

CHAPTER 1

1.0 INTRODUCTION

1.1 Banana

Banana is a monocotyledonous, perennial herb within the order Zingiberales, and the family Musaceae. The Musaceae is divided into two genera: *Musa* and *Ensete*.

Musa consist of about 40 species and is distributed through India, New Guinea, Australia and Southeast Asia (Simmonds, 1962). The *Musa* genus is grouped into four sections: Eumusa, Callimusa, Rhodochlamys and Australimusa. Eumusa is the most widespread and contains the greatest number of species and forms, for it includes all the edible seedless bananas. Almost all cultivars of the edible banana are now classified under two species *M. acuminata* (AA) and *M. balbisiana* (BB), both belonging to Eumusa section. According to Simmonds (1962) most cultivated bananas were derived from natural hybridization between two diploid species *M. acuminata* and *M. balbisiana*. *Musa acuminata* surpasses *Musa balbisiana* in variability and in diversity of species, and at least nine sub species have been described (*ssp.malaccensis*, *ssp. microcarpa*, *ssp.burmannica*, *ssp. burmannicoide*, *ssp. siamea*, *ssp. banksii*, *ssp. errans*, *ssp. zebrine* and *ssp. truncate* (De Langhe, 1969), whereas *Musa balbisiana* is less diverse with no subspecies recognized. Most of edible types that are derived from these species are triploid, although diploid (AB) and Tetraploid (ABBB) cultivars are also known.

Essentially hybridization between various subspecies of polymorphic species *M. acuminata* led to a range of diploid cultivars. Diploid AAs then gave rise to triploid AAA types. Hybridization between *M. acuminata* (AA) and *M. balbisiana* (BB) gave rise to the various AAB and ABB types presently found. The majority of cultivated types are triploid with AAAs providing many sweeter dessert cultivars whereas AABs and ABBs often provide a starchier cooking type.

Musa acuminata is the most important species and wild seeded diploid forms have their center of diversity in the Malaysian region where six of the nine subspecies overlap namely, *malaccensis*, *siamea*, *truncata*, *microcarpa*, *burmannica*, and *burmannicoides*. Four of these subspecies are reported from Malaysia only, *malaccensis*, *siamea*, and *truncate* from peninsular Malaysia and *microcarpa* mainly from East Malaysia (Borneo) (Simmonds, 1962). Therefore Malaysia is the most important center of diversity for wild *Musa acuminata* forms. Among the four specific forms, *malaccensis* is the most diverse and is the progenitor of the local AA cultivated bananas (Simmonds, 1962).

1.2 Importance of Banana

Banana (*Musa* spp.) is one the world's major food crops and widely grown in developing countries (Roux *et al.*, 2001; Madhulatha *et al.*, 2004). Banana is a staple food crop for millions of people, vital to food security and ranks in the world's top four food products (Roux *et al.*, 2001). It also provides a valuable source of income through local and international trade and contributes to the livelihood of many people through crop production, processing and marketing (Resmi and Nair, 2007). According to an FAO report (2006), the total world banana export was 16.8 million tonnes with a value of 5.8 billion US \$ (Table 1.1). Banana is cultivated in more than 130 countries in the

tropics and subtropics (Resmi and Nair, 2007), with a total production of 81.2 million metric tones (Table1.2).

Table 1.1: Export quantity and value of banana (FAO, 2006).

Country	Export quantity(tonnes)	Export value(1000 US \$)
World	167,890,32	5799147
Asia	25,846,23	478105
Africa	603,098	204945
Americas	11,461,948	3076245
Oceana	151,000	74
Europe	2,139,212	2039778

Table 1.2 : Harvested area, yield and production of banana (FAO, 2007).

Country	Area (ha)	Yield (Hg/ha)	Production (MT)
Word	4410509	184249	81,263,358
Asia	2096690	223445	46,849,643
Africa	1028270	77882	8,008,400
Americas	1190214	208273	24,788,970
Oceana	83595	144834	1,210,745
Europe	11470	345485	406,500

Major banana cultivation is centered in three continents, Asia, Americas (mostly south and Central America), and Africa. India, the largest producer of banana, contributes 26% of world production (Martin *et al.*, 2007). Banana is the second most widely cultivated fruit after durian in Malaysia. According to an FAO report (2007), the total production of banana in Malaysia was 530,000 metric tonnes. Most of the cultivated areas in Malaysia grow *Pisang Berangan* and the Cavandish type, both for local consumption and export. In 2006, banana export brought country 6.8 million USD into the country (FAO, 2006).

1.3 Salinity and Effects on Plant

Salinity is considered a major environmental factor which has a limitative effect on plant growth and productivity (Allakhverdiev *et al.*, 2000; Liu *et al.*, 2000; Veeranagamallaiah *et al.*, 2007) and is known to influence many physiological and metabolic processes (Läuchli 1984; Olmos *et al.*, 1994; Liu *et al.*, 2000), such as diminution in rate of leaf surface expansion, photosynthesis, protein synthesis, and energy and lipid metabolism (Parida and Das, 2004) . It also affects crop production by interfering with nitrogen uptake, reducing growth and stopping plant reproduction. High salinity conditions result in hyperosmotic damage to most plants, and increased Na⁺ concentrations disrupt cellular processes by interfering with vital Na⁺ sensitive enzymes and by affecting essential ion transport (Yoshida, 2002).

1.3.1 Salinity Management

Plants not only are important as the main source of food for humans and animals, but also for a large number of non food products (Yoshida, 2002). Biotic stresses such as pathogens and insects and abiotic stresses such as salinity, drought, heat and cold,

chemical and oxidative stress threaten plant life and have potential devastating effects on plant growth and productivity (Mahaja and Tuteja, 2005).

Global warming and climate change, quality and quantity of crop and fruit production, progressive increasing of world population and destruction of arable land as a result of development of cities reduces crop yield through the world. The real and potential shortage of food against the specter of the growing population of the world draws world-wide attention towards the necessity of development of stress tolerant crops. According to a report by Flowers *et al.* (1997), about one-third of irrigated land is considered to be affected by salinity and highlighted the critical situation and importance of salinity management.

Salinity management strategies include several methods such as drainage, replacement of plants that cannot tolerate saline conditions by tolerant species, using plant breeding to generate more salt-tolerant crops and more recently a focus on the potential of applying plant biotechnology and genetic engineering including using technologies such as gene silencing *via* small RNAs to increase plant salt tolerance.

Drainage is one approach to reduce the movement of salt to the root zone and control salinity but is not always practiced due to its cost and efficiency (Makin and Goldsmith, 1988). Plant breeding is another strategy that can be useful tool for generating more salt-tolerant crops but until now attempts of breeders have been largely without success. More recently, biotechnology approaches have been used successfully to create transgenic salt-tolerant plants. The possibility of gene manipulation in plants to improve its tolerance to salt has opened up opportunities to use other novel genes in

future studies. One such family of genes is microRNAs which are associated with gene silencing.

1.4 MicroRNA

MicroRNAs belong to a family of non coding RNAs and were discovered by two different research groups (Lee *et al.*, 1993; Wightman *et al.*, 1993) but they were not recognized until 2001(Lau *et al.*, 2001; Lee and Ambros, 2001). MicroRNAs are small endogenous single stranded RNA of about 22 nucleotides with structural, enzymatic and regulatory functions (Hannon, 2002; Ambros *et al.*, 2003; Ambros, 2004; Bartel, 2004) responsible for post-transcriptional gene silencing by the degradation or translational inhibition of their target messenger RNAs (Ikeda *et al.*, 2006). The majority of miRNA genes exists as independent transcriptional units and is transcribed by RNA polymerase II into long primary transcripts, called pri-miRNAs (Kim, 2005). The pri-miRNAs can be quite long, more than one 1 kb and often have internal runs of uridine residues, which would be expected to prematurely terminate pol III transcription (Bartel, 2004), Then nuclear cleavage of the pri-miRNA is performed by Drosha RNase III endonuclease in animals or Dicer in plants to release a 60-70 nt stem loop intermediate, known as an miRNA precursor, or pre-miRNA (Lee *et al.*, 2002; Bartel, 2004). Drosha cleaves both strands of the stem at sites near the base of the primary stem loop and generates pre- miRNA stem loop with 5' phosphate and about 2-nt-3' overhang (Lee *et al.*, 2003). Drosha does not exist in plants, but DCL1 (Dicer) has a nuclear localization signal, suggesting it processes the pri-miRNA as well as the pre-miRNA (Reinhart *et al.*, 2002; Bartel, 2004; Kidner and Martienssen, 2005). The nuclear cut by Drosha defines one end of the mature miRNA whereas the other end processed from cytoplasmic cut by RNase III endonuclease Dicer (Lee *et al.*, 2003). DICER or DICER-like (in plants) enzymes cleave the double-stranded stem and releases miRNA/miRNA*

duplex with 2-nt-3'overhang (Bartel and Bartel, 2003). One strand of the mature miRNA, the guide strand (miRNA), subsequently becomes incorporated as single-stranded RNAs into an RNA induced silencing complex (RISC), where it guides the cleavage or translational repression of its target mRNA by base-pairing with the target (Bartel, 2004; Berkhout and Haasnoot, 2006).

1.5 Goals and Objective of the Research

The main goal of this study was to identify microRNAs related to salt stress in the banana plant (*Musa acuminata ssp. malaccensis*). The specific objectives of this project were:

1. To determine the minimal inhibitory sodium chloride (NaCl) concentration for survival of banana plantslets (*Musa acuminata ssp. malaccensis*).
2. To construct a small RNA cDNA library from salt-stressed banana plants at this concentration.
3. To characterize miRNA potentially related to salt tolerance in banana.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Taxonomy of the Genus *Musa*

Simmonds (1962) classified the family Musaceae in the order Zingiberales.

Musaceae is divided to two genera *Musa* and *Ensete*.

2.1.1 *Ensete*

The genus *Ensete* geographical distribution is mostly in Africa but a few species are also found in Asia from northeast India to the Philippines and New Guinea (Purseglove, 1972). Plants of this genus are monocarpic, non-suckering with a distinctly swollen base and they produce large-sized seeds (Samson, 1992).

2.1.2 *Musa*

The Genus *Musa* are perennial herbs which comprises 40 species and are distributed mostly in southern Asia and the Pacific (Simmonds, 1962). Generally leaves in this genus are large, long and spirally arranged. The genus *Musa* is divided into Eumusa, Rhodochlamys, Callimusa and Australimusa (Cheesman, 1974).

2.1.2.1. Eumusa

Eumusa is the largest section among other sections and has the widest geographical distribution. The basic chromosome number is $2n = 22$ and is characterized by horizontal or drooping bunches, male axes and milky or watery juice

(Stover and Simmonds, 1987). *Musa acuminata* is now classified in this section. *Musa acuminata* Colla is a variable species with a wide geographical distribution from Burma through Malaysia to New Guinea, Queensland, Samoa and Philippines (Simmonds, 1954). In addition the edible banana *Musa* AAA group were derived from *Musa acuminata* Colla (Simmonds and Shepherd, 1955).

2.1.2.2 Rhodochlamys

The basic chromosome number is $2n = 22$ in this section and characterized by having an erect inflorescences, at least at the base, with fruit pointing towards the bunch apex. Rhodochlamys mostly distributed in Northeast India, Bangladesh, Myanmar and Thailand (Hakkinen and Sharrock, 2002).

2.1.2.3. Callimusa

The basic chromosome number is $2n = 20$ in this section and mostly are distributed in Indochina, Malaya and Borneo.

2.1.2.4. Australimusa

The basic chromosome number is $2n=20$ in this section and they are distributed from Queensland to Philippines. Australimusa cultivars differ from other cultivated banana by the erect fruit bunches and the generally red juice (Stover and Simmonds, 1987).

2.1.3 General Morphology

The banana plant consists of stem or corm and inflorescence. The subterranean stem or corm bears developing suckers, the root system, the pseudostem, the leaves

whereas the inflorescence bears the flowers and subsequently the fruit. Suckers are tool of vegetative propagation. The size of the corm is dependent on the size of the plant and internally divides into two regions, a central cylinder and an apical protein (Skutch, 1932). The root system is confined mostly to the upper 40 cm soil because of good correlations bunch weight and quantity of roots produced (Stover and Simmonds, 1987).

The adventitious root system which arises from the rhizome is replaced with the primary seedling root, but in plants established from suckers the root system is adventitious from first growth (Stover and Simmonds, 1987). The rhizome system is sympodial like most rhizomatous monocotyledons (Holtum 1955). The leaf area is large and consists of a sheath, a petiol and a blade. The inflorescence is a complex spike with stout peduncles on which flowers are arranged in a nodal cluster in two rows on a transverse cushion (crown), subtended by large spathe-like bracts that are nearly ovate and usually purple-red in color.

2.1.4 Banana Tissue culture

The term “plant tissue culture” is commonly used to describe the *in vitro* and aseptic cultivation of any plant part on a nutrient medium. Most of the procedures used currently are derived from an original technique that was demonstrated by White (1943, 1963). Cox *et al.* (1960) reported the successful embryo zygotic culture, which was the earliest successful application of *in vitro* culture of *Musa*.

The *in vitro* production of plants generally consists of three stages as described by Murashige (1974)

- (I) Establishment of the aseptic culture
- (II) Multiplication of propagules
- (III) Regeneration of plant for re-establishment in soil

Recently, tissue culture techniques are becoming increasingly popular as an alternative means of plant propagation (Shah *et al.*, 2009). The use of embryo rescue, shoot-tip culture, protoplast culture, cell suspension culture and related cell culture techniques have been used to overcome limitations in banana production and multiplication.

2.1.5 Shoot–tip Meristem Culture and Multiplication

Among the tissue culture techniques introduced for banana improvement, only shoot-tip meristem culture has been developed and applied widely (Novak, 1992). Successful application of *in-vitro* shoot-tip culture of banana was reported in early 1970 from Taiwan (Ma and Shii, 1972, 1974). Hormones such as cytokinins have a vast effect on banana micro propagation and multiplication so that many scientists in the world have dedicated their research to this area.

In 1983, Cronauer and Krikorian reported the establishment of rapidly multiplying culture from excised shoot tips of bananas. Philippine Lactan and Grande Naine were two banana clones used for their experiments. They demonstrated that apices cultured on semi-solid media formed a single shoot whilst apices placed in liquid media produced clusters. Furthermore, to form multiple shoot clusters, individual shoots were

longitudinally split through the apex. The results showed that 5 mg/L BAP significantly stimulated shoot multiplication.

Arinaitwe *et al.* (2000) applied shoot-tips of banana (*Musa* spp.) for micropropagation. The modified MS medium and three cultivars of banana, Bwara (AAA-EA), Kibuzi (AAA-EA) and Ndiziwemiti were used. Different concentrations of cytokinins including 6-benzylaminopurine (BAP), thidiazuron (TDZ), zeatin ZN, isopentenyladenine (2iP) and kinetin (KN) were employed to determine the appropriate cytokinin concentration range for banana cultivars micropropagation. They demonstrated how cytokinin type, its concentration and also the banana cultivar significantly influenced shoot proliferation, so that shoot proliferation was extensively dependent on these factors. Cultivars had a better response to BAP compared to other adenine-based cytokinins (ZN, KN and 2-iP). Also the TDZ has showed high cytokinin activity, as low concentrations of TDZ (0.045, 0.23, 1, 14, 5.68, 6.81 and 9.1) considerably increased proliferation rate of Ndiziwemiti (Arinaitwe *et al.*, 2000).

Proliferation rate of Ndiziwemiti was improved to 9.5 shoots per explant by applying 9.1 mM of TDZ. The results showed an economical privilege of TDZ rather than other adenine-based cytokinins due to greater shoot proliferation response of cultivars to different TDZ concentrations (Arinaitwe *et al.*, 2000).

Gubbuk and Pekmezcu (2004) reported the use of three newly selected banana types (Alanya 5, Anamur 10 and Bozyaz 14) to study the effects of different cytokinins on shoot multiplication. Cytokinins such as BAP (5, 10, 20 and 30 μ M) and TDZ (0.4, 1, 2 and 3 μ M) were applied for the propagation stage. Similarly to determine the best combination of cytokinin/auxin for propagation, BAP and TDZ were supplemented by 1

μM IAA. The results revealed that there was better effect of TDZ on shoot proliferation and elongation, compared to BAP in all the three banana types. In addition, combinations of cytokinin with IAA increased shoot proliferation and elongation more than when BAP was used alone. The concentrations of BAP below 20 μM or TDZ below 1 μM did not show any increase in shoot proliferation, and concentrations of BAP over 20 μM and TDZ over 2 μM suppressed shoot elongation.

Shoot meristems were used by Kalimuthu *et al.* (2007) for micropropagation of *Musa sapientum*. MS medium supplemented with different concentrations of BAP and 0.2 mg/L NAA (Table 2.1).

Table 2.1 : Concentrations of BAP and NAA used by Kalimuthu *et al.* (2007) for micropropagation.

BAP concentration	0.5 mg/L	1.0 mg/L	2.0 mg/L	3.0 mg/L	4.0 mg/L	5.0 mg/L
NAA concentration	0.2 mg/L	0.2 mg/L	0.2 mg/L	0.2 mg/L	0.2 mg/L	0.2 mg/L

Their results showed that the combination of 3.0 mg/L BAP and 0.2 mg/L NAA the most suitable combination. Three sub-culturing (21 days each) carried out by Kalimuthu *et al.* (2007) for further multiplication of shoots after establishment of culture and initiation of shoot buds. They recorded an increasing multiplication up to rate to 3-fold during every sub-culture.

In 2007, Sipeh *et al.* conducted research to study the influence of different concentrations of BAP and IAA combinations on banana shoot generation. Pisang Nangka (AAA) which is one of the economically important Malaysian bananas was used for the macropropagation. The maximum mean number (5.46 ± 0.22) of shoots produced per explant was achieved when 20 mg /L BAP was followed by 0.175 mg/L IAA.

2.2 Salinity Effects on Agricultural Products

Salinity is a major abiotic stress affecting plant productivity worldwide and costs million of dollars in lost yield and damaged infrastructure (Behdani *et al.*, 2008; Meloni *et al.*, 2008). The effect of NaCl salinity on nitrogen and amino acid metabolism will damage the value of foods in two different ways. In the first way, protein synthesis might be influenced by NaCl salinity, thus amino acid metabolism will be enhanced in general. Secondly, the nutritional value of the plant product might be reduced as a result of NaCl salinity (Keutgen and Pawelzi, 2008). Due to the destructive effect of salinity on crop plants in all aspects, many research groups in the world put an effort to find effective way against salinity. So far lots of salinity related studies, as decribed in the following paragraphs, have been done on plants such as *Hordeum vulgare*, *Medicago*, *rice*, strawberry and tomato.

To study the effect of NaCl on banana cv. *Nanicao* (AAA), Ulisses *et al.* (2000) treated buds grown in MS medium with different NaCl concentrations (0, 20,40, 60, 80, 100 and 120 mM). According to their study plant regeneration was greatly inhibited by increasing of NaCl concentrations and 120 mM of NaCl was determined to be a lethal concentration.

The influence of salinity and sodicity on stigma receptivity and grain filling of rice (*Oryza sativa*) under field conditions was studied by Khan and Abdullah (2003). Their result, showed a significant reduction of pollen viabilities in all cultivars under salinity and sodicity stress conditions. Also the starch synthase activity inhibition was more significant in sensitive cultivars compared to tolerant cultivars.

Demural *et al.* (2005) studied the effects of salinity on malting barley (*Hordeum vulgare* L). Two cultivars of Kaya and Scarlet and four parameters; growth, chemical composition, superoxide dismutase and peroxidase activities were studied. The result showed reduction in growth of both cultivars as a result of salinity. Compared to Kaya, Scarlet was more efficient, in restricting access of Na⁺ and Cl⁻ into roots and conduction to leaves. The peroxidase activities of cultivars decreased in saline condition, whereas superoxide dismutase activity of leaves increased as a result of salinity.

In 2008 Behdani *et al.* conducted research to investigate the sensitivity of morphological and physiological responses of *Medicago polymorpha* L. cv. Scimitar and *Trifolium michelianum* L.cv. frontier to low levels of salinity. The results showed an increment of sodium (Na⁺) and potassium (K⁺) in both leaves and stems tissues when the salt level was raised. The sodium content in leaves, less than 80 mM, was reported to be threefold higher for *Medicago polymorpha*. However it was twofold higher for *Trifolium michelianum* when compared with the control.

Vegetative and chemical changes of strawberries (*Fragaria x ananassa* L.) under NaCl stress condition were studied by Yilmaz and Kina (2008) .Two cultivars of strawberry (Kabarla and Gloria) and three different concentrations of NaCl (500, 1000 and 1500 mg/L) were used. The results showed, increment in salt concentrations

restricted the vegetative growth of the plants and also influenced chlorophyll and malondialdehyde levels. Moreover accumulation of Na^+ in roots, crown and leaves of the plant and ratios of K^+/Na^+ and $\text{Ca}^{2+}/\text{Na}^+$ significantly affected salt tolerance ability of the plants to saline conditions. The Kabarla cultivar which had a higher ratio of K^+/Na^+ and $\text{Ca}^{2+}/\text{Na}^+$, showed more resistance to saline condition and better growth.

2.3 RNA

RNA or ribonucleic acid is a class of nucleic acids comprising a long chain of nucleotides and characterized by the presence of the sugar ribose (deoxyribose in DNA) and the organic base uracil (thymine in DNA). All types of RNA are transcribed from DNA and are divided into two groups, coding and non-coding RNAs. RNAs are generally involved in vital processes such as protein synthesis (e.g. mRNA, tRNA, rRNA), post-transcriptional modification or DNA replication (e.g. snRNA, snoRNA) and gene regulation (e.g. miRNA, siRNA).

2.3.1 RNA Isolation

Isolation of intact, good-quality RNA is vital for further applications such as RT-PCR, cDNA library construction, and gene expression studies. Generally extracting high-quality RNA is tricky due to high levels of phenolics, polysaccharides, endogenous RNases and other compounds that bind and/or co-precipitate with RNA (Azevedo *et al.*, 2003; Kansal *et al.*, 2008). So far several conventional methods for RNA isolation have been established and used.

Saghai-Marooif *et al.* 1984 used the CTAB method to extract DNA from lyophilized tissue of barley. Later the CTAB method was modified and used for RNA extraction (Kiefer *et al.*, 2000). Chomczynski and Sacchi (1987) developed a single step method for RNA isolation by acid guanidinium thiocyanate-phenol-chloroform

extraction from animal tissue. Venugoplan and Kapoo (1997) modified the original Chomczynski and Sacchi (1987) method and used this for total RNA extraction from plant. Phenol/lithium chloride and guanidinium based methods are efficient methods for herbaceous plants such as *Arabidopsis*, tomato, tobacco, potato and maize (Kansal *et al.*, 2008).

2.3.2 Non-coding RNAs

The term non-coding RNA (ncRNA) is commonly used for RNA that is not translated into a protein. Cells contain various types of noncoding RNAs, comprising components of the machinery of gene expression, such as tRNAs and rRNAs, and regulatory RNAs that affect the expression of other genes (Ambros, 2001). Small nucleolar RNAs (snoRNAs), microRNAs (miRNAs), small interfering RNAs (siRNAs) and small double-stranded RNAs are classified as under regulatory RNAs and may be processed from the introns and exons of longer primary transcripts including protein-coding transcripts (Mattick and Makunin, 2005). RNAs with regulatory functions are able to regulate gene expression at many levels of physiology and development including chromatin architecture, RNA editing, RNA stability, transcription, RNA splicing translation and turnover (Mattick and Makunin, 2005). *Pol IV* and *Pol V*, two Pol II-related, plant-specific RNA polymerases collaborate with proteins of the RNA interference machinery to generate long and short noncoding RNAs involved in epigenetic regulation (Matzke *et al.*, 2009).

RNA regulatory networks may determine most complex characteristics which play a significant role in disease and constitute an unexplored world of genetic variation both within and between species (Mattick and Makunin, 2006). Eukaryotic cells are rich in non-coding RNAs, but only a limited number of trans-acting small ncRNAs were

identified and described to regulate mRNA translation (Mattick and Makunin, 2006; Wang *et al.*, 2007).

According to Gottesman (2004) over 50 small RNAs, have been identified in *E. coli*, that equals to 1%-2% of the number of protein-coding genes. Non-coding RNA are responsible for roughly 98% of all transcriptional output in humans and other mammals (Mattick, 2001). In recent years, the number of identified functional ncRNA genes have considerably increased and over 800 ncRNAs including microRNAs and snoRNAs, were described and listed in mammals (Pang *et al.*, 2005). More than 1100 putative antisense ncRNAs and approximately 20,000 putative ncRNAs were identified from murine and human cDNA libraries (Pang *et al.*, 2005).

2.3.3 Small Nucleolar RNAs (snoRNAs)

Small nucleolar RNAs are a class of small RNA molecules which are transcribed from introns of pre-mRNAs by RNA polymerase II (Kim *et al.*, 2006), and guide the site-specific modification of nucleotides in target RNAs (Mehler and Mattick, 2006). They are known as the most abundant group of noncoding RNAs with 60-300 nucleotide in length, that combine with a set of proteins and form small nucleolar ribonucleoprotein particles (snoRNPs) (Ganot *et al.*, 1997).

Two major classes are defined for small nucleolar RNAs. One group holds the box C (RUGAUGA) and D (CUGA) motifs, whilst the other group carry the box H (ANANNA) and ACA elements (Kiss, 2002). Some small nucleolar RNAs are involved in the nucleolytic processing of rRNAs but most of them function in 2'-O-ribose methylation and pseudouridylation of rRNAs, small nuclear RNAs (snRNAs) and perhaps other cellular RNAs, like mRNAs (Bachellerie *et al.*, 2002; Kiss, 2002). Small

nucleolar RNAs not only play an important role in modification of different RNAs, but some also (snoRNAs U3, U8, U14, E1, E2 and E3) function in the cleavage of pre-rRNAs (Grandi *et al.*, 2002; Xie *et al.*, 2007).

According to Mattick and Makunin (2005) there are more than 300 different snoRNAs in humans and nearly 200 in mouse have been identified (<http://noncode.bioinfo.org.cn> and <http://www.sanger.ac.uk/Software/Rfam>) (Mattick and Makunin, 2005).

Chen *et al.* (2003), reported to have identified 120 different box C/D snoRNA genes with a total of 346 gene variants in rice, using computer-assisted analysis. In addition they revealed the discovery of 270 snoRNA in rice. Although many of the identified snoRNA genes were conserved between rice and *Arabidopsis*, almost half of them were rice specific.

2.3.4 RNA Interference (RNAi)

RNA interference (RNAi) is a sequence-specific gene-regulatory mechanism including post-transcriptional gene silencing (PTGS) virus-induced gene silencing (VIGS) , transgene induced gene silencing (TIGS) and transcriptional gene silencing (TGS) (Mello and Conte, 2004; Dorokhov *et al.*, 2006)

In plants, double-stranded RNA precursors of various kinds are processed by a Dicer protein into short (20-30 nt) fragments. One strand of the processed duplex is loaded into an Argonaute protein, enabling target RNA recognition through Watson-Crick base pairing. Once the target is recognized, its expression is modulated by one of

several distinct mechanisms, depending on the biological context (Figure 2.1) (Carthew and Sontheimer, 2009).

Fire *et al.* in 1998 was the first research group who discovered and explained the mechanism of RNA silencing induced by double-stranded RNA (RNA interference) in the nematode worm *Caenorhabditis elegans*. The sequence-specific posttranscriptional gene silencing by double-stranded RNA is conserved in plants, fungi (*Neurospora*), flies (*Drosophila*), nematode (*Caenorhabditis elegans*), and mammals (Leung and Whittaker, 2005).

2.3.5 Small Interfering RNAs (siRNAs)

Small interfering RNAs are short double stranded RNA of about 23 nt (21–25 nt) with 2 nt, 3' overhanging ends (Wadhwa *et al.*, 2004). They are derived from continuous cleavage of long double-stranded RNA by the dsRNA-specific endonuclease, Dicer (Reinhart and Bartel, 2002).

siRNAs direct the destruction of corresponding mRNA targets during RNA interference (RNAi), in animal and perhaps during other RNA-silencing phenomenon, as well as posttranscriptional gene silencing of plant and quelling of *Neurospora* (Reinhart and Bartel, 2002). The siRNA duplex are integrated into the RNA-induced silencing complex (RISC) and the siRNA guide strand pilots RISC to perfectly complementary RNA targets. Consequently target mRNA are degraded (Richard and Erik, 2009) and the level of encoded protein *via* mRNA is considerably reduced.

In plants, endogenous siRNA can also lead the transcriptional gene silencing (TGS) which was first observed during transgene and virus-induced silencing (Mello and Conte, 2004) Later centromeres, transposons, and other repetitive sequences were revealed as another source of siRNAs (Lippman and Martienssen, 2004)

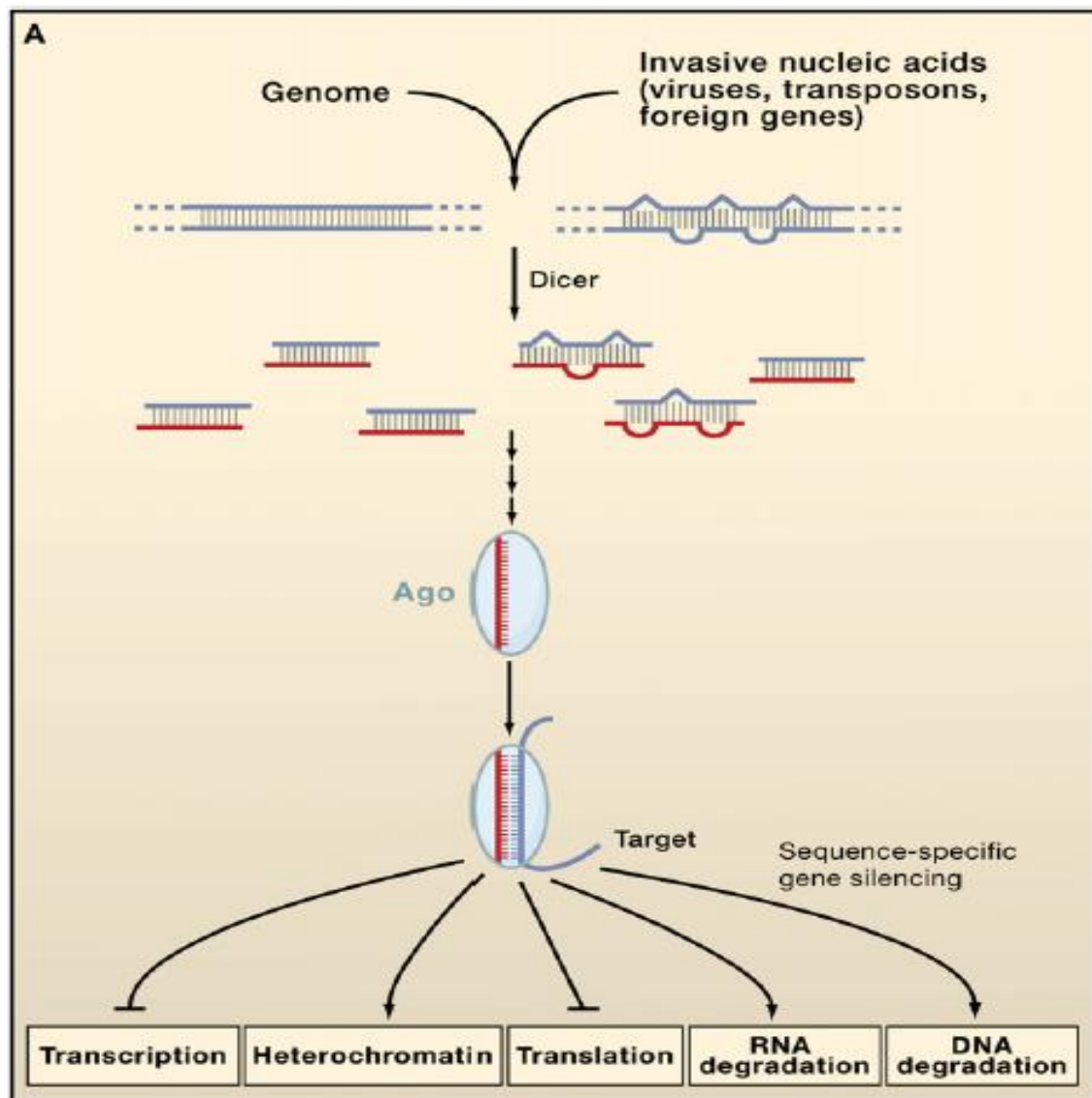
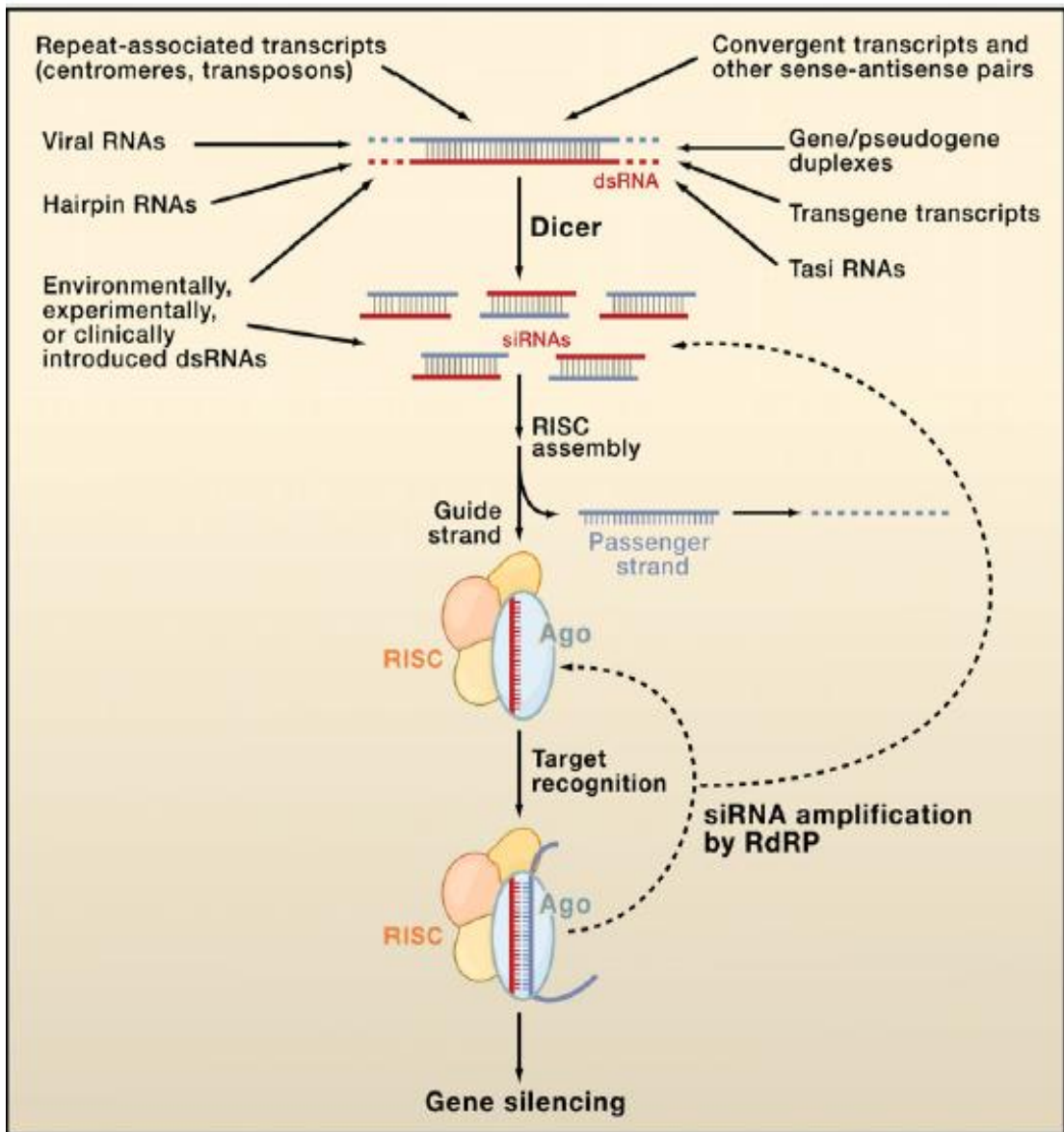


Figure 2.1 Mechanisms of silencing *via* Double-stranded RNA (Carthew and Sontheimer, 2009).



Guide strand: Red.
 Passenger strand: Blue

Figure 2.2 Biogenesis and activity of siRNA (Carthew and Sontheimer, 2009).

Golden *et al.* (2008) have uncovered multitudinous endogenous siRNAs processed from structured transcripts, and also long dsRNAs derived from convergent transcripts and apparent transposon sense-antisense pairs. Plant-specific DNA dependent RNA polymerases are found in plant siRNA pathways and are not found in animals and humans. *Pol IV* and *Pol V* are plant-specific DNA dependent RNA polymerases first discovered in *Arabidopsis* (Pikaard *et al.*, 2008; Mosher *et al.*, 2010). *Pol IV* and *Pol V* are specialized for siRNA production and transcriptional gene silencing (Ream *et al.*, 2009). siRNAs not only are restricted to posttranscriptional modes of repression but they are involved in induction of heterochromatin formation and siRNA-mediated DNA methylation (Carthew and Sontheimer, 2009).

The siRNA-mediated DNA methylation pathway in plants and involvement of two plant-specific DNA dependent RNA polymerases (*Pol IV* and *Pol V*), the RNA-dependent RNA polymerase (RDR2), DICER-LIKE3 and Argonaute proteins (AGO4 and AGO6) were described by Pikaard and Tucker (2009) and Matzke *et al.* (2009). *Pol IV* most likely generates transcripts that are used as templates by RDR2, thus producing double stranded RNAs that are cut into ~24 nt double stranded siRNAs by DCL3 (Pikaard and Tucker, 2009).

A set of endogenous siRNAs in *Arabidopsis*, which guide the endogenous mRNAs cleavage have been described by Vazquez *et al.* (2004). They have claimed, these siRNAs are different from earlier described regulatory small RNAs. Two differences have described. First, they need cosuppression pathway factors (RDR6 and SGS3) and also miRNA pathway components (AGO1, DCL1, HEN1, and HYL1). Second, these siRNAs function in repressing of the genes expression that have little

overall similarity to the genes from which they originate, a characteristic previously reported only for miRNAs.

Ho *et al.* (2007) characterized siRNAs by cloning and sequencing them from *Brassica juncea* leaves infected with Turnip mosaic virus (TuMV). It has been described that, the siRNAs with 21-22 nt long were the most abundant species in TuMV siRNA population. They believed they are derived from the same siRNA hotspots and this may demonstrate the similarity between the plant Dicer-like (DCL) enzymes. The vigorous GC bias which was detected for TuMV siRNAs against the virus genome has shown the tendency of DCL to target GC-rich regions. Dicot micro-(mi) RNAs displayed higher GC content than their DCL1 substrate RNAs, indicating that the GC bias may be ancient, and therefore may be important for the RNAi technology (Ho *et al.*, 2007).

2.3.6 MicroRNAs in Plants

A miRNA gene is transcribed as a long sequence of more than 1 kb, which is called primary miRNA (pri-miRNA) (Figure 2.3), by RNA polymerase II enzymes (Bartel, 2004; Lee *et al.*, 2004). Afterward pri-miRNA is cleaved by Dicer-like 1 enzyme (DCL1) to a stem loop intermediate known as miRNA precursor or pre-miRNA (Zhang *et al.*, 2006b). In plants dicer-like 1 enzyme (DCL1) cleave miRNAs into miRNA:miRNA* duplex in the nucleus instead of cytoplasm (Bartel, 2004). Then HASTY, the plant orthologue of exportin 5, transfer the duplex into the cytoplasm. (Zhang *et al.*, 2006b) miRNAs are unwound into single strand mature miRNAs by helicase in the cytoplasm, (Bartel, 2004). Lastly mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) and direct the translational repression or cleavage of its target mRNA by base-pairing with the target mRNA (Bartel, 2004;

Dugas and Bartel, 2004). Other than DCL1, HUA ENHANCER1 (HEN1) is also required for miRNA biogenesis in plants and post-transcriptional gene silencing (PTGS), which has two dsRNA-binding domains and a nuclear localization signal (Park *et al.*, 2002; Boutet *et al.*, 2003). Despite the close similarity of miRNAs biogenesis and functional mechanism in both animals and plants, plant miRNAs display some differences. The stem-loop structures of plant pre-miRNAs are larger and more variable in compared to animal pre-miRNAs (Yang *et al.*, 2007). Moreover the mature plant miRNAs pair to their target sites with near-perfect and unlike animals miRNAs they normally identify a single target site in the coding region and direct the mRNA to cut (Yang *et al.*, 2007).

miRNAs were first discovered in *Caenorhabditis elegans* (Lee *et al.*, 1993) , and so far many of them have been discovered in diverse species of living organisms, as well as plants. Over 700 miRNAs have been reported to identified in plants (Yang *et al.*, 2007), since the first discovery of miRNAs in *Arabidopsis* in 2002 (Reinhart and Bartel, 2002). Bartel and Bartel in 2003 listed some miRNAs which were identified in *Arabidopsis*. The functions of some of them were recognized and confirmed, such as miR156 which is responsible for floral organ identity and flowering time (Schwab *et al.*, 2005), miR160 which is responsible for auxin signaling and root development (Wang *et al.*, 2005) and miR164 which controls the boundary in meristem, organ formation, separation and petal number (Schwab *et al.*, 2005). Also miR172, 173 and 399 were confirmed to be responsible respectively for specification of flower organ identity and flowering time (Schwab *et al.*, 2005), directing ta-siRNA biogenesis (Allen *et al.*, 2005) , and phosphate-starvation response (Fujii *et al.*, 2005).

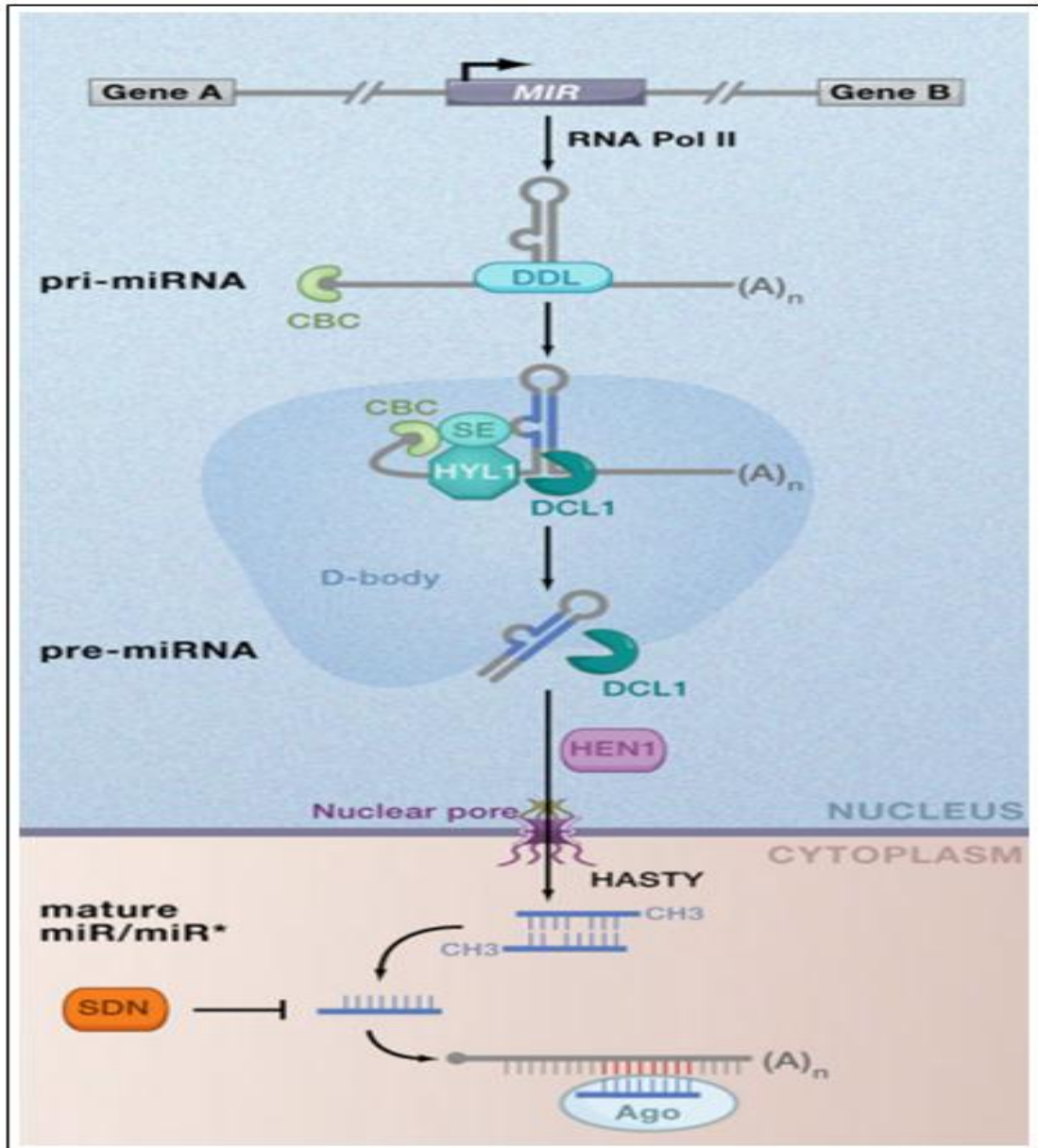


Figure 2.3 Model for miRNA biogenesis and activity in plants (Voinnet, 2009).

Palatnik *et al.* (2003) reported the JAW locus in *Arabidopsis*. JAW locus generates a microRNA that is able to direct mRNA cleavage of a number of TCP genes controlling leaf development. Overexpression of wild-type and microRNA-resistant TCP variants revealed the point that mRNA cleavage was adequate to minimize the TCP function. It was concluded that the existence of TCP genes with microRNA target sequences in a broad range of species demonstrate control of leaf morphogenesis *via* miRNA-mediated and is preserved in foliage with different leaf shapes.

Through an activation-tagging approach Aukerman and Sakai (2003), illustrated how that over expression of miRNA 172 (miR172) in *Arabidopsis* will cause early flowering and disorders the floral organ identity specification.

APETALA2 (*AP2*) and *AGAMOUS* (*AG*) are two floral homeotic genes which specify the identities of perianth and reproductive organs, respectively, for flower development in *Arabidopsis* (Zhao *et al.*, 2007b). miR172 is normally expressed in a temporal manner, consistent with its proposed role in flowering time control (Aukerman and Sakai (2003). The distinct functions *AG* and miR172 in flower development and their independent role in the negative regulation of *AP2* were demonstrated by Zhao *et al.*(2007b). It was exposed that *APETALA2* (*AP2*) which is target gene of miR172 downregulated by miR172 *via* translational mechanism rather than by RNA cleavage. Moreover gain-of-function and loss-of-function analysis depicted that two of the *AP2*-like target genes function as floral repressors, and this strongly support the idea that flowering time regulates by miR172 *via* downregulating *AP2*-like target genes.

Sunkar and Zhu (2004) reported the identification of new miRNAs related to abiotic stresses in *Arabidopsis*. It was explained how stresses such as cold, NaCl,

dehydration and ABA regulate miRNAs. According to their results miR393 was strongly upregulated by all four (NaCl, dehydration, ABA and cold) treatments. MiR397b and miR402 were slightly upregulated by all the stress treatments whereas miR319c was upregulated only by cold stress. Among miRNA which were regulated by stresses only miR389a was downregulated by all of the stress treatments.

Low-phosphate stress has a considerable influence on the target ubiquitin conjugating enzyme (UBC *) mRNA (Fujii *et al.*, 2005). Fujii *et al.* (2005) reported, reduction of (UBC *) mRNA as a result of low-phosphate stress greatly induced the miR399. They observed uppression of UBC mRNA accumulation under low-phosphate stress in transgenic plants with constitutive expression of miR399.

In 2005 Xie *et al.* reported the constructing of small RNA libraries from wild-type *Arabidopsis* (*Arabidopsis thaliana*) and mutant plants (rdr2 and dcl3). Their library consisted of thirty-eight distinct miRNAs corresponding to 22 families.

Zhao *et al.* (2007), studied microRNA expression under drought stress conditions in rice by using oligonucleotide microarray. They identified two miRNA, associated with drought stress. In addition, miR-169g was the only member of miR-169 family induced by drought stress. Also the induction of miR-169g was higher in roots than in shoots.

Zhang *et al.* (2006a) reported 188 maize miRNAs from 29 miRNA families. Homologs and secondary structures were used by Zhang and his colleagues for identification of miRNAs from EST (<http://www.ncbi.nlm.nih.gov/nucest>). Twenty eight miRNAs out of the 188 maize miRNAs were identified in at least one EST. In

addition they claimed to identify a total of 115 potential targets for 26 miRNA families. Most of the targets were transcription factors which were responsible for organ development in maize, such as leaf, shoot and root development. Moreover, these maize miRNAs were found to be engaged in other cellular processes, such as signal transduction, stress response, sucrose and cellulose synthesis, and ubiquitin protein degradation pathway.

A small RNA library consisting of roughly 40,000 small RNA sequences was made for *Brassica napus* by Wang *et al.* (2007). Eleven conserved miRNA families were identified by analyzing 3025 sequences from the small RNA library. They have found in a F1 hybrid *B.napus* line and its four double haploid progeny that showed marked variations in phenotypes majority of the conserved miRNAs were expressed at the same levels. Also it has been reported that several of them were differentially expressed between *Arabidopsis* and *B.napus*. In addition, it was detected the expression level of miR169 was high and prominent in young leaves and stems, whilst in roots and mature leaves they were untraceable.

To create computational prediction of potential miRNAs and their targets in *Brassica napus*, Xie *et al.* (2007a) studied potential miRNAs in *Brassica napus*. They sought for potential miRNAs in *B. napus* by using identified miRNAs in *Arabidopsis*, rice and other plant species. EST (<http://www.ncbi.nlm.nih.gov/nucest>) and GSS (<http://www.ncbi.nlm.nih.gov/nucgss>) databases were used. Identification of 21 potential miRNAs and 67 potential targets in *B. napus* were reported.

Yin *et al.* (2008) identified 21 conserved miRNAs in the EST (<http://www.ncbi.nlm.nih.gov/nucest>) and GSS (<http://www.ncbi.nlm.nih.gov/nucgss>)

databases by using a computational homology search in tomato. Their results demonstrated that the well-conserved tomato miRNAs have preserved homologous target interactions among different plant species.

Lu *et al.* (2008) reported identification of 68 putative miRNA sequences, classified into 27 families as a result of cloning of small RNAs from abiotic stressed tissues of *Populus trichocarpa*. Amongst the 68, nine families were novel, increasing the number of the known *Populus trichocarpa* miRNA families from 33 to 42.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Sample Collection

Seeds were collected from the fruit of wild species of *Musa acuminata ssp. malaccensis* gathered from Rimba Ilmu which is a botanical garden located in University Malaya, Kuala Lumpur, Malaysia.

3.2 Tissue Culture Medium Preparation

Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) was used as a tissue culture medium for samples. For preparation 1 liter of MS medium, first stock solutions were prepared. Based on stocks, macroelements, microelements, iron and organic supplement (vitamins) were added to approximately 700 ml dH₂O. Then 30g sucrose was added and the solution was adjusted to final volume of 1 liter with dH₂O. Gel-rite, (2g/L) as gelling reagent was used for solidification. The pH was adjusted to 5.8 using NaOH or HCl. Sterilization by autoclaving was carried out at 15 psi and 121°C for 15-30 min.

3.3 Embryo Culture

Banana fruit skins were peeled off and the seeds were removed. Surface sterilization was carried out by dipping, successively in 70% ethanol for 3 min, 20 % (v/v) commercial bleach (NaOCl), solution containing 0.2% tween-20 for 20 min and rinsing three times with sterile distilled water under a laminar flow (Ssbuliba *et al.*, 2006). Then seeds were air dried to dry the slippery mucus layer on the seed coat under

laminar flow. Mushroom shaped embryos were exposed by cracking seed coats and removed carefully using forceps and needle.

Ten Separated embryos were cultured on 9 cm diameter Petri dishes containing 40 ml MS medium and the longitudinal axis of the embryo was laid flat on the medium, halfway embedded. Cultured embryos were placed in darkness until germination. After germination cultures were transferred to lighted conditions (white fluorescent light, 2000 lux) of 16 h photo period at $26\pm 2^{\circ}\text{C}$.

3.4 Shoot Multiplication

3.4.1 Plant Material

Plantlets which were generated from embryo culture after 3 sub-cultures (every one month) were used for shoot multiplication. Each shoot apex had a length of approximately 1.5 cm after roots and leaves were removed.

3.4.2 Shoot Induction

Shoot apices (approximately 1.5 cm length) were placed onto MS medium (pH 5.8) supplemented with 30g/L sucrose, 2 g/L gel-rite and BAP. Three different concentrations of BAP, 3mg/L, 5mg/L and 7mg/L, were used to study effect of BAP on shoot induction. Prepared shoot apices were placed sequential in 3, 5 and 7 mg/L of BAP. Cap jars (150 ml) with roughly 30 ml of MS media were used. The cultures maintained at $26\pm 2^{\circ}\text{C}$ on 16 hour photo period (white fluorescent light, 2000 lux) cycle.

3.4.3 Multiplication

Multiplication was carried out by subdividing shoot clusters and sub culturing these individual pieces on fresh media every 4 weeks. After 12 weeks and two subcultures, single colonies which had been placed in 5mg/L BAP were ready for multiplication. A single shoot cluster was cut longitudinally through the apex into four pieces. Each piece was placed in regeneration medium (MS with 5 mg/L BAP).

3.4.4 Rooting

Explants were transferred to hormone-free MS media (Basal MS media) for root initiation. They were maintained in hormone-free MS media for 3 weeks. The individual shoots were used in salinity experiment after producing expanded leaves and roots.

3.5 Salinity Experiment

Clonal plantlets of the same physiological age and most similarity in shape, size and in number of leaves were chosen. Different concentrations of NaCl were used (Table 3.1). After 3 weeks, the plantlets were transferred to MS medium with 10 different NaCl concentrations containing 30 g/L sucrose and 2 g/L gel-rite. The pH was adjusted to 5.8 and 50 ml of medium were dispensed in to 300 ml jars.

Samples were maintained in a growth chamber at $26\pm 2^{\circ}\text{C}$ on 16 hour photo period for a duration of one month. This experiment was carried out three different times with a total of 150 plantlets. After four weeks, plant samples (root and shoots) from surviving plantlets were collected. Samples were divided to two equal parts. One part of samples was dried in oven for cation analysis and the other parts were kept in -20°C for proline analysis. After the determination 120 mM NaCl as the lethal concentration, fresh plantlets were put in 0 mM of NaCl as a control and 100 mM NaCl

as a concentration lower than lethal for 24 hour. Roots were subsequently frozen in liquid nitrogen and stored at -80°C until used for RNA extraction.

Table 3.1 : Different concentrations of NaCl which were used for salinity experiment.

NaCl Concentration	Number of plantlets
0 mM (control)	5 plantlets
60 mM	5 plantlets
80 mM	5 plantlets
100 mM	5 plantlets
120 mM	5 plantlets
140 mM	5 plantlets
160 mM	5 plantlets
180 mM	5 plantlets
200 mM	5 plantlets
220 mM	5 plantlets

3.5.1 Proline Extraction

Proline extraction was carried out as described by Bates *et al.* (1973). The following steps were involved in the extraction of proline.

One hundred mg of frozen plant material (root and shoots) were excised from plantlets after treatment at 0, 60, 80 and 100 mM of NaCl for one month with 3 replicates used for each concentration. Samples were homogenized in 1.5 ml of 3% sulphosalicylic acid and the residue was removed by centrifugation. One hundred μ l of the extract was treated with 2 ml glacial acetic acid and 2 ml acid ninhydrin (1.25 g ninhydrin warmed in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid until dissolved) for 1 h at 100 °C and the reaction was then terminated in an ice bath. The reaction mixture was extracted with 1 ml toluene.

The chromophore-containing toluene was warmed to room temperature and its optical density was measured at 520 nm with a spectrophotometer (ST-SP1104, SASTEC). The amount of proline was determined from a standard curve in the range of 0–100 μ gml⁻¹.

3.5.2 Cation (Na, Mg, K, Ca), Measurement

Four elements (Na, Mg, K, and Ca) were extracted based on Moraghan (1993). Elements were measured *via* atomic absorption. Three replicates were used for each element in this experiment. Plantlets which were treated in 0, 60, 80 and 100 Mm NaCl for one month were dried in oven at 104 °C for 24 h.

One hundred mg of dried sample (mixture of root and shoots) were mixed with 9 ml HNO₃ and 1ml HCl and heated in a water bath at 95°C for 1h. After cooling down to room temperature samples were filtered with Whatman paper and diluted by dH₂O to 25 ml. Then the concentrations of Na, Mg, K and Ca were determined by Atomic Absorption Spectrometer (AAAnalyst 400, Perkin Elmer, USA).

3.6 Data Analysis

Data analysis were carried out using Microsoft office excel 2007 and Minitab. ANOVA was performed on the data.

3.7 RNA Extraction

RNA was extracted using a modified CTAB method based on the Kiefer *et al.* (2000) protocol. Roots which were obtained from salt treatment stage were frozen and kept in -80 °C were used for RNA extraction.

3.7.1 CTAB Method

A liquid N₂ frozen banana root (300 mg), was ground with a pre-chilled mortar and pestle under liquid nitrogen and the powdered tissue was put into a 2 ml Eppendorf tube containing 1 ml pre-warmed extraction buffer (temperature of the extraction buffer must be ~65°C), plus 20 µl B-mercaptoethanol and mixed with vortex. Subsequently 500 µl C/I was added into the tube and vortexed (1,800 rpm) at room temperature for 10 min. Samples were then centrifuged for 5 min at 4 °C and 18,000 g. The supernatant was transferred to a 2.0 mL Eppendorf tube, and after adding 250 µl C/I, vortexed at room temperature for 2 min, it was then centrifuged for 2 min at 4°C and 18,000 g. The supernatant was transferred to a 2.0 mL Eppendorf tube and after addition of 2 volumes ice cold isopropanol, incubated for 5 min on ice, and then centrifuged for 5 min at 4 °C

and 18,000 g. The supernatant was discarded and the pellet washed with 1 ml 70% cold ethanol (v/v) (-20°C) and centrifuged for 5 min at 4°C and 18,000 g. The supernatant was aspirated and the pellet air-dried for 10-20 min. The dried pellet was dissolved in 15-50 µl DEPC treated H₂O (depending upon the size of pellet).

3.7.2 Modified CTAB Method

Liquid N₂ frozen banana root (300 mg), was ground to a fine powder with a pre-chilled mortar and pestle under liquid nitrogen and the powdered tissue was put into a 2 ml Eppendorf tube containing 1 ml pre-warmed extraction buffer (~65 °C), 20 µl β-mercaptoethanol plus 10 µl proteinase K and mixed with vortex and kept at 42 °C for 15 min. Subsequently 500 µl phenol and 500 µl C/I were added into the tube and vortexed (1,800 rpm) at room temperature for 10 min, and then centrifuged for 5 min at 4°C and 18,000 g. The supernatant was transferred to a 2 ml Eppendorf tube, after adding 500 µl C/I and vortexed at room temperature for 2 min, was centrifuged for 2 min at 4°C and 18,000g. The supernatant was transferred to a 2.0 ml Eppendorf tube, 2 volumes of ice cold isopropanol was added and incubated for 5 min on ice. Subsequently it was centrifuged for 5 min at 4 °C and 18,000 g. The supernatant was discarded and the pellet washed with 1 ml 70% cold ethanol (v/v) (-20°C) and centrifuged for 5 min at 4 °C and 18,000 g. The supernatant was aspirated and the pellet air-dried for 10-20 min. the dried pellet was dissolved in 15-50 µl DEPC treated H₂O (Depending on the size of pellet).

3.7.3 DNase Treatment

To degrade DNA contamination from total RNA, DNase treatment was carried out. Total RNA obtained from RNA extraction was then treated with DNase using Deoxyribonuclease I (Invitrogen, USA). RNA sample (1 µg), was treated with 1µl 10x

DNase I reaction buffer and 1 μ l DNase I, amplification grade (1 U/ μ l), and was adjusted to final volume of 10 μ l with DEPC-treated water. The reaction mixture was incubated for 15 min at room temperature. Then DNaseI was inactivated by the addition of 1 μ l of 25 mM EDTA solution to the reaction and subsequently heated for 1 min at 65°C.

3.7.4 Optical Density (OD) 260/280 Assay

This assay was carried out in order to determine the concentration and purity of the samples in solution. The assay is based on the fact that double strand DNA or RNA respectively at concentrations of 50 μ g/ml and 40 μ g/ml have an optical density reading of 1.0 when measured at 260 nm in cuvet with 1 cm light path. The amount of UV radiation absorbed by solution of DNA/RNA is directly proportional to amount of DNA/RNA in the sample. A 50 μ l diluted sample (1 μ l sample+49 μ l dH₂O or DEPC treated H₂O) was measured in an ultraviolet spectrophotometer (Bio photometer, Eppendorf, Germany).

To determine the purity of the DNA/RNA samples the ratio of their absorbance at 260 nm and 280 nm (260/280) was measured, where the 260 nm reading is indicative of DNA/RNA concentration and the 280 nm reading indicates the protein contamination. The best purities are indicated in the range of 1.8 to 2.0.

3.7.5 TBE Buffer Preparation

TBE buffer (1X) was used in both Agarose gel preparation and loading of Samples. To make 100 ml 1x TBE 10.8g Tris base, 5.5g Boric acid and 4ml of 0.5M EDTA were dissolved with stirring in 85 ml nuclease-free water. The final volume was adjusted to 1 liter with nuclease-free water.

3.7.6 Agarose Gel Preparation

Three different percentages 1, 2 and 3% of molecular biology and LE Analytical Grade agarose were used for the electrophoretic separation of nucleic acids.

For preparation of 1% agarose gel with diameter of 3 mm, 0.15g agarose was mixed with 15 ml TBE which was prepared with nuclease-free water in a conical flask and weighed. Then the solution was microwaved until it dissolved and weighed again followed by replacement of evaporated water with nuclease free water. After cooling the solution to about 60°C, the solution was stained with Ethidium bromide (0.5 µl) and poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

3.7.7 Loading of Samples

3.7.7.1 RNA Samples

Based on optical density, 1µg of RNA was mixed with equal amount of 2X loading dye (Fermentas, Canada), heated for 10 min at 70°C and placed on ice for 2 min to prevent the reformation of secondary structures before loading onto a gel. Running voltage ranging between 80-100 V was used for duration of 40-60 min. One % LE analytical grade Agarose (Promega), was used for preparing the gel and 1% TBE was used for running of the gels.

3.6.7.2 DNA samples

Based on optical density 1µg of DNA was mixed 1/5 with 6X loading dye (Fermentas, Canada) and loaded onto the gel. Running voltages ranging between 100-120 V were used for a duration of 20 min to 1 h. Different percentages (1, 2.5 and 3%)

of LE analytical grade Agarose (Promega), were used for gel preparation and 1% TBE was used for running of the gels.

3.8 miRNA cDNA Library Construction

To construct the cDNA library, miRNAs of salt stressed plantlets were isolated from total RNA and converted to cDNA and PCR products *via* MicroRNA Discovery™ Kit (System Bio Sciences, USA). Subsequently PCR products with size of approximately 400bp (including primer size) were ligated into the vector (pCR4-TOPO, 39.56 bp) and transformed to the host cells (TOP10 Chemically competent *E. coli*, Invitrogen, USA). The cloned bacteria are then selected, commonly through the use of antibiotic selection. Ampicillin was used as a selective antibiotic and white colonies were picked for PCR confirmation and construction of the library. Sequencing and BLAST analysis (section 3.9) were subsequently carried out in order to analyzing the colonies.

3.8.1 Adaptor Ligation, Reverse Transcription PCR (RT PCR) and PCR

MicroRNA Discovery™ Kit (System Bio Sciences, USA) was used for isolation of microRNAs. Total RNA was used for this stage. This small RNA amplification system includes 3 steps:

1. A degenerate adaptor mixture is ligated to both the 5′- end and 3′- ends of total RNA.
2. Reverse transcription of the RNA using a primer complementary to the attached adaptor.
3. PCR amplification of the cDNA.

Table 3.2 : Sequences of adaptors and primers used in adaptor ligation, RT and PCR step.

Upper Strand Primer sequence	5' - P ACTCTGCGTTGATACCACCTGCTT - 3'
Lower Strand Primer sequence	3' - r N r T r G r AGACGCAACTATGGTGACGAA (NH ₂) - 5'
	3' - r N r N r T r G r AGACGCAACTATGGTGACGAA (NH ₂) - 5'
	3' - r N r N r N r T r G r AGACGCAACTATGGTGACGAA (NH ₂) - 5'
	3' - r N r N r N r N r T r G r AGACGCAACTATGGTGACGAA (NH ₂) - 5'
RT and PCR Primer sequence	5' - AAGCAGTGGTATCAACGCAGAGT - 3'

N = G/C/U/A

3.8.2 Gel Extraction

Amplified cDNAs from the PCR step were run on 3% LE analytical grade Agarose gel and desired fragments which were between 200-300bp cut under UV light. The cut fragments were purified by gel extraction using a kit (Qiagen, Germany).

3.8.3 Cloning

A PCR 4-TOPO cloning kit (Invitrogen, USA) was used to clone the amplified cDNA after gel extraction. Ligations followed the kit instructions. One Shot TOP10 chemically competent *E.coli* (Invitrogen, USA) was used for transformation. Consequently 100 µl transformed cells were diluted in 1ml LB broth and 100 µl of dilution dispensed on onto LB agar with 50µg/ml Ampicillin plus 50µg/ml X-gal and incubated at 37°C overnight (16 h). Colonies collections were performed by sub culturing the white colonies onto the selective plates containing 50µg/ml Ampicillin plus 50µg/ml X-gal. All white colonies (200 colonies) were selected to construct small RNA library. After overnight incubation at 37°C, 10 clones were chosen for screening by PCR.

3.8.4 Colony PCR

PCR was carried out in total volume of 12.5 µl as below:

1. Control DNA Template (100 ng) 1 µl
 2. 10x PCR Buffer 1.25 µl
 3. 50 mM dNTPs 0.125 µl
 4. Forward and Reverse PCR Primers (0.1 µg/µl each) 0.25 µl
- Water 9.375 µl
5. *Taq* Polymerase (1 unit/µl) 0.25 µl

Sequence of the M13 Forward and Reverse primers:

M13 Forward 5'-GTAAAACGACGGCCAG-3'

M13 Reverse 5'-CAGGAAACAGCTATGAC-3'

Table 3. 3 : Amplification cycling parameters.

Step	Time	Temperature	Cycles
Initial Denaturation	10 minute	94°C	1x
Denaturation	1 minute	94°C	25x
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1x

3.9 Plasmid Isolation

One of the quickest and cleanest ways to isolate plasmid DNA from bacteria is to use the Qiagen plasmid purification kit. Plasmid kit was used in this study is based on modified lyses procedure, followed by binding of plasmid DNA to Qiagen Anion-Exchange Resin under appropriate low-salt and pH conditions. Plasmid DNA was isolated by using plasmid purification mini kit (Qiagen, Germany) followed kit instruction. The DNA pellets were dried *via* the DNA plus (Heto, Denmark) for 5 min. Pellets were dissolved in sterile dH₂O and kept overnight at 4°C in order to better digestion.

3.10 Sequencing and Analysis

Isolated plasmids, after reading OD, were diluted to 200 ng and sent to First Base Laboratories for sequencing. Fragment sequences (~ 200 bp), were separated from Topo vector and aligned with mature stress related micro RNAs from miRBase (www.mirbase.org). Clustal W (www.ebi.ac.uk/Tools/clustalw2/index.html) and BioEdit (BioEdit Sequence Alignment Editor Versions 7.0.5.3 and 7.0.9.0) were programs which were used for alignment. MiRBase search (<http://www.mirbase.org/search.shtml>) BLAST analysis were use data analysis. BLAST analysis included non-human non mouse EST (<http://www.ncbi.nlm.nih.gov/sites/nucest>), *Oryza sativa* EST (*indica* cultivar group and *japonica* cultivar group) and nucleotide collection (nr/nt) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

PsRNA Target (<http://bioinfo3.noble.org/psRNATarget/>) was used to identify putative miRNA targets. The banana EST data which were used for target identification in this study are from a UM project led by Prof. Rofina Yasmin Othman and contains

different data including banana virus sequences and are part of the data available to the Global *Musa* Genomics Consortium (<http://www.musagenomics.org/>).

CHAPTER 4

4.0 RESULTS AND DISCUSSION I

4.1 *In-vitro* Zygotic Embryo Culture

Contamination of embryo extracted from seeds were greatly reduced when the seeds were treated with a combination of 70% ethanol for 3 min and 20 % (v/v) commercial bleach (NaOCl) compared to treatment of seeds with 70% ethanol or commercial bleach (NaOCl) alone (Table 4.1).

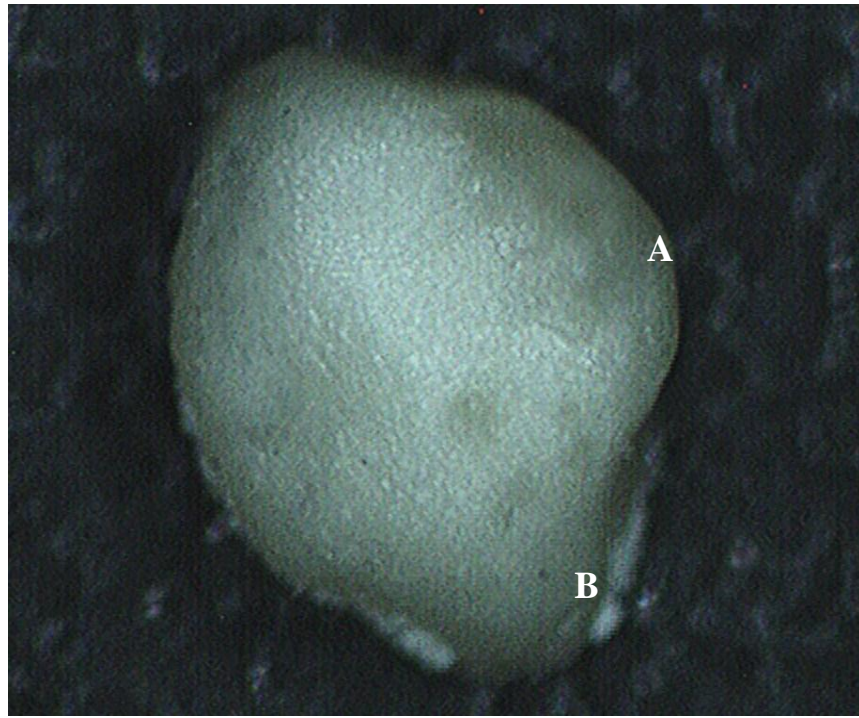
The embryos were observed to have a mushroom shape, creamy color and were divided into two parts, a meristematic portion and a haustorium (Figure 4.1). The meristematic portion is a stalk-like structure on the top of the embryo and the haustorium is a flat rounded portion at the bottom (Figure 4.1). Germination of embryos took place a few days (3-5) after initial culture. Embryos were yellow and swollen as an early sign of germination. These changes were similarly observed by Afele and De Langhe (1991) and according to their report those embryos which remained creamy never germinated. Shoot primordia and root primordia first appeared respectively from the lateral tissues of the meristematic end and the apical tissue of the meristematic end. After about three weeks, the plant-like structure appeared, consisting of the prominent shoot, which bore at its base an adventitious root system. The swelling of the whole embryo followed by the appearance of shoot and root primordia defined the process of seed germination.

**Table 4.1 : Comparison of different sterilizing reagents was used for seeds
Sterilization.**

Sample		Treatment	Contamination %
1	Rep.1	Combination of 70% and 20 % (v/v) commercial bleach (NaOCl)	10
	Rep.2		0
	Rep.3		10
	Rep.4		0
	Rep.5		10
2	Rep.1	70% Ethanol	50
	Rep.2		50
	Rep.3		40
	Rep.4		60
	Rep.5		50
3	Rep.1	20 % Commercial bleach (NaOCl)	60
	Rep.2		50
	Rep.3		60
	Rep.4		60
	Rep.5		50

Each sample was included 10 embryos.

Rep = replicate



Magnification 60 x. Original size, 1mm.

Figure 4.1 Creamy and mushroom shape embryo with meristematic end (A) and haustorium end (B).

4.1.1 *In-vitro* Embryo Germination Rate

The germination rate of *in vitro* zygotic embryos culture was observed to increase to 92 % after soaking of the seeds in water (for periods of 2 and 3 days), compared to 72 % germination when no water treatment was used (Table 4.2). Therefore the results imply that soaking of seeds, before culturing of embryo efficiently enhances germination percentage.

The seed germination rate in *Musa* generally is very low under natural conditions. Low seed germination of *Musa* can be because of a malformed embryo, unusual softening of the seed coat and the absence or missing of embryo (Vuylsteke and Swennen, 1993). *In vitro* embryo culture can improve the low rate of germination more than 90%. An increased germination rate after soaking in water was reported by Afele and De Langhe (1991), when they soaked *Musa balbisiana* seeds for 3,5 and 9 days. They suggested that this might be evidence of dormancy in banana seeds and that soaking in water could remove the inhibitors which exist in banana seeds.

Table 4.2 : Embryo germination percentage of *Musa acuminata ssp. malaccensis* under different conditions.

Treatment Sample	Lighting	Gelling Agent	Embryo Orientation	Soaking in sterile water	% embryo germination*
1	24 h dark	Gelrite	Longitudinal axis of the embryo was laid flat on the medium, halfway embedded.	0 h	72%
2	24 h dark	Gelrite	Longitudinal axis of the embryo was laid flat on the medium, halfway embedded.	24 h	92%
3	24 h dark	Gelrite	Longitudinal axis of the embryo was laid flat on the medium, halfway embedded.	48 h	92%

* Based on mean of data's. N=50

4.2 Shoot Induction and Multiplication

The shoot induction experiment included a total of 30 plantlets which were divided to three groups of 10 and placed respectively in 3, 5, 7 mg/L BAP.

No shoots were observed for all replicates (10 replicates) for concentration of 3 mg/L BAP after four weeks (Figure 4.2A), whereas at 5mg/L BAP (8 out of 10 plantlets produced one shoot) and 7 mg/L BAP (9 out of 10 plantlets produced one shoot) new shoots appeared after four weeks (Figure 4.2 B, C) (Appendix J). The results observed for 5mg/L BAP are consistent with Mante and Tepper's (1983) finding of one shoot initial per explant in 28 days.

Although there was no considerable difference between the use of 5 and 7 mg/L BAP for initiation of shoot induction (Figure 4.3), it was shown that using 7 mg/L BAP for longer periods could induce abnormalities of the shoots and stems. The results show that plantlets which were placed in 5 mg/L BAP after 4 months and three subcultures (every one month) produced clusters of distinct and normal shoots and stems (Figure 4.4). But abnormality was observed in plantlets which were grown in 7mg/L BAP within the same period and number of sub-culturing (Figures 4.5). Abnormality of colonies manifested in different shapes. As can clearly be seen in Figure 4.5A stems were very thick and thicknesses were not the same for each clone. Stems were not separated and produced a mass (Figure 4.5B) and were also thin and twisted (Figure 4.5C).

The results suggested that using 5mg/L BAP had the best effect on shoot induction compared to other concentrations tested. Due to the abnormalities which were induced by 7 mg/L BAP and the null effect of 3 mg/L BAP on shooting, 5mg/L BAP was chosen as the best concentration for multiplication.

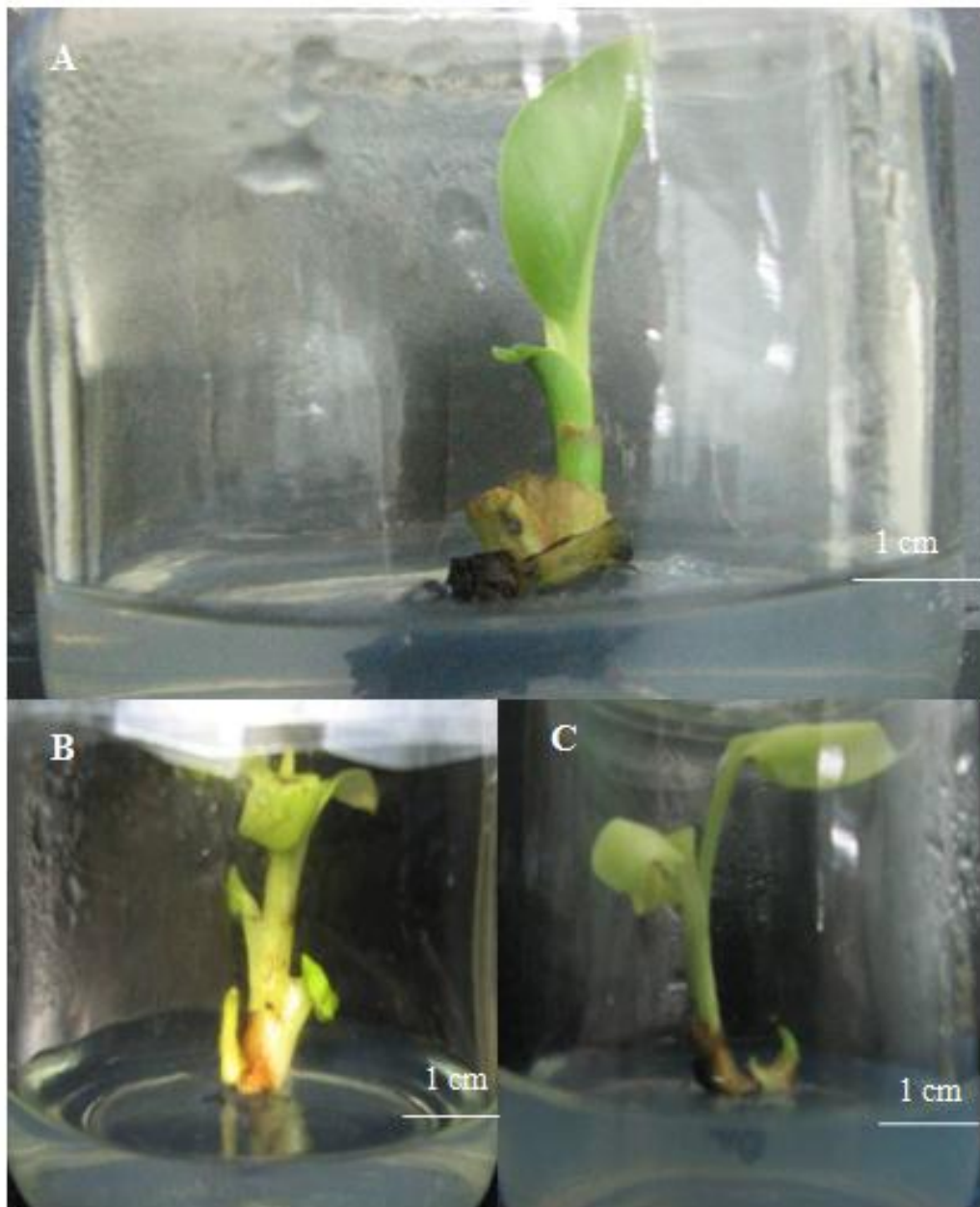
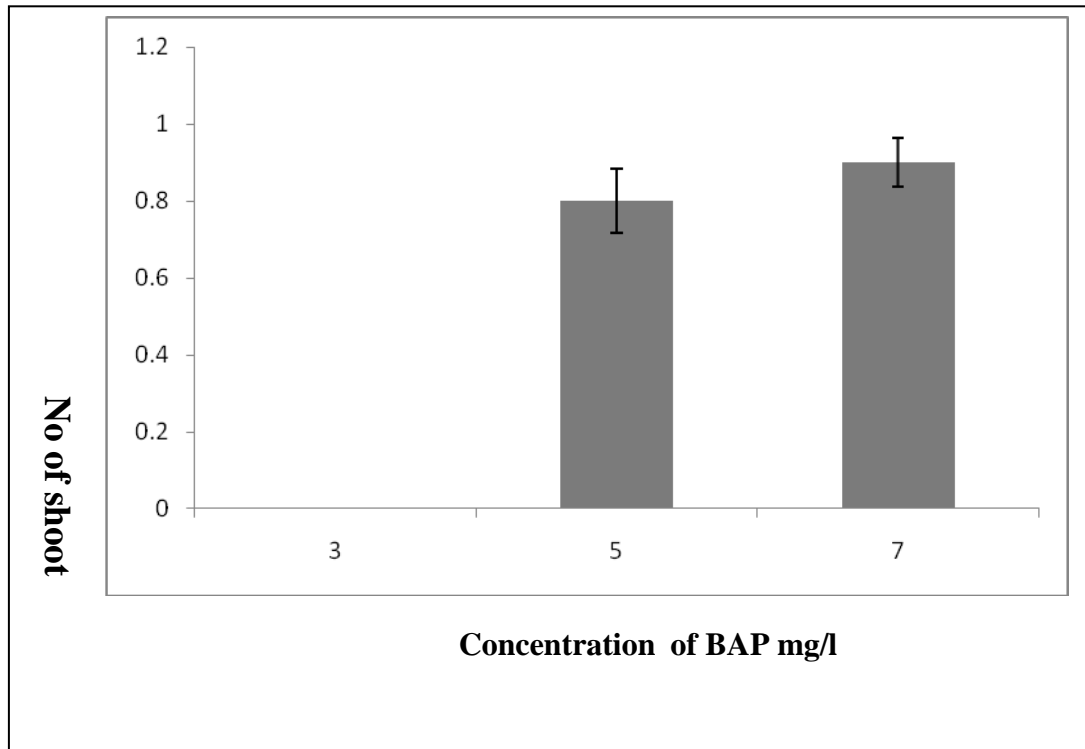


Figure 4.2 New shoot induction in 3, 5 and 7 mg/L of BAP.

A. No shoot appeared in 3 mg/L BAP after four weeks. **B.** One shoot appeared in 5 mg/L BAP after four weeks. **C.** One shoot appeared in 7 mg/L BAP after one month.



Error bar shows SD.

Figure 4.3 New shoots appearance at three different BAP concentration (3, 5 and 7mg/L) after four weeks. The values are the average of 10 replicates for each concentration of BAP.



Figure 4.4 Shoot induction after four month in 5 mg/L BAP. All plantlets are almost similar and distinct from each other and stems have approximately same size.

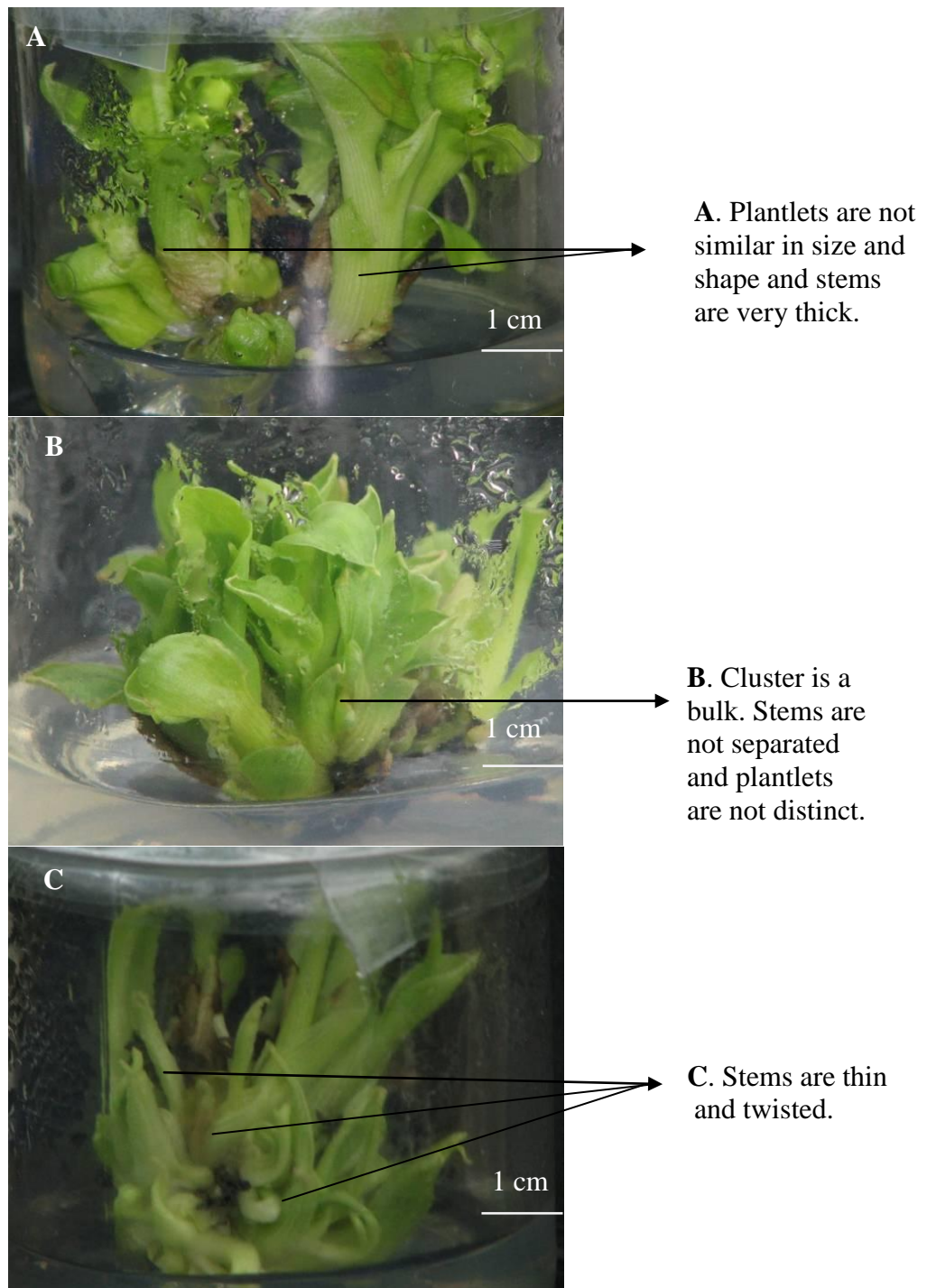


Figure 4.5 Shoot induction abnormality in 7mg/L BAP.

Shoot apices were prepared with a length of approximately 1.5cm from plantlets generated from embryo culture (Figure 4.6A). Shoot apices were placed in 5 mg/L BAP for shooting purposes (Figure 4.6B). Slightly before shoot initial development explants were swollen and the first visible shoot appeared from the meristem after 4 weeks (Figure 4.6C). Similar swelling in the explants before shoot initial development was observed by Mante and Tepper (1983) in *Musa textilis* Nee. After 12 weeks, shoot clusters (Figure 6.1E) were cut longitudinally through the apex to individual pieces (single shoot) (Figure 6.1F). Each single shoot was separately placed in 5mg/L BAP. In this stage 12 single shoots were used and after four weeks each piece produced a clone with average of 10.2 shoots per clone (Table 4.3).

Cronauer and Krikorian (1984) produced multiple shoot cultures from the dessert banana (*Philippine lacatan* and *Grande naine*) and plantain by cutting small shoot longitudinally through the apex. By using 5mg/L BAP they achieved an average of 9.1 new shoots per single shoot. This result is almost similar to the results achieved in this study which showed an average of 10.2 per shoot. The slight difference can be interpreted as a difference between species.

Table 4.3 : Number of new shoots was produced per single shoot after four weeks.

SampleNo	1	2	3	4	5	6	7	8	9	10	11	12	Mean of samples
No of shoots after 4 weeks	10	10	11	9	10	10	12	9	10	10	10	11	10.2

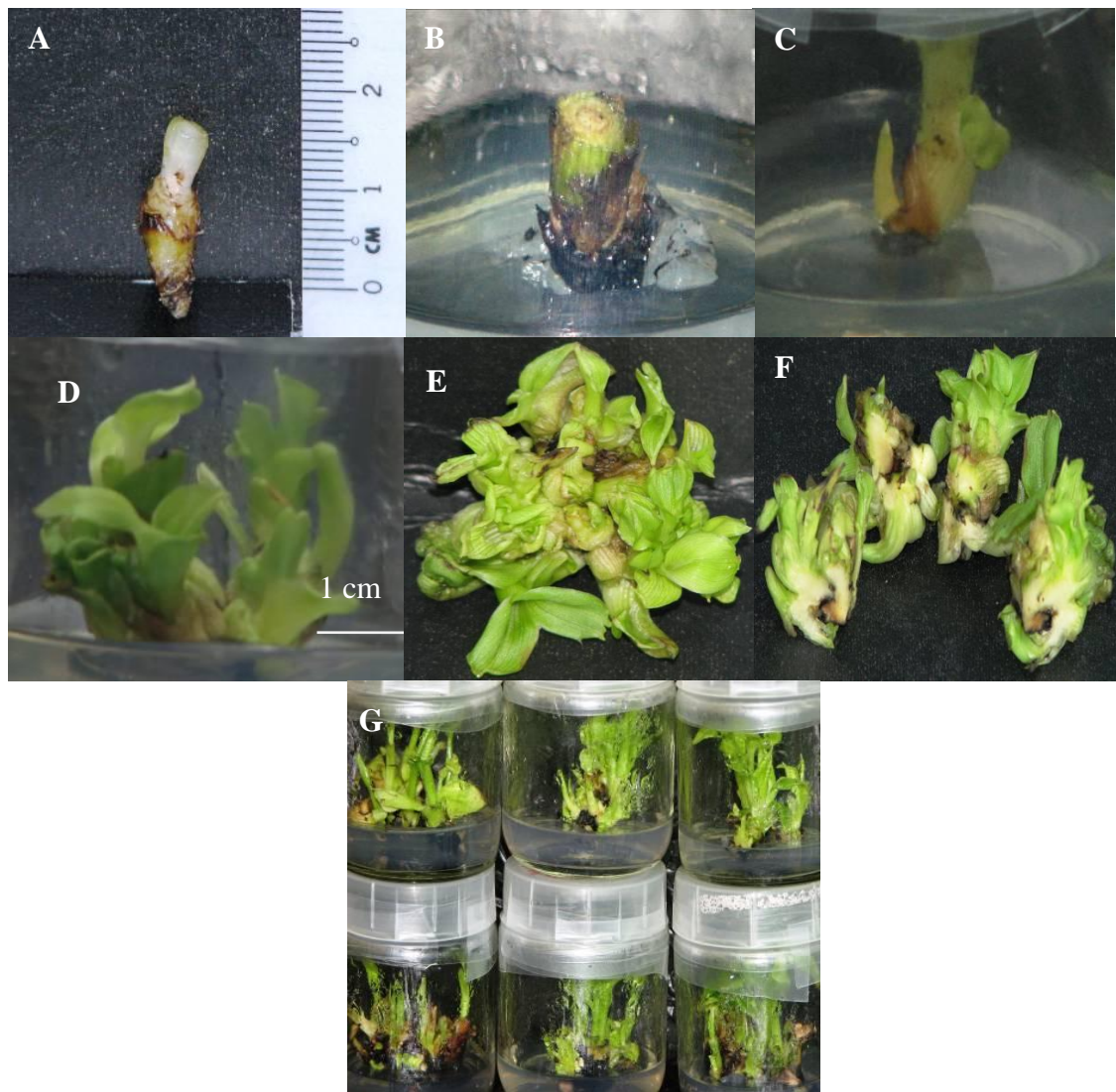


Figure 4.6 Multiplication of *Musa acuminata ssp. malaccensis*.

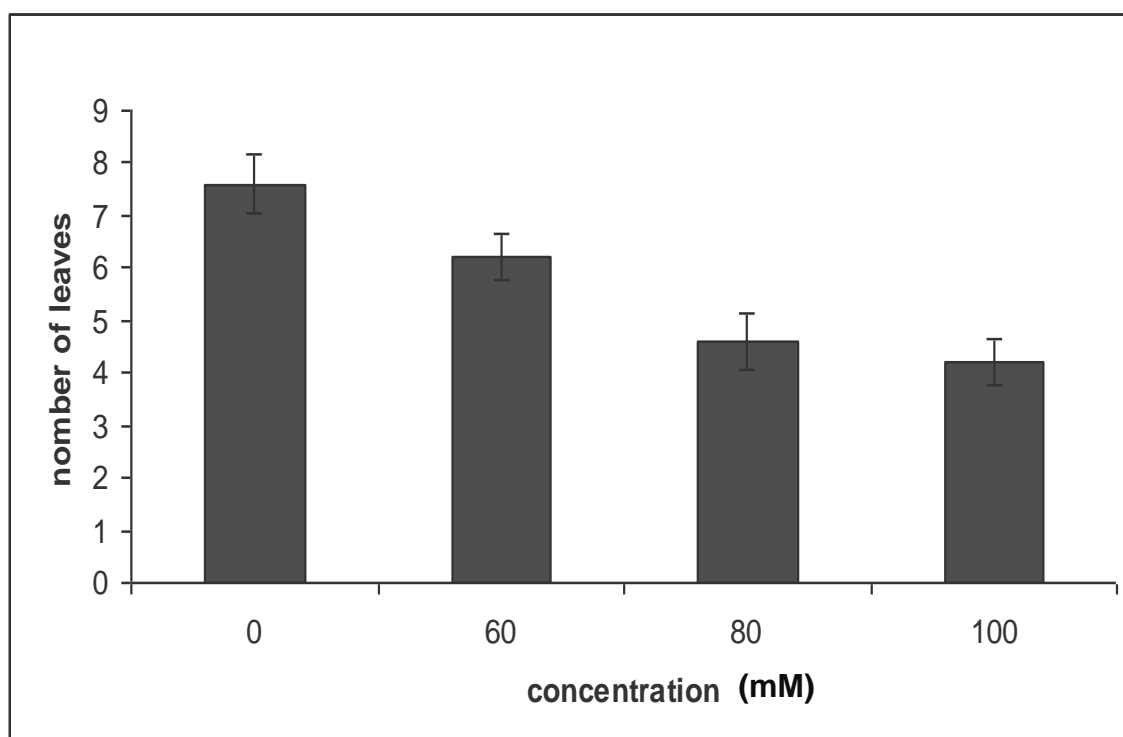
A. Shoot apex was produced with length of approximately 1.5cm after roots and leaves were removed. **B.** Shoot apex was placed in MS medium with 5mg/L BAP. **C.** First visible shoots after 4 weeks. **D.** Colony (shoot cluster) after 8 weeks. **E.** Colony (shoot cluster) after 12 weeks. **F.** Cutting a single shoot longitudinally through the apex. **G.** Each piece produced a new clone (shoot cluster) with average of 10.2 shoot per clone after 4 weeks, (N= 12).

4.3 Salt Stress Assessment

4.3.1 General Effect of Salinity on *Musa acuminata ssp. malaccensis*

The initial effect of salinity on the plants appeared as a general wilting and twisting of leaves, 3-5 days after exposure of plantlets to saline conditions. Differences in the time of appearance of salinity associated symptoms were dependent on NaCl concentrations. Chlorosis and necrosis appeared on leaf surfaces 5-8 days after exposure to saline conditions. General growth of plants was considerably inhibited by increasing NaCl concentrations. Production of new leaves was considered as a positive indicator of plant growth. The study showed that the number of new leaf production was greatly reduced by elevation of NaCl concentration (Appendix K). The results were derived from observation of 50 plantlets over a period of one month. The analysis of variance (ANOVA) showed a significant difference (at $p < 0.05$) in number of plant new leaf production with increasing NaCl concentrations (Appendix L). Figure 4.7 shows that there was a significant difference in the number of new leaves appearing in the control group compared to those at 60 mM of NaCl. The difference between 60 and 80 mM NaCl was also significant. The number of new leaf production at 100 mM NaCl was lower than 80 mM NaCl but the difference was not significant. Growth ceased at concentrations higher than 100 mM NaCl and all plantlets died at concentrations of 120 mM of NaCl. Expansion of roots was observed at lower concentrations (60 and 80 mM NaCl). At 60 and 80 mM NaCl plants produced aerial roots which had upward expansion (Figure 4.8). These roots contained secondary root and they were hairy compared to the roots observed in control samples. Root elongation, aerial and hairy root production *via* plants under saline condition are considered as the plants adaptive response to salinity (Balibrea *et al.*, 2000). With further increasing of NaCl concentrations decreases were observed in root expansion and development. Roots

growth at 60 and 80 mM NaCl suggests the plants attempted to adapt and bear the saline conditions. Salinity has diverse negative effects on plants. It has limitative influence on plant growth and productivity (Allakhverdiev *et al.*, 2000; Veeranagamallaiah *et al.*, 2007), and affects many physiological and metabolic processes (Läuchli 1984; Olmos *et al.*, 1994). Inhibition and stunting of growth under saline conditions were reported as common effects of salinity on plants (Cherian *et al.*, 1999; Takemura *et al.*, 2000). Similarly in this study the growth of *Musa acuminata ssp. malaccensis* plantlets was significantly affected as a result of increasing of the salt concentration in the growth media. Developing of root system and rising aerial and hairy root is understood as a plant effort for adaptation for survival.



Error bar shows SD.

Figure 4.7 New leaf production with increasing in salt concentration after one month.

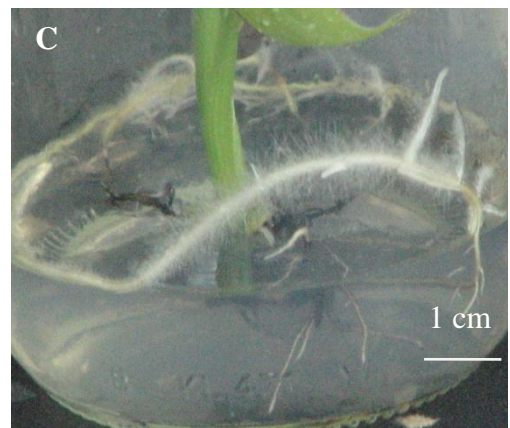


Figure 4.8 Expansion of root system at 60 and 80 mM of NaCl.

A. Root expansion on the control medium. **B.** Root expansion at 60 mM. Roots formed secondary hairy roots. **C.** Root expansion at 80 mM. Roots formed secondary hairy roots.

4.3.2 Determination of Lethal Concentration of NaCl for *Musa acuminata ssp. malaccensis*

The results of this experiment were collected from a total of 150 plantlets after one month. The experiment was conducted in triplicates (each time 50 plantlets) at 10 different concentrations, 0 mM as a control concentration and 60, 80, 100, 120, 140, 160, 180, 200 and 220 mM NaCl. In spite of the intensive damage of salinity on exterior parts of plantlet such as general wilting, chlorosis, necrosis, all plantlets survived at 60, 80 and 100 mM of NaCl after one month. All plantlets died at concentrations of 120 mM of NaCl. Destructive influence of high concentrations (General wilting and twisting of leaves followed chlorosis, necrosis and burning of leaf margin) could clearly be seen, 15 days after exposing of plantlets to 120 mM of NaCl concentration (Figure 4.9B). Plantlets died after 30 days after exposure to 120 mM of NaCl (Figure 4.9C). The same results were obtained in the higher concentrations from 140 to 220 mM where all plantlets were observed to have died.

Ulisses *et al.* (2000) used different concentrations of NaCl (0, 20, 40, 60, 80, 100 and 120 mM) to select salt tolerant banana buds (*spp. Nanicao*). They also reported that all buds died at 120 mM of NaCl. The results of this study reaffirmed their finding that 120 mM was determined to be a lethal concentration of NaCl for *Musa acuminata ssp. malaccensis*. Accordingly 100 mM of NaCl was determined as the minimal inhibitory sodium chloride (NaCl) concentration for survival of banana plantlets (*Musa acuminata ssp. malaccensis*) and used for RNA extraction and construction of salt stress small RNA library.

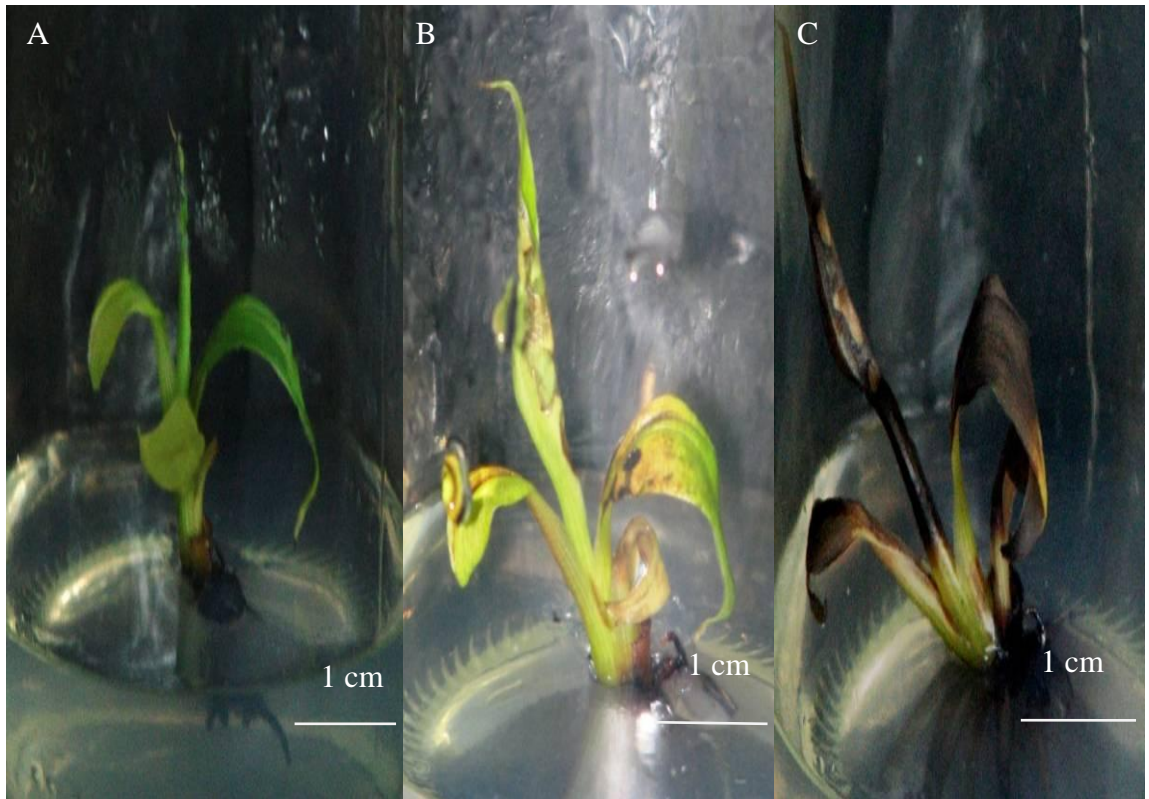


Figure 4.9 Plantlets in MS medium with 120 mM NaCl.

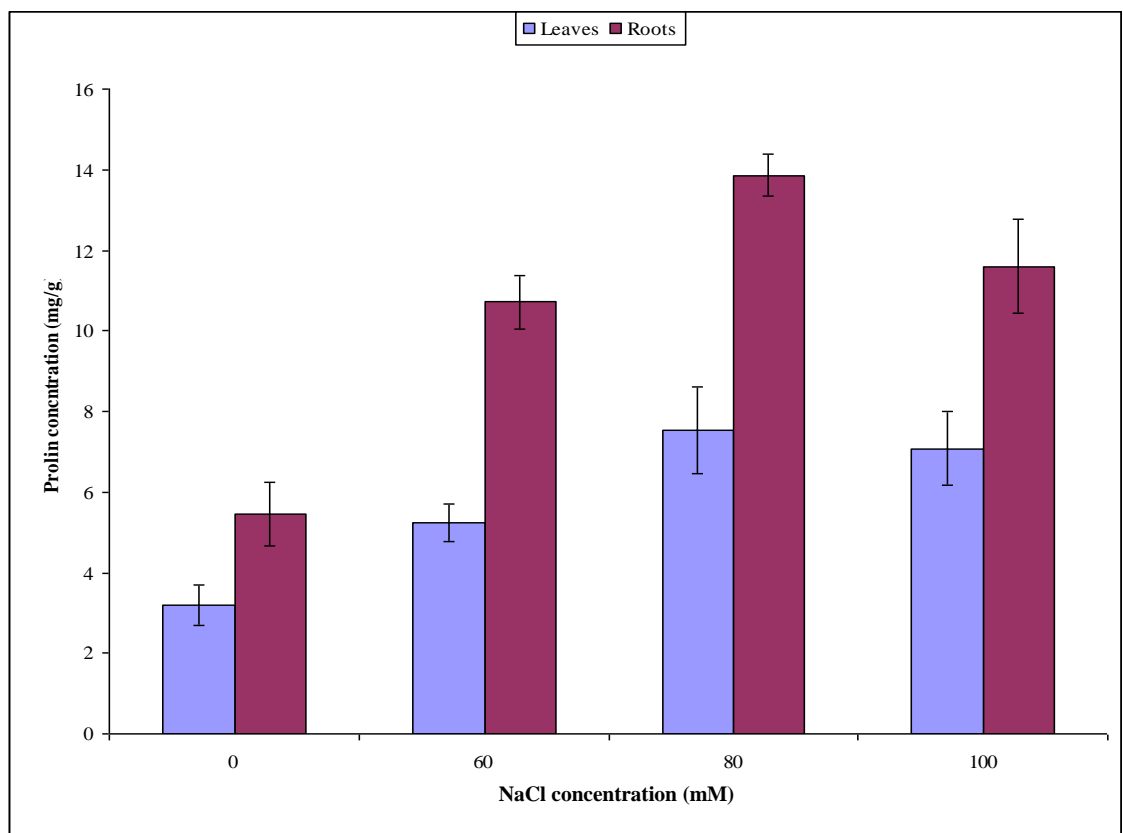
A. Plantlet immediately after being placed in salty 120 mM of NaCl. **B.** Plantlet after 15 days in 120 mM of NaCl. **C.** Plantlet after 1 month in 120 mM of NaCl (died).

4.3.3 Proline Assay

The concentration of proline increased significantly in both root and leaves at 60 and 80 mM of NaCl concentration compared to the control (Figure 4.10). Analysis of variance (ANOVA) showed a significant difference (at $p < 0.05$) in proline concentrations with increasing NaCl concentrations (Appendix M). The highest amount of proline accumulation in both root and leaves was recorded at 80 mM of NaCl. An increase of proline in the roots was observed in 60 mM NaCl and it reached a peak of 15 mg/g at 80 mM. With further increment of NaCl to 100 mM, the concentration of proline was significantly lowered in roots. The concentrations of proline in leaves also showed significant increases at 60 and 80 mM NaCl compared to the control. Similar to roots, the highest concentration of proline in leaves was obtained at 80 mM NaCl. Further increase in NaCl reduced concentration of proline.

The study observed that in the banana samples tested increases in the concentration of NaCl influenced the concentration of proline in the roots and leaves. Similar increment of proline concentration in response to NaCl increase was reported by Van Diggelen *et al.* (1987), Ewing *et al.* (1995), Parida and Das (2005), Naidoo and Kift (2006) and Keutgen and Pawelzik (2008) in different plants. Accumulation of proline is a defence strategy for many plants against salinity and reflects a plant's vital ability for salt tolerance and adaptation to salt conditions (Molinaria *et al.*, 2004; Meloni *et al.*, 2008). Proline accumulation is a general response of plants to many kinds of stresses such as, salinity, drought, heavy metal toxicity and high temperature (Siripornadulsil *et al.*, 2002). Organic metabolites such as proline are known to play an important role in osmoregulation and osmotolerance (Sharma *et al.*, 1998) and serve as good indicators for studying the effects of abiotic stresses on plants as shown in this study.

Increasing proline concentrations at 60 and 80 mM NaCl clearly shows the attempt of the plants to adapt itself to saline conditions. The plants in this study could tolerate the elevation of salt concentrations until 80 mM NaCl but with further increase to 100 mM NaCl the plants ability to resist the higher concentration of NaCl reduced and eventually plants died at 120 mM of NaCl. This result supports the observation of the previous experiment (Determination of lethal concentration of NaCl for *Musa acuminata ssp. malaccensis*).



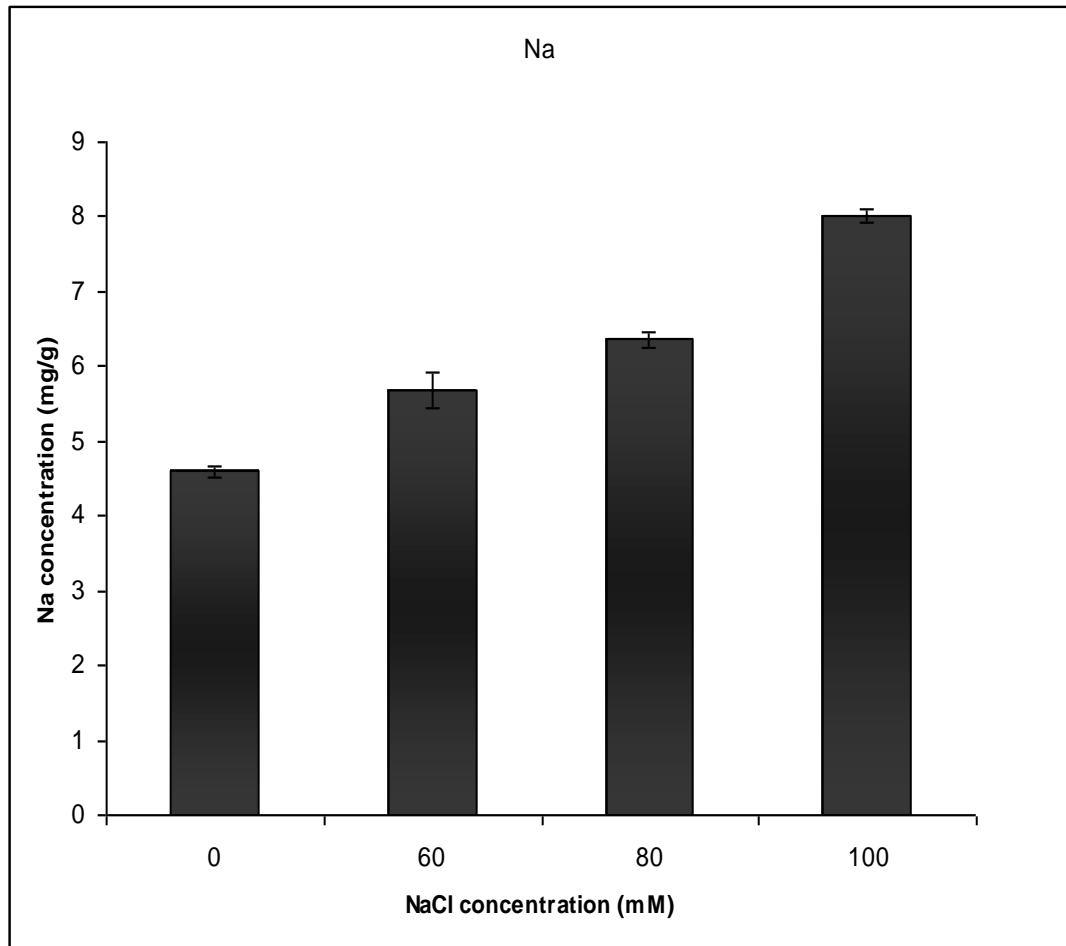
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Figure 4.10 Proline concentrations in the roots and leaves of *Musa acuminata ssp. malaccensis* with increasing of NaCl concentration.

4.3.4 Elements measurement (Na^+ , K^+ , Ca^{++} , Mg^{++})

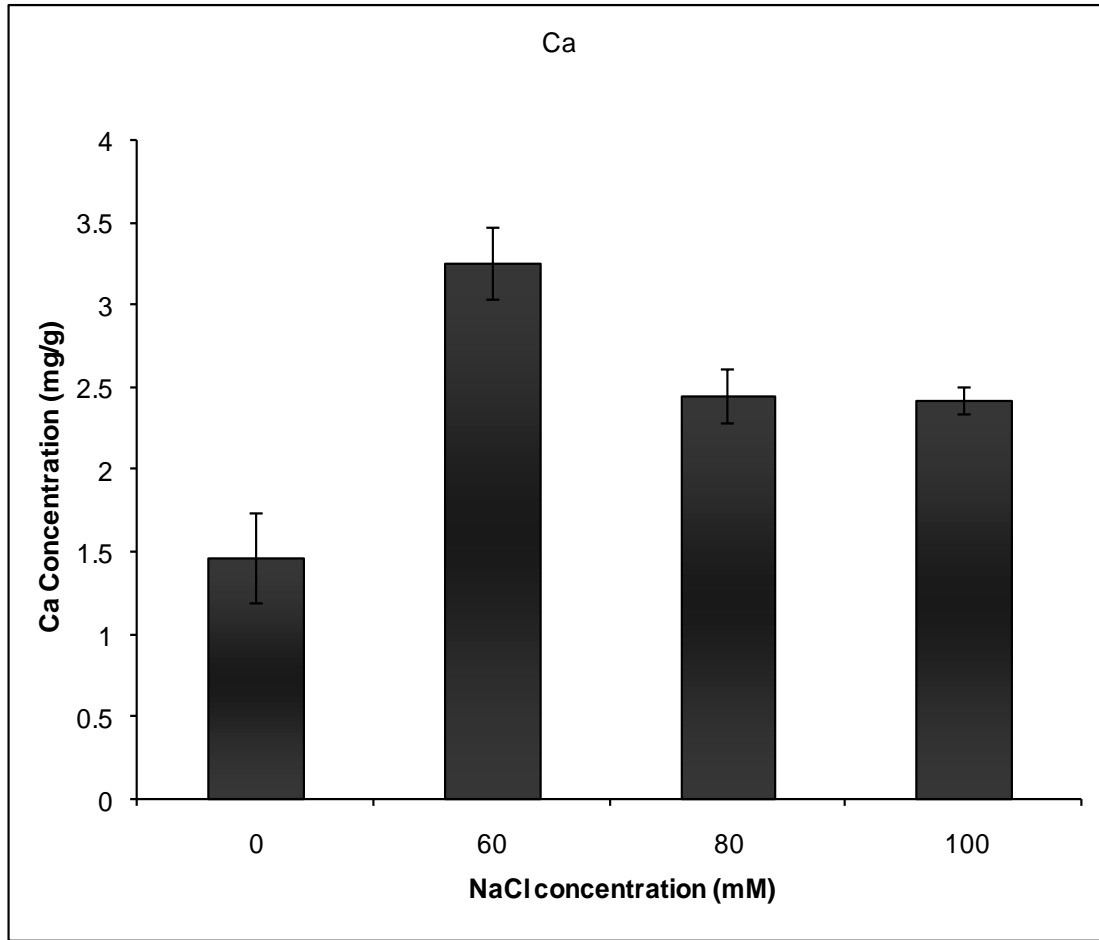
Elevating NaCl concentrations in the medium resulted in increasing Na^+ and decreasing K^+ , Ca^{++} and Mg^{++} concentrations in the plants. The Na^+ content was increased in 60, 80 and 100 mM NaCl treatment (Figure 4.11). Gadallah (1999), Khan *et al.* (1999), Khan (2001), reported an increment in Na^+ levels by increasing NaCl treatment in a variety of plants. An increment was reported in barley (*Hordeum vulgare* L.) (Demural *et al.*, 2005), strawberries (*Fragaria x ananassa* L.) (Yilmaz and Kina, 2008) and legumes (*Medicago polymorpha* L. cv. Scimitar and *Trifolium michelianum* L. cv. Frontier) (Behdani *et al.*, 2008).

With increasing of NaCl levels decreasing K^+ , Ca^{++} and Mg^{++} contents observed in all concentrations (60, 80 and 100 mM of NaCl). Ruiz *et al.* (1999) reported to falling concentration of K^+ , Ca^{++} content in citrus as a result of salinity. Decreasing K^+ , Ca^{++} and Mg^{++} levels in a number of plants in relation to increase in NaCl levels were reported by Khan *et al.* (1999, 2000). Ashraf (2002) reported decreasing K^+ , Ca^{++} and Mg^{++} levels in cotton by increasing NaCl. Figure 4.12 shows decrease in Ca^{++} in relation to increasing salt levels in this study. At 60 mM an increase in Ca^{++} was observed in comparison with the control but, with further increase of NaCl to 80 and 100 mM, a decline was observed in the Ca^{++} concentration. A similar result was seen with K^+ . Figure 4.13 shows decreasing K^+ concentration in relation to NaCl elevation. At 60 mM an increase in K^+ was observed compared to the control but, K^+ concentration decreased with further increment of NaCl. Figure 4.14 shows decreasing Mg^{++} in relation to increase on NaCl concentrations.



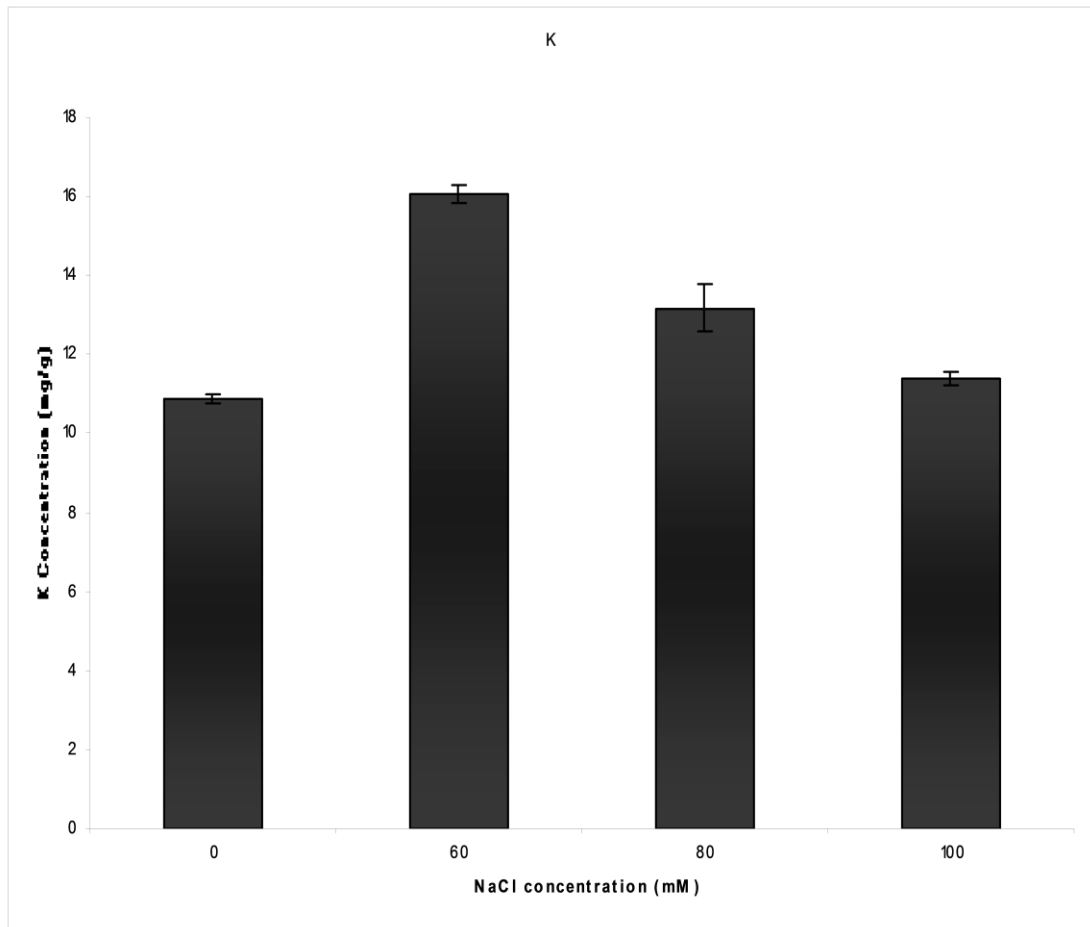
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Figure 4.11 Na⁺ levels in banana plants grown on media containing increasing concentrations of NaCl.



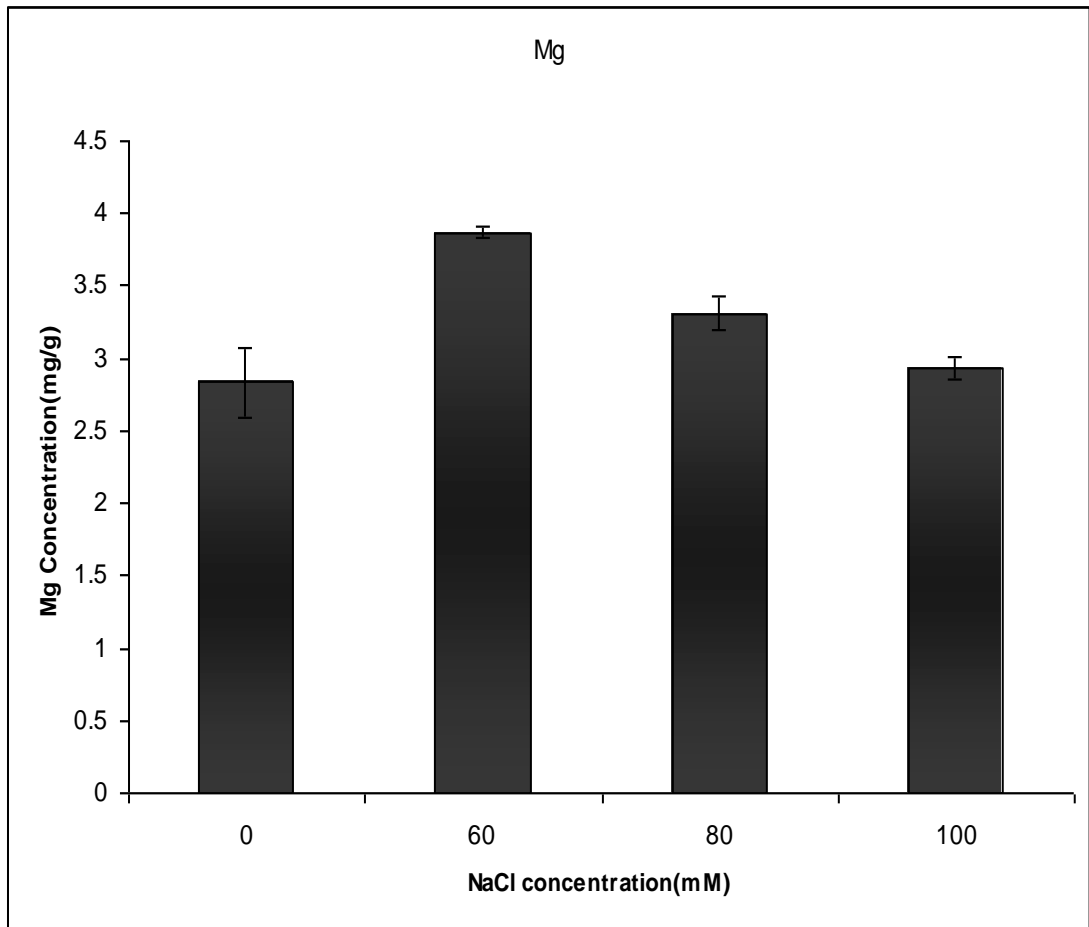
Error bar shows SD.

Figure 4.12 Ca^{++} levels in banana plants grown on media containing increasing concentrations of NaCl.



Error bar shows SD.

Figure 4.13 K⁺ levels in banana plants grown on media containing increasing concentrations of NaCl.



Error bar shows SD.

Figure 4.14 Mg⁺⁺ levels in banana plants grown on media containing increasing concentrations of NaCl.

The increase in Ca^{++} , K^+ and Mg^{++} concentrations at 60 mM NaCl compared to the control and the decrease between the 60 and 80 mM NaCl treatment suggests it to be a result of the plants adaptive response to increasing salt concentrations in the media. Plantlets which were suddenly exposed to high concentrations of NaCl try to absorb these ions at a higher rate because ratios of K^+/Na^+ and $\text{Ca}^{++}/\text{Na}^+$ affect salt tolerance of plant. But with further increases in NaCl concentration, the plants could not withstand high concentration of ions and probably discharged them. The capability to keep a high K^+ cytosolic concentration in the face of increasing competition from Na^+ is a key determinant in salinity tolerance (Naidoo and Kift, 2006). Effect of $\text{Ca}^{++}/\text{Na}^+$ and K^+/Na^+ on plant salt tolerance has been explained by Yasser (2007) and Yildiz *et al.* (2008). Yildiz *et al.* (2008) reported a higher ratio of $\text{Ca}^{++}/\text{Na}^+$ and K^+/Na^+ in tolerant cultivar of strawberry. Yasser (2007) also reported that these ratios were higher in salt tolerant genotypes of green pea. These ratios are again a useful indicator of a plants ability to respond to salinity.

CHAPTER 5

5.0 RESULTS AND DISCUSSION II

5.1 RNA Extraction

5.1.1 Comparison of RNA Quality from CTAB and Modified CTAB Methods

RNA extraction from the roots of *Musa acuminata ssp. malaccensis* was conducted with CTAB (adapted from Kiefer *et al.*, 2000) and CTAB modified methods. Isolating good-quality and intact RNA is crucial for further applications such as RT-PCR. High levels of phenolic compounds, carbohydrates, or other compounds that bind and/or coprecipitate with RNA are strong barriers to high quality RNA achievement (Salzman *et al.*, 1999; Kansal *et al.*, 2008). Figure 5.1, lane one shows RNA extracted from roots with CTAB (Kiefer *et al.*, 2000) protocol. Both 28s and 18s are detectable and intact. The A260/280 ratio was 1.70 and A260/230 was 1.60. The low ratio of A260/230 is because of high level of polyphenol and polysaccharide. Generally the rate of phenolic compounds in banana is high (Titov *et al.*, 2006). To solve this problem and also to achieve higher purity (higher A260/280 ratio) the CTAB method was modified. Phenol followed by Chloroform/Isoamylalcohol (C/I) plus proteinase K were used to precipitate protein and gain higher purity. Also 4% polyvinylpyrrolidone (PVP) was used to reduce the concentration of phenolic compounds. Figure 5.1 lane-2 shows RNA extracted from a root sample with the CTAB modified method. The modified CTAB method considerably enhanced the purity of RNA. The A260/280 for RNA extracted with CTAB modified method was 1.95 and 260/230 was 2.0. Gasic *et al.* (2004) reported all RNA samples with A260/280 ratio range from 1.91-2.02 are considered as pure from protein contamination. They also mentioned that RNAs with A260/230 ratio

of 2.0 and higher are very pure and clean from polysaccharide and polyphenol contamination.

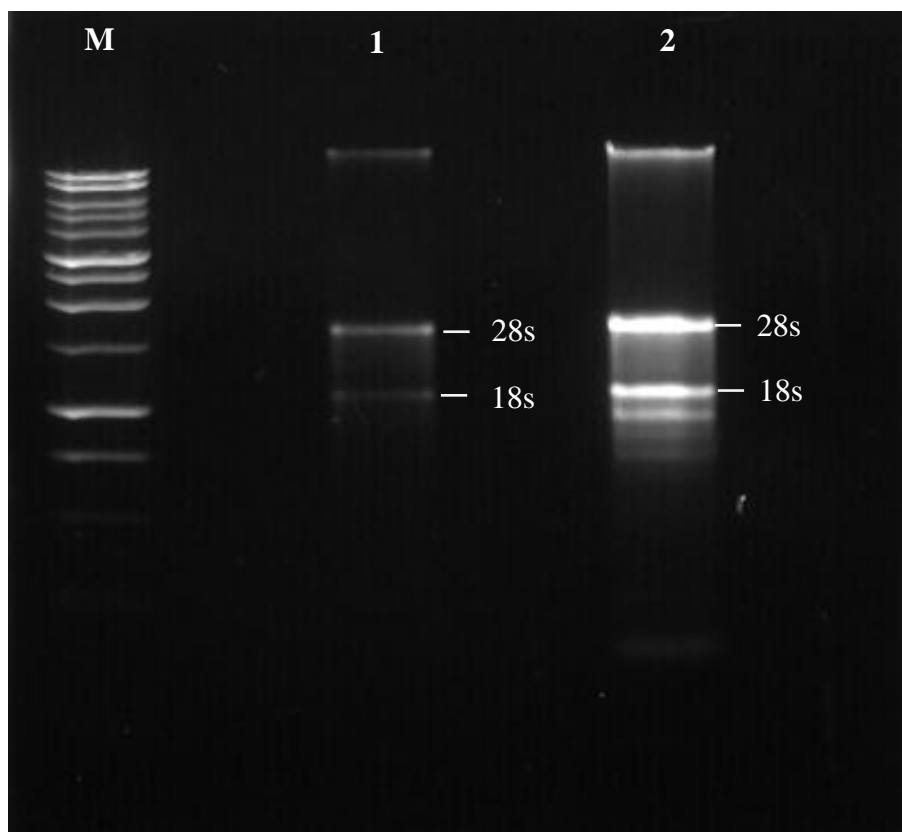


Figure 5.1 Comparison of RNA extraction with modified CTAB method and CTAB (Kiefer *et al.*, 2000), method.

Lane 1: RNA from roots of *Musa acuminata ssp. malaccensis* with CTAB method using Chloroform/Isoamylalcohol (C/I).

Lane 2: RNA from roots of *Musa acuminata ssp. malaccensis* with CTAB modified method using Phenol/Chloroform/Isoamylalcohol (P/C/I). **M:** 1 kb ladder (Fermentas).

Viewed on, 1% Agarose gel.

Salzman *et al.* (1999) reported that using 1% of soluble PVP is sufficient for removing phenolic compounds from plant tissues. The plant tissues used in their experiments was from *Vitis vinifera*, *Griffonia simplicifolia*, *Albizia procera*, *Raphanus sativus* and *Abies procera*. But in this study with *Musa acuminata ssp. malaccensis*, even with utilizing 2% of PVP the ratio of A260/230 was low and around 1.60 whereas employing 4% PVP could improve the ratio of 260/230 to 2.0. The difference can be interpreted as arising from the different type of plants.

On the other hand RNAs isolated *via* the CTAB modified method were enriched in RNA smaller than 18s rRNA. As it can clearly be seen in Figure 5.1 lane-2, the bands below 18s rRNA is distinct and visible. Even small RNAs are detectable at the lower part of the gel.

5.1.2 RNA Extraction from Root Samples

RNA extractions from roots of plantlets from control medium (MS medium without salt treatment) and from MS medium which was supplemented with 100 mM of NaCl after 24 h are shown in Figure 5.2. The A260/280 for RNA from control medium (Figure 5.2, lane-1), was 1.96 and 260/230 was 2.1 whilst For the RNA from 100 mM concentration of NaCl (Figure 5.2, lane-2), A260/280 was 1.93 and A260/230 was 2.0.

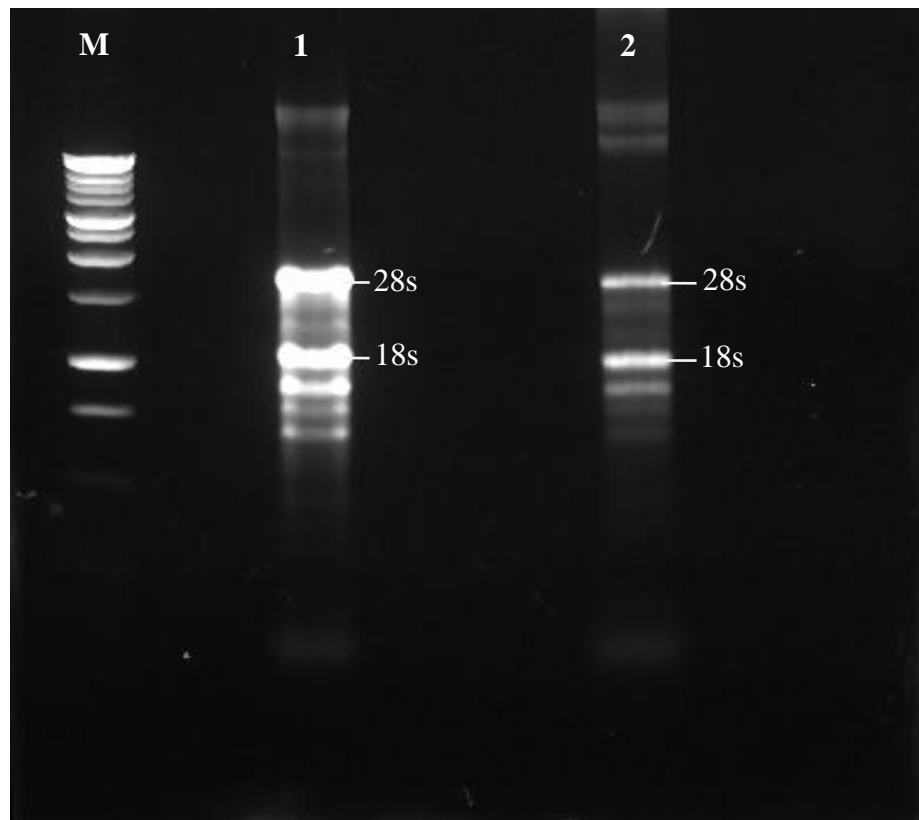


Figure 5.2 RNA extractions from roots of *Musa acuminata ssp. malaccensis* grown in 0 mM and 100 mM NaCl.

Lane 1: RNA from roots of *Musa acuminata ssp. malaccensis* after 24 h on MS medium containing 0 mM of NaCl. **Lane 2:** extracted RNA from roots of *Musa acuminata ssp. malaccensis* after 24 h on MS medium containing 100 mM of NaCl. **M:** 1 kb ladder (Fermentas). Viewed on, 1% Agarose gel.

5.2 DNase Treatment

Figure 5.3 shows banana root RNA samples before and after DNase treatment. Lane-1 shows RNA with DNA contamination before DNase treatment. After DNase treatment there is no visible band of DNA (Figure 5.3, lane-2).

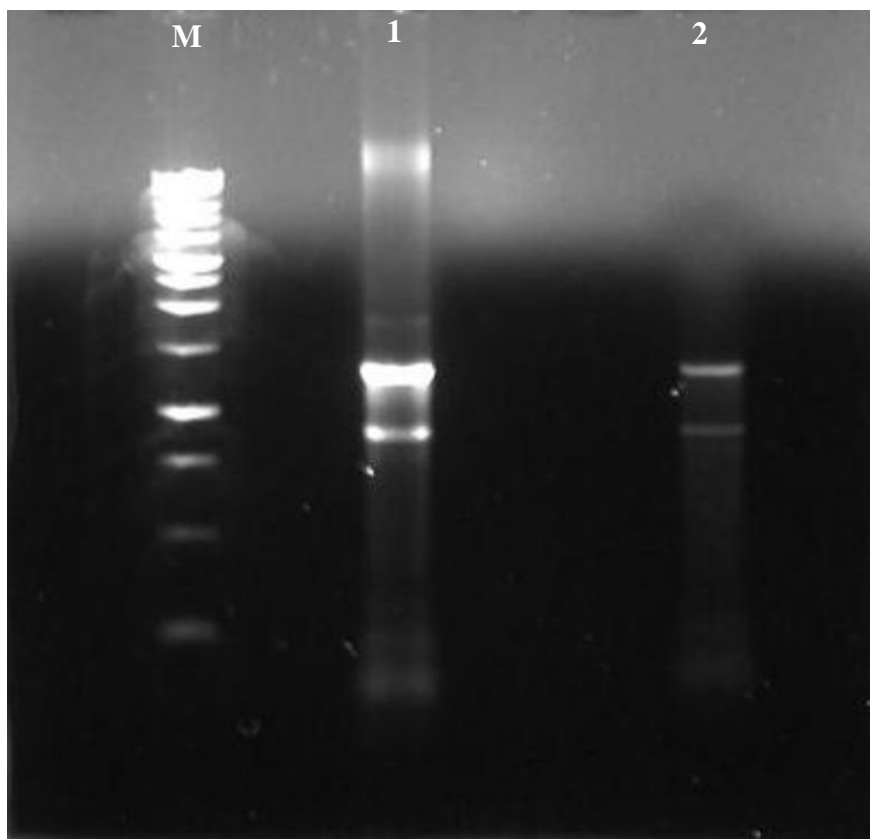


Figure 5.3 RNAs before and after DNase treatment.

Lane 1: RNA before DNase treatment. **Lane 2:** RNA after DNase treatment

M: 1 kb ladder (Fermentas). Viewed on, 1% Agarose gel.

5.3 MicroRNA Library Cloning

First-strand cDNA synthesis and PCR amplification were carried out following the MicroRNA discovery kit (System Biosciences) instructions with adaptor ligation. The PCR products had a size of approximately 200bp (Figure 5.4). Figure 5.4a shows the PCR product from non-treated root sample with size of ~200bp while Figure 5.4b shows the PCR product from treated root sample by 100 mM of NaCl with size of ~200bp.

This result is comparable with results which were highlighted in the kit manual. The size of amplified cDNA reported in the kit manual was around 1.80 bp. The slight difference in the product size is normal and because of using a different type of organism. Different animal tissues were used in the kit whereas roots of *Musa acuminata ssp. malaccensis* were used in this experiment.

Mosher *et al.* (2009) employed mirVana miRNA isolation kit (Ambion) to purify small RNAs. This kit was designed just for small RNAs isolation and for further cDNA amplification it is necessary to use a different kit or conventional method. But MicroRNA discovery kit (System Biosciences) provides all steps including adaptor ligations, cDNA synthesis and PCR amplifications in one kit.

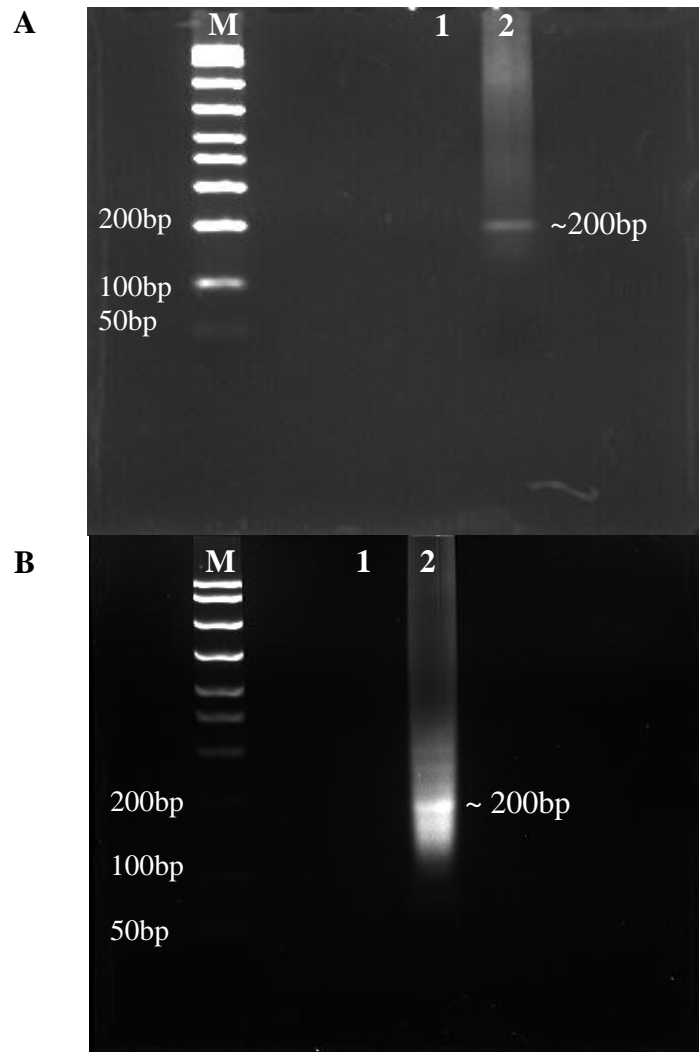


Figure 5.4 PCR products with size of ~200bp.

A: Lane 1: negative control. **Lane 2a:** PCR product from non-treated sample.

B: Lane 1: negative control. **Lane 2b:** PCR product from NaCl treated sample.

M: 50 bp amplisize DNA ladder (Bio-Rad). Viewed on, 2.5 % Agarose gel.

5.3.1 Gel Extraction

Figure 5.5 shows non-specific bands of PCR products before purification. Non specific bands were detected (~250bp and 700bp, Figure 5.5A and ~ 300bp, Figure 5.5B) while samples were run on 3% LE analytical grade Agarose with gel extraction specific wide well. Figure 5.5A shows the PCR product from non-treated sample with size of ~200bp. Figure 5.5B shows the PCR product from treated sample by 100 mM of NaCl with size of ~200bp. Specific bands with size of ~200 bp were excised under UV light and purified. After purification exact product sizes were achieved and no non-specific bands were detected (Figure5.5.C).

5.3.2 Colony Screening

Purified PCR products with size of ~200 bp were cloned into pCR4-TOPO vector. From the 100 µl plated, a total of 200 white colonies were produced. The titre of The 1 ml library stock was therefor calculated to be 2000. White colonies which were expected to carry inserted small RNA clones were screened using M13 forward and M13 reverse primer to confirm the insert size. PCR showed the size of ~400 bp for samples which includes the expected insert size (~200 bp) plus vector size (170 bp) (Figure 5.6).

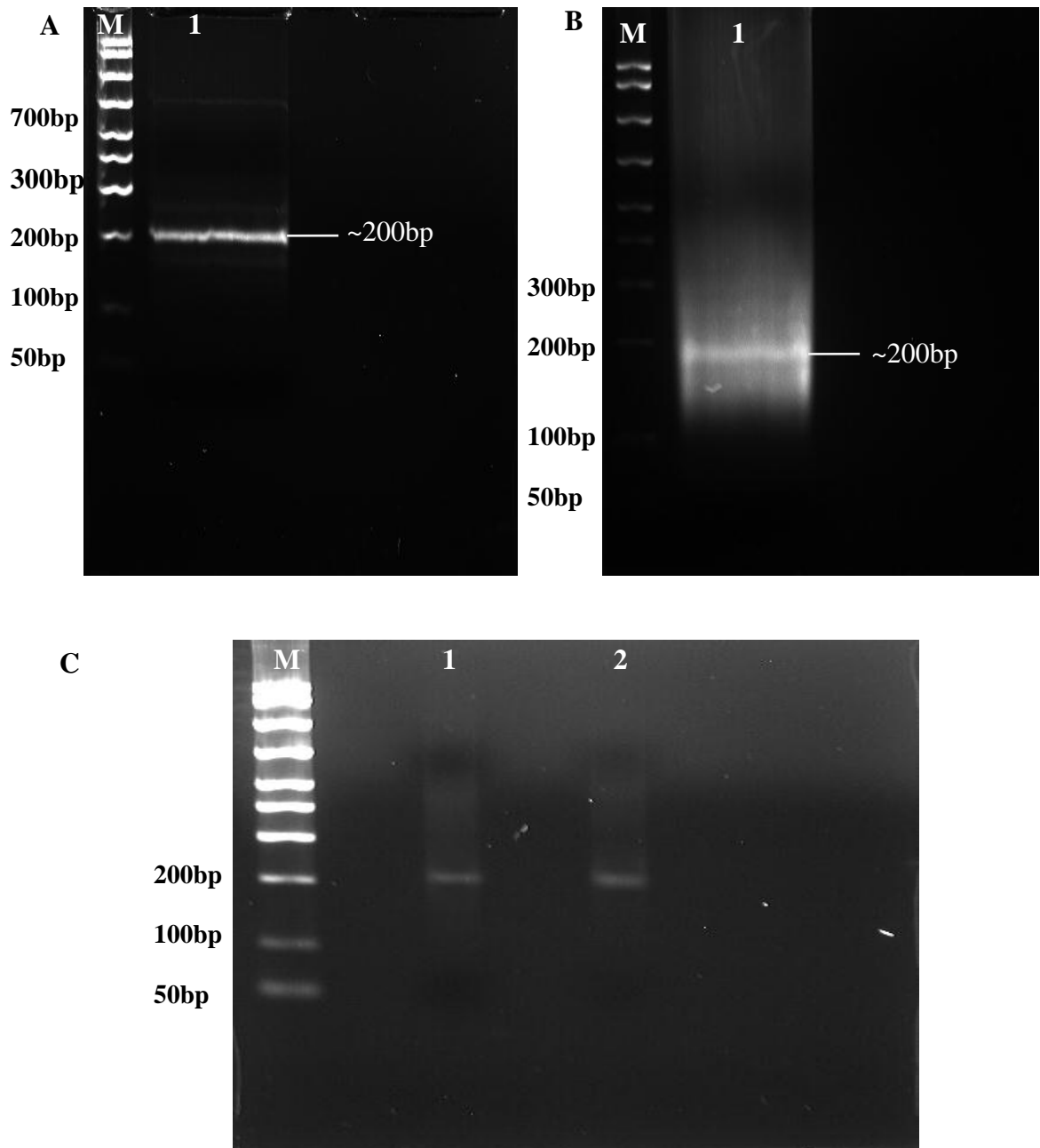


Figure 5.4 PCR products before and after purification.

A: Lane 1: PCR product from non-treated plant RNA. Viewed on, 3% Agarose gel.

B: Lane 1: PCR product from NaCl treated sample. Viewed on, 3% Agarose gel.

C: Lane 1: PCR product from non-treated sample with size of ~200bp after purification

C: Lane 2: PCR product from treated sample by 100 mM of NaCl with size of ~200bp.

C: Viewed on, 2.5 % Agarose gel.

M: 50 bp amplisize DNA ladder (Bio-Rad).

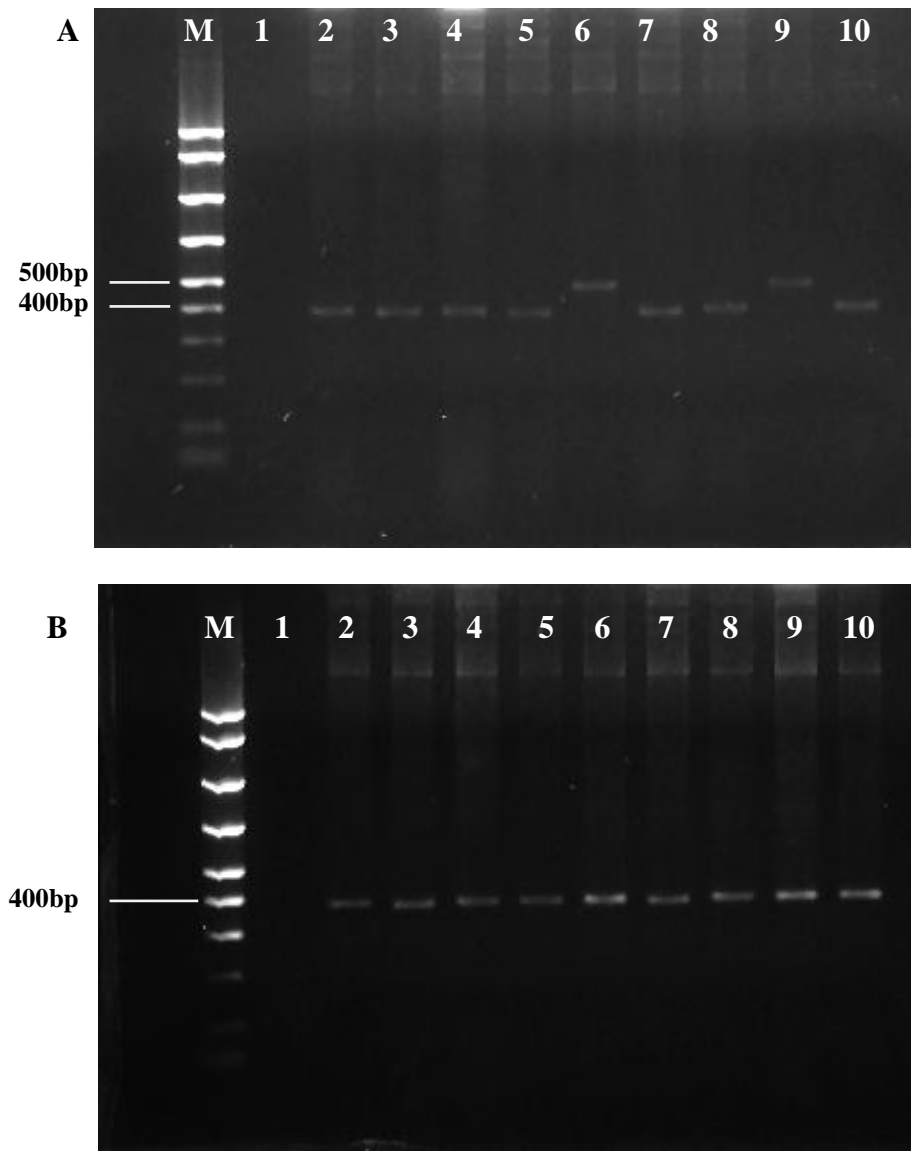


Figure 5.5 Colony PCR screening of library.

A: Lane 1: Negative control. Lane 2-10: Non-treated samples.

B: Lane 1: Negative control. Lane 2-10: NaCl-treated samples.

M: 50 bp amplicon DNA ladder (Bio-Rad). Viewed on 1% Agarose gel.

5.3.3 Plasmid Extraction

Plasmid of white colonies which were expected to carry the PCR products (~400 bp) was extracted using a purification kit (Qiagen). Figure 5.7 shows plasmid DNA isolated from white colonies derived from NaCl treated banana plantlet cDNA library.

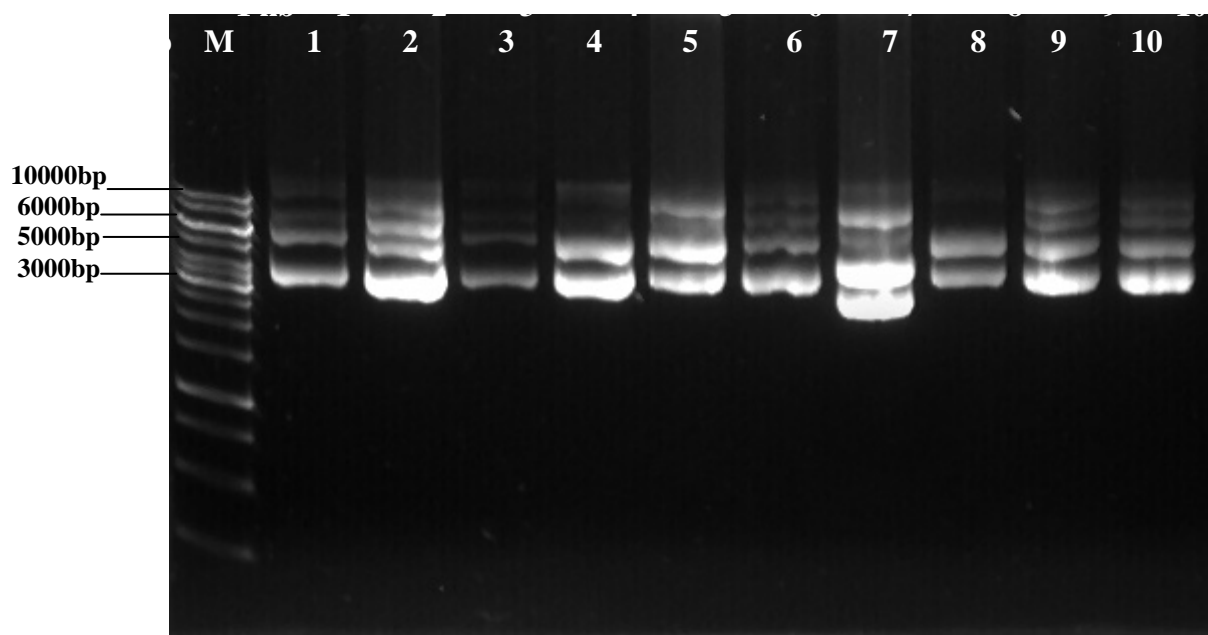


Figure 5.6 Isolated plasmids of white colonies derived from NaCl treated banana Plantlet cDNA library.

Lane 1-10: plasmids isolated from white colonies.

M: 1 kb DNA ladder (Fermentas). Viewed on, 1% Agarose gel.

5.4 Sequence Data Analysis

Two miRNA libraries from salt-stressed and non-salt stressed banana root samples were constructed. A total of 50 white colonies, 5 from none stressed sample as a control and 45 from salt-stressed samples which had been confirmed to have inserted sequences *via* PCR were selected for plasmid extraction and sequencing. Nucleotide BLAST was used in this study for data analysis. Nucleotide collection (nr/nt) was used to search for all potential stress related miRNA precursors and gene sequences. Nucleotide collection (nr/nt) is a non redundant data base and contains all Gene Bank and PDB sequences except Expressed Sequence Tags, Sequence Tagged Sites, Genomic Survey Sequences and unfinished High Throughput Genomic Sequences (Gustave and Janet, 2009). EST is a short sub-sequence of a transcribed cDNA sequence and Plant EST was used to search for all potential stress related gene sequences in plants.

5.4.1 Results of Data Analysis against Nucleotide Collection (nr/nt)

DNA sequences from the selected library clones (45, salt stressed samples and 5 controls) with length range 180-250 were analyzed using BLAST against the non-redundant nucleotide database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). One sequence (clone-12 sequence of miRNA library clones), showed high similarity (91%) with the identity score of 108/119 to the precursor of microRNA 393 from *Oryza sativa Japonica* (Figure 5.8) (Appendix N). No significant matches were found for miRNA precursor or stress related gene sequences for the other sequences.

```

> gb|HM139753.1| Oryza sativa Japonica Group cultivar Padi Kasalle
precursor microRNA
393 gene, complete sequence
Length=685

Score = 154 bits (83), Expect = 2e-34
Identities = 108/119 (91%), Gaps = 6/119 (5%)
Strand=Plus/Plus

Query  38  TGGGGAAGCATCCAAAAGAATCGCATTGATCCTTCAAAGCTCTCGCTCGCTTCCATGGCG 97
      |||
Sbjct  279  TGGGGAAGCATCCAAAGGGATCGCATTGATCCTTCAATCGCTCTCGCTCGCTTCCATGGCG338

Query  98  GTCGTCGCCTTTCTACAAGCAGTCTTGACGGATCATGCGATCCTTTTGGATTGCTTCCT 156
      |||
Sbjct  339  GTCGTCG--T--CTACAAGCAG-CTTGACGGATCATGCGATCCTTTTGGAG-GCTTCCT 391

```

Figure 5.7 Nucleotide collection (nr/nt) blast alignments for clone-12

5.4.2 Results of Data Analysis against Plant EST

(<http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantESTBLAST.shtml>)

DNA sequences from the selected library clones (45, salt stressed samples and 5 controls) with length range 180-250 were analyzed using BLAST against the NCBI plant EST database. From the EST database results three different types of gene sequences were found, salt stress, drought stress and BAP treated related gene sequences. Salt stress associated genes found in this study were included AJ853689.1, EX452105.1, EX451719.1, EX451695.1, EX451687.1, EX451718.1, EX451698.1, EX451678.1, EX451675.1, EX451663.1, EX451650.1, AJ853684.1 and AJ853637.1 from *Oryza sativa indica*, CF332208.1, CF332014.1, CF331927.1, CF331207.1, CF331108.1, CF330429.1, CF330199.1, CF330169.1, CF329645.1, CF328946.1, CF328814.1, CF328813.1, CF328430.1, CF328176.1, CF327316.1 and CF326953.1 from *Oryza sativa Japonica* and AU312469.1 from *Hordeum vulgare*.

Salt stress related gene sequences from the small RNA library included clone-1, clone-16, clone-25, clone-32, clone-33, and clone-39 (Appendix O) and drought stress related gene sequences were included clone-16, clone-25, clone-28, clone-32 and clone-39 (Appendix O). Clone-1 showed 86% similarity (identity score of 181/212) to AJ853689.1 from *Oryza sativa (Indica cultivar-group)* salt stress associated EST (Figure 5.9) and EST AU312469.1 with identity score of 165/193 from a salt stressed cDNA library from root of *Hordeum vulgare* (Figure 5.10). No matches to any salt stress related EST (AJ853689.1 and AU312469.1) were observed for sequences from control samples. Figure 5.11 shows the conserved regions of the sequence from clone-1 with AJ853689.1 and AU312469.1. High similarity between the salt stress related gene from *Oryza sativa* and *Hordeum vulgare* to *Musa acuminata ssp. malaccensis* (clone-1

sequence of miRNA library clones) would support that it is a homologue of this sequence however, there is no function assigned to this sequence at this time. Clone-16 showed 99% similarity (identity score of 119/121) to EST EX452105.1, EX451719.1, EX451695.1 and EX451687.1 (Figure 5.12A) and 98% similarity (identity score of 118/121) to EST EX451718.1, EX451698.1, EX451678.1, EX451675.1, EX451663.1 and EX451650.1 from *Oryza sativa* (*Indica* cultivar group) salt stress library (Figure 5.12B). Clone-25, clone-33 and clone-39 showed high similarity, respectively 99% (identity score of 88/89), 91 % (identity score of 80/88) and 99% (identity score of 109/110) to AJ853684.1 from *Oryza sativa* (*Indica* cultivar-group) salt stress associated EST (Figure 5.13). Clone-39 also showed 89 % (identity score of 103/115) similarity to AJ853637.1 from *Oryza sativa* (*Indica* cultivar-group) salt stress associated EST gene (Figure 5.14). Clone-32 showed high (99%) similarity to EST CF330848.1 and CF330239.1 (Figure 5.15A) and 98% similarity to EST CF332208.1, CF332014.1, CF331927.1, CF331207.1, CF331108.1, CF330429.1, CF330199.1, CF330169.1, CF329645.1, CF328946.1, CF328814.1, CF328813.1, CF328430.1, CF328176.1, CF327316.1 and CF326953.1 (Figure 5.15B) from *Oryza sativa Japonica* Rice callus plasmid cDNA library. High similarity of the salt stress related gene from *Oryza sativa* to *Musa acuminata ssp. malaccensis* (clone-16, clone-25, clone-32, clone-33 and clone-39) would support that it is a homologue of this sequence however, there is no function assigned to these sequences at this time. Presence of drought stress related genes in a salt stress derived library is not unexpected as salt stress has an effect on plant water uptake. Inhibition of root water uptake capacity is one of the primary responses of plants to salt stress (Boursiac *et al.*, 2005). The ability of plants to take up water is reduced under high salinity conditions and this rapidly causes reductions in growth rate, along with a suite of metabolic changes identical to those caused by water stress (Munns 2002).

Sequence entry CI308356 from *Oryza sativa* (*Japonica* cultivar-group) BAP treated callus was annotated as a BAP treated related gene sequence. Amongst the other sequences from the miRNA library, clone-3 sequence of miRNA library clones showed the highest similarity to EST CI308356 (*Japonica* cultivar-group) from BAP treated callus (98%: Figure 5.16) (Appendix O). Appearance of BAP related gene sequences in the library can be justified as the plantlets were grown in the presences of BAP in MS medium before they were placed under stress conditions. BAP related gene sequences also were detected from the control sequenced clones.

```

>| emb|AJ853689.1| AJ853689 Oryza sativa (indica cultivar-group) salt
stress associated
EST Oryza sativa Indica Group cDNA clone OsSsp130, mRNA
sequence.
Length=658

Score = 211 bits (114), Expect = 4e-54
Identities = 181/212 (86%), Gaps = 10/212 (4%)
Strand=Plus/Plus

Query 4 CTATCTACTTCTGGTA-CAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCG 62
||| ||||||||||| | |||||||||||||||||||||||||||||||||||||||
Sbjct 84 CTAGCTACTTCTGG-AGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCG 142

Query 63 GGAACGTATTACCCGCAACATTCTGATTTGCGATTACTAGCGATTCCGACTTCATGGAGT 122
||||||||||||| || ||||||| ||||||||||||||||||||||| |||
Sbjct 143 GGAACGTATTACCCGTGACGTTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGT 202

Query 123 CGAGTTGCAGACTCCAATCCGGACTTA-GATGCACTTTC-TGAGATTCGCTCCCCCTCGC 180
||||||||||||| | ||||||| | ||| | ||| | |||| ||||| |||||
Sbjct 203 CGAGTTGCAGACTGCGATCCGGACT-ACGAT-CGGTTTTATGGGATTAGCTCCACCTCGC 260

Query 181 AGGCTCG-CTTCCTCTGTAT-GCACCATTGT 210
||| | | |||| |||| | |||||||
Sbjct 261 -GGCTTGCAACCCTTTGTACCG-ACCATTGT 290

```

Figure 5.8 EST alignment results for clone- 1.

```

>| dbj|AU312469.1| AU312469 6 days salt stressed cDNA library, root
Hordeum vulgare .cDNA clone BGP2B1, mRNA sequence.
Length=478

Score = 195 bits (105), Expect = 1e-46
Identities = 165/193 (86%), Gaps = 7/193 (3%)
Strand=Plus/Plus

Query 21 AACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTACCCGCAA
80
||| ||||||||||| |||||||||||||||||||||||||||||||||||||||
Sbjct 14 AACCAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTACCCGCGG
73

Query 81 CATTCTGATTTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAAT
140
| | ||||| ||||||||||||||||| ||||||| | ||||||||||| | ||||
Sbjct 74 CGTGCTGATCCGCGATTACTAGCGATTCCAACCTTCATGCACTCGAGTTGCAGAGTGCAAT
133

Query 141 CCGGACTTAGATGCACTTTCTG-AGATTCGCTCCCCCTCGCAG-GCTCGCTTCCTCTGT
198
||| ||| ||||| |||| | ||||| |||| | ||||| | ||||||| ||| ||||
Sbjct 134 CCGAACTGAGATGG-CTTT-TGGAGATTAGCTCACACTCGC-GTGCTCGCTGCCCACTGT
190

Query 199 -ATGCACCATTGT 210
| ||||||||
Sbjct 191 CAC-CACCATTGT 202

```

Figure 5.9 EST alignment results for clone-1.

```

gi|75980887|gb|AJ853689.1|AJ85      CACTCCGTGGTAACCGTCTCCCGAGGGTTAGACTAGCTACTTCTGGAGC 100
gi|51862609|gb|AU312469.1|AU31      -----GACTTCGGGAAA 12
1st_BASE_352085_sample_1_M13F_      -----AAGCTATCTACTTCTGGTAC 20
                                         ***** **

gi|75980887|gb|AJ853689.1|AJ85      AACCCA-CTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT 149
gi|51862609|gb|AU312469.1|AU31      AACCCA-CTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT 62
1st_BASE_352085_sample_1_M13F_      AACCCA-CTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT 69
                                         ** *** *****

gi|75980887|gb|AJ853689.1|AJ85      ATTCACCGTGACGTTCTGATTTCACGATTACTAGCGATTCCGACTTCACGC 199
gi|51862609|gb|AU312469.1|AU31      ATTCACCGGGCGTGTGATCCGCGATTACTAGCGATTCCAACCTTCATGC 112
1st_BASE_352085_sample_1_M13F_      ATTCACCGCAACATTCTGATTTCGCGATTACTAGCGATTCCGACTTCATGG 119
                                         ***** * * ***** *****

gi|75980887|gb|AJ853689.1|AJ85      AGTCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAG 249
gi|51862609|gb|AU312469.1|AU31      ACTCGAGTTGCAGAGTGCAATCCGAACTGAGATGGCTTTTG-GAGATTAG 161
1st_BASE_352085_sample_1_M13F_      AGTCGAGTTGCAGACTCCAATCCGGACTTAGATGCACTTTCTGAGATTTCG 169
                                         * ***** * * ***** *** *** * * * *

gi|75980887|gb|AJ853689.1|AJ85      CTCCACCTCGCGGCTTGGCAACCCCTTGTACCGACCATTGTAGCACGTGT 299
gi|51862609|gb|AU312469.1|AU31      CTCACACTCGCGTGTCTGCTGCCACTGTACCACCATTTGTAGCACGTGT 211
1st_BASE_352085_sample_1_M13F_      CTCCCCCTCGCAGGCTCGCTTCCCTCTGTATGCACCATTTGT----- 210
                                         *** ***** * ** *** *** *****

gi|75980887|gb|AJ853689.1|AJ85      GTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCACCTTCC 349
gi|51862609|gb|AU312469.1|AU31      GTAGCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCACCTTCC 261
1st_BASE_352085_sample_1_M13F_      -----

```

Figure 5.10 Clustal W multiple alignments: Clone-1, AJ853689 and AU312469.

A

```
>| gb|EX452105.1| SSH002525 Salt stress SSH library Oryza sativa Indica
Group cDNA,
mRNA sequence.
Length=176

Score = 213 bits (115), Expect = 1e-54
Identities = 119/121 (99%), Gaps = 0/121 (0%)
Strand=Plus/Plus

Query 21 TTCTTGGTCAGGCCGGCATTGTGAAGAAGGAACACATCAAGATTCATGGTTTCTGAGCAA 80
      |||||||
Sbjct 39 TTCTTGTTCAGGCCGGCATTGTGAAGAAGGAACACATCAAGATTCATGGTTTCTGAGCAA 98

Query 81 CTGCCAAAACCATTGCAAAGACTATAGTTTGGGGTGGAGTATACTTGGTTGTGTACATGC 140
      |||
Sbjct 99 CTGCCAAAACCATTGCAAAGACTATAGTTTGGGGTGGAGTATACTTGGTTGTGTACCTGC 158

Query 141 C 141
      |
Sbjct 159 C 159
```

B

```
>| gb|EX451718.1| SSH001963 Salt stress SSH library Oryza sativa Indica
Group cDNA,
mRNA sequence.
Length=212

Score = 207 bits (112), Expect = 5e-53
Identities = 118/121 (98%), Gaps = 0/121 (0%)
Strand=Plus/Plus

Query 21 TTCTTGGTCAGGCCGGCATTGTGAAGAAGGAACACATCAAGATTCATGGTTTCTGAGCAA 80
      |||||||
Sbjct 75 TTCTTGTTCAGGCCGGCATTGTGAAGAAGGAACACATCAAGATTCATGGTTTCTGAGCAA 134

Query 81 CTGCCAAAACCATTGCAAAGACTATAGTTTGGGGTGGAGTATACTTGGTTGTGTACATGC 140
      |||
Sbjct 135 CTGTCAAACCATTGCAAAGACTATAGTTTGGGGTGGAGTATACTTGGTTGTGTACCTGC 194

Query 141 C 141
      |
Sbjct 195 C 195
```

Figure 5.11 EST alignment results for clone-16.

A

```
> emb|AJ853684.1| AJ853684 Oryza sativa (indica cultivar-group) salt
stress associated
EST Oryza sativa Indica Group cDNA clone OsSsp125, mRNA
sequence.
Length=855

Score = 161 bits (87), Expect = 4e-39
Identities = 88/89 (99%), Gaps = 0/89 (0%)
Strand=Plus/Plus

Query 1 AATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACC 60
|||||
Sbjct 623 AATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAANAGGCCCGCACC 682

Query 61 GATCGCCCTTCCCAACAGTTGCGCAGCCT 89
|||||
Sbjct 683 GATCGCCCTTCCCAACAGTTGCGCAGCCT 711
```

B

```
> emb|AJ853684.1| AJ853684 Oryza sativa (indica cultivar-group) salt
stress associated
EST Oryza sativa Indica Group cDNA clone OsSsp125, mRNA
sequence.
Length=855

Score = 121 bits (65), Expect = 7e-27
Identities = 80/88 (91%), Gaps = 0/88 (0%)
Strand=Plus/Plus

Query 1 AATCACCTTGCACCACATCCCCCTTTCACCAACTGGCGTGGTACCGAAGAGGCCCGCACC 60
|||||
Sbjct 623 AATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAANAGGCCCGCACC 682

Query 61 GATCGCCCTTCCCAACAGTTGCGCAGCC 88
|||||
Sbjct 683 GATCGCCCTTCCCAACAGTTGCGCAGCC 710
```

C

```
> emb|AJ853684.1| AJ853684 Oryza sativa (indica cultivar-group) salt
stress associated
EST Oryza sativa Indica Group cDNA clone OsSsp125, mRNA
sequence.
Length=855

Score = 200 bits (108), Expect = 9e-51
Identities = 109/110 (99%), Gaps = 0/110 (0%)
Strand=Plus/Plus

Query 1 AACCCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGT 60
|||||
Sbjct 602 AACCCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGT 661

Query 61 AATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCT 110|
|||||
Sbjct 662 AATAGCGAANAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCT 711
```

Figure 5.12 EST alignment results for clone-25(A), clone-33 (B) and clone-39(C).

> emb|AJ853637.1| AJ853637 Oryza sativa (indica cultivar-group) salt stress associated

EST Oryza sativa Indica Group cDNA clone OsSsp78, mRNA sequence.

Length=933

Score = 145 bits (78), Expect = 4e-34

Identities = 103/115 (90%), Gaps = 5/115 (4%)

Strand=Plus/Plus

```
Query 1 AACCT-GGCGTTACCCAACTTAATCGCCTTGCAGCACAT-CCCCCTTTCGCCAGCTGGC 58
      ||||| || |||||||||||||||||||||||||||||||||||||||||
Sbjct 816 AACCTGGGGGTTACCCAACTTAATCGCCTTGCAGCACATNCCCCCTTTCGCCAGCTGGG 875

Query 59 GTAATAGCG-AAGAGGCCCGCACCG-ATCGCCCTTCCCAACAGTTGCGC-AGCCT 110
      ||||| || || ||||||| || ||||||||||||||| ||||| || |||||
Sbjct 876 GTAATAACGAAAAAGGCCCGCNC CGAATCGCCCTTCCCAANAGTTGGGCCAGCCT 930
```

Figure 5.13 EST alignment results for clone-39.

A

```
> gb|CF330848.1| NACL--06-L19.g1 Rice callus plasmid cDNA library
(NACL) Oryza
sativa Japonica Group cDNA clone NACL--06-L19, mRNA sequence.
Length=512
```

```
Score = 163 bits (88), Expect = 4e-39
Identities = 90/91 (99%), Gaps = 0/91 (0%)
Strand=Plus/Plus
```

```
|
Query 92 TTTTTTTTTTTTGGCGCTCTCAAAGTAGCATATATTATTCAATCTTGATGAAAGAGGGACA 151
      |||
Sbjct 4 TTTTTTTTTTTTGGCGCTCTCAAAGTAGCATATATTATTCAATCTTGATGAAAGAGGGACA 63

Query 152 CTAGCCATCATCTATAGTGATGGTGCCTCTT 182
      | |||
Sbjct 64 CAAGCCATCATCTATAGTGATGGTGCCTCTT 94
```

B

```
> gb|CF332208.1| NACL--08-K01.b1 Rice callus plasmid cDNA library
(NACL) Oryza
sativa Japonica Group cDNA clone NACL--08-K01, mRNA sequence.
Length=484
```

```
Score = 158 bits (85), Expect = 2e-37
Identities = 90/92 (98%), Gaps = 1/92 (1%)
Strand=Plus/Minus
```

```
Query 92 TTTTTTTTTTTT-GCGCTCTCAAAGTAGCATATATTATTCAATCTTGATGAAAGAGGGAC 150
      |||
Sbjct 431 TTTTTTTTTTTTGGCGCTCTCAAAGTAGCATATATTATTCAATCTTGATGAAAGAGGGAC 372

Query 151 ACTAGCCATCATCTATAGTGATGGTGCCTCTT 182
      ||
Sbjct 371 ACAAGCCATCATCTATAGTGATGGTGCCTCTT 340
```

Figure 5.14 EST alignment results for clone-32.


```
> dbj|CI044090.1| CI044090 Oryza sativa (japonica cultivar-group) callus Oryza
sativa Japonica Group cDNA clone 011-M055R-D09 3', mRNA sequence.
Length=545
```

```
Score = 285 bits (154), Expect = 6e-74
Identities = 158/160 (98%), Gaps = 0/160 (0%)
Strand=Plus/Minus
```

```
Query 25  CGTGACGCCCAGGCAGGCGTGCCCTTAGCCGGATGGCCTCGGGCGCAACTTGC GTTCAA 84
          |||
Sbjct 544  CGTGACGCCCAGGCAGGCGTGCCCTCGGCCGGATGGCCTCGGGCGCAACTTGC GTTCAA 485

Query 85  GACTCGATGGTTCACGGGATTCTGCAATTCACACCAGGTATCGCATTTCGCTACGTTCTT 144
          |||
Sbjct 484  GACTCGATGGTTCACGGGATTCTGCAATTCACACCAGGTATCGCATTTCGCTACGTTCTT 425

Query 145  CATCGATGCGAGAGCCGAGATATCCGTTGCCGAGAGTCGT 184
          |||
Sbjct 424  CATCGATGCGAGAGCCGAGATATCCGTTGCCGAGAGTCGT 385
```

Figure 5.15 EST alignment results for clone-3.

5.4.3 Results of Analysis Based on Known microRNA Genes Related to Salt Stress

A total of 50 white colonies from salt-stressed (45 clones) and non salt-stressed (5 control clones) banana root miRNA libraries were sequenced. A list of mature miRNA sequences related to salt stress were obtained from miRBase. An alignment strategy was engaged to find candidate mature miRNA from *Musa acuminata ssp. malaccensis* among the sequenced library clones as used by Zhang *et al.* (2009). Sequences were aligned with three miRNA families, miR393, miR397, miR402 which were reported to be related to abiotic stresses including salt stress (Sunkar and Zhu, 2004; Liu *et al.*, 2008; Sunkar, 2010). Table 5.1 shows members of miR393, miR397, miR 402 families (<http://www.mirbase.org>).

Plant miRNAs are conserved in their mature miRNA sequences, rather than the complete precursor sequences (Zhang *et al.*, 2006c). According to Qiu *et al.*, (2007) and Wang *et al.*, (2007) miRNA sequences with 85% (3 mismatches) similarity to registered miRNA from database can be considered as new miRNAs. Wang *et al.* (2007) reported identification of miRNA by allowing a maximum three mismatches with their homologues in *Arabidopsis* or rice. Also some other reports (Cheng Xiang Qiu *et al.*, 2007; Wang *et al.*, 2007; Xie *et al.*, 2007a), have emphasized a maximum three mismatches are allowed (85 % similarity to registered database) for prediction of mature miRNA from previously known plant mature miRNAs.

Table 5.1 : MicroRNA family (miR393, miR397, mi402) sequences from miRBase**(<http://www.mirbase.org>).**

No	MicroRNA Family	Sequence	Size
1	>ath-miR393aMIMAT0000934	UCCAAAGGGAUCGCAUUGAUCC	22n
2	>ath-miR393bMIMAT0000935	UCCAAAGGGAUCGCAUUGAUCC	22n
3	>bna-miR393MIMAT0004447	UCCAAAGGGAUCGCAUUGAUC	21n
4	>ghr-miR393MIMAT0014334	UCCAAAGGGAUCGCAUUGAUCU	22n
5	>gma-miR393MIMAT0007362	UCCAAAGGGAUCGCAUUGAUC	21n
6	>mtr-miR393MIMAT0001647	UCCAAAGGGAUCGCAUUGAUC	21n
7	>mtr-miR393bMIMAT0011087	UCCAAAGGGAUCGCAUUGAUC	21n
8	>osa-miR393MIMAT0000957	UCCAAAGGGAUCGCAUUGAUC	21n
9	>osa-miR393bMIMAT0001078	UCCAAAGGGAUCGCAUUGAUCU	22n
10	>ptc-miR393aMIMAT0002015	UCCAAAGGGAUCGCAUUGAUC	21n
11	>ptc-miR393bMIMAT0002016	UCCAAAGGGAUCGCAUUGAUC	21n
12	>ptc-miR393cMIMAT0002017	UCCAAAGGGAUCGCAUUGAUC	21n
13	>ptc-miR393dMIMAT0002018	UCCAAAGGGAUCGCAUUGAUC	21n
14	>rco-miR393MIMAT0014186	UCCAAAGGGAUCGCAUUGAUC	21n
15	>sbi-miR393MIMAT0001426	UCCAAAGGGAUCGCAUUGAUC	21n
16	>sbi-miR393bMIMAT0011348	UCCAAAGGGAUCGCAUUGAUC	21n
17	>vvi-miR393aMIMAT0006557	UCCAAAGGGAUCGCAUUGAUC	21n
18	>vvi-miR393bMIMAT0005708	UCCAAAGGGAUCGCAUUGAUC	21n
19	>zma-miR393aMIMAT0001747	UCCAAAGGGAUCGCAUUGAUCU	22n
20	>zma-miR393bMIMAT0013999	UCCAAAGGGAUCGCAUUGAUCC	22n
21	>zma-miR393cMIMAT0014000	UCCAAAGGGAUCGCAUUGAUCU	22n
No	Micro RNA family	Sequence	Size
1	>ath-miR397aMIMAT0000946	UCAUUGAGUGCAGCGUUGAUG	21n
2	>ath-miR397bMIMAT0000947	UCAUUGAGUGCAGCGUUGAUG	21n
3	>bdi-miR397MIMAT0012180	UCAUUGAGUGCAGCGUUGAUG	21n
4	>bna-miR397aMIMAT0005600	UCAUUGAGUGCAGCGUUGAUGU	22n
5	>bna-miR397bMIMAT0005601	UCAUUGAGUGCAGCGUUGAUGU	22n
6	>osa-miR397aMIMAT0000980	UCAUUGAGUGCAGCGUUGAUG	21n
7	>ptc-miR397aMIMAT0002038	UCAUUGAGUGCAGCGUUGAUG	21n
8	>ptc-miR397cMIMAT0002040	UCAUUGAGUGGAGCUUUGAUG	21n
9	>sbi-miR397MIMAT0011359	UCAUUGAGUGCAGCGUUGAUG	21n
10	>vvi-miR397aMIMAT0006561	UCAUUGAGUGCAGCGUUGAUG	21n
11	>vvi-miR397bMIMAT0006562	UCAUUGAGUGCAGCGUUGAUG	21n
12	>zma-miR397aMIMAT0014018	UCAUUGAGCGCAGCGUUGAUG	21n
13	>zma-miR397bMIMAT0014019	UCAUUGAGCGCAGCGUUGAUG	21n
14	>rco-miR397MIMAT0014193	UCAUUGAGUGCAGCGUUGAUG	21n
No	Micro RNA family	Sequence	Size
1	>ath-miR402MIMAT0001003	UUCGAGGCCUAUUAACCUCUG	22n

From the Clustal W alignment and miRBase search results only one clone (Clone-12 sequence of miRNA library clones) out of 45 sequenced library clones showed more than 85% similarity to a known stress related miRNA. Clone-12 included the 22 nt fragment (5' UCCAAAAGAAUCGCAUUGAUCC 3') with 90.9 % similarity (two mismatches) to ath-miR393 and ath-miR393a (from *Arabidopsis thaliana*) and zma-miR393b (from *Zea mays*), as shown in Table 5.2.

Within this potential mature miRNA sequence (clone-12) is a shorter 21nt sequence (5' UCCAAAAGAAUCGCAUUGAUC 3') also reported for some miRNA from the same family (Table 5.1). Both 22 nt and 21 nt sequences are possible candidates for the miR393 in *Musa acuminata ssp. malaccensis* (Figure 5.17). The 21 nt sequence from clone-12 also showed two mismatches) with bna-miR393.(from *Brassica napus*), gma-miR393 (from *Glycine max*), mtr-miR393, mtr-miR393b (from *Medicago truncatula*), osa-miR393 and (from *Oryza sativa*), ptc-miR393a, ptc-miR393b, ptc-miR393c, ptc-miR393d (from *Populus trichocarpa*), rco-miR393 (from *Ricinus communis*) sbi-miR393, sbi-miR393b (from *Sorghum bicolor*), vvi-miR393 and vvi-miR393b (from *Vitis vinifera*) (Table 5.2). Also clone-12 showed 86.4 % similarity (3 mismatches) to ghr-miR393 (from *Gossypium hirsutum*), osa-miR393b (from *Oryza sativa*), zma-miR393a and zma-miR393c (from *Zea mays*). Figure 5.18, 5.19 and 5.20 show Clustal W alignments of clone-12 with miRNA from miRBase. From the Clustal W alignment and miRBase search results, no significant matches were seen (less than three mismatches) also for miRNA related to 397 and 402 families and also other kind of stresses such as cold, drought and dehydration.

Clone-12 sequence of miRNA library clones.

AAGCAGTGGTTATCAACGCAGAGTCGTGTGGATTCGATGGGGAAGCA **TCCA**
AAAGAATCGCATTGATCCTTCAAAGCTCTCGCTCGCTTCCATGGCGGTCGTC
GCCTTTCTACAAGCAGTCTTGACGGATCATGCGATCCTTTTGGATTGCTTCCT
TATCTGGGCGTCACGCACTCTGCGTTGATAACCACTGCTT

Clone-12 sequence of miRNA library clones.

AAGCAGTGGTTATCAACGCAGAGTCGTGTGGATTCGATGGGGAAGCA **TCCA**
AAAGAATCGCATTGATCCTTCAAAGCTCTCGCTCGCTTCCATGGCGGTCGTC
GCCTTTCTACAAGCAGTCTTGACGGATCATGCGATCCTTTTGGATTGCTTCCT
TATCTGGGCGTCACGCACTCTGCGTTGATAACCACTGCTT

Figure 5.16 Shows two possible miR393 sequences of clone-12. Possible candidate

mature miRNA sequences are highlighted.

Table 5.2 : Similarity of clone-12 sequence of miRNA library clones to miR393 family from miRBase.

NO	Micro RNA	Similarity To Original MicroRNA From miRBase	Micro RNA sequence	Size
12	ath-miR393a	20/22	UCCAAAAGAAUCGCAUUGAUCC	22n
	ath-miR393b	20/22	UCCAAAAGAAUCGCAUUGAUCC	22n
	bna-miR393	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	ghr-miR393	19/22	UCCAAAAGAAUCGCAUUGAUCC	22n
	gma-miR393	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	mtr-miR393	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	mtr-miR393b	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	osa-miR393	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	osa-miR393b	19/22	UCCAAAAGAAUCGCAUUGAUCC	22n
	ptc-miR393a	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	ptc-miR393b	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	ptc-miR393c	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	ptc-miR393d	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	rco-miR393	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	sbi-miR393	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	sbi-miR393b	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	vvi-miR393a	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	vvi-miR393b	19/21	UCCAAAAGAAUCGCAUUGAUC	21n
	zma-miR393a	19/22	UCCAAAAGAAUCGCAUUGAUCC	22n
	zma-miR393b	20/22	UCCAAAAGAAUCGCAUUGAUCC	22n
zma-miR393c	19/22	UCCAAAAGAAUCGCAUUGAUCC	22n	

CLUSTAL 2.0.12 multiple sequence alignment

```
ath-miR393bMIMAT0000935 -----TCC 3
zma-miR393bMIMAT0013999 -----TCC 3
ath-miR393aMIMAT0000934 -----TCC 3
1st_BASE_360237_sample_12_M13F AAGCAGTGGTTATCAACGCAGAGTCGTGTGGATTCGATGGGGAAGCATCC 50
                                                                    ***

ath-miR393bMIMAT0000935 AAAGGGATCGCATTGATCC----- 22
zma-miR393bMIMAT0013999 AAAGGGATCGCATTGATCC----- 22
ath-miR393aMIMAT0000934 AAAGGGATCGCATTGATCC----- 22
1st_BASE_360237_sample_12_M13F AAAAGAATCGCATTGATCCCTTCAAAGCTCTCGCTCGCTTCCATGGCGGTC 100
                                                                    ***,*,*****

ath-miR393bMIMAT0000935 -----
zma-miR393bMIMAT0013999 -----
ath-miR393aMIMAT0000934 -----
1st_BASE_360237_sample_12_M13F GTCGCCTTTCTACAAGCAGTCTTGACGGATCATGCGATCCTTTTGGATTG 150
```

Figure 5.17 Alignment results *via* Clustal W for microRNA 393 family: ath-miR393a, ath-miR393b and zma-miR393b. Clone-12.

CLUSTAL 2.0.12 multiple sequence alignment

```

vvi-miR393aMIMAT0006557 -----TCC 3
vvi-miR393bMIMAT0005708 -----TCC 3
sbi-miR393bMIMAT0011348 -----TCC 3
sbi-miR393MIMAT0001426 -----TCC 3
rco-miR393MIMAT0014186 -----TCC 3
ptc-miR393dMIMAT0002018 -----TCC 3
ptc-miR393cMIMAT0002017 -----TCC 3
ptc-miR393bMIMAT0002016 -----TCC 3
ptc-miR393aMIMAT0002015 -----TCC 3
osa-miR393MIMAT0000957 -----TCC 3
mtrmiR393bMIMAT0011087 -----TCC 3
mtr-miR393MIMAT0001647 -----TCC 3
gma-miR393MIMAT0007362 -----TCC 3
bna-miR393MIMAT0004447 -----TCC 3
1st_BASE_360237_sample_12_M13F AAGCAGTGGTTATCAACGCAGAGTCGTGTGGATTGGGGAAGCATCC 50
                                                                ***

vvi-miR393aMIMAT0006557 AAAGGGATCGCATTGATC----- 21
vvi-miR393bMIMAT0005708 AAAGGGATCGCATTGATC----- 21
sbi-miR393bMIMAT0011348 AAAGGGATCGCATTGATC----- 21
sbi-miR393MIMAT0001426 AAAGGGATCGCATTGATC----- 21
rco-miR393MIMAT0014186 AAAGGGATCGCATTGATC----- 21
ptc-miR393dMIMAT0002018 AAAGGGATCGCATTGATC----- 21
ptc-miR393cMIMAT0002017 AAAGGGATCGCATTGATC----- 21
ptc-miR393bMIMAT0002016 AAAGGGATCGCATTGATC----- 21
ptc-miR393aMIMAT0002015 AAAGGGATCGCATTGATC----- 21
osa-miR393MIMAT0000957 AAAGGGATCGCATTGATC----- 21
mtrmiR393bMIMAT0011087 AAAGGGATCGCATTGATC----- 21
mtr-miR393MIMAT0001647 AAAGGGATCGCATTGATC----- 21
gma-miR393MIMAT0007362 AAAGGGATCGCATTGATC----- 21
bna-miR393MIMAT0004447 AAAGGGATCGCATTGATC----- 21
1st_BASE_360237_sample_12_M13F AAAAGAATCGCATTGATCCTTCAAAGCTCTCGCTCGCTTCCATGGCGGTC 100
                                                                ***.*.*****

```

Figure 5.18 Alignment results *via* Clustal W for microRNA 393 family: bna-miR393, gma-miR393, mtr-miR393, mtr-miR393b, osa-miR393, ptc-miR393a, ptc-miR393b, ptc-miR393c, ptc-miR393d, rco-miR393, sbi-miR393, sbi-miR393b, vvi-miR393a, and vvi-miR393b. Clone-12.

CLUSTAL 2.0.12 multiple sequence alignment

```

zma-miR393aMIMAT0001747 -----TCC 3
zma-miR393cMIMAT0014000 -----TCC 3
osa-miR393bMIMAT0001078 -----TCC 3
ghr-miR393MIMAT0014334 -----TCC 3
1st_BASE_360237_sample_12_M13F AAGCAGTGGTTATCAACGCAGAGTCGTGTGGATTCGATGGGGAAGCATCC 50
                                                                    ***

zma-miR393aMIMAT0001747 AAAGGGATCGCATTGATCT----- 22
zma-miR393cMIMAT0014000 AAAGGGATCGCATTGATCT----- 22
osa-miR393bMIMAT0001078 AAAGGGATCGCATTGATCT----- 22
ghr-miR393MIMAT0014334 AAAGGGATCGCATTGATCT----- 22
1st_BASE_360237_sample_12_M13F AAAAGAATCGCATTGATCCTTCAAAGCTCTCGCTCGCTCCATGGCGGTC 100
                                                                    ***,*,*****

zma-miR393aMIMAT0001747 -----
zma-miR393cMIMAT0014000 -----
osa-miR393bMIMAT0001078 -----
ghr-miR393MIMAT0014334 -----
1st_BASE_360237_sample_12_M13F GTCGCCTTCTACAAGCAGTCTTGACGGATCATGCGATCCTTTTGGATTG 150

```

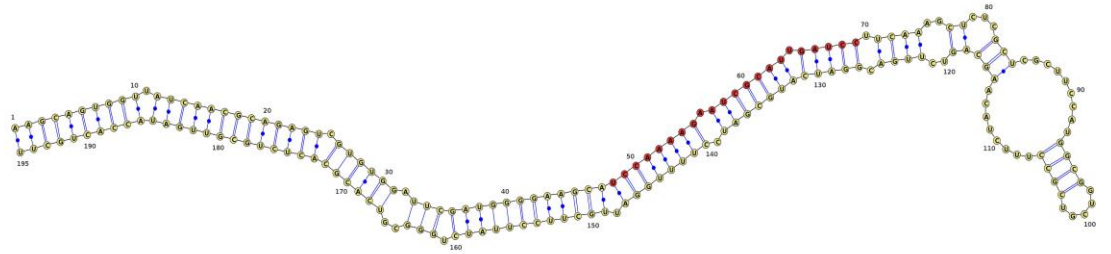
Figure 5.19 Alignment results *via* Clustal W for microRNA 393 family: ghr-

miR393, osa-miR393b, zma-miR393a and zma-miR393c.Clone-12.

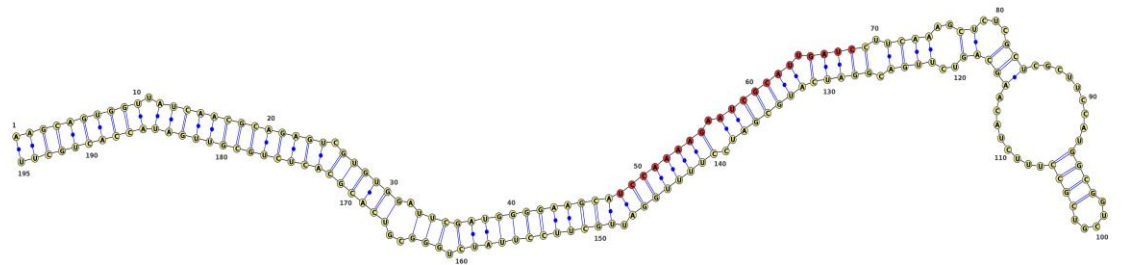
The Secondary structures with minimal folding free energies (MFEs) of sample-12 were predicted and generated using MFold (Zuker, 2003). Figure 5.21 shows the predicted stem-loop structures of miRNA 393 precursor. Any candidate sequence for mature miRNA should be able to fold into a hairpin structure and also should contain the ~22 nt mature miRNA sequence in one arm of the hairpin (Ambros *et al.*, 2003). The result of this part of study showed clone-12 sequence was able to perform hairpin structure contained 22 mature miRNA sequence in one arm. Ambros *et al.* (2003) mentioned that the fold back structure should not contain large internal loops or bulges, which have not seen from hairpin structure of precursor of miR393 which was reported in current study. Also calculated A+U content for miR 393 with 22 nt was 59 % and for miR 393 with 21 nt was 62% which were higher than G+C contents. According to Zhang et al (2006c) mature miRNAs contain more A+U nucleotides than G+C.

Table 5.3 : Specifications of two possible candidates of mac-miR393.

MicroRNA Gene	miRNA Sequence (5'→3')	Fold back	Length
mac-miR393	UCCAAAAGAAUCGCAUUGAUCC	Yes	22n
mac-miR393	UCCAAAAGAAUCGCAUUGAUC	Yes	21n



Musa-miRNA-393a



Musa-miRNA-393b

Figure 5.20 Predicted stem-loop structures of *Musa* 393 miRNAs precursor. The mature miRNAs are indicated by red color.

5.4.4 Target prediction for miR393 *Musa acuminata ssp. malaccensis*

The sequence of mature mac-miR393 (for both 21 nt and 22 nt sequences) was input into psRNA Target (<http://bioinfo3.noble.org/psRNATarget/>) (Dai et al., 2010) and searched against a banana ESTdatabase. One putative target sequence with a score of ≤ 3 for miR393 was predicted (Table 5.4). Xie and Zhang (2010) considered the following special characteristics in alignments and filtering results, ≤ 4 mismatches between the miRNA and its targets, ≤ 2 consecutive mismatches in the alignment region, no mismatches between bases 10-11, and ≤ 1 mismatch from bases 1-9. The psRNATarget result showed no mismatches between bases 10-11 and also no bases from 1-9. This target is specific for banana and has not reported for miR393 from other plant before.

Table 5.4 : Target prediction for mac-miR 393

Micro RNA	Banana EST ID	Target description	Expectati on	Plant
mac-miR393	600103026T13	Not annotated	3	Banana
Alignment result :				
<pre> miRNA 21 CUAGUUACGCUAAGAAAACCU 1 : : : : : : : : : : : : Target 474 GAUUGAUGGGAUACUUUUGGA 494 </pre>				

Note: The banana EST data are from a UM project led by Prof. Rofina Yasmin Othman and contains different data including banana virus sequences and are part of the data available to the Global *Musa* Genomics Consortium (<http://www.musagenomics.org/>).

CHAPTER 6

6.0 CONCLUSION

In the present study two main objectives were followed. Firstly, the minimal inhibitory sodium chloride (NaCl) concentration for survival of banana (*Musa acuminata ssp. mallancensis*) was determined. Secondly, a small RNAs library was established from salt-stressed banana plants. This library was used for identification of potential, salt stress associated genes from *Musa acuminata ssp. mallancensis*. These included homologues of *Oryza sativa* and *Hordeum vulgare* salt stress related gene sequences (ESTs) and a candidate miR393 (mac-miR393) which is a salt stress related microRNA, reported from other plants species.

A total of 10 different NaCl concentrations were used (60, 80, 100, 120, 140, 160, 180, 200 and 220 mM) and 120 mM was determined as the lethal concentrations. All examined plantlets were died at 120 mM of NaCl. Although saline conditions of up to 100 mM imposed severe damage to plantlets but, plants survived and 100 mM of NaCl was determined to be a minimal inhibitory sodium chloride (NaCl) concentration for survival of (*Musa acuminata ssp. malaccensis*).

Total RNA was extracted from salt-stressed banana plants with a modified CTAB method. Subsequently small RNA was isolated and converted to cDNA using MicroRNA discovery kit (System Biosciences). PCR products with size of approximately 200 bp were cloned to PCR 4-TOPO cloning kit (Invitrogen) and white colonies were selected. Plasmids of white colonies which were confirmed to have an insert were extracted and 50 were sent for sequencing. A total of 50 sequences (45

samples plus 5 controls) were compared against EST databases using BLAST. Three different types of gene sequences (Salt stress, drought stress and BAP treated related) were found, with high similarity to salt stress related gene from *Oryza sativa* and *Hordeum vulgare*, drought stress and BAP treated related from *Oryza sativa*. Therefore it is suggested these small RNA library clones may be homologues from *Musa acuminata ssp. malaccensis*. Also mac-miR393 was found as a potential salt stress related miRNA homologue in *Musa acuminata ssp. malaccensis*.

There are some suggestions for improving the data. Performing high throughput sequencing of small RNA was used and suggested by Barakat *et al.* (2007), Fahlgren *et al.* (2007), Yao *et al.* (2007), Sunkar *et al.* (2008) and Liang *et al.* (2010), for miRNA identification in plants. In the current study high throughput sequencing was not performed because of high cost and limited budget. The whole constructed salt stress small RNA contained an estimated 2000 recombinant clones however; only 45 colonies were sequenced and studied in this work. There are possibilities to find new miRNA candidate if more library clones are sequenced, Furthermore newly identified miRNA can be studied to identify targets and gene expression.