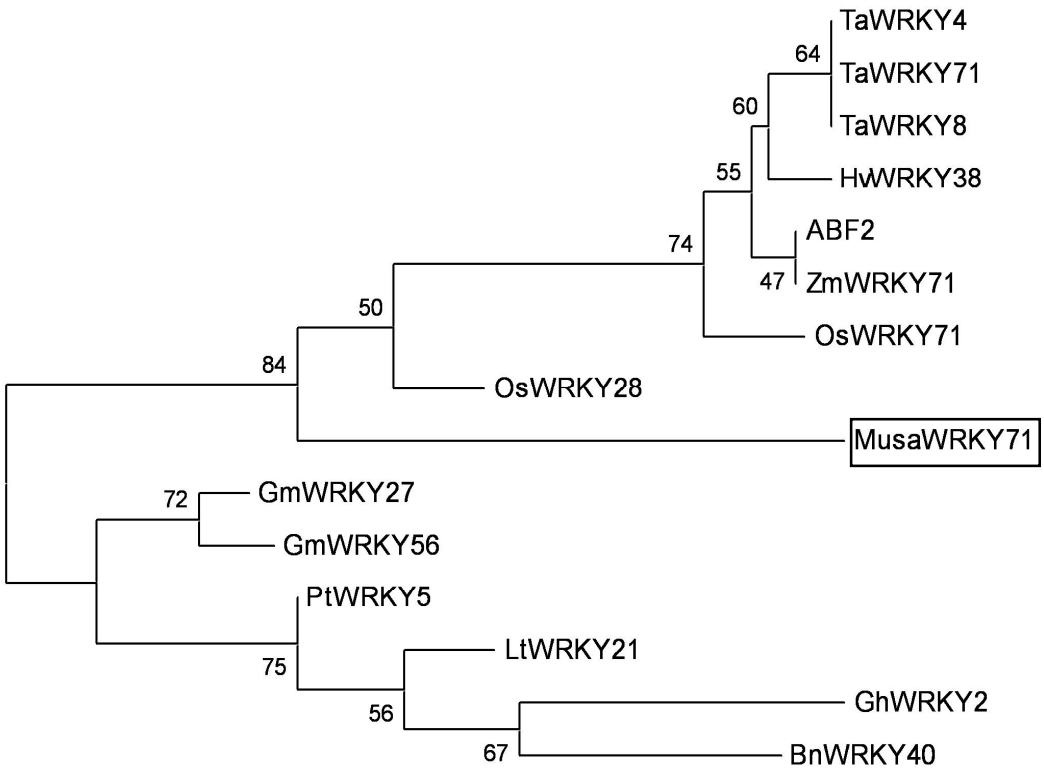

Figure 3.3 Phylogenetic analysis of MusaWRKY71 derived from cv. *Karibale Monthan* in context with other closely related WRKY proteins. The amino acid sequences used to build up the phylogenic tree were: OsWRKY71 (AAT84158), ZmWRKY71 (ACG28515), HvWRKY38 (AAS48544), TaWRKY71 (ABN43177), ABF2 (CAA88331), LtWRKY21 (AAW30662), OsWRKY28 (DAA05093), TaWRKY8 (ABC61128), TaWRKY4 (ACD80365), GhWRKY2 (ABI23959), GmWRKY27 (ABC26917), PtWRKY5 (ACV92007), GmWRKY56 (ABY84656) and BnWRKY40 (ACQ76806). The bootstrapped tree (with 1000 replicates) was constructed using ClustalW2 and MEGA 5 software.

3.3.2 Cellular localization of *MusaWRKY71*

Based on the presence of defined nuclear localization signals in the primary protein sequence of MusaWRKY71 and the results obtained for subcellular localization of MusaWRKY71 using WoLF PSORT prediction software, nuclear localization of MusaWRKY71 was predicted. To confirm the localization of the MusaWRKY71 protein at the cellular level, an expression cassette comprising of *MusaWRKY71*::GFP fusion cDNA driven by CaMV 35S promoter was constructed (p1302-*MusaWRKY71)* and transformed into banana cells. As shown in Figure 3.4 c-f, GFP fluorescence was prominently present in the nuclei of the p1302-*MusaWRKY71* transformed banana cells whereas in cells transformed with pCAMBIA-1302 GFP fluorescence was observed throughout the entire cell (Figure 3.4 a-b). These results conclusively indicate that MusaWRKY71 is localized in the nucleus. Further the presence of *MusaWRKY71-GFP* fusion cDNA was shown by RT-PCR (Figure 3.4 g)

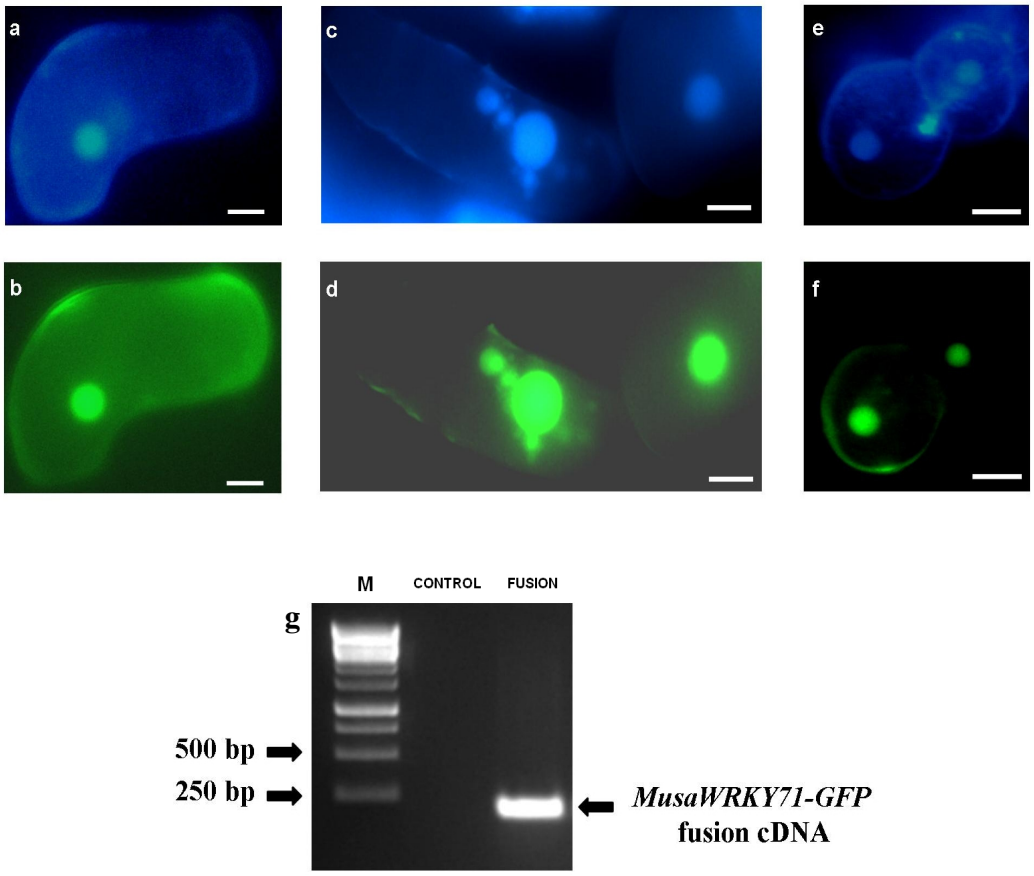

Figure 3.4 Nuclear localization of MusaWRKY71 in banana cv. *Rasthali* suspension culture cells. Banana cells transformed with pCAMBIA-1302 (a-b) and p1302-*MusaWRKY71* (c-d and e-f) were visualized under a fluorescent microscope. Nuclei of the banana cells were stained using Hoechst 33258 stain (a, c, e). Note the predominant concentration of GFP fluorescence in the nuclei of p1302-*MusaWRKY71* transformed cells proving the nuclear localization of MusaWRKY71 protein. White bars correspond to 100 µm. Presence of *MusaWRKY71-GFP* fusion cDNA in the transformed cells was demonstrated by the presence of a ~ 200 bp long fragment amplified with primers specific for *MusaWRKY71* (forward) and GFP (reverse), which was absent in controls (g)

3.3.3 Copy number determination of *MusaWRKY71*

Southern blotting of banana cv. *Karibale Monthan* genomic DNA digested with *Eco*RI and *Xho*I restriction endonucleases indicated that *MusaWRKY71* is present in the genome as a single copy gene (Figure 3.5).

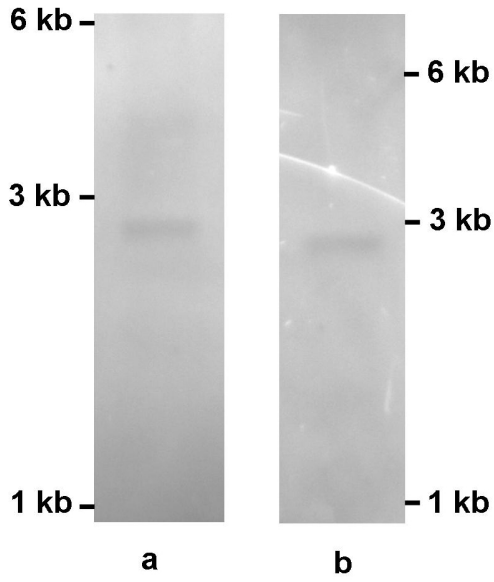

Figure 3.5 Southern blot analysis of *MusaWRKY71* gene in cv. *Karibale Monthan*. Genomic DNA restricted with *Eco*RI (a) and *Xho*I (b) was filter hybridized with DIG-labeled probes against *MusaWRKY71* gene. Approx. positions of the DNA marker bands are indicated. Presence of single hybridization band in genomic DNA restricted with two different enzymes proves that *MusaWRKY71* gene is present in a single copy in banana genome.

3.3.4 Expression profiles of *MusaWRKY71* under different stress stimuli

Semi-quantitative RT-PCR was performed for expression profiling of *MusaWRKY71* using total RNA isolated from stressed and non-stressed leaves and roots of banana cv. *Karibale Monthan* plantlets (Figure 3.6). When banana plantlets were subjected to cold, dehydration, or oxidative (H_2O_2 or methyl viologen) stress, or were exposed to NaCl, ABA, ethylene, salicylic acid or methyl jasmonate, there was a substantial induction in the levels of *MusaWRKY71* transcripts as compared to untreated controls. The pattern and quantum of expression of *MusaWRKY71* as detected by semi-quantitative RT-PCR and quantified by densitometry of ethidium bromide stained bands differed significantly between leaves and roots for different treatment conditions. Expression of *MusaWRKY71* mRNA peaked at 24 hr in both leaves and roots under cold stress and declined thereafter. Similarly, in dehydration stress and NaCl, ABA, ethylene or MeJa treatment *MusaWRKY71* transcripts accumulated quickly to a maximum level in 5 hr and then slowly declined in both leaves and roots. In case of H_2O_2 treatment, there was a significant increase in the levels of *MusaWRKY71* transcripts in the leaves of banana plantlets 2 hr after the treatment whereas in roots there was no change of expression of *MusaWRKY71*. Also, heat stress failed to induce the expression of *MusaWRKY71* in both leaves and roots of banana plantlets. Finally, salicylic acid treatment elicited the best induction of *MusaWRKY71* which did not decline even at 48 hr in leaves whereas there was a gradual decrease in the levels of *MusaWRKY71* in roots after a peak at 5 hr. These results indicate that *MusaWRKY71* gene might be a part of the complex signaling networks regulating abiotic and biotic stress responses of banana plant.

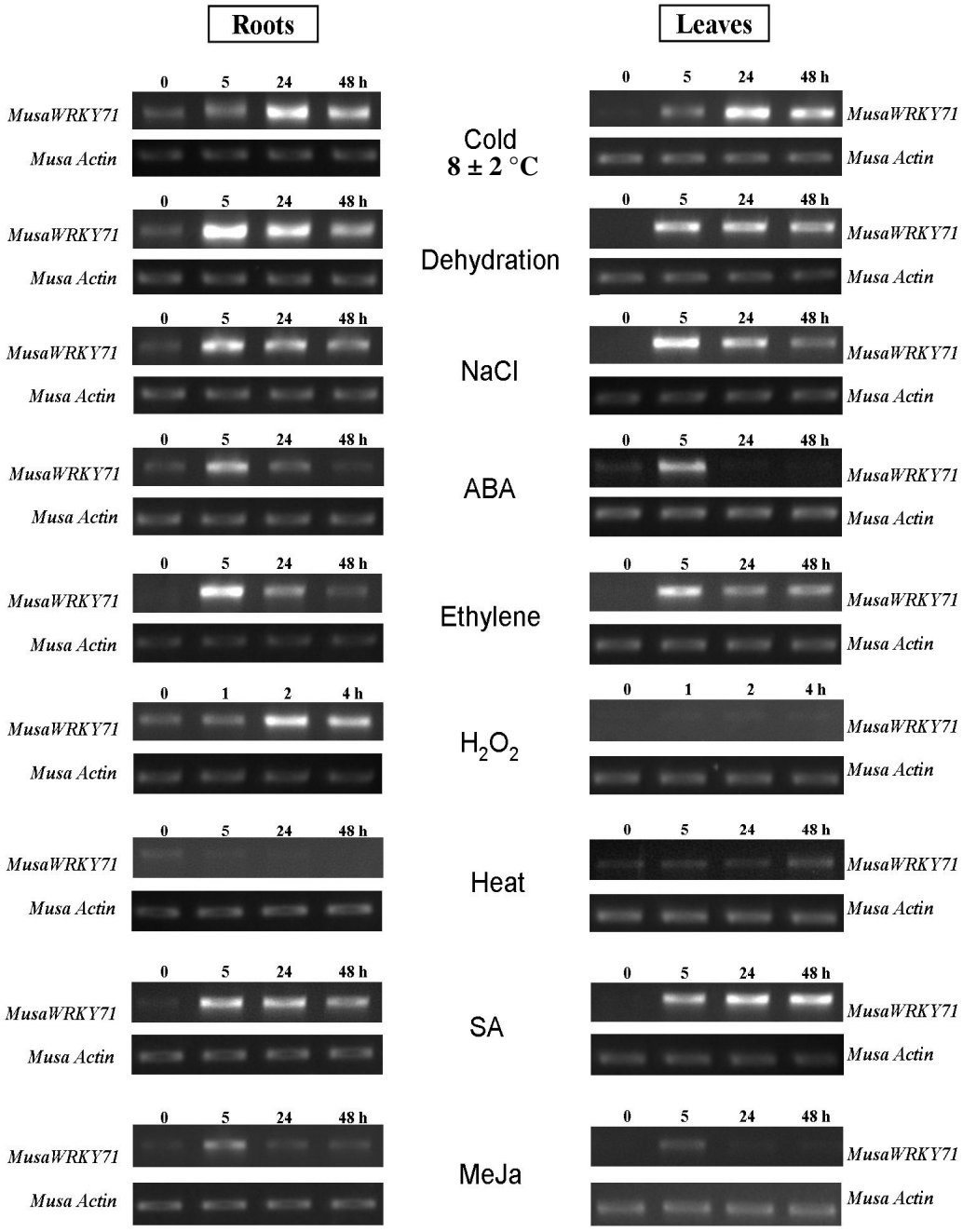

Figure 3.6 Expression profiling of *MusaWRKY71* in cv. *Karibale Monthan* under various stress stimuli. (a) semi-quantitative RT-PCR analysis of expression of *MusaWRKY71* in leaves and roots of banana cv. *Karibale Monthan* at different time points after various stress treatments as visualized on a 1.5 % agarose gel. Note the immediate induction of *MusaWRKY71* transcripts in all stress conditions tested. Banana *actin* gene was used as an internal standard to normalize the variation in the amount of cDNA template used.

3.3.5 Detection of putative cis-acting elements in the 5'-proximal region of *MusaWRKY71*

To elucidate the regulation of *MusaWRKY71* in different stress conditions and to obtain possible clues for its functions, a 918-bp fragment present upstream of *MusaWRKY71* coding sequence was isolated from banana cv. *Karibale Monthan* genomic DNA using TAIL-PCR (Figure 3.7 a). A number of putative cis-acting elements involved in stress-responsive gene regulation were identified using online PlantCARE and PLACE software indicating MusaWRKY71 might be involved in multiple stress responsive signaling pathways (Figure 3.7 b)

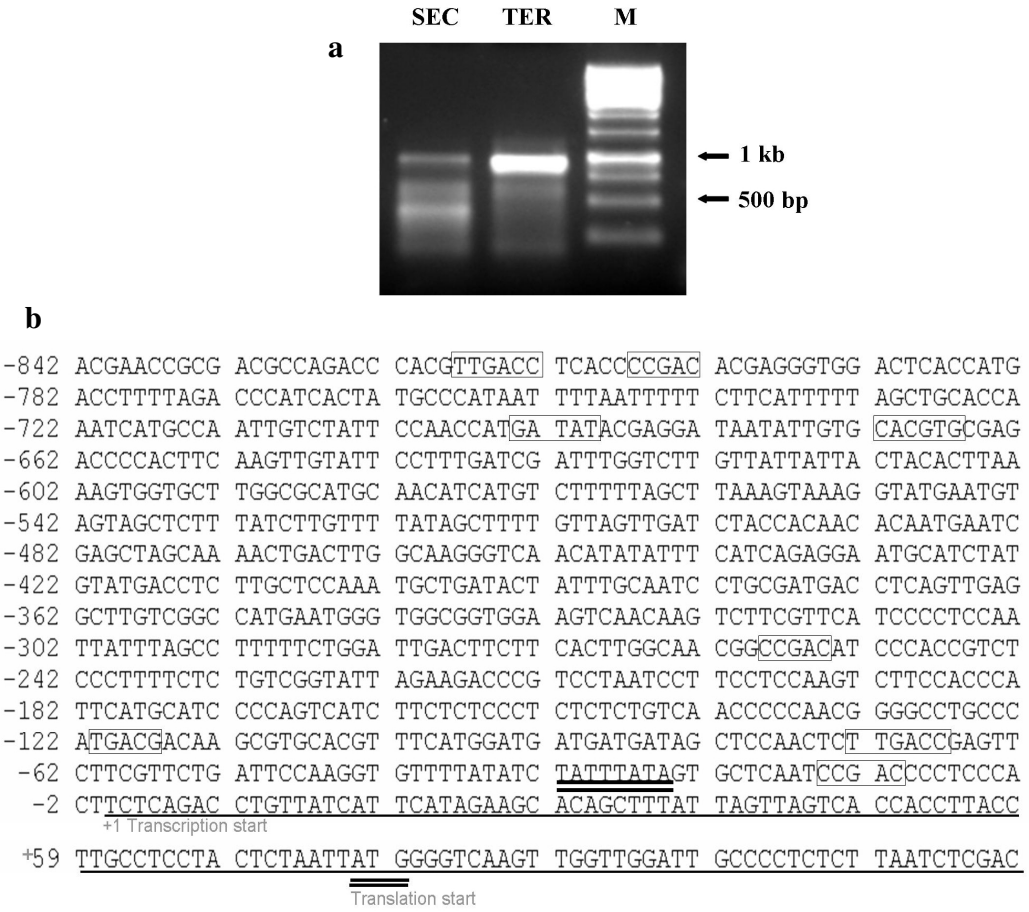

Figure 3.7 TAIL-PCR and nucleotide sequence of the 5' upstream region of *MusaWRKY71* in cv. *Karibale Monthan* (a) products of the secondary and tertiary TAIL-PCR reaction. (b) sequence of the band obatained in tertiary reaction. The predicted transcription and translation start sites are indicated. The underlined region is the 5' region of the *MusaWRKY71*mRNA. Probable TATA-box and the translation initiation codon are double underlined. ABRE (CACGTG), LTRE/DRE/C repeats (CCGAC), Box-W1 (TTGACC), TGACG-motif (TGACG) and GATAT-motif (GATAT) are boxed

3.3.6 Generation of *MusaWRKY71* overexpressing banana plants

Banana cv. *Rasthali* embryogenic cells derived from 7 days old subcultured suspension cultures were cocultivated with *Agrobacterium* harboring p1301-*MusaWRKY71* plant expression vector designed to overexpress *MusaWRKY71* in a constitutive manner in the transgenic banana plants (Figure 3.8 a). Three to four weeks after cocultivation, whitish translucent embryos developed on banana embryo induction medium supplemented with hygromycin (5 mg l^{-1}). Secondary embryos also developed from these primary embryos upon subculturing onto fresh medium of the same composition (Figure 3.8 b). These embryos were subcultured onto embryo germination medium containing BAP for efficient germination. The germinating embryos were then transferred on to banana multiplication medium to facilitate multiple shoot induction (Figure 3.8 c). The clonal shoots developed for each transgenic line were separated and rooted on MS medium supplemented with NAA (Figure 3.8 d). Rooted plantlets were acclimatized in a contained greenhouse (Figure 3.8 e).

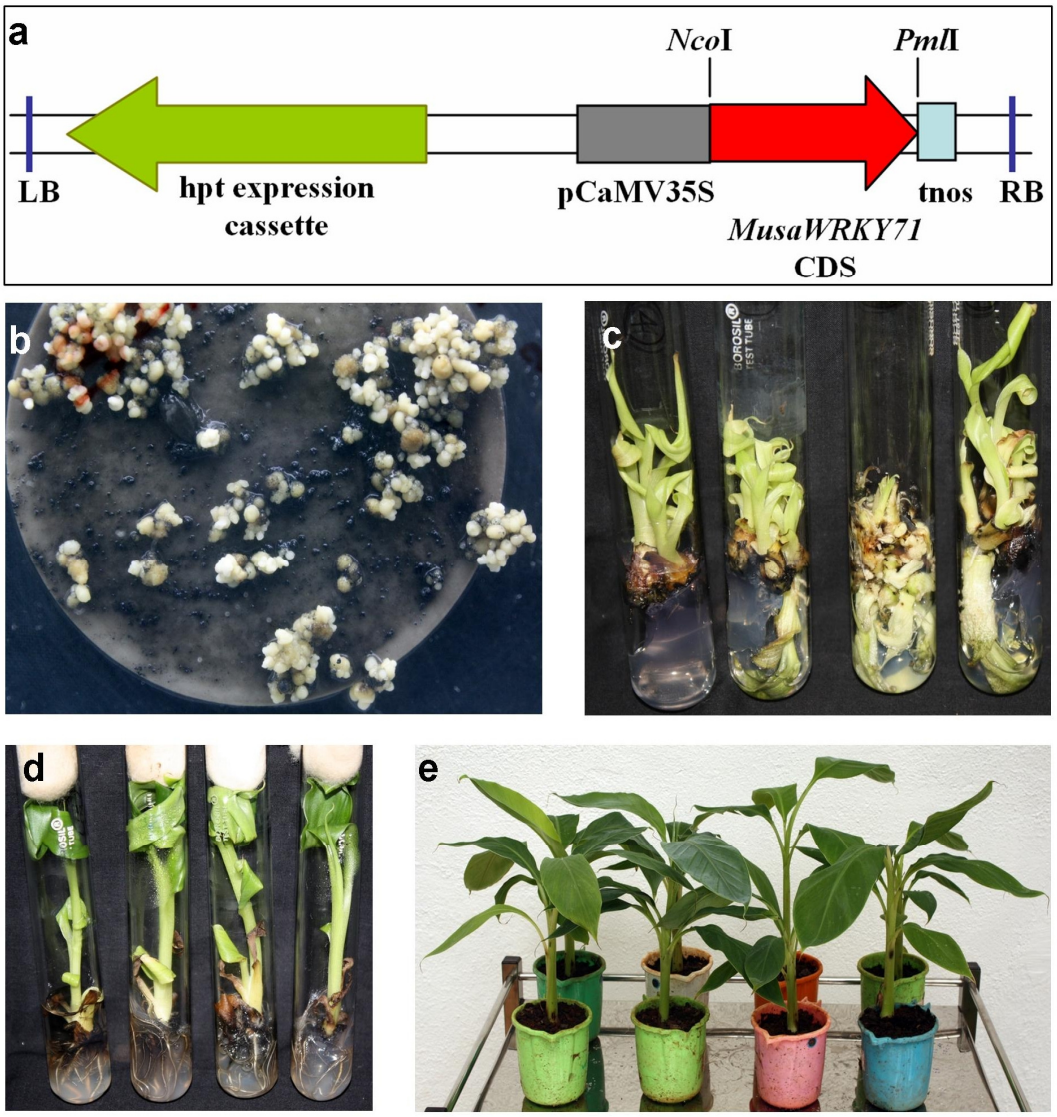

Figure 3.8 Generation of transgenic banana cv. *Rasthali* plants overexpressing *MusaWRKY71*. (a) T-DNA region of binary vector p1301-*MusaWRKY71* designed to constitutively overexpress *MusaWRKY71* in transgenic banana plants. (b) Transformed embryos on embryo induction medium. (c) Transgenic multiple shoots on multiple shoot induction medium. (d) Transgenic rooted plantlets on rooting medium. (e) Transgenic hardened plants in greenhouse (2-months old).

3.3.7 Molecular analysis of the transgenic plants

Seven transgenic lines were observed to grow successfully upon repeated subcultures in hygromycin containing medium. Genomic DNA PCR analysis of these putative *MusaWRKY71* overexpressing lines showed a single 788-bp amplified fragment derived from *hygromycin phosphotransferase* coding sequence whereas it was absent in untransformed control plants (Figure 3.9 a). Four randomly selected lines (W2, W3, W8 and W22) were characterized by Southern blotting wherein restriction digested genomic DNA was probed with a DIG-labeled probe generated using *hygromycin phosphotransferase* gene coding region. Restriction enzyme *BamH*I was used to restrict the genomic DNAs as it is expected to cut the T-DNA region of the overexpression vector (p1301-*MusaWRKY71*) only once and therefore the number of chemiluminescent bands obtained can directly be taken as the number of T-DNA copies transferred to banana genome in these transgenic lines. T-DNA copy numbers in the range of 1 to 4 were found in these four lines (Figure 3.9 b). Also, bands obtained in Southern blots performed using probes directed against *MusaWRKY71* coding sequence correlated well the number of bands obtained using *hygromycin phosphotransferase* coding sequence as template for probe preparation (Figure 3.9 b). Quantitative real-time RT-PCR was employed to estimate the exact quantum of overexpression of *MusaWRKY71* transcript in the four selected lines. *MusaWRKY71* overexpression, determined by using banana *EF1α* gene for expression normalization, was found to be 1.231 times (relative to the expression of *MusaWRKY71* in untranformed control) in W2, 2.395 times in W3, 2.789 times in W8 and 2.610 times in W22 line (Figure 3.9 c).

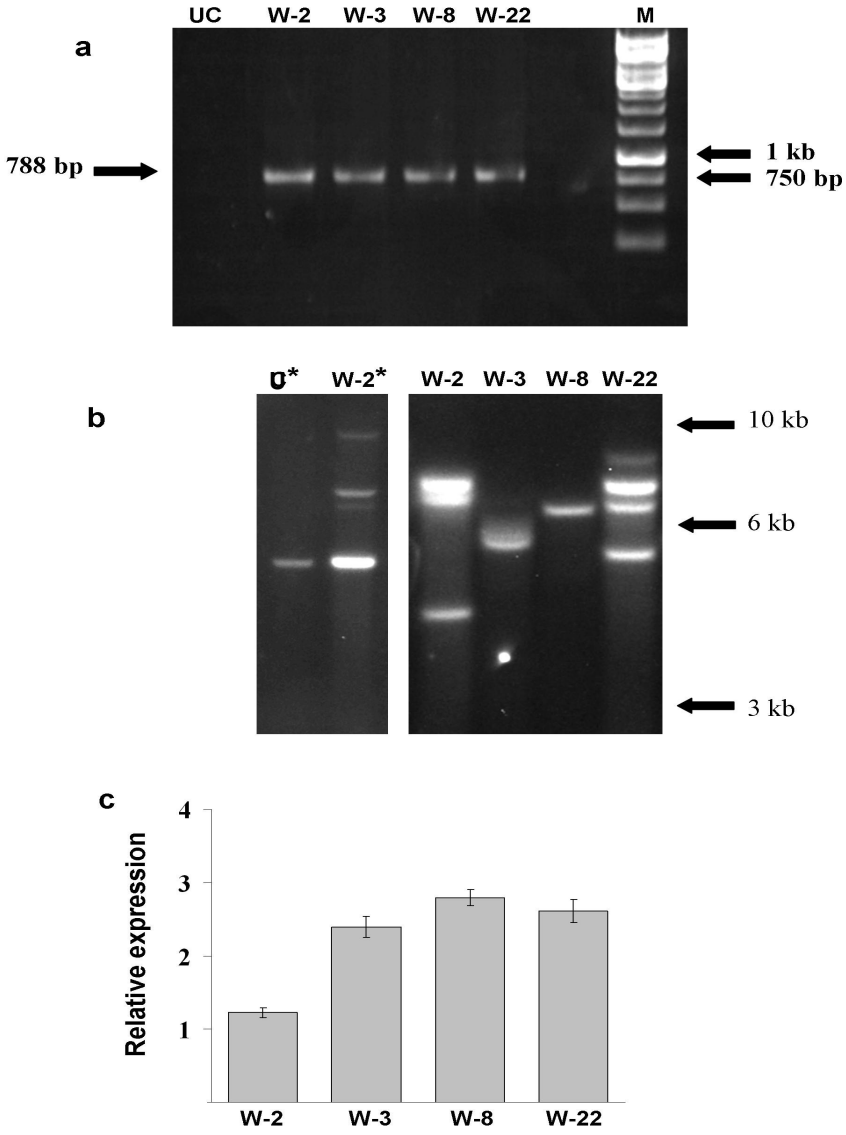

Figure 3.9 Molecular analysis of putatively transgenic banana plants overexpressing *MusaWRKY71*. (a) genomic DNA-PCR analysis of untransformed control (UC) and the transgenic lines (W2, W3, W8 and W22). (b) Southern blot analysis of p1301-*MusaWRKY71* transformed banana lines using probe against *hpt* gene. Lanes UC* and W-2* show Southern blot of control and W-2 line using probes against *MusaWRKY71* gene sequence (c) Real time quantitative RT-PCR analysis of the selected transgenic lines (W2, W3, W8 and W22) for determination of the exact quantum of *MusaWRKY71* overexpression in transgenic banana lines. All gene expression values have been normalized against banana *EF1α* cDNA expression levels. Expression of *MusaWRKY71* in untransformed plants has been assumed to be 1 for estimating the level of overexpression of *MusaWRKY71* in different transgenic lines. Values are mean ± SE.

3.3.8 Detached leaf stress tolerance assays for *MusaWRKY71* overexpressing lines

Transgenic banana plants overexpressing *MusaWRKY71* were observed to be phenotypically normal and indistinguishable from the untransformed plants in both in vitro and ex vitro conditions. To assess the stress hardiness of *MusaWRKY71* overexpressing plants, we used detached leaves from greenhouse grown transgenic and untransformed control plants in stress assays. Detached leaves along with their petioles were exposed to 10 μM methyl viologen or 350 mM NaCl in 1/10 MS medium for 7 days. Leaves derived from *MusaWRKY71* overexpressing transgenic plants showed significantly less damage by the simulated oxidative or salt stress treatments as compared to untransformed control leaves which showed marked browning and chlorosis respectively, in oxidative and salt stress application (Figure 3.10 a, Figure 3.11 a). Oxidative stress exerted through ROS is a common constituent of most abiotic as well as biotic stress conditions. Methyl viologen accepts electrons from photosystem I in the presence of light and transfers the same to oxygen to generate ROS. Treatment with methyl viologen and NaCl is a standard procedure employed to examine improved tolerance against oxidative and salt stress in plants and the fact that detached transgenic leaves remained green and looked better than equivalent controls even after 7 days in methyl viologen or NaCl solution clearly proved that *MusaWRKY71* overexpression in these transgenic plants resulted in an improved ability to tolerate these stress conditions. Photosynthetic efficiency quantified in the form of maximum quantum efficiency of Photosystem II and denoted as physiological parameter Fv/Fm [ratio of variable fluorescence (Fv) over

the maximum fluorescence value (Fm)] is a sensitive marker of overall plant wellbeing and exposure to any form of abiotic or biotic stress stimuli causes reduction in photochemical quenching of energy within Photosystem II leading to a lowered Fv/Fm. The stressed leaves derived from untransformed control plants displayed the lowest Fv/Fm in both the stress assays suggesting deficient photosynthetic functions in these leaves as compared to the *MusaWRKY71* overexpressing transgenic leaves (Figure 3.10 b, Figure 3.11 b). The damage caused to the cellular membranes (including chloroplastic membranes) during detached leaf assays was also quantified by Thiobarbituric Acid Reactive Substances (TBARS) assay which estimates the level of malondialdehyde (MDA) formed by lipid peroxidation of cellular membranes. Untransformed control leaves showed the maximum damage to cellular membranes as the MDA levels were found to be the highest in these leaves (Figure 3.10 c, Figure 3.11 c). Constitutive overexpression of *MusaWRKY71* in transgenic plants thus enhanced the capacity to scavenge the reactive oxygen species, thereby minimizing the damage inflicted by these free radicals on the target cellular membranes of transgenic banana plants.

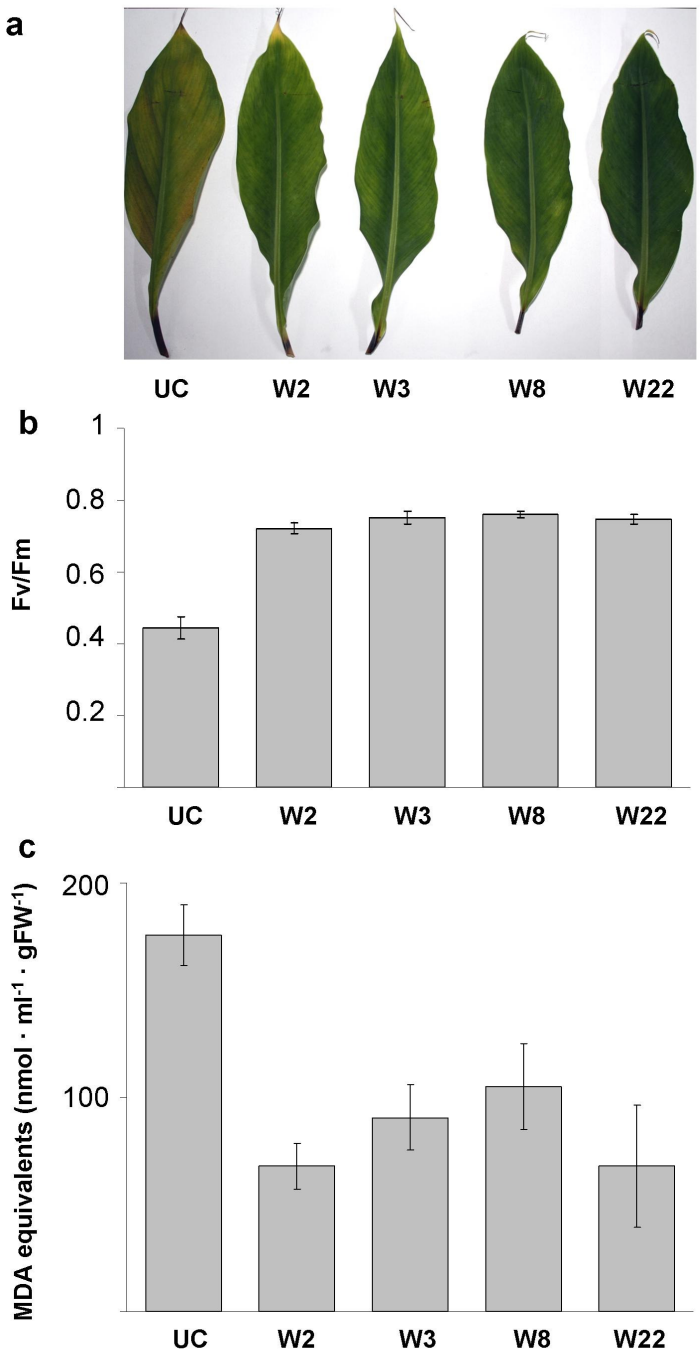

Figure 3.10 Detached leaf oxidative stress assay of p1301-*MusaWRKY71* transgenic plants. (a) detached banana leaves derived from greenhouse maintained transgenic (W2, W3, W8 and W22) and control plants (UC) after exposure to simulated oxidative stress (10 µM methyl viologen in 1/10 MS basal medium for 7 days). (b) photosynthetic efficiency (measured as Fv/Fm ratio) of untransformed and p1301-*MusaWRKY71* transgenic leaves exposed to methyl viologen. (c) MDA levels in untransformed and p1301-*MusaWRKY71* transgenic leaves exposed to methyl viologen.

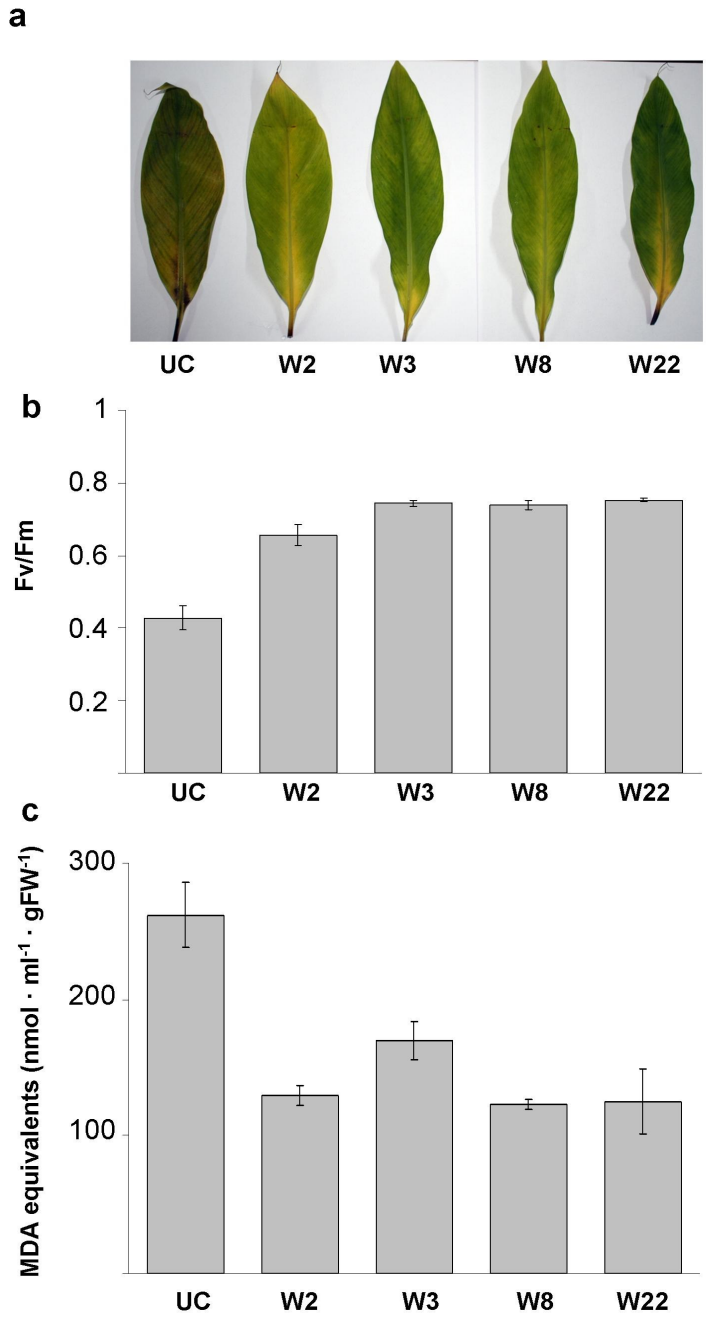

Figure 3.11 Detached leaf salt stress assay of p1301-*MusaWRKY71* transgenic plants. (A) Detached banana leaves derived from greenhouse maintained transgenic (W2, W3, W8 and W22) and control plants (UC) after exposure to simulated salt stress (350 mM NaCl in 1/10 MS basal medium for 7 days). (B) Photosynthetic efficiency (measured as Fv/Fm ratio) of untransformed and p1301-*MusaWRKY71* transgenic leaves exposed to salt. (C) MDA levels in untransformed and p1301-*MusaWRKY71* transgenic leaves exposed to salt.

3.3.9 Differential expression of putative MusaWRKY71 target genes in *MusaWRKY71* overexpressing lines

Although *MusaWRKY71* is induced in response to elicitor molecules of biotic stress response pathways like ethylene, salicylic acid and methyl jasmonate, the *MusaWRKY71* overexpressing plants were found to be equally susceptible to the infection of *Fusarium oxysporum* f. sp. *cubense* as the untransformed control plants in preliminary test assays. Thus, to gain clues for understanding the role of *MusaWRKY71* in biotic stress responses of banana plant, we attempted to identify those defense related genes whose expression is modulated by *MusaWRKY71* overexpression. Among the most probable downstream targets of WRKY proteins are other *WRKY* genes (*WRKY* genes are known to be cross regulated by other members of the wider *WRKY* gene family in a plant species), *PR protein* genes, *NPR1* genes and *chitinase* genes. We studied the differential regulation of these gene families in *MusaWRKY71* overexpressing plants by using specific primers in quantitative RT-PCR reactions (Figure 3.12). Among the 67 *WRKY* genes analyzed, differential expression was observed in six genes. Whereas the expression of GSMUA_Achr4G02800_001 and GSMUA_Achr10G06050_001 was upregulated respectively by 2.45 and 1.74 folds, the expression of GSMUA_Achr7G14140_001 GSMUA_Achr7G25400_001, GSMUA_Achr4G07230_001 and GSMUA_Achr4G03660_001 was down regulated respectively by 1.47, 1.71, 0.97 and 1.67 folds. Similarly, among the 20 *PR protein* genes tested, three genes were found to be up regulated in *MusaWRKY71* overexpressing plants. The expression of GSMUA_Achr6G17070_001, GSMUA_Achr4G23100_001 and

GSMUA_Achr2G13240_001 was respectively up regulated by 1.64, 1.9 and 2.9 folds. Further, among the 26 chitinase genes tested the expression of only GSMUA_Achr3G26900_001 was found to be upregulated by 1.6 fold in *MusaWRKY71* overexpressing plants. No difference in expression of *NPR1* genes was noticed between the *MusaWRKY71* overexpressing and untransformed control plants. Further, all the genes wherein differential expression was noticed had WRKY protein binding W-box like sequences [(C/T)TGAC(T/C)] in their 1 kb proximal promoter regions.

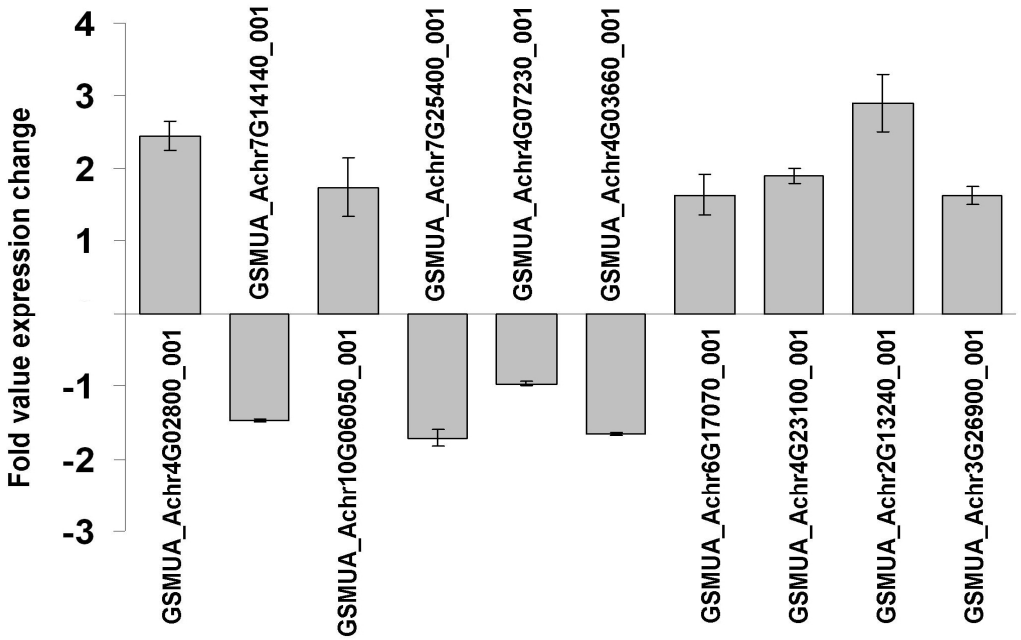

Figure 3.12 Differential regulation of putative MusaWRKY71 downstream genes in *MusaWRKY71* overexpressing plants. Six *WRKY* genes (GSMUA_Achr4G02800_001, GSMUA_Achr7G14140_001, GSMUA_Achr10G06050_001, GSMUA_Achr7G25400_001, GSMUA_Achr4G07230_001, GSMUA_Achr4G03660_001), 3 *PR protein* genes (GSMUA_Achr6G17070_001, GSMUA_Achr4G23100_001, GSMUA_Achr2G13240_001), and 1 *chitinase* gene (GSMUA_Achr3G26900_001) showed differential expression in the transgenic plants. All gene expression values have been normalized against banana *EF1α* cDNA expression levels. The x-axis represents the expression level of *MusaWRKY71* in control conditions. Values are mean ± SE.

3.4 Discussion

WRKY proteins are important transcription factors taking part in diverse plant processes and stress response pathways. *Arabidopsis* and rice have served as the focus plants for studies relating to the functions of *WRKY* genes. In spite of the enormous economic importance associated with banana, very few studies have been carried out to document the role of various transcription factors in biotic and abiotic stress responses of banana. In the present study, we isolated a novel WRKY transcription factor from a robust edible cultivar of banana namely banana cv. *Karibale Monthan*. This novel WRKY transcription factor named MusaWRKY71 based upon its high similarity with WRKY71 proteins of related monocots species like rice, wheat and maize contains a single WRKY domain along with defined nuclear localization signals. Further, nuclear localization of MusaWRKY71 has been established by making a MusaWRKY71::GFP fusion protein and demonstrating its presence in the nuclei of banana cells. Southern blotting of the restricted genomic DNA indicated that the *MusaWRKY71* gene was present in a single copy in the banana genome.

As is true with most of the members of *WRKY* gene family, *MusaWRKY71* was found to be inducible in response to a variety of abiotic and biotic stress conditions. *MusaWRKY71* mRNA transcripts were found to be induced in cold, salt and dehydration stress. Presence of several low temperature response element binding sites (CCGAC) and GATAT-motif (demonstrated to be present in majority of *Arabidopsis* promoters which are long term up-regulated in response to cold stress) in the 5' proximal region of *MusaWRKY71* gene also point towards the same. These

findings are in conformity with the induction of *Hv-WRKY38* gene in barley in response to cold and dehydration stress [89]. Several of the soybean WRKY proteins studied (including *GmWRKY6, GmWRKY17, GmWRKY21, GmWRKY41*, *GmWRKY54* and *GmWRKY27*) were found to get induced in response to salt or cold stress [90]. Further, the significance of this induction was demonstrated when *GmWRKY21*- transgenic *Arabidopsis* plants were shown to be tolerant to cold stress and *GmWRKY54*- transgenic *Arabidopsis* plants demonstrated to be tolerant to salt and drought stress. *MusaWRKY71* also gets induced in both roots and leaves in response to external ABA application. Presence of a single ABA response element binding site (CACGTG) in 5' flanking region of *MusaWRKY71* gene can be directly correlated with ABA inducibility of *MusaWRKY71* gene. ABA mediated induction is important as several ABA inducible proteins have been shown to confer abiotic stress tolerance in transgenic plants overexpressing them [91]. Reactive oxygen species like H_2O_2 have been demonstrated to influence the expression of many plant genes to help the plant to cope with adverse environmental changes [92]. Treatment of banana plantlets with H_2O_2 resulted in an increase in the expression level of *MusaWRKY71* indicating the overlap and convergence of various stress response pathways generating reactive oxygen species in banana cells. Salicylic acid, jasmonic acid and ethylene constitute three important signaling molecules taking active part in plant biotic stress responses [93]. In most cases, salicylic acid mediates the signaling cascade associated with responses against biotrophic agents and the establishment of systemic acquired resistance [94] whereas the jasmonic acid and ethylene associated cascade is involved in responses towards insect herbivores, wounding and

necrotrophic pathogens [95]. The final biotic defense responses reflect complicated positive or negative interactions between the two abovementioned cascades. As *MusaWRKY71* is induced in response to all these three signaling molecules in both leaves and roots, it can be presumed to play an important role in biotic responses of banana plant. Furthermore, the high homology of MusaWRKY71 with other defense related WRKY71 proteins gives an indication towards similarity in functional roles also.

When *MusaWRKY71* was overexpressed in transgenic banana plants using a constitutive promoter, transgenic banana grew normally in vitro in the presence of hygromycin and no gross abnormalities or stunting effects were noticed. Leaves derived from these transgenic plants performed better in the abiotic stress assays as demonstrated by lower phenotypic damage and positive membrane damage and photosynthetic efficiency parameters as compared to controls. When these plants were subjected to Fusarium wilt assay by treating the roots of the plants with a pathogenic isolate of *Fusarium oxysporum* f sp *cubense*, we did not observe any difference between the untransformed controls and the four transgenic lines indicating that overexpression of *MusaWRKY71* was not sufficient to modulate the plant defenses to achieve Fusarium tolerance. Failure to observe any difference with respect to biotic stress exerted by Fusarium, and our previous observation regarding the salicylic acid, methyl jasmonate and ethephon inducibility of *MusaWRKY71* led us to look for differential expression of genes which are known to be involved in biotic stress response pathways. Among the genes which are known downstream targets of WRKY proteins, genes of *WRKY* [82], *PR proteins* [96], *chitinases* [97] and

NPR1 proteins [98] have been studied in detail in relation to biotic stress response pathways. Our results showing differential expression of a total of 10 genes out of a total of 122 tested from the above four groups indicated that although *MusaWRKY71* by itself is unable to prevent a fatal biotic interaction, but it still controls the expression of other genes related to biotic stress. Together with the fact that *MusaWRKY71* overexpressing plants were tolerant towards oxidative and salt stress, the differential expression of several biotic stress related genes pointed towards a role for this protein in the interface between the abiotic and biotic stress response pathways of banana plant.

Over the last decade, several studies detailing overexpression and down regulation of select specific *WRKY* genes have been conducted in different plant species. *OsWRKY72* overexpressing plants were retarded and displayed early flowering as well as reduced apical dominance along with an enhanced gravitropism response [99]. Further, the plants were more sensitive towards salt and osmotic stress. *Arabidopsis* ABO3, a WRKY transcription factor was found to mediate plant responses to ABA and drought tolerance, as an ABA overly sensitive mutant (*abo3*) was hypersensitive to ABA in seedling establishment and growth [100]. *CaWRKY1* overexpressing transgenic plants showed heightened hypersensitive cell death in response to tobacco mosaic virus and *Pseudomonas syringe* pv. *Tabaci* [101]. *CaWRKY1* was in fact observed to act as a regulator to turn off systemic acquired resistance once the pathogen challenge has diminished. Overexpression of *OsWRKY89* in transgenic rice increased lignification and reduced internode length [102]. It also enhanced resistance to the rice blast fungus and the tolerance to UV-B

irradiation. Constitutive overexpression of *VvWRKY2* in transgenic tobacco plants reduced the susceptibility towards different fungal pathogens indicating the role for *WRKY* genes in fungal tolerance in plants [103]. The varied phenotypes and physiological effects resulting from modulation of *WRKY* expression in transgenic plants showed the multiple roles of these proteins in plants. Further, in our study at least one gene from the three out of four groups of genes (*WRKY*, *PR proteins* and *Chitinases*) was found to be differentially regulated. But among the nine *NPR1* genes tested, none of the genes were found to have differential expression in the transgenic lines. In contrast, the rice plants overexpressing *OsWRKY71* (the closest homologue of *MusaWRKY71*) showed enhanced expression of *OsNPR1* and *OsPR1b* as well as improved resistance towards rice bacterial blight [104]. Thus, it appears that even among related monocot species like rice and banana, the regulation of defense signaling pathways involving *WRKY* and *NPR1* genes can be quite divergent.

In conclusion, *MusaWRKY71* is proposed to be an important member of the *WRKY* gene family in banana which has the capacity to cross regulate other *WRKY* gene members as well as other genes involved in biotic stress signaling pathways. Further, since overexpression of this gene provides the transgenic plants with an improved oxidative and salt stress tolerance, it may well be a significant link in the interface between abiotic and biotic stress pathways. Further studies involving microarray experiments based on the newly uncovered banana genome sequence are warranted in future to fully establish the multi faceted roles played by *MusaWRKY71* gene in banana stress responses.

Table 3.1 Primers used for studies on *MusaWRKY71*

Primers used in cloning and TAIL-PCR

Amplicon / Description	Primer sequence (5' to 3')
Forward primer for 3' RACE of *MusaWRKY71*	ATGGGGTCAAGTTGGTTGGAT
MusaWRKY71 full length CDS	ATGGGGTCAAGTTGGTTGGAT
	TTAGCTAGTTCGTAGGGGAAGC
MusaDHN-1 sequential nested primers for TAIL-PCR	CTTGTTCTCTTCGGTTGCCAGAAC
	AGCCGACGTTGAGGTCGAGA
	AGGGGCAATCCAACCAACTTGA
MusaWRKY71 full length CDS	TGACCATGGGGTCAAGTTGGTTGG
	GATACTAGTGCTAGTTCGTAGGGGAAGC A
Banana *actin* full length CDS	ATGGCTGACGGCGAGGATA
	TCAGAAGCACTTCATGTGGACAA
Arbitrary degenerate primers for TAIL-PCR	NTCGASTWTSGWGTT
	NGTCGASWGANAWGAA
	WGTGNAGWANCANAGA
	AGWGNAGWANCAWAGG
	TGWGNAGSANCASAGA
	STTGNTASTNCTNTGC
	GTNCGASWCANAWGTT
	WGCNAGTNAGWANAAG
	AWGCANGNCWGANATA

Banana *WRKY* genes and primers

Gene (banana genome notation)	Primer sequence (5' to 3')
GSMUA_Achr3G00510_001	AGCTTGGAGCACCTCGTCT
	GGAAGGAGGAGTTCGTGGAC
GSMUA_Achr3G13440_001	GATCGGCTTGGTTGGATCT
	GAGGTGTACTTCGCCGTGAT
GSMUA_Achr1G27980_001	CTGCTGCTGAGCTTTCGAG
	AGCTCGCTGAATTGCTTCTG
GSMUA_Achr10G01150_001	GCTGATGACGACTGGGATCT
	ACTCCACCTCGACTGTTGCT
GSMUA_Achr3G29310_001	GGAAGGAGTGGAAGACGCTAA
	AGAAGATGCTGTGGTTGAAGC
GSMUA_Achr3G09940_001	AGCCTCTGTCCACCAGAAAG

	GGAGTCTGCACCACCACTCT
GSMUA_Achr5G16460_001	ATTTAGAAGCCGAGCTGAACC
	TCGTGTTCTTCGGGTCGAT
GSMUA_Achr1G17160_001	CCTCGTCAGGTTCACAGGTC
	GGTCGTCGTTCTTCCTTCTCT
GSMUA_Achr11G08290_001	CCAATCCTCTCGTCCTGAAG
	TTGGAGGAGGAGATGTCTCAG
GSMUA_Achr4G01600_001	CAAACCTTGCCATAGCTGGA
	AGCTGAGGTCCTAGAGGAAGC
GSMUA_Achr1G04770_001	TCTCCTTCTACGCCGAGAGA
	ACTGAAGAGCTCGGTGATGC
GSMUA_Achr6G22160_001	TCATCAAAGCCCCGCAACT
	CTCGCTGTACTGCGTGAAGG
GSMUA_Achr5G16750_001	CAACTTCCTAGCGAGGACCA
	GATTGGGTTTGGAGCGTCT
GSMUA_Achr4G14270_001	TCAACGTCCGACACCAAATA
	TCAACCTCGCTTGTTGTCTG
GSMUA_Achr10G22020_001	AGTTACCCTCCCCTGAAGGA
	CCACCAAGAGCAAGTCGTC
GSMUA_Achr9G22950_001	GGAGGATGGACGACCAGAT
	CTCTACCGGGGGTACGAAG
GSMUA_Achr10G24080_001	AAGAGAACAGGAGGCTTACCG
	CGGTTCGCACATAAACCTTC
GSMUA_Achr5G09430_001	TCCTCCTCGAGCCCACTA
	GTCGAAGTATCCGGAGCTGA
GSMUA_Achr4G16840_001	GTGGATGAGCAAAACGAACC
	ACCATCTTCCGTAACGCTGT
GSMUA_Achr10G11580_001	GCGATCCAAGGAAGATGGTA
	TGATGTTGTTTGTGAAGAAACTGA
GSMUA_Achr4G13970_001	GCAGCAGCCTTTAGAAATCG
	ACCAAATTTCCTGCAGTCGT
GSMUA_Achr10G15290_001	CACGTTGGATCTCACCCAAA
	AAGTTCGGGTCGGCAGTTAT
GSMUA_Achr4G20600_001	TGACGGTTTCCAAGTTCAGG
	CCGAGACTCAGGGTCGTTG
GSMUA_Achr6G31840_001	CAGCACTCGCCAGAAGTTT
	ATGTCTCCCACCGTCAACA
GSMUA_Achr6G16760_001	AAATGGCATGACTGCTGCTAC
	ACCTCGCTTGTTGTTTGCAC
GSMUA_Achr5G04150_001	AGGCTTGACCAACCTTGCTA
	CTAGAGGTAGCCTGCTCATCATC
GSMUA_Achr7G00610_001	AGCGGGTCTAATGGTTCAAA
	GATGCTGATTGGTTGTTTCTGA
GSMUA_AchrUn_randomG17930_001	CCAAGTGCAACGTGAAGAAG
	TTTTCGAGAGTAGGGACGATG
GSMUA_Achr7G26450_001	AATCCTAACCTAGGCTCTCTGGA

	TCTGAGAAGAAGCTGCCATGT
GSMUA_Achr3G15690_001	CAGAACCGCCTACCTCCTC
	CCGAGGTAGTGGAAGCAGAA
GSMUA_Achr7G15930_001	GTTCATGTCGTCCGTCACC
	TCACCCGTGATTTCCTTCTC
GSMUA_Achr10G08720_001	CACTCCAACTCCTCCCAAGA
	AGCTGGCAACACTCCTATCC
GSMUA_Achr8G20660_001	GCTGGCCATGGAAGAGTTAG
	GATCAGATGATGCTGGTCCAC
GSMUA_Achr3G29850_001	ACCTTGGTGTTGGCATCAG
	GCGGCCGAATAGTACATATCA
GSMUA_Achr11G17620_001	TGACGCAGTCCGTGAACC
	GGACTTGGGAAGCTGAGGAG
GSMUA_Achr6G02330_001	AGCAGAAGCAGCAGCAAATC
	TCCAAAGTTGCTCCAGTGTG
GSMUA_Achr5G21910_001	CTTCGCTCAATGGAGCACCT
	CGCGAGGGTAAGACTCTTCG
GSMUA_Achr1G23560_001	CAACCTGGACTTCACCAACC
	ATCATCGAGGGATCGCTTCT
GSMUA_Achr9G21560_001	CCTTCACTTCCTCCAACAGC
	TTCATCGAGTCGGACAACTG
GSMUA_Achr7G24840_001	GAACCACCACCACAAGCAC
	GCGAACAAAGAGTCCTCCTC
GSMUA_Achr6G27090_001	GTAGTCGCAGGAGCAAGACC
	TCCCTCTCCCTCTTCTCTCC
GSMUA_Achr4G02800_001	CACACACCTAGTGGCACCTC
	TGCCGTGATCAAAAGAGAATAGT
GSMUA_Achr9G06750_001	GTGGTGATGGCGGTAAGAAC
	CCTTTCTTCCTCGGCTTGTT
GSMUA_Achr7G14140_001	CCTTCTCCTTCCCTTCGACT
	AATGATCTCGGTGAGGTCAGA
GSMUA_Achr4G14990_001	CAACAGTCAGAGGGAGCACA
	GCCGTGAATGAAGGAAGGA
GSMUA_Achr3G27880_001	GGTGTCGTCGGCCCATAAG
	TTTTTCCCGGTCCTTCCTGT
GSMUA_Achr10G06050_001	TGGCTGATCTGATATCCAAGG
	GCTTCGAAACTTTGGGCTTA
GSMUA_Achr5G04870_001	TTGACCCTCGACTTCACGA
	GAGGAGAGCGGAGGCTTG
GSMUA_Achr11G10010_001	CTCCAACACAGCACCCATC
	GGACCCAGTCCAGCCATAG
GSMUA_Achr8G10740_001	TGAACACGAAGGAGTTCCAA
	ACTCCTCCTTTCTTATCTCTGACG
GSMUA_Achr9G05460_001	CGAGGAGGAGGAAGAAGAGG
	ATTGCTGTTAGCCAGCCAAG
GSMUA_Achr8G19810_001	TCCTCTCCTGCCTCTGTCTC
	GCCCATGAAGAGCAGGTC
GSMUA_Achr6G05710_001	CCCGATGAGCACTTTGTGTA

	CCACTGAGTCGAGCATGAAG
GSMUA_Achr7G25400_001	TCATCAGAACCCTGCAACAG
	AAGGAAGGAACGATGTCCTG
GSMUA_Achr4G15540_001	CTGATCACCGAGGAGGAGTC
	GAACAGAGAGTCGTCGTCACC
GSMUA_Achr10G23420_001	CCATGGAGCACCTCATCAGT
	TGAAATCCACCGTCAACGTC
GSMUA_Achr4G07230_001	AGTAATTTGCCCTGTGGACTG
	TCCTAGGCTCAGTCAATGTCC
GSMUA_Achr6G13630_001	ACCGACGGACTGATGAACTC
	GCATCACCAAGTGGGTCTTT
GSMUA_Achr8G14610_001	GGTCGGATGCTCTTTTCGTA
	AACATGTGTGACGGATCCAA
GSMUA_Achr2G15200_001	CGTTCCCTGGTATGCCTGTA
	TTATTGGCGCTGCTCTTGTT
GSMUA_Achr7G19340_001	TGAGCACAACCATGGACATC
	TTAGTTTTGAGCCGGTGGAT
GSMUA_Achr3G05670_001	CTGATCTCACCGTCTCCAAGTT
	CGATCTTGCCGTTGGAGAT
GSMUA_AchrUn_randomG17570_001	CCAGATTCCGTCCACCTTT
	GTTGGCAGTGTCGGATGAC
GSMUA_Achr4G03660_001	AGACCCAAGACCGAGCTTTAG
	TCATGCCACTGTTGCTTCTC
GSMUA_Achr11G17950_001	CGCAATCTAAAGGAAGAGCCTA
	CTGGCTTTGTTTCGGTAAGC
GSMUA_Achr6G05880_001	GTCAAGGTTCTCAGGGTTGC
	TTCTACTGGCAGTGCTCCTG
GSMUA_Achr8G01730_001	GCAAGGATGAGAAGGACGAC
	CTGCCTCGGCAAGTATATGG

Banana *PR-Protein* genes and primers

Gene (banana genome notation)	Primer sequence (5' to 3')
GSMUA_Achr9T16540_001	GAAGGCGACGGCTCTGTT
	ACCGTCAGCACACATCCTC
GSMUA_AchrUn_randomT10460_001	GCGGGATGGACTTCTACGA
	CGTCGAAGGCGTAGCTGTA
GSMUA_Achr9T08440_001	GCAGCACATGGTGAAGACA
	GCGTGAGATGACTCCATCG
GSMUA_Achr6T17070_001	CCTCCGAACTATGATGTCGTC
	TCTGCAGGAAGGCGTTGTA
GSMUA_Achr3T08250_001	TCCGTAAGGCAGCTCAACTT
	CCGGGCAAGAACTTGTAGG

GSMUA_Achr3T08230_001	AAGGAGCGGCTGGACTTT
	ATGATTCCGGTCACCGTCT
GSMUA_Achr10T02850_001	AGGGTGCTACACGGAGGTT
	AGGTGCTTGTGCCATCATC
GSMUA_Achr6T31500_001	ATGCCGCCAACTTCGAGAT
	ACGTGGGGCTGAAGTCCATA
GSMUA_Achr10T11230_001	GGAGCTCCTCCGGACTATG
	CTCCTCCCATCTCCCGTAA
GSMUA_Achr7T21980_001	ACGATCCTGCCGAAGAGAG
	CGTCTCCTTCCTCGTCACC
GSMUA_Achr3T15660_001	CTCACGTAGATGGCCGTTC
	CTTGCCTTGTCCCCAGAAG
GSMUA_Achr2T08710_001	TTCTCAGGGGCGTTACGG
	TGGCAGAGGAGGAGTCGAA
GSMUA_Achr2T13210_001	CACCACCTTAGCGCAAAAC
	CGTCGATTCCGGTGTAGTC
GSMUA_Achr2T13220_001	GGTCGGCAAATTACGCTTC
	CGAAGAGGTTCTCCCCGTA
GSMUA_Achr5T27850_001	ATCCGACAGCCGTCGTTC
	TCCCAATCTCCTGGACGTG
GSMUA_Achr8T26540_001	ACGCTGGTAAATCGTCTCAAA
	GGAGAAGGAGGCGGTATCA
GSMUA_Achr4T23100_001	AGGTCCTCAAACCCAGCTT
	GCTGGTTGGCGTAATTCTG
GSMUA_Achr4T31970_001	CAATGCTCCGACACCGTCT
	AGCCGTAGGCTTGGCACT
GSMUA_Achr7T16510_001	CTCCTCCCCCTGCTCTTC
	TCGCCTGTCTGGCAGCTA
GSMUA_Achr2T13240_001	GGTGTGTGCTATGGCCTTG
	CCAGAAGATGTTCTCCCCATAA

Banana *chitinase* genes and primers

Gene (banana genome notation)	Primer sequence (5' to 3')
GSMUA_Achr7T20770_001	CAGCAAGACCTCCTGTGGAT
	GCTCCAGGTATTCTGGATGG
GSMUA_Achr9T02370_001	ATCGTGACGCAGAGCTTCTT
	CATCGCAATAGTCCCTCGAT

GSMUA_Achr6T21330_001	ATGAGACCGGACATTTCTGC
	GCGGTCTTGAAGGAGATGAC
GSMUA_Achr8T24270_001	ATGAGCCCAAGCCAAGACTA
	AGGCTGCTTCTTCTTCATCG
GSMUA_Achr3T14710_001	TCGTGCCATGAGGTCATTATC
	TAAAGAGATCGCCGATAGCAGT
GSMUA_Achr6T36390_001	GAAACTGGAAGCCCACAAAG
	GATGGATTGAAAGGCACCTG
GSMUA_Achr3T06330_001	CCACCAAGAACGACACATTG
	GATGCGGTAGAAGGATCGAA
GSMUA_Achr3T26910_001	CACGAAGAAGAGGGAGATCG
	CGGCCCGTAGTTGTAGTTGT
GSMUA_Achr3T25100_001	CTCGGTAAGGCTCTCCATTC
	ATCCAGCTGAGAGGAGCATT
GSMUA_Achr5T19050_001	ATGGGAGTTTGGGAGACAAC
	GACCGTAGGTTCCGAAGTGT
GSMUA_Achr8T23500_001	CACCTCGGTCTCTTCTTTCC
	AGATCAACCTTCAGCCCATC
GSMUA_Achr8T16460_001	GATCTGTGGGTGAGCTTTGA
	CCTGTGGTGCTACAGAAGGA
GSMUA_Achr1T07320_001	CCCGGCAAGAAGTACTATGG
	CACTCCAACCCTCCGTTAAT
GSMUA_Achr6T30280_001	AGACGTGGAAGCTCAAGGAC
	CCGCTGGGCATAGTTTATCT
GSMUA_Achr8T27900_001	TGGGACCAAGGTGTACCTCTC
	CGTAGTTGGACGCAGTCTTGA
GSMUA_Achr8T27880_001	GCGAAGCAGCTCTACGACTT
	AGCACCTGGTTGGTGAGGT
GSMUA_Achr8T32980_001	AGGTGTTCCTCGGACTCACT
	GACGACGCCACCAAAGTAG
GSMUA_AchrUn_randomT11990_001	AGCCAAGACTACTGCGATCC
	ATCATGAGCTGAAGGCTGCT
GSMUA_Achr9T25580_001	TATGGGCAGGTCATCGACTA
	GAAGAGTCTGCAGCCCAGAT
GSMUA_Achr3T26900_001	TCTGGATGACTCCTCAGTCG
	TGGCTGTAGCAGTCCAAGTT
GSMUA_Achr9T16770_001	TCAGATCTCCCATGAGACCA
	CCACTGTTTCCGGATTCTTC
GSMUA_Achr7T23350_001	CGGATACCAGGAGGCATTT

	CGAGCAAGAAGGCTTGAGAT
GSMUA_Achr8T27910_001	CTACGTGTGGGTGCAGTTCT
	AACACTGCTCTTCACTTTCTCG
GSMUA_Achr3T26890_001	TCTGGATGACTCCTCAGTCG
	AATGTGGCTGTAGCTGCTGT
GSMUA_Achr3T22670_001	AGCAACAGAGGCAAGAAGGT
	GGCAGTACGTGCGATATGAG
GSMUA_AchrUn_randomT12350_001	CTCATGCATTGGCGTCTACT
	GCAGAACCACCCAAGAAACT

Banana *NPR1* genes and primers

Gene (banana genome notation)	Primer sequence (5' to 3')
GSMUA_Achr3T25880_001	CATTTCTTCCCACGGTGTTC
	CAGCCTTGCTCCTAACTGCT
GSMUA_Achr7T09190_001	GAACAGTGGAACTCGGGAAG
	TACAATGCCGACCGATGTT
GSMUA_Achr6T00950_001	GGCACGAATGAGAGCACTTT
	ACAAGGCAGACCTGTCGAAT
GSMUA_Achr2T16670_001	CTAGAACAGTGGAACTTGGGAAG
	CCCTACAGACGTCGATGATG
GSMUA_Achr4T32060_001	CGGCGATATTTTCCTCACTG
	CTTGCAAACGTGTGATGCTT
GSMUA_Achr8T05490_001	GCAACTTGGGATGCATTTCT
	TAGAAGACGAAGCGGCAGAG
GSMUA_AchrUn_randomT01860_001	CCAGGATGGAAGCTCTATCAA
	GATGAAGCAGAGGCAGAACC
GSMUA_Achr5T16220_001	TGACGTCCGACTTCCTCTTC
	CCACCACTTGTGTGATTTGC
GSMUA_Achr5T02120_001	CGCTATTTTCCCAACTGTTCA
	AATCCTTAGGCAGCGATGAG

Chapter 4

Studies on *MusabZIP53,* a bZIP transcription factor gene from banana

4.1 Introduction

Being sessile organisms, plants are continuously exposed to different abiotic stresses like as high salt, cold and drought which in effect seriously impact the optimal plant growth and developmental processes. In order to respond to these challenges to their well being, plants have developed multiple mechanisms which work at physiological, cellular and molecular levels and enable the plant to tide over these adverse environmental conditions [73]. Plant hormone abscisic acid (ABA) plays an especially vital role in the signaling processes associated with these abiotic stresses in plants. Generally when a plant encounters adverse environment conditions, ABA levels increase and this leads to a chain of signal transduction events wherein multiple pathways are modulated to finally reorient the internal milieu of a plant cell such that it now has enhanced capacity to overcome the stress period. Most genes which are observed to respond to drought, high salt and cold stress treatments are also up regulated by exogenous ABA application [105]. On the other hand, there are other genes which have been observed to get induced under abiotic stress conditions but do not respond to exogenous ABA treatments [106]. The above said two groups of genes are therefore recognized as being part of either ABA-dependent or ABA-independent stress response pathways in plants. Concurrently, promoter analyses of common abiotic stress inducible genes in different plants systems have led to the identification of two major cis-acting elements: the ABA-responsive element [(C/T)ACGTGGC] which is recognized by ABA-dependent mechanisms [24] and the DRE/CRT element

[A/GCCGACNT] which is recognized by ABA independent framework [107]. Modulation of gene expression through ABA-responsive elements is predominantly regulated by basic leucine zipper protein (bZIP) transcription factors [106]. bZIPs constitute a large family of transcription factor proteins having a highly conserved bZIP domain composed of two invariate structural features: a basic domain (rich in basic amino acids) required for sequence specific DNA binding, and the leucine zipper domain which contains at least 3 - 4 repeats of leucine at every 7^{th} position (heptad leucine repeat) that mediates dimerisation through hydrophobic bonding [108]. Most bZIP proteins can form homodimers and are able to bind DNA having ACGT core sequence. Outside of this bZIP domain, bZIPs also contain other regions which may work as transcriptional activators and these include proline-rich, glutamine-rich and acidic domains [109]. bZIP proteins are spread throughout the plant kingdom where they are involved in a multitude of physiological processes including but not limited to seed maturation and germination [110], photomorphogenesis and light signaling [111], flower development and fertility [112], senescence [113] and abiotic stress responses [114,115].

In *Arabidopsis* and rice respectively, 61 and 84 members of *bZIP* gene family have been identified since the completion of their genome sequences. In contrast, 123 *bZIP* like genes have been identified in banana genome [88]. Despite its status as one of the most important food and fruit crops of the world, research in banana stress response networks has lagged way behind comparable species like rice. In the last decade however, several concerted efforts have been made to understand the stress response regulons of banana. One such study reported stress-inducible expressed

sequenced tags (ESTs) from the stress tolerant Brazilian banana variety named 'Cachaco' (http://www.ncbi.nlm.nih.gov). Realizing the important role played by bZIP proteins in abiotic stress pathways in well studied plants like rice and *Arabidopsis*, a putative *bZIP* gene was identified from the 'Cachaco' EST library (Genbank accession no. DN239778) based on the assumption that comparative analysis of stressed and non-stressed tissue derived EST data sets can provide reasonable prediction about differential expression of moderately abundant ESTs [80].

Close homologs of this putative *bZIP* transcription factor gene have been documented to be stress-inducible in related plant species and in several cases overexpression of these *bZIP* genes have led to improved abiotic stress tolerance in transgenic plants [27]. Further, specific *bZIP* genes have also been documented to be involved in sugar regulated transcriptional control of multiple metabolic enzymes in plant cells [116]. This sucrose mediated control is exerted through an untranslated ORF (uORF) present in the 5' UTR sequence of the specific *bZIP* genes [117]. Apart from definite involvement in abiotic stress response pathways and sugar signaling in plants, specific bZIP genes have been documented to play important roles in plant senescence [113]. Since plant senescence is generally related with browning of the ageing plant tissue, polyphenol oxidases (PPOs) have been proposed to play important roles in these senescence processes [118]. PPOs are especially important in context of banana fruit where any level of chilling injury imparts a PPO mediated browning and undesirable odour [119]. Induction of *bZIP* coding genes in response to cold stress together with their involvement in plant senescence points towards a

possible correlation between bZIP proteins and chilling induced PPO activity which needs thorough investigation.

As part of this doctoral study, detailed characterization of the identified *bZIP* gene was performed (Genbank accession no. DN239778) by profiling its expression in response to different abiotic stress conditions by northern blotting followed by its overexpression using a strong constitutive promoter in transgenic banana plants. Differential expression of several genes was observed in the transgenic banana plants. Further during the course of these overexpression studies, it was concluded that this *bZIP* gene takes part in sucrose homeostasis and exerts a coordinated control over multiple PPO coding genes in banana thereby establishing that this *bZIP* gene (*MusabZIP53*) is an important link between abiotic stress, ABA signaling and PPO activity.

4.2 Materials and methods

4.2.1 Primers

The primer sequences used in this study are listed in Table 4.1

4.2.2 Amplification and sequence analysis of *MusabZIP53* gene

Total RNA was extracted from young leaves of banana cv. *Karibale Monthan* by using Concert Plant RNA Reagent (Invitrogen, USA). This RNA was cleaned up and subjected to on column DNase treatment using RNeasy Plant Mini Kit (Qiagen, Germany). Approx. five µg of this RNA was utilized in synthesis of first strand cDNA using Oligo $(dT)_{12–18}$ primer (Invitrogen, USA) and AccuScript Reverse Transcriptase (Stratagene, USA). The full-length coding sequence of *MusabZIP53* gene was amplified from this cDNA using Pfu Ultra AD DNA Polymerase

(Stratagene, USA). Putative translated protein sequence for *MusabZIP53* cDNA was obtained using ExPASy translate tool (http://au.expasy.org/tools/dna.html). This predicted MusabZIP53 protein sequence was aligned with its closest homologs (determined by Blastp program of NCBI) by using ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and Box Shade server (http://www.ch.embnet.org/software/BOX_form.html). The conserved bZIP domain of MusabZIP53 protein was identified using ExPASy prosite server (http://au.expasy.org/prosite/). Evolutionary relationships for MusabZIP53 protein were determined using MEGA 5 software. Genomic context of *MusabZIP53* gene was analyzed using the banana genome database. Further, the 5' and 3' UTRs of *MusabZIP53* were predicted based on the banana EST and transcriptome datasets available in NCBI database.

4.2.3 Expression profiling of *MusabZIP53* in banana tissues under various stress treatments by northern blotting

In vitro plantlets of banana cv. *Karibale Monthan* were hardened in the controlled greenhouse for 2 - 3 months. Uniform sized banana plants with 4 - 5 healthy leaves were exposed to cold stress at 8 ± 2°C in a plant growth chamber set at 16 h light / 8 h dark regime. For imparting drought stress, plants were uprooted and washed to remove the attached soil and then left to dehydrate on blotting sheets in the greenhouse. For ABA treatment, plants were treated with 100 µM ABA solution. Plants exposed only to sterile water were taken as experimental controls. Leaf and root samples derived from treated plants were immediately frozen in liquid nitrogen and stored at -80°C freezer. Three independently samples for each treatment were

mixed uniformly before the RNA extraction. Total RNA was extracted from the treated tissues using Concert Plant RNA Reagent (Invitrogen, USA) and RNeasy Plant Mini Kit (Qiagen, Germany) as described before and subsequently resolved using a 1.2 % (w/v) FA-MOPS agarose gel. These RNA samples were allowed to slowly migrate in 1X MOPS buffer at an applied field strength of 4-5 V/cm for 2 hours. Subsequently, the RNA containing gel was washed twice with 20X SSC and then the RNA was allowed to be transferred onto positively charged nylon membrane by capillary transfer in 20X SSC buffer. RNA was subsequently cross linked to the membrane by baking at 120°C for 30 min. DIG-labeled DNA probes targeted against *MusabZIP53* cDNA sequence were used to probe the nylon membrane. Prehybridization was done at 60°C for 2 hours followed by hybridization with DIG-labeled DNA probes at 55°C overnight. After the hybridization, the nylon membrane was washed twice each with 2X SSC and 0.1% (w/v) SDS at room temperature and 0.1X SSC and 0.1% (w/v) SDS at 60°C. Chemiluminescent detection of hybridization signals was performed according to the manufacturer instructions (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Applied Science, Germany) in a chemiluminescent enabled gel documentation system.

4.2.4 Determination of cellular localization of MusabZIP53 protein

In order to study the localization of MusabZIP53 protein in transiently transformed banana cells, a *MusabZIP53::GUS* fusion cDNA was prepared using pCAMBIA-1301 as base vector. Firstly *MusabZIP53* cDNA without the native stop codon was amplified from banana cv. *Karibale Monthan* leaf derived cDNA. *Zea mays* polyubiquitin promoter along with 5'UTR was amplified from *Zea mays* genomic

DNA. These two fragments were then inserted into pCAMBIA-1301 vector using appropriate restriction enzymes in a three way ligation reaction such that *p*Zm*UBI-*MusabZIP53** replaced the CaMV 35S promoter in pCAMBIA-1301. The newly constructed vector p*Zm*UBI-*MusabZIP53*::*GUS*-1301 was electroporated into *Agrobacterium tumefaciens* strain EHA105 and then used to transform banana embryogenic suspension culture cells. Five days post cocultivation with *Agrobacterium*, the transiently transformed banana cells were stained with GUS buffer [47] for 1 hr at 37°C. Cellular localization of the MusabZIP53::GUS fusion protein in banana cells was photographed in a light microscope. Banana cells transformed with pCAMBIA-1301 unmodified vector were also stained with GUS buffer and later photographed as controls.

4.2.5 Construction of plant expression vector for overexpression of *MusabZIP53*

Plant expression cassette for overexpressing *MusabZIP53* in transgenic banana plants was assembled in the MCS of pCAMBIA-1301 plant expression vector. Initially, the nos 3' UTR amplified using pBI121 plant binary vector as template was inserted in *Sac*I and *Eco*RI restriction sites in MCS of pCAMBIA-1301. Later, the *Zea mays* polyubiquitin promoter amplified using *Zea mays* genomic DNA and the *MusabZIP53* cDNA amplified from the banana cv. *Karibale Monthan* leaf derived cDNA were inserted into the modified pCAMBIA-1301 expression vector by performing a three-way ligation reaction in which restriction sites specific for *Hin*dIII, *Pst*I and *Kpn*I restriction enzymes were utilized. The newly constructed binary vector was denoted as p*MusabZIP53*-1301. This expression vector, having the expression cassette [pZmUbi-*MusabZIP53*-nos] was later sequenced using

appropriate primers to confirm the sequence of the cloned *MusabZIP53* coding region. p*MusabZIP53*-1301 binary vector was the electroporated into *Agrobacterium tumefaciens* strain EHA 105 and subsequently used for transformation of banana embryogenic cells.

4.2.6 *Agrobacterium*-mediated genetic transformation of banana embryogenic suspension culture cells, generation of transgenic banana plants and their phenotypic assessment

Overnight grown culture of *Agrobacterium tumefaciens* strain EHA105 harboring p*MusabZIP53*-1301 binary vector was resuspended at an $OD_{600\ nm}$ of 0.1 in the morning and grown further till an $OD_{600\ nm}$ of 0.6 - 0.8 was obtained. This culture was centrifuged at 6,500*g* for 10 min and then the *Agrobacteria* were resuspended in M2 medium [48] added with 100 µM ACS. This bacterial suspension was utilized for transforming banana cv. *Rasthali* embryogenic suspension culture cells as described previously [49]. Five to seven days post-subculture cells (0.5 ml PCV) were sieved through a 85-µm sieve before being cocultivated with *Agrobacterium* for 30 minutes with intermittent shaking. These cells were then aspirated onto glass filter discs with the help of Buchner apparatus and then transferred on to semi-solid M2 medium added with 100 µM ACS. The plates containing the cells on glass filter discs were incubated in dark for three days at 25 ± 1^{o}C. After three days, the cells along with the filter discs were transferred to fresh semi-solid M2 medium supplemented with cefotaxime (400 mg l^{-1}). Three days hence the cells were removed from the filters and cultured on banana embryo induction medium supplemented with cefotaxime (400 mg l^{-1}) and hygromycin (5 mg l^{-1}). Embryo development could be observed in three to

four weeks and then the developing embryos were subcultured on the same medium for three subculture cycles of three weeks each along with hygromycin selection. Fully grown embryos were transferred to MS medium added with BAP (0.5 mg l^{-1}) for efficient germination. The germinated embryos were subsequently subcultured onto banana multiplication medium [50] to obtain multiple copies of the same transformed line. Individual shoot were isolated from the multiple shoot cultures and then transferred to MS medium supplemented with NAA (1 mg l^{-1}) for efficient root development. Multiple shoot cultures and the rooted plantlets obtained after transformation with p*MusabZIP53*-1301 were compared with equivalent controls to analyze the effects of overexpression of *MusabZIP53* on the growth and development of transgenic banana tissues. Photographic evidence of representative samples was accordingly recorded at all of these stages. The complete putatively transformed plantlets obtained were later hardened in the greenhouse using soilrite mix and then used for all the analysis.

4.2.7 Molecular analysis of transgenic banana plants

Genomic DNA isolated from leaves of different transgenic lines (using GenElute plant genomic DNA miniprep kit, Sigma, USA) was used as template in PCR reactions together with primers targeted against *hygromycin phosphotransferase* gene present within the two T-DNA borders of p*MusabZIP53*-1301 binary vector. Genomic DNA sourced from untransformed control banana plant was used as controls in these PCR reactions. Results obtained from genomic DNA PCR of the putatively transformed banana lines were further confirmed by Southern blot analysis. Approx. 20 μg of genomic DNA isolated from the leaves of four putatively

transformed lines and untransformed control plants was restricted with *Kpn*I restriction enzyme before being resolved in a 0.8 % (w/v) TAE agarose gel. This DNA was then transferred onto positively charged nylon membrane by capillary transfer using 20X SSC buffer. Subsequent to the cross linking of this DNA on to the nylon membrane by baking at 120°C for 30 min, DIG-labeled probes targeted against *hygromycin phosphotransferase* gene were used to probe the membrane. Stringency washes and chemiluminescent detection of hybridization signals was performed according to the manufacturer instructions (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Applied Science, Germany) in a chemiluminescent enabled gel documentation system.

Overexpression of *MusabZIP53* transcript in transgenic banana plants was confirmed by northern blotting. Total RNA isolated from transgenic leaves as well as from leaves of untransformed control plants were used for this northern blot which was done as described above for expression profiling of *MusabZIP53* gene in different abiotic stress conditions. Three independently grown leaf samples for each line were mixed uniformly before the RNA extraction.

4.2.8 Assessment of stress tolerance characteristics of *MusabZIP53* overexpressing lines

Leaf disc stress tolerance assays were performed to ascertain response of *MusabZIP53* overexpressing lines towards cold, drought and salt stress conditions. Leaf discs cut out of leaves derived from untransformed control plants and the four transgenic lines were exposed to cold (8°C) for 14 days, 250 mM mannitol for 1 week and 250 mM NaCl for 1 week in 1/10 MS media in a 16h light/ 8h dark regime. The

leaf discs were subsequently photographed to demonstrate and record the differential damage in the two groups of samples. Further, the level of stress damage was quantitatively estimated by determining the malondialdehyde (MDA) levels in the treated leaf discs. All the leaf discs experiments were performed in groups of five leaf discs and each treatment was repeated at least thrice.

4.2.9 Analysis of differentially expressed genes in *MusabZIP53* overexpressing banana plants

To determine the genes which are directly or indirectly regulated by *MusabZIP53* gene in banana plants, we identified genes coding for dehydration response element binding (DREB) proteins, late embryogenesis abundant (LEA) proteins, anti-oxidant enzymes, aquaporins, PPOs, and Aux/IAA proteins from banana EST and transcriptome databases maintained at NCBI and also from the recently released banana genome database. Several other putative *MusabZIP53* targets were identified based on previous studies wherein close homologs of *MusabZIP53* (*AtbZIP11*, *AtbZIP53*, *TBZ17*) were overexpressed in *Arabidopsis* and tobacco [116, 110, 117]. In total, 189 genes were identified based on the above criteria and subsequently specific primers for these genes were designed for use in real time quantitative RT-PCR reactions wherein leaf cDNAs derived from one of the *MusabZIP53* overexpressing transgenic lines (B4) and untransformed control plant were used as template. Three independently grown leaf samples for transgenic and untransformed plants were mixed uniformly before the RNA extraction. Total RNA extraction and cDNA preparation for these studies were performed as described before. REST-MCS software was used to calculate relative expression values for these genes [43] and

primers specific to banana *EF1α* gene [87] were used for normalization of expression values.

4.2.10 Analysis of sucrose content and sucrose mediated regulation of MusabZIP53 protein

Since the 5'UTR of *MusabZIP53* shows significant homology with an uORF documented to be involved in sucrose signaling in other plants, sucrose levels were estimated in leaves of *MusabZIP53* overexpressing plants using a modified anthrone method [120]. Herein, 500 mg of leaf tissue was first crushed under liquid nitrogen, followed by addition of 10 ml 80 % (v/v) ethanol to the crushed leaves. This mixture was collected in test tube and heated in a boiling water bath for 15 min. Subsequently, the mixture was made up to 50 ml with 80 % (v/v) ethanol. 1 ml of this solution was then added with 9 ml of freshly prepared Fehling's solution and heated in a boiling water bath for 30 minutes. Subsequently, 1 ml of this solution was added with 10 ml of freshly prepared anthrone reagent and incubated at 40°C for 30 minute. Finally the absorbance was read at 610 nm. A standard curve of sucrose was prepared using the same protocol to calculate the exact amount of sucrose in the transgenic and control leaves. Sucrose estimation was repeated thrice with independent samples for each line and untransformed control plant.

To validate the role of the conserved uORF in sucrose signaling, we gave sucrose starvation treatment to banana embryogenic cells for 24 hours followed by isolation of total RNA and cDNA preparation as described before. Expression levels of two genes (coding for an aspargine synthetase and a PPO enzyme) which were shown to be highly induced in *MusabZIP53* overexpressing transgenic banana plants

were checked before and after the sucrose starvation treatment to ascertain the effect of sucrose starvation on the translation level of MusabZIP53 protein in banana cells.

4.2.11 Analysis of PPO gene transcription in abiotic stress and protein activity

Since multiple PPO coding genes were shown to be up regulated in *MusabZIP53* overexpressing transgenic plants, possible correlation between cold stress, ABA treatment, *MusabZIP53* up regulation and PPO gene induction was investigated by studying the expression pattern of the four PPO coding genes (up-regulated in *MusabZIP53* overexpressing transgenic banana plants) in response to ABA treatment (100 μM for 24 hrs) and cold stress (8°C for 24 hrs). Total RNA isolated from the leaves of these treated plants was used to make cDNA as described above and this was then used in real time quantitative RT-PCR reactions using primers specific for the four PPO coding genes mentioned above. Calculation of relative expression values and banana *EF1α* gene based normalization of expression values was performed as described above. Further, PPO activity in the leaves of *MusabZIP53* overexpressing transgenic banana plants together with untransformed controls was determined using catechol as a substrate as described before [121]. Also, PPO activity in ABA treated and cold stressed leaves of control banana plant was determined similarly. PPO activity estimation was repeated thrice with independent samples for each line and untransformed control plant and also for cold and ABA treatment.

4.3 Results

4.3.1 Amplification and sequence analysis of *MusabZIP53* gene

A novel *bZIP* transcription factor gene was identified from banana EST library (Genbank accession no. DN239778) based on its differential abundance in contrasting

sets of abiotic stress tissue derived EST sequences. Full length coding sequence of this gene was amplified from banana cv. *Karibale Monthan* leaf derived cDNA and then sequenced. Since *AtbZIP53* is the closest homolog of this gene among all *Arabidopsis bZIP* genes, it was named as *MusabZIP53* to reflect this homology. Full length coding sequence of *MusabZIP53* consists of 435 nucleotides. A 493 bp long 5' UTR and a 225 nucleotide long 3' UTR of *MusabZIP53* gene were also identified after analysis of several *MusabZIP53* homologous sequences sourced from EST and transcriptome libraries of banana maintained at NCBI (Figure 4.1). *MusabZIP53* cDNA encodes a protein consisting of 144 amino acids, having a molecular weight of 16.46 kDa and a theoretical pI of 8.11. The bZIP domain of MusabZIP53 protein, similar to its close homologs, possesses a basic domain which is rich in basic amino acids and a leucine zipper domain having heptad leucine repeat. Multiple sequence alignment of MusabZIP53 protein sequence with its closest homologs indicated that apart from the respective bZIP domains, these homologs share very little homology with each other indicating specific function for each of these proteins in the respective species (Figure 4.2 and 4.3). *MusabZIP53* gene and all the close homologs have long 5' UTRs. Investigations in close homologs like *AtbZIP53* have revealed the presence of conserved uORFs in these long 5' UTRs [117]. In fact, a highly conserved uORF was identified in the 5' UTR of *MusabZIP53* gene also. The significance of this uORF in context of regulation of MusabZIP53 activity is dealt with later. Further, no intron was identified in either the coding or untranslated regions of *MusabZIP53* gene when its mRNA sequence was compared with its

genomic complement available in the banana genome database (locus identifier GSMUA_Achr11T05140_001).

gtggcttctggtttatccttggctggggtccttcttaggatacacctccgggtttaaaatct

gatctttcggggttgattttggcttttgggttgttgtcttttagccccgatcctcagaat

ccgatctttggaatcgacccttccgtttagagagcttgcgcttctcaaaatctgttattt

ggaggccaatcttttcttgaaagatctgttatttcggtgttggtctttgttcttgcaagt

aattagaggggctgatgatcttgatgttttcgtctctaattctgtcgaaaaccagtttc

taccctttatgaatcttgctactctcagcgtctgcttcctccactccttctccgtggtcc

tcctctactggttctatgtgatctcatgaactaagtctctttgcgtgatccagtcatttc

atttcatttttttccgttcaaaccaatttctattagcttagaattagagaacctcccctg

*ctaaagagt*atgtcttccattccggtccgccgcgcttcgagttctgaaggagactcgcag

M S S I P V R R A S S S E G D S Q

ccgacgtccgatgagaggaagagaaagaggatgatttcgaacagggagtccgcaagaagg

P T S D E R K R K R M I S N R E S A R R

tctaggatgaggaagcagcagcatctcgatgatctgataaaccaagccgagcagctcaag

S R M R K Q Q H L D D L I N Q A E Q L K

aaccaaaacagccagatcgacgtgcagatcaatctggcgacacagcagtacgtcaaggtg

N Q N S Q I D V Q I N L A T Q Q Y V K V

gaatctgagaacgctattctaagggctcagctgagtgaattgacagagagactgcactcg

E S E N A I L R A Q L S E L T E R L H S

atcaactctgttctccgtttcattgaggaggtcagtggaatggccatggacataccggag

I N S V L R F I E E V S G M A M D I P E

ataccagatcctctcctgaagccattgcagcttccccgtgcggcacaaccaatcatggcc

I P D P L L K P L Q L P R A A Q P I M A

aatgctgacatgttgcagttctgaaatgtcattgttgtgtaaggtctatctagcatgtgt

N A D M L Q F -

ctccattgattgggctattagttccttgttttgctgcttatgctttcatcttgttgagtc

gcagtaaattagcttttctttggttcgagtcagtaagtgatgtacggttgtgtgcttat

tgtaattttacctttcagcaaatgttgctttggctgctcaaaaaaaaaaaaaaaaaaaa

aaaaaaaaa

Figure 4.1 Sequence analysis of *MusabZIP53* gene isolated from banana cv. *Karibale Monthan*. mRNA sequence of *MusabZIP53* along with its predicted protein sequence. Note the unusually long 5' UTR**.**

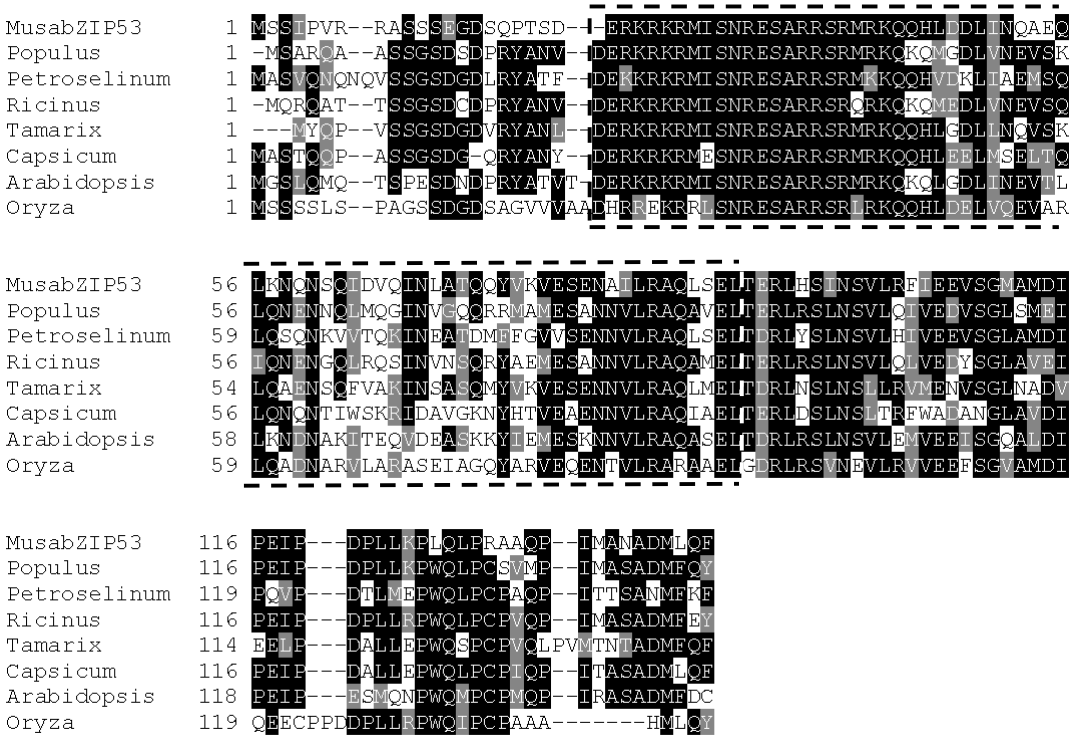

Figure 4.2 Alignment of MusabZIP53 protein sequence derived from cv. *Karibale Monthan* with closely related sequences. Sequences from *Populus trichocarpa* (EEE80784), *Petroselinum crispum* (CAC00657), *Ricinus communis* (EEF47269), *Tamarix hispida* (ACN71235) and *Capsicum annuum* (ABB17073), *Arabidopsis thaliana* (NP_191801) and *Oryza sativa* (BAE16260) have been used. The bZIP domain is boxed and shows relatively high homology as compared to the flanking regions.

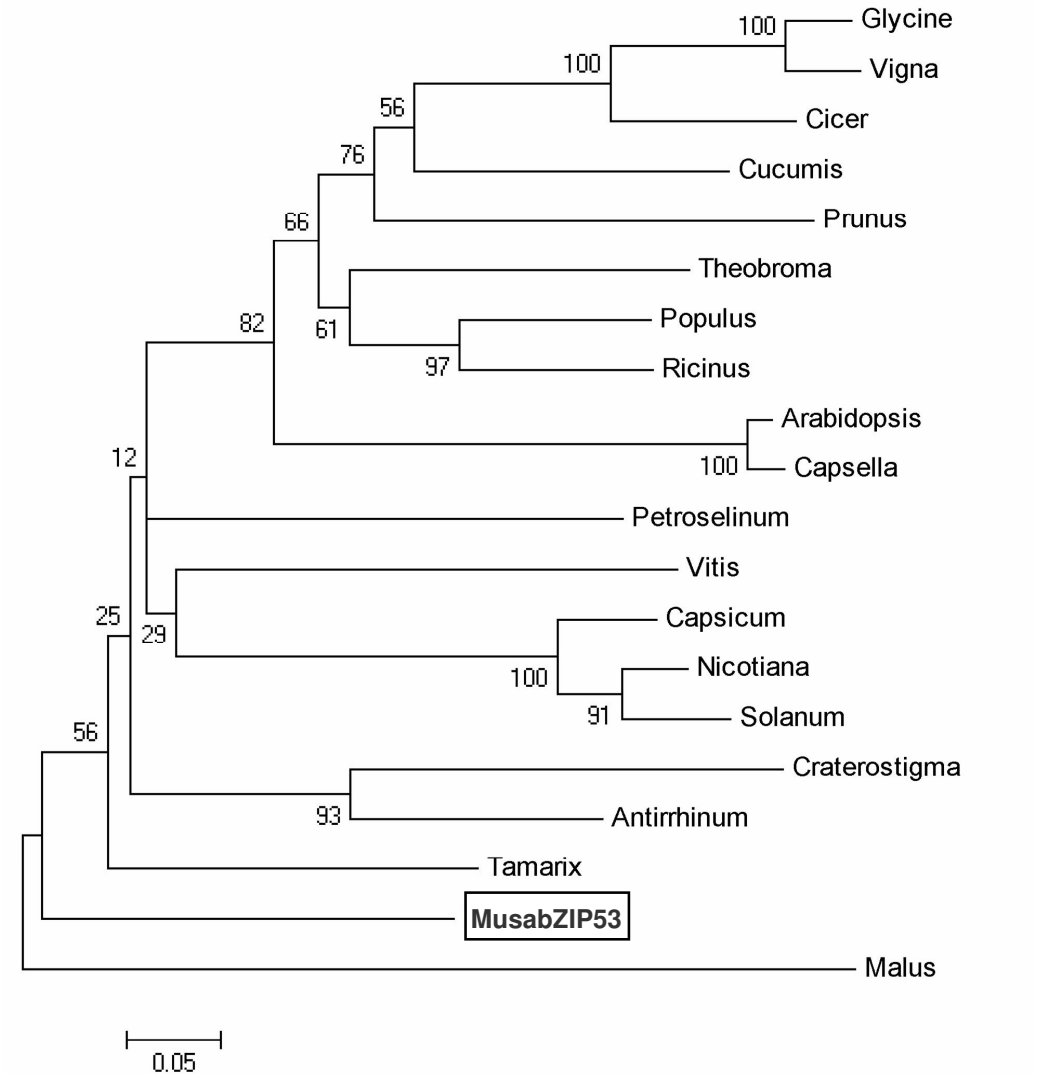

Figure 4.3 Phylogenetic relationship of MusabZIP53 derived from cv. *Karibale Monthan* with other closely related bZIP sequences. Sequences from different plant species namely *Populus trichocarpa* (EEE80784), *Petroselinum crispum* (CAC00657), *Ricinus communis* (EEF47269), *Tamarix hispida* (ACN71235), *Capsicum annuum* (ABB17073), *Nicotiana tabacum* (AAK92214), *Arabidopsis thaliana* (NP_191801), *Theobroma cacao* (EOX95259), *Capsella rubella* (EOA25509), *Solanum lycopersicum* (NP_001234339), *Cicer arietinum* (XP_004494422), *Vitis vinifera* (XP_002276485), *Cucumis sativus* (XP_004152226), *Craterostigma plantagineum* (AAZ72654), *Glycine max* (NP_001237222), *Malus domestica* (ADL36616), *Antirrhinum majus* (CAA74022), *Vigna unguiculata* (BAM93582), *Prunus persica* (EMJ03088) have been used. This bootstrapped tree with 1,000 replicates was constructed using ClustalW2 and MEGA 5 tools.

4.3.2 Expression profiling of *MusabZIP53* in banana tissues under various stress treatments by northern blotting

Since *MusabZIP53* was initially identified from the banana EST library based on its differential abundance in abiotic stress tissue derived contrasting EST datasets, northern blotting was performed to confirm its abiotic stress inducibility. *MusabZIP53* was found to be strongly induced in cold and drought stressed banana leaves as well as roots (Figure 4.4 a and b). This in turn validated the assumption that differential abundance in abiotic stress tissue derived contrasting EST datasets indicates stress responsive behavior of a particular gene. Further, the northern blots also indicated that the steady state levels of *MusabZIP53* gene in control untreated conditions were quite low as compared to its abiotic stress induced levels. To confirm the involvement of ABA in the cold and drought induced expression of *MusabZIP53*, we checked the expression levels of *MusabZIP53* in ABA treated leaf and roots of banana. ABA treatment strongly induced the levels of *MusabZIP53* transcripts in banana tissues indicating that the cold and drought inducibilty of *MusabZIP53* is probably ABA dependent (Figure 4.4 c).

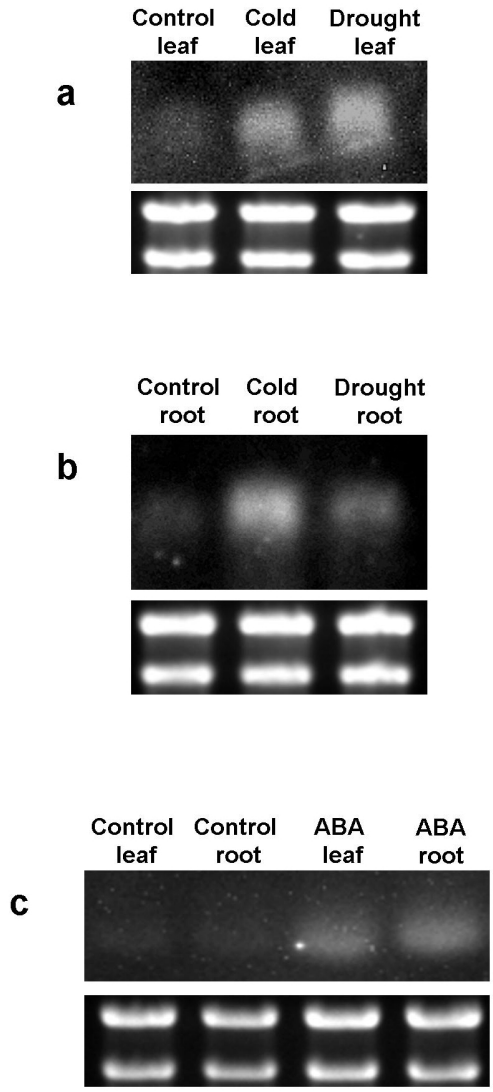

Figure 4.4 Expression profiling of *MusabZIP53* in cv. *Karibale Monthan* under different abiotic stress stimuli. Northern blotting was performed using total RNA isolated from root and leaf of cold (8 ± 2°C) and drought stressed banana plants. *MusabZIP53* was found to be induced in both leaf (a) and root (b) tissues of stressed plants. Similarly, *MusabZIP53* transcript was found to be induced in both leaf and root of banana plants treated with 100 µM ABA (c). rRNA bands have been shown to depict equal loading of total RNA in each lane.

4.3.3 Determination of cellular localization of MusabZIP53 protein

To confirm the nuclear localization of MusabZIP53, an expression cassette comprising of *pZmUbi-MusabZIP53*::*GUS*-nos (p*Zm*UBI-*MusabZIP53*::*GUS*-1301) was prepared in pCAMBIA-1301 wherein the *MusabZIP53* coding sequence without stop codon and GUS coding sequence were fused in frame. When this construct was transformed into banana embryogenic cells, GUS staining was clearly localized to nuclear regions in cells transformed with p*Zm*UBI-*MusabZIP53*::*GUS*-1301 whereas in pCAMBIA-1301 transformed cells the GUS staining was distributed uniformly in the whole cytosol indicating that the MusabZIP53 protein is nuclear localized in banana cells (Figure 4.5).

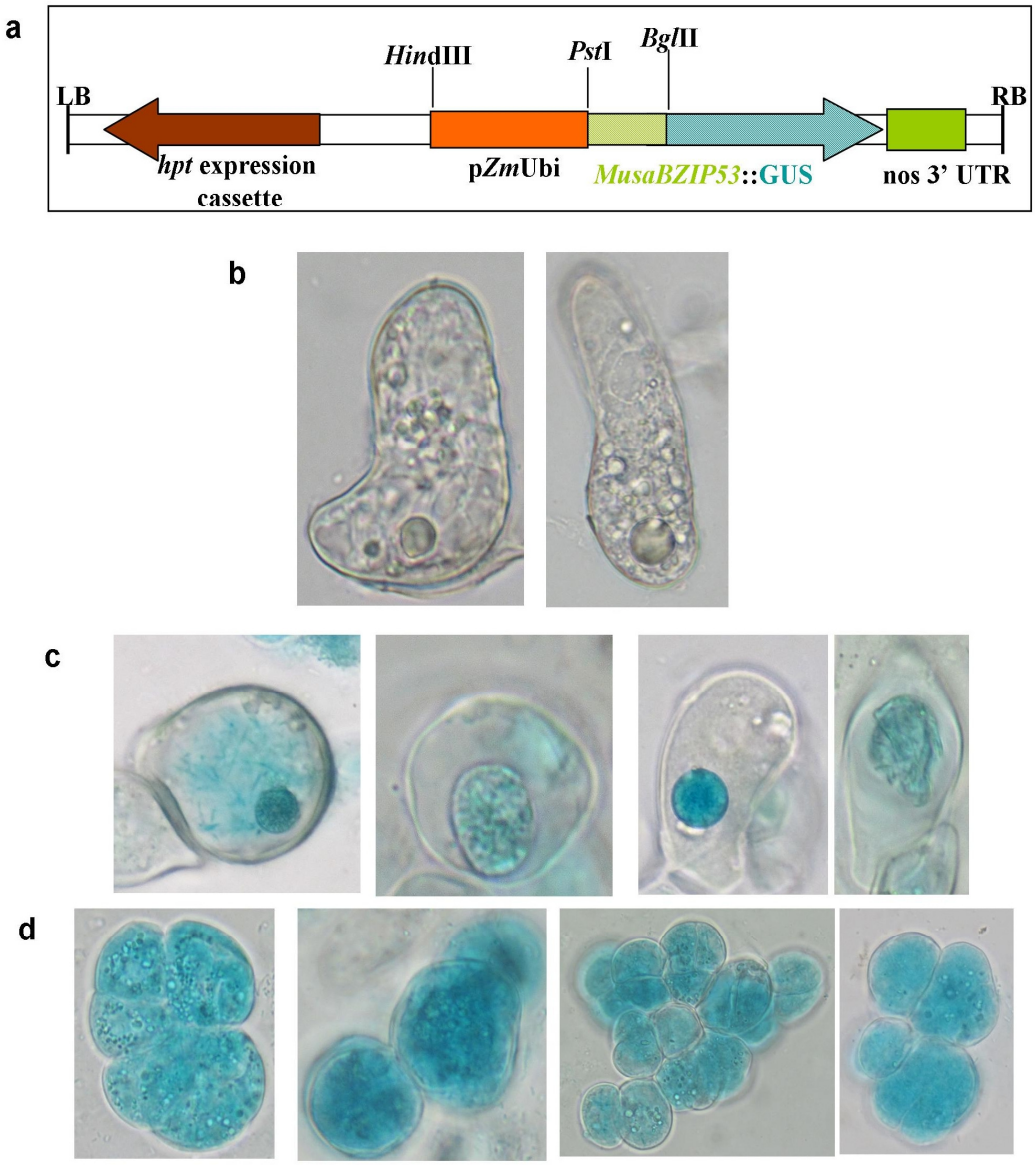

Figure 4.5 Cellular localization of MusabZIP53::GUS fusion protein in transiently transformed banana cells. (a) schematic representation of T-DNA region of plant binary vector p*Zm*UBI-*MusabZIP53*::*GUS*-1301 designed to express a MusabZIP53::GUS fusion protein in banana cells. (b) banana suspension culture cells with prominent nuclei. Cell size ranged from 20-100 µM. (c) banana cells transformed with p*Zm*UBI-*MusabZIP53*::*GUS*-1301 binary vector after GUS staining. Presence of the fusion protein in nuclei of these cells is clearly visible. (d) GUS stained banana cells transformed with pCAMBIA-1301 unmodified vector showing cytosol localized GUS protein.

4.3.4 Generation of transgenic banana plants and their phenotypic assessment

Banana cv. *Rasthali* embryogenic suspension culture cells were genetically transformed using *Agrobacterium tumefaciens* harboring a plant binary vector designed to overexpress *MusabZIP53* (p*MusabZIP53*-1301) constitutively in transgenic banana plants (Figure 4.6 a-e). Three to four weeks from transformation, several whitish embryos developed on banana embryo induction medium supplemented with hygromycin (5 mg l^{-1}). These embryos were later subcultured on fresh medium of the same composition to obtain secondary embryos. The embryos so generated were cultured on embryo germination medium to facilitate the emergence of first shoots from them. These were later subcultured on to banana multiplication medium having BAP for multiple shoot induction. Since each emerging shoot represented a unique transformation event, multiple shoot induction was necessary to generate multiple copies of the same transformation event. These shoots were then separated and put for rooting on MS medium added with NAA. Multiple copies of each transformation event were hardened in a contained greenhouse using soilrite mix.

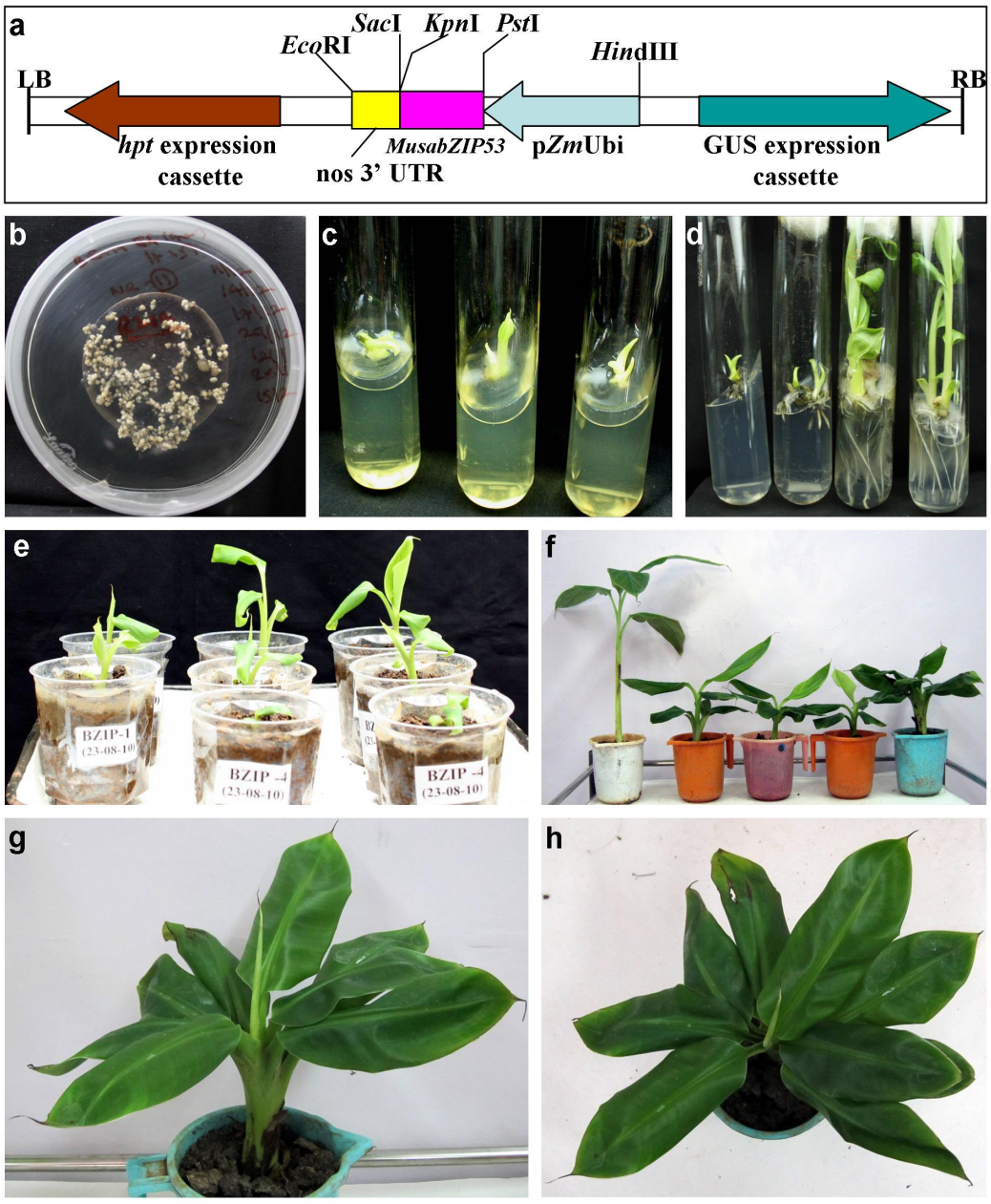

Figure 4.6 Generation of transgenic banana cv. *Rasthali* plants overexpressing *MusabZIP53*. (a) T-DNA region of binary vector p*MusabZIP53*-1301 designed to constitutively overexpress *MusabZIP53* in transgenic banana plants. (b) transformed embryos on embryo induction medium. (c) transgenic multiple shoots on multiple shoot induction medium. (d) transgenic rooted plantlets on rooting medium along with untransformed control plantlets (e) transgenic hardened plants in greenhouse (2-months old). (f) 2 years old transgenic plants together with a 6 months old untransformed control plant. (g), (h) close-up of the dwarfed transgenic plants.

Severe stunting was observed in putatively transformed transgenic tissues at all stages of transformation/ regeneration protocol. The embryos were slow to emerge from the transgenic tissues and they were significantly lower in number as compared to the equivalent controls. Further, multiple shoot induction and rooting of these putatively transgenic tissues was also slow. Subsequently, the hardened plants were observed to be severely dwarfed as compared to controls (Figure 4.6 f-h). In the absence of vertical growth of banana pseudostem in these plants, there was increase in the girth of these plants overtime. The root growth was also severely stunted. The leaves of these transgenic plants showed a very typical morphology in that they were significantly thicker than the control leaves of the same length and a transverse section demonstrated that the number of aerenchyma were almost double in the transgenic leaves of the same size as the control leaf (Figure 4.7). Further, due to the overall stunting, the leaves appear to form a stiff rosette on top of the plant.

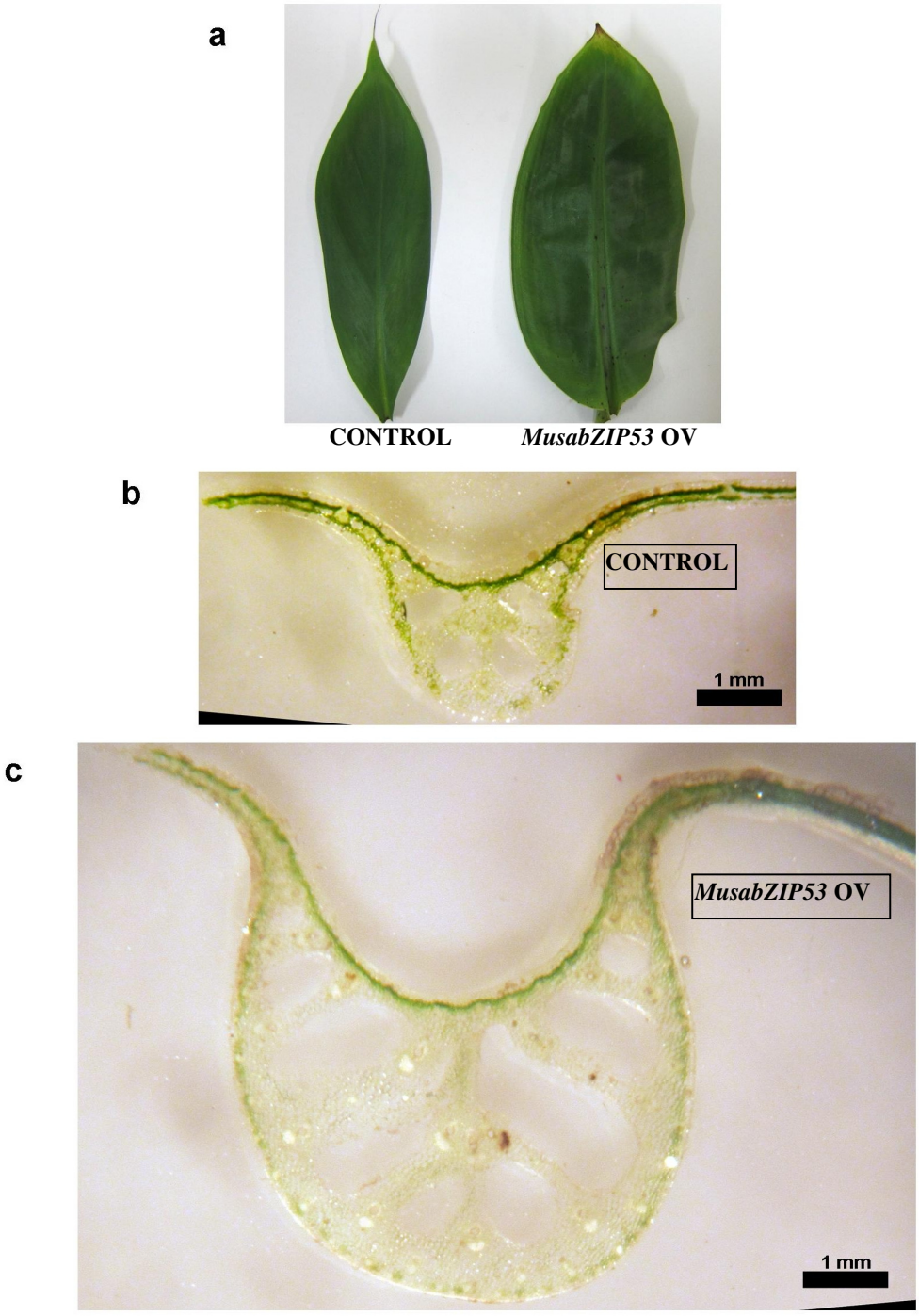

Figure 4.7 Effect of growth retardation on the leaf phenotype in *MusabZIP53* overexpressing banana plants. (a) leaf derived from a 3 months old normal untransformed banana plant (left) with a transgenic leaf derived from 3.5 years old *MusabZIP53* overexpressing plant. Notice that although these leaves are of same length, the transgenic leaves are very thick with greater number of aerenchyma in the midrib, (b) and (c).

4.3.5 Molecular analysis of transgenic banana plants

Since the growth of transformed tissues was slow and stunted from early stages, only four putative transgenic banana lines could be successfully regenerated and established in the contained greenhouse. Genomic DNA PCR analysis of the four *MusabZIP53* overexpressing lines displayed a single 788-bp fragment derived from *hygromycin phosphotransferase* coding sequence present on the T-DNA of the transformation vector whereas it was absent in genomic DNA derived from untransformed control plants (Figure 4.8 a). To further confirm the transgenic nature of these transgenic banana plants, Southern blotting of restricted genomic DNA derived from these transgenic lines was undertaken using a DIG-labeled probe generated using *hygromycin phosphotransferase* gene coding region. Restriction enzyme *Kpn*I was used to restrict these genomic DNAs as it is expected to cut the T-DNA of the overexpression vector only once and therefore the number of bands visible after chemiluminescence imaging can directly be recorded as the copy number of the T-DNAs integrated in the banana genome in these transgenic lines. All the four transgenic lines analyzed had a single copy of T-DNA integrated into the banana genome. The different sizes of the bands observed in these four lines established that these transgenic lines were derived from independent transformation events (Figure 4.8 b). Further, to ascertain whether the T-DNAs have indeed integrated into transcriptionally active euchromatin regions of banana genome and *MusabZIP53* is actually being overexpressed in the transgenic lines, northern blotting was performed using DIG-labeled DNA probes targeted against *MusabZIP53* coding sequence. In the total RNA derived from untransformed control leaf, a single band corresponding to

the basal level of native *MusabZIP53* gene expression was noticed. In contrast, in all the four transgenic lines, in addition to this *MusabZIP53* native transcript, a smaller transcript (derived from the integrated T-DNAs) was visible at a much higher intensity confirming the expression of *MusabZIP53* from the constitutive expression cassette carried on the T-DNA. Since the native *MusabZIP53* gene has an unusually long 5' UTR, the native *MusabZIP53* band is of significantly higher nucleotide size than the one derived from the T-DNA wherein the mature 5' UTR derived from *Zea mays* polyubiquitin promoter is smaller in size (Figure 4.8 c).

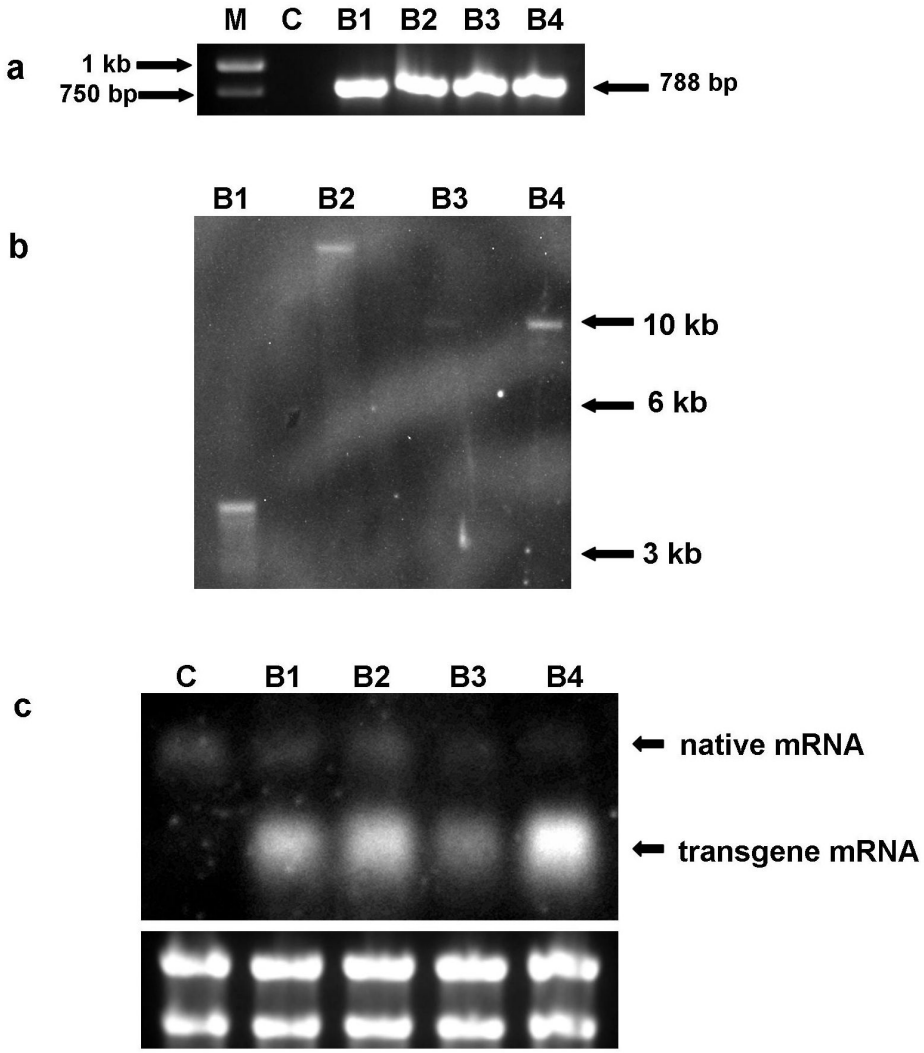

Figure 4.8 Molecular analysis of *MusabZIP53* overexpressing transgenic banana plants. (a) genomic DNA-PCR analysis of untransformed control and transgenic plants. A 788-bp amplification band derived from *hygromycin phosphotransferase* gene carried on the T-DNA was amplified from genomic DNA derived from p*MusabZIP53*-1301 transformed banana lines (B1, B2, B3 and B4) whereas it was absent in untransformed banana plants (C) (b) genomic Southern blot analysis of *MusabZIP53* overexpressing banana plants (B1, B2, B3 and B4). Genomic DNA derived from young leaves was digested with *Kpn*I before being immoblised and filter hybridized with DIG-labeled probes generated using *hygromycin phosphotransferase* gene sequence as template. Approx. positions of the DNA marker bands are indicated. (c) northern blot analysis of *MusabZIP53* overexpressing transgenic lines using probe against *MusabZIP53* protein coding sequence. Note the presence of two bands in each of the transgenic lines (B1, B2, B3 and B4), one corresponding to the native copy of *MusabZIP53* gene and the other shorter and denser one derived from the integrated T-DNA. The total RNA derived from the untransformed control plant (C) shows only one native *MusabZIP53* band.

4.3.6 Assessment of stress tolerance characteristics of *MusabZIP53* overexpressing lines

Since the transgenic plants were stunted and very slow growing as compared to the controls, whole plant assays for abiotic stress tolerance were not considered in the case of *MusabZIP53* overexpressing plants. Instead, uniform sized leaf discs cut out from transgenic and control leaves using a leaf borer were used for abiotic stress tolerance test. For all the three assays performed (cold, drought and salt stress) leaf discs derived from transgenic plants performed better as indicated by visual assessment of the damage done to the leaf discs (Figure 4.9 a-c). The visual assessment was further confirmed by estimating the levels of MDA in the leaf discs post stress treatment. The MDA levels were found to be highest in the control leaf discs indicating maximum damage in control untransformed leaves (Figure 4.9 d).

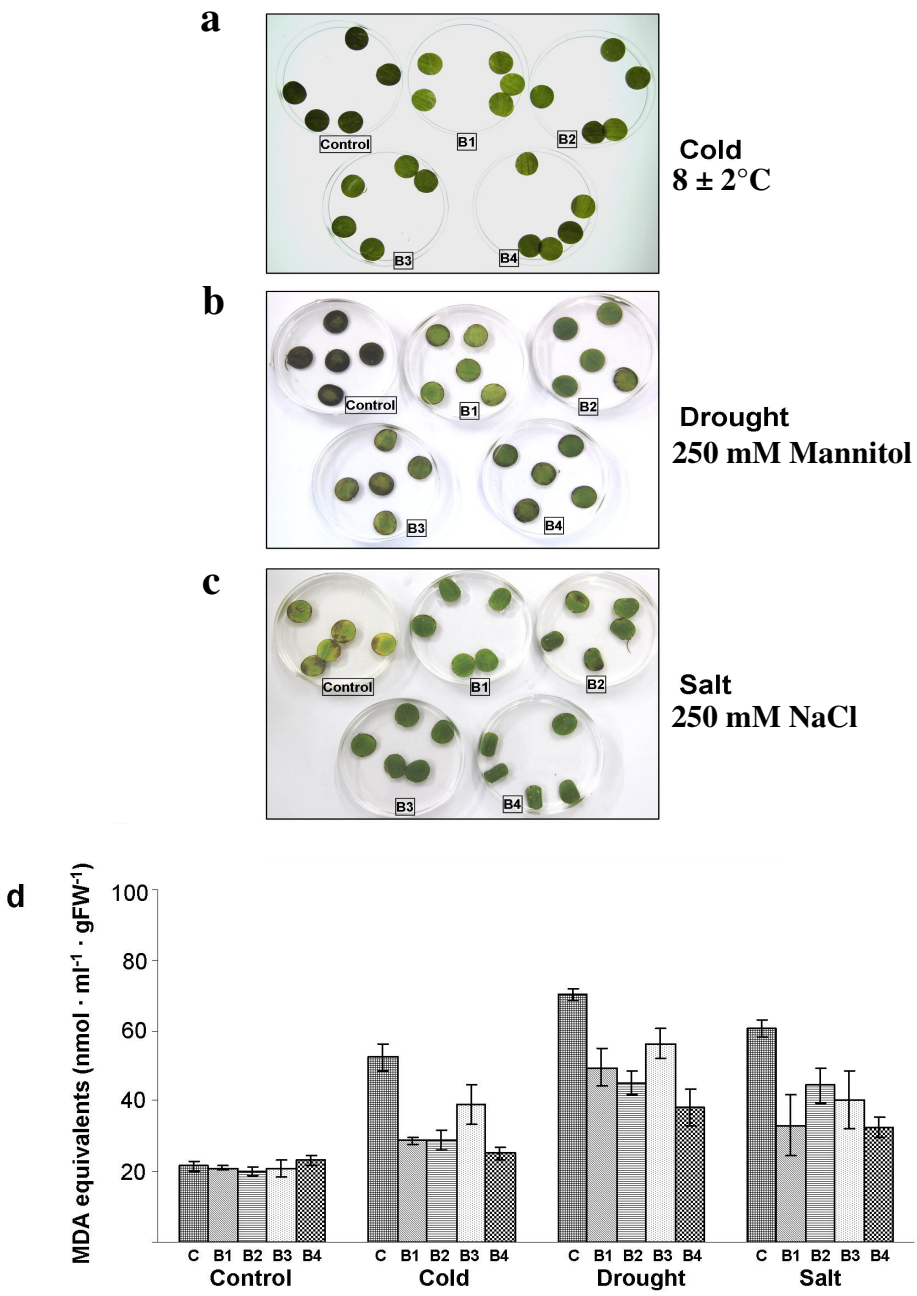

Figure 4.9 Leaf disc assay for abiotic stress tolerance of *MusabZIP53* overexpressing banana lines. Leaf disc derived from *MusabZIP53* overexpressing transgenic banana plants (B1, B2, B3 and B4) and untransformed controls (Control) were exposed to cold stress (a), drought stress (b) and salt stress (c) before being photographed. (d) MDA equivalents in leaf discs of *MusabZIP53* overexpressing lines and untransformed control plants subjected to cold, drought and salt stress. Data represents mean ± SD.

4.3.7 Analysis of differentially expressed genes in *MusabZIP53* overexpressing banana plants

In order to gain clues for understanding the role of *MusabZIP53* in the stunting effects and also the abiotic stress tolerance noticed in the transgenic banana plants, we attempted to identify genes whose expression is significantly modulated by *MusabZIP53* overexpression in these plants. Since bZIP transcription factors are known to modulate expression of multiple gene families, we investigated differential expression of genes known to be involved in abiotic stress responses in other well studied model plants like *Arabidopsis* and rice. Accordingly, genes coding for DREB proteins, LEA proteins, PPOs, different anti-oxidant enzymes and those coding for aquaporins and Aux/IAA proteins were identified from banana EST and transcriptome databases maintained at NCBI and also from the recently released banana genome database. Apart from that, based on previous studies on related bZIP proteins, genes coding for several proteins involved in amino acid metabolism were identified from different banana sequence databases and investigated.

Among the 92 DREB protein coding genes tested, we could detect differential expression in 10 genes. Similarly, among the 30 LEA protein coding genes, 8 PPO coding genes, 7 anti-oxidant protein coding genes, 13 aquaporin coding genes and 23 Aux/IAA protein coding genes, respectively 2 LEA protein coding genes, 4 PPO coding genes, 3 anti-oxidant protein coding genes, 1 aquaporin coding genes and 3 Aux/IAA protein coding genes were differentially regulated in *MusabZIP53* overexpressing plants. Further, among the 16 genes coding for proteins involved in amino acid metabolism, 2 genes coding for asparagine synthetases were noticed to be

highly up-regulated in Musa*bZIP*53 overexpressing plants. In total, 25 genes were found to be differentially regulated out of a total of 189 tested. Also most of these genes were up regulated whereas only 5 were found to be down-regulated (Figure 4.10).

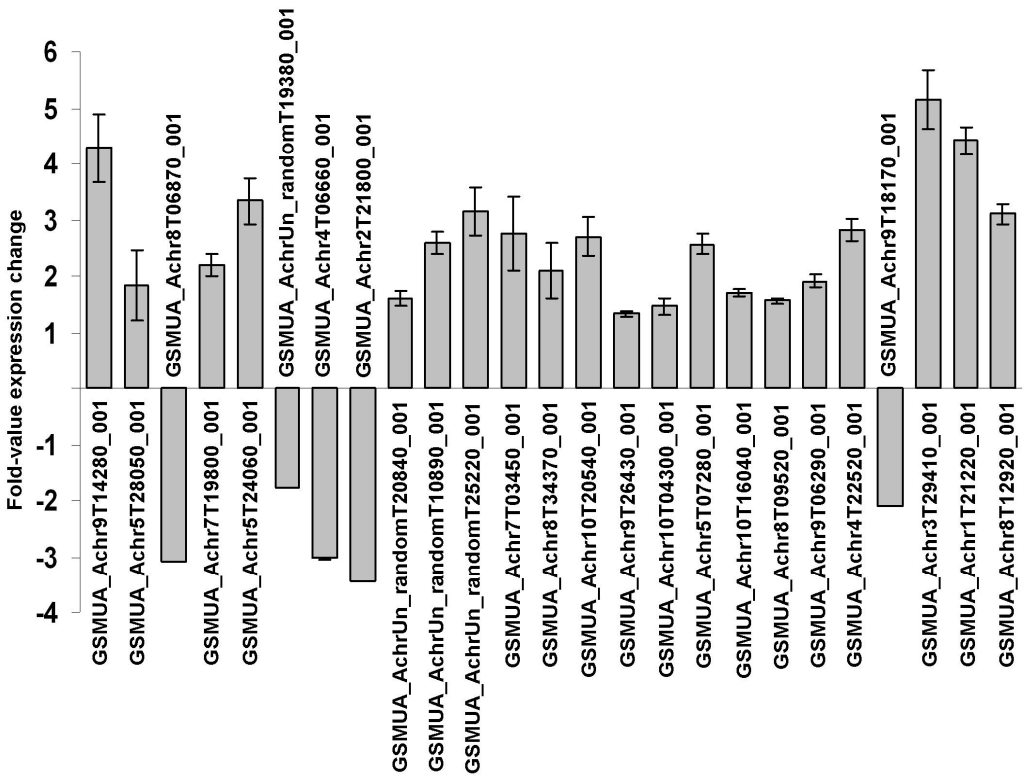

Figure 4.10 Differential expression of genes known to be involved in abiotic stress reponses in *MusabZIP53* overexpressing transgenic banana lines. In total, 25 genes out of a total of 189 tested were found to be differentially expressed. Banana genome database locus identifiers have been used to depict each differentially regulated gene. From left to right, the first 10 genes are DREB protein coding genes followed by 4 PPO coding genes, 2 LEA protein coding genes, 3 anti-oxidant protein coding genes, 3 Aux/IAA protein coding genes, 2 asparagine synthetase coding genes and one aquaporin coding gene. The gene expression values have been normalized against banana *EF1α* cDNA expression levels and the x-axis represents the expression level of the respective gene in untransformed control plant. Values are mean ± SE

4.3.8 Analysis of sucrose content and SIRT (sucrose induced repression of translation) based regulation of MusabZIP53 protein

Plants overexpressing close homologs of *MusabZIP53* have been reported to have elevated sucrose levels in their leaves. Further, these genes have also been reported to possess a highly conserved uORF in their long 5' UTRs (Figure 4.11 a). These studies have postulated that these *bZIP* genes possessing this uORF are involved in sucrose sensing such that their translation is slowed down if the amount of cellular sucrose increases and vice versa. In our studies also, we observed the levels of sucrose were higher by approx. 40 % in the transgenic leaves as compared to the equivalent controls (Figure 4.11 b). Further, to prove that sucrose indeed affects the translation of *MusabZIP53* gene, we used banana embryogenic suspension culture cells growing in modified MS medium with 4.5 % (w/v) sucrose. When these cells were starved of sucrose for 24 hrs, we noticed that the transcript levels of two genes which are up regulated in untreated *MusabZIP53* overexpressing leaves were induced to significant levels indicating that sucrose starvation indeed leads to elevated translation levels of MusabZIP53 in banana cells leading to induction of the two genes as described above (Figure 4.11 c).

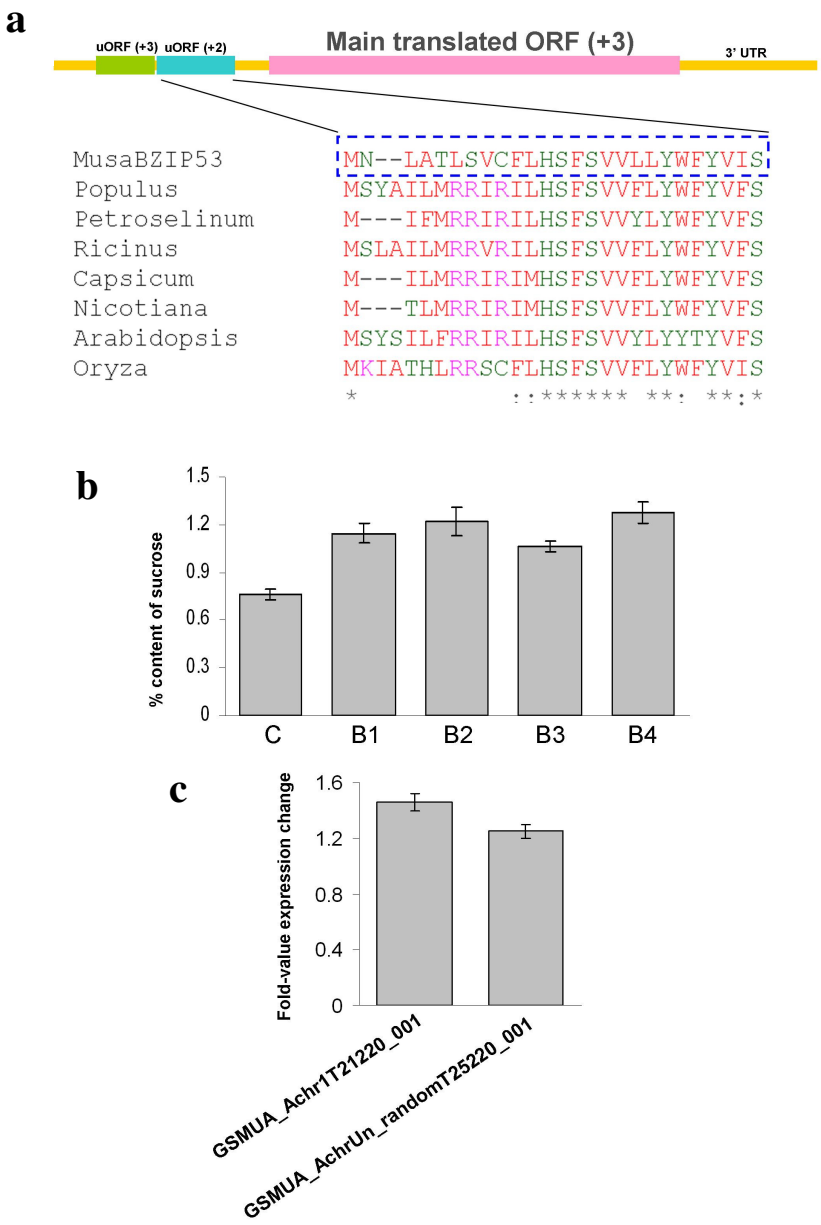

Figure 4.11 SIRT in *MusabZIP53* gene and enhanced sucrose content in *MusabZIP53* overexpressing lines. (a) schematic representation of the structure of *MusabZIP53* gene with its conserved uORF present in the 5' UTR and its homologous sequences from other related plant species. (b) enhanced sucrose contents in the transgenic banana lines (B1, B2, B3 and B4) as compared to the untransformed controls. (c) elevated transcript levels of two putative targets of *MusabZIP53* protein in sucrose starvation conditions. GSMUA_Achr1T21220_001 codes for asparagines synthetase enzyme and GSMUA_AchrUn_randomT25220_001 codes for a PPO enzyme. The gene expression values have been normalized against banana *EF1α* cDNA expression levels and the x-axis represents the expression level of the respective gene in untransformed control conditions (4.5 % w/v sucrose concentration). Values are mean ± SE.

4.3.9 Analysis of PPO gene transcription in abiotic stress and protein activity

Out of a total of eight PPO coding genomic loci detected in banana genome database, we could determine sequences corresponding to only four of these in the extensive EST and transcriptome databases of banana maintained at NCBI. Further, we could not detect the other four PPO sequences by RT-PCR using our banana tissues probably indicating that these four are not expressed to detectable levels. Further, all the four PPO sequences detected in EST and transcriptome databases were found to be up regulated in *MusabZIP53* overexpressing plants (Figure 4.10). Since all the four expressed PPO coding genes were up regulated in the transgenic *MusabZIP53* overexpressing plants, we wanted to investigate whether the *MusabZIP53* gene exerts a coordinated control of the total PPO activity in banana and whether these PPO genes are in any way related to abiotic stress response pathways in banana plant. Hence, PPO coding gene transcript levels were determined in cold and ABA stressed banana leaves. All the four PPO coding transcripts demonstrated to be up regulated in *MusabZIP53* overexpressing plants were found to be induced in ABA treated banana leaves also whereas in case of cold stressed leaves, two of these were induced to high levels whereas the other two were induced to moderate levels (Figure 4.12 a). To validate the correlation between elevated transcript levels and increased protein activity, total PPO activity was determined in the leaves of four transgenic plants and in the untransformed controls and also in cold and ABA treated untransformed banana plants. Approx. 20 - 25 % higher PPO activity was noticed in all the four transgenic lines indicating higher transcript levels do lead to increased protein activity, although increased transcript levels could not be linearly correlated with increase in protein activity (Figure 4.12 b). Further, higher PPO activity was noticed

in ABA and cold stressed banana leaves as compared to the equivalent untreated controls and this increase in PPO activity was commensurate with the higher PPO activity in untreated *MusabZIP53* overexpressing plants (Figure 4.12 b). These results are significant since alteration of PPO activity in response to abiotic stress (most commonly cold temperatures) affects the visual and sensory quality of banana fruits.

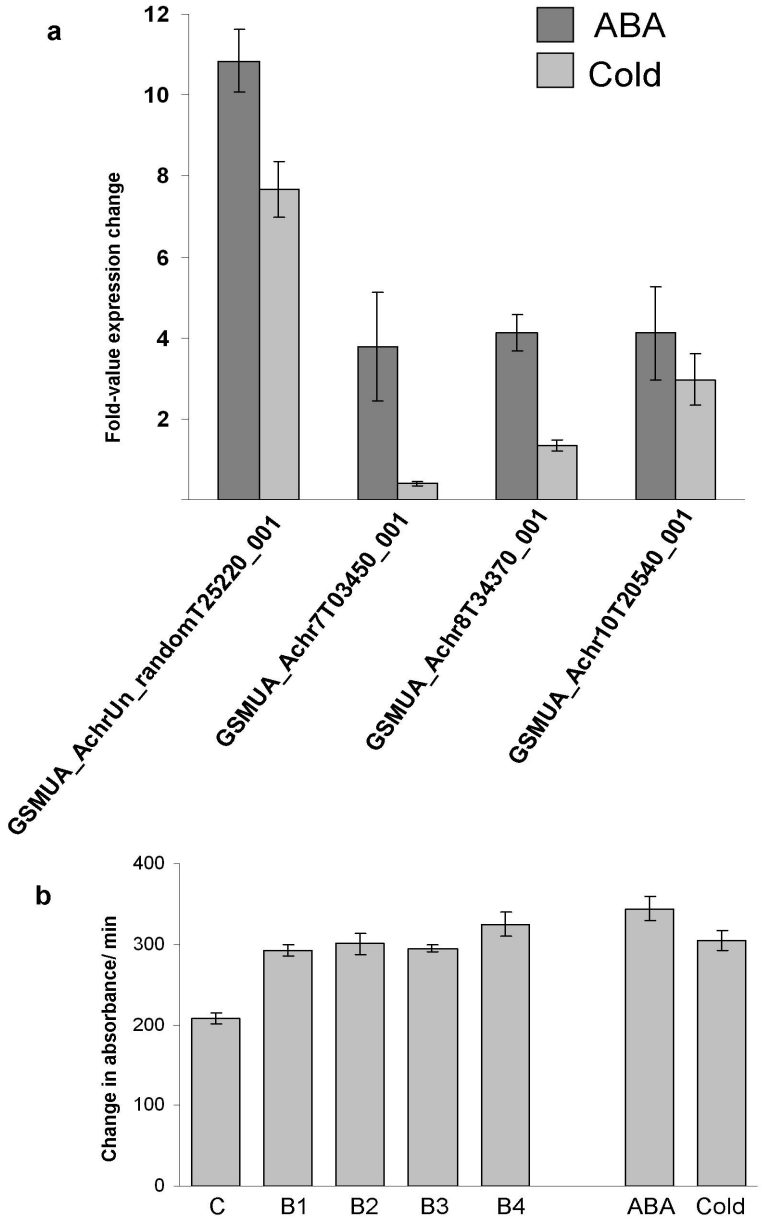

Figure 4.12 Analysis of PPO gene transcriptional levels in abiotic stress and protein activity. (a) all the four PPO coding genes found to be expressed in banana tissues (GSMUA_AchrUn_randomT25220_001, GSMUA_Achr7T03450_001, GSMUA_Achr8T34370_001, GSMUA_Achr10T20540_001) are induced in response to ABA or cold treatment. The gene expression values have been normalized against banana *EF1α* cDNA expression levels and the x-axis represents the expression level of the respective PPO coding gene in control conditions. Values are mean ± SE (b) total PPO activity is higher in all the transgenic lines generated as compared to equivalent controls. Further, total PPO activity increase in ABA and cold treated leaves is in line with increased transcription of PPO coding genes. Values are mean ± SD.

4.4 Discussion

bZIP transcription factors have been implicated in several important cellular processes and hence investigations into the roles played by various members of *bZIP* gene family in plants is important. Many of these bZIP transcription factors have been in the past associated with abiotic stress responses wherein their overexpression has resulted in improved abiotic stress tolerance in the transgenic plants. *ThbZIP1* gene from *Tamarix hispida* enhanced the salt stress tolerance in transgenic tobacco plants [27]. Transgenic *Arabidopsis* plants overexpressing three soybean *bZIP* genes displayed better tolerance to salt and freezing stress as compared to respective controls [28]. Transgenic tobacco plants overexpressing a wheat *bZIP* gene *Wlip19* showed significantly improved freezing stress tolerance [122]. *Arabidopsis bZIP* gene *ABF3* improved abiotic stress tolerance in rice without stunting the growth of transgenic rice plants [123]. *OsABF1* and *OsABF2 bZIP* transcription factor genes from rice have been shown to be positive regulators of abiotic stress tolerance [114,115]. As is evident, in most cases these investigations have involved genes derived from important model plants like *Arabidopsis* or staples like rice while the equally significant plants like banana, with huge food security role and trade potential, have been largely left untouched. However, probably as a result of realization of the importance of banana in global food paradigm, several studies have been recently undertaken to investigate banana abiotic stress signaling and its mitigation. These studies have mostly focused on characterization of specific genes predicted to be involved in abiotic stress responses. These include characterization of *MusaDHN-1* (the present study), *MusaSAP1* [121], *MusaWRKY71* (the present

study), *MpRCI* [42], and *MpAsr* [62, 124]. A *bZIP* gene, *MabZIP3*, from banana was recently reported to be responsive to methyl jasmonate, abscisic acid, chilling stress and pathogen *Colletotrichum musae* [125]. The present study deals with the characterization of another important abiotic stress inducible *bZIP* gene from banana. As our results demonstrate, *MusabZIP53* plays an important part in different abiotic stress response pathways in banana which ultimately lead to a coherent response upon the onset of abiotic stress.

MusabZIP53 was initially identified based on its differential abundance in the two contrasting EST datasets derived from abiotic stress tolerant banana cultivar 'Cachaco'. Since it was found to be more abundant in the EST library derived from stressed 'Cachaco' tissue, we predicted it to be involved in abiotic stress tolerance in banana plant. Similar predictions based on such libraries have been validated by us in the past by performing detailed studies on multiple genes wherein overexpression of full length coding sequences of these genes resulted in transgenic banana plants displaying improved tolerance towards multiple abiotic stress stimuli (the present study). Once *MusabZIP53* was confirmed to be significantly induced under different abiotic stress stimuli by northern blotting, we postulated that a positive correlation must exist between *MusabZIP53* up regulation and abiotic stress tolerance in banana plants. Towards this end, *MusabZIP53* was constitutively overexpressed in transgenic banana plants using highly efficient *Zea mays* polyubiquitin promoter. Although the resulting transgenic plants were severely stunted and dwarfed with a very different phenotype than the untransformed controls, abiotic stress assays performed using leaf discs confirmed that these plants tolerate abiotic stress conditions much better than

the equivalent controls. Genes belonging to multiple gene families reported to be involved in abiotic stress perception and tolerance were observed to be differentially regulated in *MusabZIP53* overexpressing banana plants. These included genes coding for DREB proteins, LEA proteins, aquaporins, anti-oxidant enzymes, PPOs and others. Further, the fact that a single *bZIP* gene was able to regulate genes coding for several different protein classes indicated that this *bZIP* gene probably acts higher up in the abiotic stress regulatory networks to simultaneously affect several cellular processes in the aftermath of stress perception in banana plants.

Although several studies have previously reported growth stunting as a side effect of overexpression of potent transcription factor genes in plants, *MusabZIP53* overexpressing plants were very unique in the sense that even after 3.5 years of growth in rich organic manure supplemented soil mix with ample growing room, they did not raise even 30 cm in height. To couple that, the stunting effect was even more pronounced in the leaves which were rather very stiff and arranged in a uniform rosette around the pseudostem. Although, we were not able to pinpoint the definite reasons behind this severe stunting, simultaneous expression of multiple genes which otherwise express only under strong abiotic stress conditions may be responsible for this type of stunting phenotype. The differential regulation of several DREB transcription factor genes in these transgenic plants was a clear indicator of this fact. Since each transcription factor gene is expected to modulate the expression of several effector genes at the cellular level, the cellular homeostasis in these plants is strongly altered. The transcriptional load arising out of expression of several genes and proteins, which normally are required only for smaller periods of time immediately in

the aftermath of stress perception and whose expression is mostly maintained at lower insignificant levels in unstressed conditions, probably induces a strong inhibitory effect on plant growth and development. Even the *MusabZIP53* gene itself is maintained at very low cellular levels in the banana plants under control conditions and gets induced to high levels only under stressed conditions. Since the duration of abiotic stress in natural conditions is quite short, the effect of induction of these stress specific effector genes does not significantly affect the growth trajectory of the plants under stress. In contrast, in *MusabZIP53* overexpressing plants, since the transcript levels of *MusabZIP53* are always high owing to its constitutive overexpression, the target genes and proteins which are otherwise only momentarily induced are also up regulated constitutively. This probably explains the extreme dwarf phenotype that is observed in the transgenic *MusabZIP53* overexpressing plants. Even so, *Arabidopsis* homolog of one of the DREB genes found to be up regulated in *MusabZIP53* overexpressing plants (referred to as TINY), is known to cause similar stunting of transgenic plants when it is overexpressed [126]. Therefore in our study where several DREB genes are simultaneously differentially expressed, such stunting effect is but expected.

Another unique feature of these transgenic plants was that they had almost 40 % higher sucrose content as compared to equivalent controls. Similar results were reported in studies wherein close homologs of *MusabZIP53* [117] were overexpressed in transgenic plants. These two genes share a conserved uORF in the 5' UTR with *MusabZIP53* and have been implicated in cellular responses to low energy conditions such as darkness, low sugar and stress conditions. According to [117] in tobacco, in

low cellular energy conditions, *TBZ17* transactivates asparagine synthetase gene and induces metabolic reprogramming, thereby turning on the sucrose synthesis pathway. Further, if the end product sucrose reaches an upper limit, the excess sucrose acts via the conserved uORF to suppress the translation of TBZ17 in a feedback manner. This phenomenon was referred to as sucrose induced repression of translation. On the other hand, in plants overexpressing *TBZ17* without the linked uORF, even if sucrose concentration reaches an upper threshold, there is no translation repression and the *TBZ17* translation continues increasing the total sucrose content of the cells [117]. The same feedback loop appears to be conserved in banana also with *MusabZIP53* sharing the critical conserved uORF with *TBZ17* and *AtbZIP53*. Similar to *TBZ17* mediated up regulation of asparagine synthetase coding gene in tobacco, we also found that two banana asparagine synthetase coding genes are highly up regulated in the transgenic *MusabZIP53* overexpressing plants. And finally like the *TBZ17* and *AtbZIP53* overexpressing tobacco and *Arabidopsis* plants, the banana plants overexpressing *MusabZIP53* were also stunted and the leaves were thicker than the controls. Lastly, since a very strong constitutive promoter (derived from *Zea mays* polyubiquitin) was used in our studies, the level of stunting was very severe in our case.

During the course of our studies aimed at investigating the differentially expressed genes in *MusabZIP53* overexpressing plants, we noticed that all the four PPO coding genes (out of a total of eight such genomic loci) which are expressed in banana plants, are induced in *MusabZIP53* overexpressing plants. Since there have been no reports of induction of PPO coding genes in plants overexpressing close

homologs of *MusabZIP53*, we found the coordinated up regulation of all the four expressed PPO coding genes in our transgenic plants very interesting. Role of PPOs in oxidation of plant flavonoids has been studied in detail in the past [127] and a positive correlation is known to exist between stress tolerance and induction of PPOs. Since PPOs are especially important in context of banana owing to the negative sensory and visual characteristics they impart to the banana fruit [119], we wanted to investigate the correlation between abiotic stress induction of *MusabZIP53* and the up regulation of these PPO coding genes in the transgenic plants overexpressing *MusabZIP53*. As expected, all the four PPO coding genes that are induced in *MusabZIP53* overexpressing plants were also found to be induced in cold as well as ABA treated control untransformed banana plants. Further, the total PPO activity was also higher in the stressed tissues as compared to the controls. Taken together, these results indicate that probably *MusabZIP53* plays an intermediary role between the perception of abiotic stress and up regulation of PPO coding transcripts. In one of earlier studies, we had reported a similar up regulation of one of these PPO coding genes in transgenic plants overexpressing a zinc finger containing stress associated protein gene, *MusaSAP1* [121]. This transcript was also demonstrated to be inducible in response to methyl jasmonate treatment and wounding stress as was the case with *MusaSAP1* gene itself. Results of the present study are unique in the sense that all four expressed PPO gene transcripts are induced to significant levels in the transgenic banana plants indicating major role for *MusabZIP53* gene in regulation of PPO activity in banana plants.

In conclusion, the study presented here proved that *MusabZIP53* gene plays important roles in abiotic stress related signaling in banana. Further, it also takes part in sucrose homeostasis in banana plant through a linked uORF present in its 5'UTR. Also, our findings pertaining to the coordinated induction of all four expressed PPO coding genes by *MusabZIP53* gene (in effect controlling the total cellular PPO activity) assign a hitherto unknown role for *bZIP* genes in plants.

Table 4.1 Primers used for studies on *MusabZIP53*

Primers used in cloning and for probe preparation:

Amplicon / Description	Primer sequence (5' to 3')
Amplification of full-length cDNA of *MusabZIP53*	AATTCTGCAGATGTCTTCCATTCCGGTCCG CCGCGCTTC
	AATTGGTACCTCAGAACTGCAACATGTCAG CATTGGCCATGATTGG
Amplification of *MusabZIP53* cDNA without the native stop codon for generation of GUS fusion protein	AATTCTGCAGATGTCTTCCATTCCGGTCCG CCGCGCTTC
	CGGAGATCTACGAACTGCAACATGTCAGC ATTGGCCATGATTGG
Amplification of nos 3' UTR	CTACCGAGCTCGAATTTCCCCGATCGTT
	CGGCCAGTGAATTCCCGATCTAGTAACA
Amplification of *Zea mays* polybuiquitin promoter	AATTAAGCTTCCGGTCGTGCCCCTCTCTA
	AGCTCTGCAGAAGTAACACCAAACAACAG G
Real time quantitative amplification of banana *EF1α*	CCGATTGTGCTGTCCTCATT
	TTGGCACGAAAGGAATCTTCT
Amplification of *hpt* gene	GTCCTGCGGGTAAATAGCTG
	ATTTGTGTACGCCCGACAGT

DREB protein coding genes and primers:

Gene*/ NCBI Accessions	Primer sequence (5' to 3')
ES436343	CTCTGATGACCCGGTGGT
	CTGCTGATTGAGATCAGGGTTT
ES432749	AAGCAGCACGGTGGAGTC
	GTCAGACGGTGGTGGTTCAT
FL659594	AGGCCCCTTCCTCCTGAT
	ATCAGCGCCCTCCAGTTTA
FF559848	CCACAAGAGATGCTCAATGTCA
	TTGGGGGTTTTAACCTCACTTT
ES436780	GACACCGCGCTCTTCTACC
	GAACCGTTTCTGATCTCCTTCC
ES433029	GAGGAGAAGGAAGGAGATCAGC
	TGAGATCTGGATGGCTTGTTTC
FF560353	GGAGGTGTGAGGAGGGATTC
	CCACCCTCATCTTCTAATTCCAC
ES431610	GGCTAAGACCAACTTCCCAATC
	AAGCAGCGCCTAGGAACAAG
ES433424	CAGGGATAAAACCAGCTCCAC
	CCCAGCAGCACTTTCCTTAGT
FF561178	ATCAGGAGATTTTAGTGGATCTGC
	ATAGGCTCTTGCAGCCTCTTC
ES435265	GGCCGCGGAGATAAGAGA
	GCGTAGTCGCTGGGGTTC
ES437095	GAGCGATCATCTCTGACTTCG
	TTCCTCGGGTCTCTTATCTCC

ES432514	CCAGTAGTCAGAGCAGCACCT
	GACTTCGCGTCGACAAAGA
FL662828	ACGGGTCGAAGAAGAAGAAGA
	ACTGCAGGTCCATCAGATTCA
FF561371	TCAAATGTAGCCAAAGCACCT
	GAAAGAGTTGCTTCCCTGGTC
FF560746	GGCTTCTTGGAAGAGAAGGTG
	TGTCTGAGGGTTATCGTGTCC
ES435892	CAGTTCACAGAAACAGGGGAAG
	TCCCATGATGGATACTTGTGC
FF561432	CGCCCACAAGAAAGTCTCAA
	ACAAAGGAGTTGCTTCCCTGA
ES432610	GCCGGATTCGGTTCCATA
	TGCAGCCTTCGTTGAAGC
FF559386	AGCATTCCGACCATCCAAAT
	TGATCTCAGGGGTTTTGTCCT
FF558748	CTTCCGAAACTAGCTGCATCA
	AATTCATCCCAGCCACCAC
ES436488	GCTCCGGCTACTGCTTCC
	GCGGGCCGTATCTCCTTC
FF560823	GAGTTCGATGACGAGGAGGAG
	ATGGACGTTGGCGGATTC
ES431669	GGAGCTCTAGCTTCGGGAGA
	CCTGCTGCTGCTGTTGAGA
ES432382	GCTGCCCCTCAATGAAAAC
	GGGCACAGTTGCAGATGAA
ES433867	CTCAGACGCAGCTACCCTTC
	CGTACCCCTCGGTACTTCC
HN248085	ATGATGATCCGGACACTTCC
	CCACATCTGGTCGGGTTC
HN248835	ATTACCGAGGGGTGAGGAAG
	TGCGGGAAGTTGGTCTTG
HN260232	TACGCTGCGGAGATACGG
	GAGGACTCCACCGTGCTG
HN240556	CGGCCTTCGACTACACCTT
	AATCGCCAAACTCCACCTC
HN253812	GGTTTGATGGATCTGCTGCTA
	GATGATGCAGTTGCTGCTGT
HN243470	GGAGATCGGGTTTGAGGTG
	GTCTCCCGTGAGCCTGATT
HN248321	GAAGTGGGCGGCTGAGAT
	CTCTTCCAGCTTGGGTTCG
HN250386	ATGCAAGTCAGGCAAAAGGA
	TTATGGCCACGAATCCTACG
HN260101	CCGAGAACAAGGGATCGAC
	GAAGTTGGTTTTCGCCTTGG
HN253732	GTACAAGGGAGTGCGATTGAG
	CTCGTACCGGCTCAGCTT
HN261004	CGGCGGAGATAAGGGACT
	CCTCCTCCTTAACGAGTGCTT
HN241935	ACTCCAAAGCCGAGATCGT
	CCTTCAACGGGAAGTGTTTCT
HA107944	GATCCTGACGCCACTGATTC

	GCCTGACTCCCTTAAACTTGG
GSMUA_Achr5T25620_001	ACACCCACAGCTTCTTCCAG
	ATGCAGTTTGACCTCCATGC
GSMUA_AchrUn_randomT13500_001	AAACGATGGACCACGAAAAG
	TGGTTCTCCCAAAGGTTGTT
GSMUA_Achr4T12060_001	CGGAGATCAGGGACTCGAC
	TGACCTCCTCCTCTTCCTGA
GSMUA_Achr2T05810_001	GCCAAGAAGTCAAAGGGACA
	TATGGAGCACTCAGCACCTG
GSMUA_Achr9T07890_001	TCGTGAACCTGGAATCATCA
	ACATTGTTCCCTCCATTTGC
GSMUA_Achr5T09470_001	TCCAAGATCCAGGCCATCT
	CGAGCACTTCCCAGATCAAT
GSMUA_Achr4T23620_001	CTCTTCGACACGATCGTCAG
	GGGAAGGTTGAGGTCGAGAT
GSMUA_Achr1T22650_001	AAATGGGTGAATCGTCTTGC
	CATGGTGGAAAACACCCTTT
GSMUA_Achr4T14060_001	AGCGGAGTCTTTGAGGTTGA
	TTTTGGAATCGGAGTTACCG
GSMUA_Achr7T19320_001	CTCCTTGCCATCGAATTGTC
	AGTCCAGCAGCTCTTCAATCA
GSMUA_Achr3T21460_001	TGTACGGCGGAAGCTATCA
	CATCCATGCATTGTTCTGCT
GSMUA_Achr5T04670_001	ACGGGATTAGCATGTTGGAG
	CCTCAGCTTGCCTCCTTTTA
GSMUA_Achr2T20760_001	CTCGGAACCCAAGGACATC
	CACAGAAAGGGATCCCACAA
GSMUA_AchrUn_randomT02200_001	GGCCACTCACTTACCAGCAA
	CATCGGCGAGTAGAGTTCGT
GSMUA_Achr4T28640_001	AAGTGTACAGGCCGTGCTTT
	AGCGTATCTGAAGGGTCGTG
GSMUA_Achr5T05160_001	GCCGACTCCAGTTGACTCTC
	AAAGGAATTGGACCCGAAGT
GSMUA_Achr2T19400_001	GTGGGTTAACAGGCTTGCAG
	TAGTTAGCTGCTCCCCACCA
GSMUA_Achr8T03550_001	AGTAGCCAGAGCAGCACCAT
	GAAGAGTCGGAGTCGCTTTG
GSMUA_Achr3T08170_001	GCAAGAAGGCAAAGGTCAAC
	CAAAACTCCAGAGCATCTCCA
GSMUA_Achr8T12230_001	TCATGGCTGGTCTAGCCTCT
	CTATTTTGCCTCGAGTTGTGC
GSMUA_AchrUn_randomT20840_001	TTGGCCAATTCCTCAAAAAG
	TGGAGTCCCAGTCAATCTCC
GSMUA_Achr3T29290_001	CCAAGGCGAAGACCAACTT
	CACGGTCTTCCTCTCCTGTT
GSMUA_Achr4T12070_001	GAGATCCGTGACCCCAGTC
	CAAGCACTCCAGTTCGATCA
GSMUA_Achr1T01380_001	AGGTGGAATCGAGAAGCAAG
	AACTCTCTCCACCGGGAAAT
GSMUA_Achr3T05800_001	GCAAAGGTGAATTTCCCTGA
	GTTCATCTGAGCCTCCTTCG
GSMUA_Achr3T23710_001	AAGACCAACTTCCCCTACCC

	CAGCCATCACAACAGGAGAG
GSMUA_Achr8T21030_001	CCGCCAGAAATCCTGTATTC
	CATCTGCCATGTCCAGTTTG
GSMUA_Achr6T33140_001	TTCCTTTGGTGGTCGATTTC
	AAGAGCAGCTGTCACTGTGG
GSMUA_Achr3T16850_001	GCCTTTTGGGAAGTCAGACA
	TTACTGGTGCCCAGAGATCA
GSMUA_Achr11T12650_001	AACTTCCCGGAGTCGGTAGA
	GCCAGCTCCAACCACATCT
GSMUA_Achr10T24170_001	TCTCTTGAAGCAGCACCTCA
	AGGTTGGAGTCAAGCAGCTC
GSMUA_Achr3T01650_001	GCCAAGCTCCAGACCATCTA
	GATGGGTACTTCCGCAACAC
GSMUA_Achr9T13760_001	CTCATGTGCGGATCCAAGG
	TGAGCTCCTCGATCATCTCCTC
GSMUA_Achr11T22020_001	GAGCCGAAGGAGAAGTCGTA
	CTGCTGCTCGTCTCACTCTC
GSMUA_AchrUn_randomT10890_001	AGCATGGGTGAATCGGTTAG
	TGGCTTCACACCTATGTTGC
GSMUA_Achr11T15810_001	GTCAACTTCCCCAACGAGGT
	CGCCTCAATCCCAATATTCC
GSMUA_Achr3T15150_001	AGGCAACTGATGCAGGAAAA
	TTCCCTAACCTGTCCGTGTC
GSMUA_Achr8T33590_001	CCCTAAGGCCAAGACCAACT
	ACACCGGCGCTCTTAAATCT
GSMUA_Achr4T06170_001	CCTCAGTAGCACCGTGGAAT
	CTGGTGGGAGGAGATTGAGA
GSMUA_Achr6T28550_001	TTCCAGCCATCTATGATCCA
	GTCGACTGCTGTGGCAGATA
GSMUA_AchrUn_randomT26940_001	GACGAGATCCTAGCGGAAGA
	AACTGCTGTTGTTCGAGCTG
GSMUA_Achr6T28760_001	TTCGGAAAGTCGGACACAG
	CTTTGAGCAATTCTGCGTGT
GSMUA_Achr4T26080_001	AACATTCCATCTGCCGTAGC
	CCTGACATGCTGGTGAAGAA
GSMUA_Achr11T03210_001	CTCAACTTCCCCGACGAGAT
	CGGCATGTCGAAGATTTCAT
GSMUA_Achr8T14110_001	GACAACAACACCCGAAATGG
	TAGAAGACGCGTCAGCATCC
GSMUA_Achr2T05880_001	GCAACACCCAGACCTTCTTC
	TACACGTTCCTGCCACCATA
GSMUA_AchrUn_randomT23040_001	CTGCTCACTCTCCACCAACA
	CCCGTAACCATTACCACCAA
GSMUA_Achr8T09110_001	TGGTAACATGGCCAGCAGTA
	GCAGCCATGGTGTTAGACCT
GSMUA_Achr11T02840_001	GCTGAGGCAAGCAAAAACAG
	GTGCAATGGAGCATCATCAG
GSMUA_Achr1T15350_001	ACCTGGGGAGGAGGAGGAG
	CACCGGGGAAGTTGAGGAC
GSMUA_Achr9T28840_001	GGACCAACTCGTCGACATCT
	AGCCTCGATCGGGAATAAGT

GSMUA_Achr1T02070_001	CCTTTCGGGAAGACTGACAC
	TCGGTGGACTAGAGGAATGC
GSMUA_Achr10T04860_001	ATCATCAAACGCAGCAAACA
	GTTGGGTCACCACCAGTTTT

PPO coding genes and primers:

Gene*/ NCBI Accessions	Primer sequence (5' to 3')
GSMUA_Achr6T29370_001	GAGGAGAAGGAGGAGGTGCT
	GCCTTGAGATCCTCCAACAG
GSMUA_AchrUn_randomT25220_001	ACGACGAGAACGAGAAGCTC
	GTCTCGTTGGTGGTCACCTT
GSMUA_AchrUn_randomT22730_001	ATGAAGACATGGGCGACTTC
	CAGCATCTGACTTGCCTTGA
GSMUA_Achr8T34370_001	TTCGACCGCGACTACTTCAT
	CTTTCGGGACGATGGTGAC
GSMUA_Achr7T03450_001	TCAAGTTCGACGTCTTCGTG
	CGACGGACACCTTTCCTTT
GSMUA_AchrUn_randomT22740_001	CGGACGGATACAAGGAAAAC
	AGGCAGTCGCTCATTTTGAC
GSMUA_Achr7T03560_001	ACTCCCAAGCTCACCAAGG
	CACGATGACGTCGAACTTGA
GSMUA_Achr10T20540_001	TTCGATGTGTTCATCAACGTC
	TGTCACCCAGATGCTGTCAT

LEA protein coding genes and primers:

Gene*/ NCBI Accessions	Primer sequence (5' to 3')
GSMUA_Achr8T30100_001	CTGGAGCCTAAAGCTACAAAGG
	TGTTGAGCTTCTTCAGCCTTC
GSMUA_Achr9T26840_001	GGAAAATCCAACAGGAGGAAC
	GCAAGTGAATCACCTTCTCGT
GSMUA_Achr4T32740_001	GGATTCTCTTGCATGCCTTC
	CCTGCCGAATAAGGGTCATA
GSMUA_Achr1T19670_001	GCCGAGATCAACAAGCAAGA
	GAGCCGACGTAGGGGTTGT
GSMUA_Achr5T01540_001	CGGGGGTGGAAGAAACTATT
	AGATAGTGGCCCAACAGCAT
GSMUA_Achr11T05130_001	CGCAAAAGGCTAATCTGGAG
	TTGTAGCGAGCGGCAATAGT
GSMUA_Achr10T12880_001	GGGCTATCACTTGCTCACATT
	CAAGCCTGCAATTACCATCA
GSMUA_Achr4T11310_001	CATAACCGGCATGCTCCAC
	ACCTTCTCCACGAATCCCTTC
GSMUA_Achr7T15490_001	GACGTTCCTGGAAGTGATTT
	ACCAACACATATAAGGCGTAAA
GSMUA_Achr9T09720_001	TCCCAGACTGGTTGGAAATG
	CGCCAGATAGGGTAAACCAA
GSMUA_Achr9T26430_001	TTCCCTGACTGGTTGGAGAT
	CGCCAGATAGGGTAAACCAA

GSMUA_Achr11T16760_001	TGGAGAAGATGCAGGAGGAT
	TTTCTCCTCGGTCTTGTGGT
GSMUA_Achr6T34630_001	AGATGCCCGGAGCACTAAT
	ATGCAGGAATAGGTGCCAGA
GSMUA_Achr5T02900_001	AGACTGACCTCCCCGGTCAA
	CCCAGTCCACCTGCGAGAG
GSMUA_Achr7T27190_001	CAACGACGGCTGGTAACAA
	TCCCTCGTTGCTTCTTTGTC
GSMUA_Achr3T25520_001	ACAGGGGTAAATACCGTGCTG
	GGACGCATCTCCAGGTTTG
GSMUA_Achr1T13650_001	GATCCGGCTACCGGGTACTA
	TGACAGCATCGTTCGTTCAT
GSMUA_Achr9T16350_001	CCATCTGCCAGCTCTCCTAC
	TAGAGAGCGGGATGGTGAAG
GSMUA_Achr6T20420_001	TGGTTAACCGGAGGGGATA
	TTGCTTGTGGGAGAGAGTCA
GSMUA_Achr5T09440_001	CATCTGCGAGATCTCCTACACC
	GAGAGGGGGATGGTGAAGTC
GSMUA_Achr11T18110_001	CTCTCTCTCGGGTGCTGCT
	AGTTGGCGGGCCTGTAGTA
GSMUA_Achr1T16570_001	AGTCCGGCATGGACAAGAC
	CCCTCGACAGGGTAGAATCC
GSMUA_Achr10T21680_001	GCAATAGAGGCAGCCAAGAG
	ACGCCAATGGTTTCCAGTAG
GSMUA_Achr4T19200_001	GGAGAAGGTGAAGGACATGG
	GACTGGTAAGGGGAACACGA
GSMUA_Achr4T14830_001	TGTCTTCATGGACCGACAAG
	CAGCTTGGTCTTGTCCTCGT
GSMUA_Achr5T16860_001	CCAAGGACAAGGGATCTGAG
	AGCTCTTCACAGCATGAGCA
GSMUA_Achr7T15580_001	CGAGACTGTCGTCCCTGGT
	GCTCTCCTCCCGACTCCTC
GSMUA_Achr10T04300_001	CAATAGCAAACCTCCACCTGA
	CCCTTCCCCTGATCATATCC
GSMUA_Achr3T30940_001	GATCGCAACGACGACAAAAC
	CAACGTTGTTGGTTAAGCCTTG
GSMUA_Achr1T17700_001	CAAGTTCTTTGGGCTGTCG
	CTCATCTTGCTGGTGCAGTC

Aquaporin coding genes and primers:

Gene*/ NCBI Accessions	Primer sequence (5’ to 3’)
FF561783	GAGGGGAAAGAGGAGGATGT
	GTAGAAGGACCAGGAGCTGAG
FL659825	GGTTGGGAGCAAACAAGTTC
	GTAGAAGGACCAAGAGGTGAGC
ES436988	CTCTCAGATCGCCATTGGAC
	GACACCGCCACGAACAAG
FF559745	GCCGCTTCGATGACTCTTT
	GATGTTGAAGCCCACCGATA
ES431237	AGCTGGGAAGCAGAAAGGA
	CTACCATCACCGCCACCA
FL650336	ATGGCGAAGGACATCGAG

	GGTCCGACTGGTGCTTGT
FL649971	ATGGCGAAGGATGTGGAG
	GGTCGGACTGGTGCTTGTA
DN239603	CGGAGCACGGGAAGTACA
	GTCGGTCGGGTTCACGTT
DN239593	TGCCGATCACTCAGATAGCC
	ATGTTAGCTCCCACGGACAC
ES432704	CCACCGTGAGGTGTACGAG
	GCGGAAGAGGGTGATGTTG
ES436452	CTTCGCATAACGATCGGAAC
	GGCTACTGCGACGTAGAGG
FL667907	GAAGGAGGTGAACGTGGAG
	CGCGTTCTGTTCCTTGTG
DN238269	TTGGGCAATTTGACGACTCT
	CAGCTTGTCTGCAAACCACA

Anti oxidant proteins coding genes and primers:

Gene*/ NCBI Accessions	Primer sequence (5' to 3')
JK537910 (Catalase)	TGGACCACCAACTCTGGG
	GGACTCCCACGCTCATGAAT
FL667088 (Peroxiredoxin)	GACGAAGGTGTCTCCTCCAA
	CTGCAGTTTTTCAGCCCATC
FL667313 (Peroxiredoxin)	AAGGGCGTTGATGAGATCCT
	TGCTCCACCTTCTTCGATGT
FF558218 (Thioredoxin reductase)	GTTCCAGGAGGGCAGCTAAT
	AGTGGCTCCTGTTGCGATAA
JK544457 (Ascorbate peroxidase)	CTCATCGCCGAGAAGAACTG
	GGTCCTCCGGTAACTTCGAC
JK535321 (Ascorbate peroxidase)	ATGGCATGATGCTGGAACTT
	TTGGGCAAACTGATGAATCC
FL667540 (Ascorbate peroxidase)	TCAAGGAGCTGCTCAAGACC
	TAGCACTGGCCAACTGGAAC

Aux/IAA protein coding genes and primers:

Gene*/ NCBI Accessions	Primer sequence (5' to 3')
ES436589	ACTGTGGCTCTCAAGGAATGA
	TTGCACTTCTCCATTGCTCTT
FL647788	GAAAACAAGGGCTAGGGTGTC
	ATCACCAACAAGCATCCAGTC
FL663734	CCTCAGCACTCGAGAAGATGT
	CTCTTGCAGGAGTTGGTGAAC
DN239045	AGGGGGTTCGCTGAGACTAT
	CCTTGCTTCCCTTCTCAGAGT
FF562258	TGGAATGCCTCTATGTCAAGG
	CAAGCATCCAATCACCATCTT
FL658218	CTGGCTGTCCAATCTGAGAAG

	ACCCACAGCTTGATGAAACAC
FL666597	TGTAGCCAAGAAACAGGTCGT
	TCCTTCTGCACCACCTAAAGA
ES434565	AGGACCTCAAAATTGCTGGAT
	ATGGGAACCTAGAAAGGACCA
DN239791	AAGAGGGGGTTTGTGGAGAT
	TCCCTTCTCAGAGTGGACAGA
FL664138	ATGGGGAATGTTCTTGGAAAC
	GTAGGGTCAAAACGATGCAAA
FF557659	GAACAAAATGCAAGGTGGTTCA
	TCCAGGGATCATCTCCAACA
FF557313	GCAGAGCAACTCAGCGGATA
	TGCACCTTTGTGCAACTCCT
FF559342	GGATCGGTTGGGAGGTCTCT
	TTCAGCACATCCTCAGGTGAA
FL649597	CCATGGCCTTGGAGAAGATG
	TGCTCTTGGTGCGAGTCCTA
FF558559	TGTGGTGCACATGGAATGTC
	TGGACTTCTCCATGACCCTTG
FF559468	GCCGGTGTTCTGCAAGCTA
	CGTGGAAGCAACAGCAGGTA
FF558832	GGATGACATGATGTTGGTTGG
	ACTGGTGGCAGCTTCCTCTT
FL649932	ACTGTGGCTCTCAGGGGATG
	TTGCATTTCTCCATGGCTCTT
FL658549	CAACAAAAGCTGGTTGGTTGTG
	TTCGGTTCTTTGCCAGCTATT
FF562359	GAACCGGTTGGAAGTTGGTC
	TCATCAAATTGGCCTCTCCA
ES432713	TGCATTCCCCTGAAAATGTG
	ATCGCCGACCAGAAGAATGT
FL648295	TTTGTCGACAGGGAGAACGA
	CCTGTTTTCCCATTTTCAGCA
FF561993	CCCTGAAGGGAACACTGACC
	GCAGATGCATCACCGAGAGT

Miscellaneous banana homologs of *AtbZIP53*, *TBZ17* responsive genes and their primers:

Gene*/ NCBI Accessions	Primer sequence (5' to 3')
GSMUA_Achr9T15390_001 (Asparagine synthetase)	TCTTTCCGAAGAATGCTGCT
	GCCTCCACCATAGCATCTTG
GSMUA_Achr5T28200_001 (Asparagine synthetase)	AAATGATGCAGAACGCGAAG
	GGATCGTACGCCGAAACAT
GSMUA_Achr9T04460_001 (Asparagine synthetase)	TTCGAAAGCTTCTTCCCACA
	TCAACCAGTGAGCCCTGAAG
JV369830 (Asparagine synthetase)	ACTGGATACACGCAATAACACTTAC
	CCGGTTTTGCTGGAGTACAAG
JV315011 (Asparagine synthetase)	CAAGCTTCGTTCGCTTTACC
	AGGATTCGCTCTGGGAGGTAT

GSMUA_Achr11T21250_001 (Proline dehydrogenase)	CACAACGTTCGATCTGGACA
	ACTGTGGAGGTGCAAAGGAGT
GSMUA_Achr5T20640_001 (Proline dehydrogenase)	AGCATCCAGGAAACACACCA
	TCACCTGGAAACCTGCATTC
GSMUA_Achr11T21240_001 (Proline dehydrogenase)	GTTCATCCGAGCATCCAAGA
	TGGAAACCAGCATTCCTGAG
JV375224 (Proline dehydrogenase)	CCCATAACGTCGAATCAGGA
	CCTCGATTTTCTTCGGCTCT
JV313799 (Proline dehydrogenase)	ATATGTCGACCGAAGGCAAG
	TTCTCGTTCCCGTTCTGGAT
JV333266 (Proline dehydrogenase)	TGTGGTTGCGAAGATCACAG
	CCTCGCACGTTTTACATAGCC
GSMUA_Achr3T29410_001 (Asparagine synthetase)	TCGTTCGGAGCTTCTCACTT
	TAATCCTTGCTGCCGTCTTC
GSMUA_Achr1T21220_001 (Asparagine synthetase)	CCAGGGCTTACCAGGAATTT
	TCTGCTCCTGCTGAATGCT
JV352031 (Asparagine synthetase)	GAGATTGCACGTCAGCGATA
	ACATGTCTGTGCCCATCGTA
JV351202 (Asparagine synthetase)	AAACCCGACTCCGAAGAGTT
	GGGTTTGAGCTCGGATTCTT
JV344777 (Asparagine synthetase)	TCGTCGGTGAATATCATCTCG
	GTGTCGCTGACATGCAACC
GSMUA_Achr6T17480_001 (Sucrose-phosphate synthase)	CTCGCGTACTCGGTCAAAG
	AGCTGCTCGTGATCGGTATC
GSMUA_Achr4T16070_001 (Sucrose-phosphate synthase)	GTGGACTTCGATGCCATCTT
	CCTGCGGTTCTCAGTAGCTC
GSMUA_Achr9T22510_001 (Sucrose-phosphate synthase)	GAGCTCCGGAAGCATATGAG
	CAGTCTTCTGCACTCCACCA
GSMUA_Achr4T06050_001 (Sucrose-phosphate synthase)	ATGAGAATCCAGGCACTTCG
	GCTCGCTTTGGGCTGTATTA

* Banana genome database locus identifiers

Chapter 5

Conclusions and future work

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

1. *MusaDHN-1* transcript is induced in a variety of abiotic stress conditions in banana plants and its constitutive overexpression using a strong promoter, like *Zea mays* polyubiquitin promoter, in transgenic banana plants imparts multiple abiotic stress tolerance without compromising on any important vital growth parameters. This study was the first to report characterization of a banana gene using transgenic banana plants. Further, *MusaDHN-1* dehydrin gene characterized in this study can potentially be used to develop abiotic stress tolerant lines in other important crop plants.

2. *MusaWRKY71* is an important member of the *WRKY* gene family in banana and it possess the capacity to cross regulate other *WRKY* gene members as well as other genes involved in biotic stress signaling pathways. The plants overexpressing this transcription factor were found to have improved oxidative and salt stress tolerance. Further, since this gene was found to regulate multiple genes involved in biotic stress perception and mitigation, it may well be a significant link in the interface between abiotic and biotic stress pathways in banana. Further studies involving microarray experiments based on the newly uncovered banana genome sequence are warranted in future to fully establish the multi faceted roles played by *MusaWRKY71* gene in banana stress responses.

3. *MusabZIP53* gene plays important roles in multiple cellular networks involved in abiotic stress related signaling in banana as indicated by its induction in response to different abiotic stress conditions and by the

differential regulation of various genes related to abiotic stress perception and mitigation in *MusabZIP53* overexpressing plants. Further, it also contributes to the maintenance of sucrose homeostasis in banana plant through a linked uORF present in its 5'UTR. Also, our findings pertaining to the coordinated induction of all four expressed PPO coding genes by *MusabZIP53* gene (in effect controlling the total cellular PPO activity) assign a hitherto unknown role for *bZIP* genes in plants.

4. Among the three genes characterized as part of this study, the *MusaDHN-1*dehydrin gene is the most suited to develop abiotic stress tolerant banana plants. Being an effector protein in contrast to the regulatory nature of MusaWRKY71 and MusabZIP53 transcription factor proteins, MusaDHN-1 works as a molecular shield and directly protects the various cellular macromolecules from the effects of dehydration. As is evident from our results, *MusaWRKY71* and *MusabZIP53* transcription factor genes are involved in multiple regulatory pathways in banana plant and hence constitutive overexpression of these genes might not be the most appropriate way to develop stress tolerant banana plants. Further, native regulatory elements (promoter and 3' UTR) can be used to overexpress *MusaDHN-1* in transgenic banana plants to create cis-genic banana plants with desired characteristics.

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