CHAPTER-II

REVIEW OF LITERATURE

The present review is an attempt at bringing together some of the finding on sesame and prediction of microRNAs (miRNAs) and their targets in plants by computational methods and expression analysis of miRNAs.

2.1 SESAME

2.1.1 History

Sesame seed and its oil are being utilized as important food ingredient since about 5,000 years. The name Sesame comes from Arabic word “simsim”. India was cited as the origin of sesame but it was also believed that actual origin was Africa where many wild species are found (Bedigian and Harlan, 1986). A cultivated species of Sesame, *Sesamum indicum* L., is thought to have originated in Savannas of Central Africa, spreading to Egypt, India, Middle East, China and elsewhere. According to archaeological record, sesame is one of the most ancient oilseed crop known to mankind. It was cultivated and domesticated on the Indian subcontinent during Harappan and Anatolian eras (Nayar and Mehra, 1970), (Fuller, 2003).

2.1.2 Classification and Morphology

Sesame is broad leaf summer crop of *Pedaliaceae* family with bell-shaped flowers and opposite leaves. Its classification is given in Table 2.1.

It is an annual plant that can reach 1-1.8m height. The plant prefers fertile, well drained and neutral to slightly alkaline soil. It is cultivated in tropical areas and need a growing season of 110-150 frost-free days. The optimum air temperature is 30-35°C and soil temperature never below 20°C. Flowering starts 40 days after sowing and continuous further for 40 days. The flowers develop into seed pods (capsules). Each capsule contains 60-100 seeds. The seed colour varied from white to brown, grey, golden and black. Seeds are small in size and weight is 2-3g/1000 seeds. Morphology of sesame is given in Figure 2.1.
Table 2.1: Classification of *Sesamum indicum* L.

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subkingdom</td>
<td>Tracheobionta</td>
</tr>
<tr>
<td>Superdivision</td>
<td>Spermatophyta</td>
</tr>
<tr>
<td>Division</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Subclass</td>
<td>Asteridae</td>
</tr>
<tr>
<td>Order</td>
<td>Scrophulariales</td>
</tr>
<tr>
<td>Family</td>
<td>Pedaliaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Sesamum</td>
</tr>
<tr>
<td>Species</td>
<td><em>Sesamum indicum</em> L.</td>
</tr>
</tbody>
</table>

Figure 2.1 Morphology of Sesame. (a) Flowering plant. (b) Flower and Seeds. (c) Longitudinal and Transvers sections of fruit

Sesame is a survivor crop. For about 5,000 years, it has been planted by subsistence farmers in areas that will not support the growth of other crops or under very difficult conditions with drought and/or high temperature. In some countries it is grown after the monsoon on residual moisture with no rains during its production cycle. In some countries it is grown in the monsoon season and subject to daily rains during parts of its cycle. In several countries it is the last crop that can be grown at the edge of deserts, where no crops grow. Very little sesame is grown under high input conditions, although yields improve dramatically as inputs increase (Langham, 2007).
2.1.3 Importance of Sesame

Sesame is grown for its seeds and the primary use of the sesame seed is as a source of oil for cooking. The young leaves may also be eaten in stews and the dried stems may be burnt as fuel with the ash used for local soap making but such uses are entirely subordinate to seed production (Anilakumar et al., 2010).

2.1.3.1 Sesame oil

Aromatic, amber coloured sesame oil is made from pressed and toasted sesame seeds. It has been traditionally used in South India and countries such as China and Japan. Europeans use it as a substitute for olive oil. In Chinese cuisine, it is often used as a flavour enhancer. Sesame oil is also used as salad oils, as a solvent, skin softener, in margarine making, manufacturing of soaps, perfumes, paints, insecticides, cosmetics and in pharmaceuticals industry. It has almost zero amount of erucic acid with simultaneous high content of PUFA (Poly Unsaturated Fatty Acid; about 47% oleic and 39% linoleic acid) unlike mustard oil- the most popular edible oil (Jin et al., 2001). It has highest concentrations of Omega-6 fatty adds plus Omega-9. The oil also contains magnesium, copper, calcium, iron along with vitamin E, vitamin A, vitamin B1, vitamin B2 and vitamin B6. Sesame is rich in natural antioxidants or lignans, which are both oil and water-soluble. The oil is highly stable and rarely turns rancid in hot climates as well as the foods fried in sesame oil have a long shelf life because of an antioxidant or lignan called sesamol. Sesame oil is favoured for its antioxidant and antidepressant properties in the whole world (Anilakumar et al., 2010).

The oil has wide medical and pharmaceutical application. Sesame oil is mildly laxative, and demulcent. Sesame oil is an excellent emollient and is beneficial in alleviating tension and stress. The oil is used in treatment of backache, dry skin ailments, migraines, chronic constipation, hemorrhoids, dysentery, receding gums, tooth decay, hair loss, weak bones and knees, osteoporosis, dry cough and cold, mouth ulcers, blood in the urine and stiff joints. It is useful in toning kidney, liver, anemia, dizziness, poor vision, tinnitus etc. It is anti-aging and anti-cancerous for
humans. The oil has been found to inhibit the growth of malignant melanoma in vitro and the proliferation of human colon cancer cells (Smith and Salerno, 1992).

The oil is also known to maintain high density lipoprotein cholesterol (HDL) and lower low density lipoprotein cholesterol (LDL) (Sirato-Yasumoto et al., 2001). Sesame oil has a high PUFA content. There is a reduction in blood pressure due to the presence of PUFAs, vitamin E, and sesamin (a lignan) in sesame oil. Sesame oil contains a ton of antioxidants or lignans which can minimize heat oxidation. These antioxidative compounds of sesame are biologically active and provide a variety of health benefits upon ingestion. These compounds are being researched as potential industrial antioxidants and nutraceutical and pharmaceutical ingredients (Fukuda et al., 1986).

The biological functions of sesame lignans are being investigated in both animal studies and human clinical trials. Antioxidant content in sesame may be enhanced through traditional crop breeding. Sesamin binds to and activates a receptor in the body called Peroxisome Proliferator- Activator Receptor Alpha (PPARalpha). PPARalpha is highly expressed in muscle, liver, kidneys and heart and is involved in the regulation of lipid metabolism, specifically the transcription of genes involved in the a-oxidation of fatty adds and lipogenesis. Activation of PPARalpha increases gene expression of the fatty acid oxidation enzymes and decreases gene expression of lipogenic enzymes. In other words, sesamin increases the fat burning process and decreases the storage of fat in the body (Penalvo et al., 2006). Sesame lignans (Sesamin; Sesamolin; Sesamol) are also the sources of phytoestrogens (Wu et al., 2006) (Ashakumary et al., 1999). Apart from increasing the fat oxidation, sesamin has also been proven to decrease lipogenesis by decreasing lipogenic enzymes of liver. Sesamin has been shown to decrease the lipogenic gene expression of sterol regulatory element binding protein-1 (SREBP-1), acetyl-CoA carboxylase and fatty acid synthase, that means less fat is esterified in the liver and therefore less fat synthesis (Ide et al., 2003). Sesamin has bactericide and insecticide activities. Sesamolin also has insecticidal properties and is used as a synergist for pyrethrum insecticides (Morris, 2002).
2.1.3.2 Sesame Seeds

The seed is a perfect food as there are little possibilities of causing any allergy and known as best alternative source of food to the people with milk allergies. The white seeds are extremely rich and useful in all the cases of calcium deficiency. The red/brown seeds are exceptionally rich in iron and useful for anemic conditions. Sesame seeds contain a great number of useful nutrients. A tablespoon of dried sesame seeds (9 g) is 53 kcal and contains 1.6 g proteins, 1.2 g dietary fiber, 2 g carbohydrate, 88 mg calcium, 57 mg phosphorus, 42 mg potassium, 32 mg magnesium, as well as zinc, selenium, copper, iron, sodium, riboflavin, thiamin, niacin, phytosterols, and a variety of fatty and amino acids (Anilakumar et al., 2010). Sesame seeds are used in baking, preparation of sweet meals like sesame candy, cake, confectionery and other food industries like they are used for culinary purposes also and eaten in roasted, parched or raw form. De-hulled sesame seeds are used as a nourishing food and also as flavoring agent. It is also used in holy functions/ yagna of Hindu ceremonies (Shah, 2013).

The seeds also contain two unique substances, sesamin and sesamolin. During their refinement the two phenolic antioxidants, sesamol and sesaminol are formed. Both of these substances belong to lignans and have been shown to possess cholesterol-lowering effect in humans and simultaneously prevent high blood pressure along with increase vitamin E supplies in animals (Yamashita et al., 1992).

The total phytosterol content in sesame seeds is ~400 mg/100 g (Phillips et al., 2005). Just a quarter-cup of sesame seeds supplies 74.0% of the daily value (DV) for copper, 31.6% of the DV for magnesium and 35.1% of the DV for calcium. This rich assortment of minerals translates into many medicinal properties. Lecithin, present in sesame seeds is helpful in maintaining the cholesterol fluidity and thus prevents the formation of gall stones, works great as a memory enhancer and also improves the quality of milk in feeding mothers. Copper provides relief for rheumatoid arthritis. Magnesium supports vascular and respiratory health. Calcium helps prevent colon cancer, osteoporosis, migraine, and PMS. Zinc promotes bone health. Sesame seed
consumption increases plasma y-tocopherol and enhances vitamin E activity, which is reported to prevent cancer and heart diseases (Cooney et al., 2001).

### 2.1.3.3 Roots

Roots are traditionally used in treating cough, asthma and stomach pains (Shah, 2013).

### 2.1.3.4 Leaves

Fresh leaves of sesame are used in treatment of inflamed membranes of the mouth, dry cough and hair care. The leaves have been described as highly useful in nephropathy, ophthalmopathy and dermopathy. Crushed leaves are beneficial in the treatment of dandruff and prevent premature graying of hairs and promote hair growth (Anilakumar et al., 2010).

### 2.1.3.5 Whole plant

Ash of roots, stems, leaves, flowers and fruits is soaked in water and extract is then given to cure dyspepsia (Anilakumar et al., 2010).

### 2.1.3.6 Flower

Sesame flower extracts possess tumor inhibiting effect. The effect of alcohol extract from *S. indicum* flower on tumor growth in tumourigenic mouse and on weight of immune organs showed inhibiting effect on tumor growth and had not distinct effect on weight of thymus and spleen in mice (Chakraborthy et al., 2008).

### 2.1.4 Production

India is the largest producer of Sesame seeds in the world followed by China Nigeria, Myanmar and Tanzania. Major sesame seeds producing states in India are West Bengal, Gujarat, Tamil Nadu, Andhra Pradesh, Telangana, Madhya Pradesh, Maharashtra and Rajasthan. Major sesame importing countries are Japan, China, United States, Canada, The Netherlands, France and Turkey. In 2014, the production of sesame seeds in world 6235530 T while in India production was 811000 T (FAOSTAT, 2014). Major producers of sesame seeds are listed in Table 2.2.

<table>
<thead>
<tr>
<th>Countries</th>
<th>Production (T)</th>
</tr>
</thead>
</table>
In spite of an increasing demand for this highly valued food, the production of sesame seed has not increased. It is mainly because of manual harvesting procedure. Seeds are expelled by pod explosion and so harvesting has to be carried out at maturity before the pod opens. The small size of seed is also a hindrance for mechanical harvesting. To increase the production of sesame seeds efforts for developing high yielding, shatter-resistant varieties are being made.

2.2 MICRORNAs

2.2.1 Theory

MicroRNAs are a class of recently discovered non-coding endogenous small RNAs. Plant miRNAs are endogenously expressed small RNAs processed from a larger precursor RNA. They are generally conserved and mostly found in genomic regions distinct from annotated genes.

miRNA biogenesis requires multiple steps in order to form mature miRNAs from miRNA genes. First, a miRNA gene is transcribed to a primary miRNA (pri-miRNA), which is usually a long sequence of more than several hundred nucleotides. The simplified model of miRNA biogenesis is shown in figure 2.1. This step is controlled by Pol II enzymes. Second, the pri-miRNA is cleaved to a stem loop intermediate called miRNA precursor or pre-miRNA. This step is controlled by the Drosha RNase III endonuclease in animals or by Dicer-like 1 enzyme (DCL1) in plants (Tang et al., 2003). In animals, pre-miRNAs are then transported by exportin 5 from the nucleus into the cytoplasm, followed by formation of miRNA:miRNA* duplex and mature miRNAs by another RNase III-like enzyme called Dicer. In this step, however, plant miRNAs differ from animals. Plant miRNAs are cleaved into miRNA:miRNA* duplex possibly by dicerlike enzyme 1 (DCL1) in the nucleus rather
than in the cytoplasm, then the duplex is translocated into the cytoplasm by HASTY, the plant orthologue of exportin 5. In the cytoplasm, both plant and animal miRNAs are unwound into single strand mature miRNAs by helicase. Finally, the mature miRNAs enter a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC) where they regulate targeted gene expression. This suggests that miRNA biogenesis is complicated; several enzymes are required for processing long pri-miRNA to ~20–24 nt mature miRNAs (Bartel, 2004).

In the RISC complex, miRNAs bind to target messenger RNA (mRNA) and inhibit gene expression through perfect or near-perfect complementarity between the miRNA and the mRNA. This causes post-transcriptional gene silencing (PTGS) in plants. In plants, most target mRNAs only contain one single miRNA complementary site, and most corresponding miRNAs typically perfectly complement to these sites and cleave the target mRNAs. Unlike animal miRNA targets, the complementary sites in plants can exist anywhere along the target mRNA rather than at the 3-UTR. Recently, another mechanism was identified in plant miRNA regulation. This suggested that miRNAs may be involved in more complicated mechanisms to control gene expression in plants than in animals. Plant miRNAs regulate gene expression at the post-transcriptional level not only by repression of mRNA translation but also by direct cleavage of mRNAs (Zhang et al., 2006).
Computational prediction of miRNAs and their targets

Computational prediction of miRNAs in Arabidopsis thaliana was carried out by Adai et al., (2005). The prediction was initiated by producing a candidate miRNA-target data set using an algorithm called findMiRNA, which predicts potential miRNAs within candidate precursor sequences that have corresponding target sites.
within transcripts. Then these data were passed to RNAfold for structural analysis. Using these methods, 13 different miRNAs were predicted. Targets of miRNAs encompass a wide range of transcripts, including those for F-box factors, ubiquitin conjugases, Leucine-rich repeat proteins, and metabolic enzymes, and that regulation by miRNAs might be widespread in the genome.

Zhang et al., (2005) identified and characterized new plant microRNAs using EST analysis. They found 18,694 plant expressed sequence tags (EST) in the GenBank EST databases by comparing all previously known Arabidopsis miRNAs to plant EST databases with BLAST algorithms. After removing the EST sequences with more than 2 mismatches, a total of 812 EST contigs were identified. After predicting and scoring the RNA secondary structure of the 812 EST sequences, 338 new potential miRNAs were identified in 60 plant species.

Zhang et al., (2006a) identified microRNAs and their targets in maize. Mature miRNAs and their precursor sequences were downloaded from the miRNA database miRBase. Maize GSS, mRNA, cDNA, and EST sequences were obtained from NIH GenBank nucleotide databases. Mature miRNAs were BLASTed against the genomic survey sequence. The secondary structures were predicted using the web-based computational software MFOLD. The protein coding gene database was used for BLAST analysis against predicted miRNAs for their target prediction. 188 maize miRNAs belonging to 29 miRNA families. Of the 188 maize miRNA genes, 28 (15%) were found in at least one EST. A total of 115 potential targets were identified for 26 of the miRNA families. A majority of the targets were transcription factors which play important roles in maize development, including leaf, shoot, and root development.

Global computational analysis of rice (Oryza sativa) transcriptome was carried out by Archak and Nagaraju, (2007). The predictions (684 unique transcripts) showed that rice miRNAs mediate regulation of diverse functions including transcription (41%), catalysis (28%), binding (18%), and transporter activity (11%). Among the predicted targets, 61.7% hits were in coding regions and nearly 72% targets had a
solitary miRNA hit. The study predicted more than 70 novel targets of 34 miRNAs putatively regulating functions like stress-response, catalysis, and binding. It was observed that more than half (55%) of the targets were conserved between *O. sativa indica* and *O. sativa japonica*. Members of 31 miRNA families were found to possess conserved targets between rice and at least one of other grass family members. About 44% of the unique targets were common between two dissimilar miRNA prediction algorithms.

Dryanova *et al.*, (2008) identified miRNAs and the mRNA targets of miRNAs by sequence complementarity within a DNA sequence database for species of the *Triticeae*. miRNA precursors and targets were identified in 10 related species, though the great majority of them were in bread wheat (*Triticum aestivum*) and barly (*Hordeum vulgare*). Data screening identified 28 miRNA precursor sequences from 15 miRNA families that contained conserved mature miRNA sequence within predicted stem-loop structures. The identification of 337 target sequences among *Triticeae* genes provided further evidence of the existence of 26 miRNA families in the cereals.

Jin *et al.*, (2008) computationally identified and verified microRNA in wheat (*Triticum aestivum*) using the in-house program GenomicSVM, a prediction model for miRNAs. They discovered 79 miRNA candidates. After validation of the 9 selected miRNAs, 59 wheat ESTs were predicted as their putative targets.

A bioinformatics approach for ESTs and GSS-wide prediction of novel miRNAs in *Medicago truncatula* was carried out by Zhou *et al.*, (2008). A total of 38 potential miRNAs were detected following a range of filtering criteria. After removal of 12 overlapping miRNAs that have already been deposited and 26 miRNAs belonging to 15 families were found to be new. Using the newly identified miRNA sequences and *M. truncatula* mRNA database, BLAST search was performed and 16 potential targets of miRNAs were detected. Many miRNA-targeted genes were predicted to encode transcription factors that regulate plant development, signaling, and metabolism.
In silico microRNA identification of paprika (Capsicum annuum) ESTs was carried out by Manila et al., (2009). ESTs of Capsicum annuum were obtained from dbEST at NCBI. The processed ESTs were submitted to miRNAfinder for predicting the miRNAs present in paprika. A tool named “miRseeK” was created using the standalone BLAST program. A database containing all plant miRNAs was created and maintained as the reference database. All available plant miRNAs were obtained from the miRBase. The structures were predicted using a loop-based energy model and the dynamic programming algorithm. The targets of the obtained miRNAs were predicted using the tool miRU2. 13 miRNAs with significant similarity with the plant miRNAs were predicted. The targets of the predicted miRNAs suggested that most miRNAs play an important role in plant development, signal transduction, protein degradation and in response to environmental stress.

A computational-based update on microRNAs and their targets in barley was provided by Colaiacovo et al., (2010). They had full collection of non-redundant mature miRNA sequences and used in a BLASTn search against dbEST, accepting a number of mismatch lower than 4. Matching ESTs have then been related to Unigene clusters and the corresponding annotations were recorded. In order to predict the secondary structure of the precursors, the software mfold 3.2 was used. Polymorphisms in target genes have been searched through a comparison of the ESTs belonging to the same Unigene cluster. As a result, 156 microRNA mature sequences belonging to 50 miRNA families have been found. Many previously known and several putatively new miRNA/target pairs have been identified. Candidate microRNA coding genes have been reported and genetic variation (SNPs/indels) both in functional regions of putative miRNAs (mature sequence) and at miRNA target sites has been found.

Lu and Yang (2010) carried out computational identification of novel microRNAs and their targets in Vigna unguiculata. Previous known plant miRNAs were BLASTed against the EST and GSS databases of V. unguiculata, and according to a series of filtering criteria, a total of 47 miRNAs belonging to 13 miRNA families
were identified, and 30 potential target genes of them were subsequently predicted, most of which seemed to encode transcription factors or enzymes participating in regulation of development, growth, metabolism, and other physiological processes.

Computational identification miRNAs and their target genes in tea was carried out by two different groups. The database of tea (*Camellia sinensis*) ESTs was BLASTed to previously known plant miRNAs for searching potential miRNAs. Prabu and Mandal, (2010) identified four candidate miRNAs from four families for the first time in tea. Using the newly identified miRNA sequences, a total of 30 potential target genes were identified for 11 miRNA families; 6 of these predicted target genes encode transcription factors (20%), 16 target genes appear to play roles in diverse physiological processes (53%) and 8 target genes have hypothetical or unknown functions (27%). Das and Mondal, (2010) identified 13 conserved miRNAs belonging to 9 miRNA families. A total of 37 potential target genes in *Arabidopsis* were identified subsequently for 7 miRNA families based on their sequence complementarity which encode transcription factors (8%), enzymes (30%) and transporters (14%) as well as other proteins involved in physiological and metabolic processes (48%).

In *Solanaceae* plants, microRNAs and their putative target genes were identified and characterized by Kim *et al.,* (2011). *Solanaceae* miRNAs and their target genes were identified by analyzing EST data and mRNA from five different *Solanaceae* species. Previously identified miRNAs and their pre-miRNAs in plants were downloaded from the miRNA registry database. ESTs and mRNA sequences were obtained from GenBank nucleotide databases. Prediction of potential miRNAs was carried out using BLAST homolog search. Secondary structure predictions of candidate sequences were determined using the Zucker folding algorithm with mFold. For target prediction, the identified *Solanaceae* miRNAs were used to search for their potential target genes by comparing their sequences against the protein coding nucleotide database from GeneBank with BLASTN. The results revealed the presence of 11 miRNAs in pepper, 22 in potato, 12 in tomato, 53 in two different tobacco
Review of Literature

species. The identified Solanaceae miRNAs and their target genes were deposited in the SolmiRNA database.

Sahu et al., (2011) computationally identified miRNAs in medicinal plant Senecio vulgaris. miRNAs from known plants were downloaded from the miRNA database miRBase. ESTs and GSS sequences of S vulgaris were downloaded from NCBI. BLASTN of Bioedit, a sequence alignment editor tool, was used to reveal homology between ESTs/GSS and miRNA. The RNA structure and free energy were calculated by RNA structure software in SwissProt database. Using these methods, 10 miRNAs were identified from 1956 EST and 115 GSS sequences.

Identification, characterization and expression analysis of MicroRNAs and their targets in the potato (Solanum tuberosum) was carried out by Xie et al., (2011). BLAST was used for the first screening of miRNA homolog sequences from ESTs and GSSs and also for removing repeated sequences as well as protein-coding genes. The alignment tool WATER, from bioinformatics software tool package EMBOSS was employed to identify potential conserved miRNAs and their targets and was accessed from the public EMBOSS website. Pre-miRNA hairpin structure folding was accomplished by the software RNAfold. Newly developed target prediction computational tool, called Target-align was used to align potential potato miRNAs with ESTs. Using this approach, 202 potential potato miRNAs were identified, which belong to 78 families. A total of 1094 miRNA targets were predicted and some of them encode transcription factors as well as genes that function in stress response, signal transduction, and a variety of other metabolic processes.

In silico identification of microRNAs and their targets in fiber and oil producing plant flax (Linum usitatissimum L.) was carried out by Barozai, (2012). The known plant miRNAs from the microRNA Registry were downloaded and homology search was performed using Blast algorithm from the publicly available Flax ESTs database at NCBI. The stem-loop structures were generated by applying RNA folding algorithm, MFOLD. For prediction of miRNA targets, The NCBI Blastn program and RNA-hybrid-a miRNA target prediction tool were applied. Flax mature miRNA sequences were subjected as queries. The mRNA sequences having 70%
query coverage were selected and subjected to RNA-hybrid for the confirmation. By these methods, total 26 novel miRNAs from 19 families were identified. All 26 miRNA precursor sequences showed thermodynamically stable structures and the mature miRNAs are appeared in the stem of the stem loop structure. These novel miRNAs in Flax targets transcription factors, retrotransposon, rust resistance genes, cell signaling proteins, maturase K, cellulose synthase, male sterility-related protein and many hypothetical proteins.

In-silico and in-vivo analyses of EST databases was carried out to unveil miRNAs from *Carthamus tinctorius* and *Cynara cardunculus* by Catalano *et al.*, (2012). Two complete EST datasets of artichoke and safflower were obtained. RNAHybrid analysis was carried out for searching the putative mature miRNAs and miRNA* strand. RNAfold was employed for hairpin structure prediction. The RNAhybrid program was used to identify possible binding sites present in the ESTs, following specific base pairing rules. As a result of in silico analysis, 17 potential miRNAs were identified from both the plants.

Dong *et al.*, (2012) carried out computational identification of miRNAs in Strawberry. All the known plant miRNAs were obtained from miRBase. The prediction was performed using the EST databases from NCBI. Known miRNA from the referential set was selected and its seed region (positions 2–8) aligned to the ESTs on both strands. Then, the known miRNA was taken as a pattern and PatScan was used to filter all the raw miRNAs. The secondary structures of these sequences were predicted using miRCat software. By adopting these methods, 11 potential miRNAs belonging to 5 miRNA families were identified.

In silico prediction of microRNAs in plant mitochondria was performed by Kamarajan *et al.*, (2012). The available miRNA sequences were collected from miRBase. Mitochondrial coding sequences (CDS) of 47 plants were available in NCBI’s Organelle Genome Database. The publicly available Standalone BLAST was used for the complementarity search to identify all possible miRNA targets, depending upon the sequence complementarity. The RNAhybrid tool was used to calculate the Minimum Free Energy (MFE), and to deduce the secondary structure of
miRNA:mRNA hybrid. The mitochondrial gene targets identified for miRNAs are located both in mitochondrial and nuclear compartments. This observation suggested the early origin of miRNAs. Besides, most of the targets identified can have copies in two compartments and suggest the possibility of miRNA mediated regulation.

Patanun et al., (2012) computationally identified microRNAs and their targets in cassava. All previously known plant miRNA sequences were downloaded from the miRBase and the cassava genome database was provided by Phytozome. Secondary structures of pre-miRNAs were predicted by MFOLD 3.1. The miRNA target genes were predicted by using two databases—Phytozome and the web tool psRNATarget using the Manihot esculenta (cassava) DFCI Gene index (MAESGI) release 1 as the sequence library for target search. 169 potential cassava miRNAs belonging to 34 miRNA families were identified as results. A total of 15 miRNA clusters involving 7 miRNA families, and 12 pairs of sense and antisense strand cassava miRNAs belonging to six different miRNA families were discovered. Prediction of potential miRNA target genes revealed their functions involved in various important plant biological processes.

Computational identification and analysis of novel sugarcane microRNAs was carried out by Thiebaut et al., (2012). A bioinformatics search was carried out to discover novel miRNAs that can be regulated in sugarcane plants submitted to drought and salt stresses, and under pathogen infection. By means of the presence of miRNA precursors in the related sorghum genome, 623 candidates of new mature miRNAs in sugarcane were identified. Out of these, 44 were classified as high confidence miRNAs. The biological function of the new miRNAs candidates was assessed by analyzing their putative targets. The set of bona fide sugarcane miRNA includes those likely targeting serine/threonine kinases, MYB and zinc finger proteins. Additionally, a MADS-box transcription factor and an RPP2B protein, which act in development and disease resistant processes, could be regulated by cleavage (21-nt-species) and DNA methylation (24-nt-species), respectively.

Dehury et al., (2013) identified and characterized conserved miRNAs and their target genes in sweet potato ESTs using in silico methods. Known plant
miRNAs from miRBase and EST sequences of sweet potato from NCBI were downloaded. The alignment tool BLAST was used to identify the potentially conserved miRNA. Mfold was used for secondary structure prediction. The target genes for the putative miRNAs of sweet potato were identified by using psRNATarget server. Additionally, the UEA sRNA toolkit was employed for prediction of the sweet potato miRNA target genes by incorporating Arabidopsis gene index database and ESTs of sweet potato. Using this comparative genomics-based approach, 8 potential miRNA candidates were identified. 42 target genes were predicted. Most of the newly identified miRNAs target transcription factors as well as genes involved in plant growth and development, signal transduction, metabolism, defense, and stress response.

In silico prediction of miRNAs in Curcuma longa was carried out by Rameshwari et al., (2013). A total of 12,593 ESTs were downloaded from dbEST database and processed and trimmed through SeqClean. This contig database was used to find the putative miRNAs by performing a local BLAST with the miRNAs of Arabidopsis thaliana retrieved from miRBase. The targets were scanned by hybridizing screened ESTs with the UTRs of human using miRanda software. Finally, 12 putative miRNAs were found to hybridize with the various targets of signal transduction and apoptosis that may play significant role in preventing diseases like diabetes mellitus type 2, cardiovascular disorders, Alzheimer, cancer, thalassemia by gene silencing.

Rebijith et al., (2013) performed in silico mining of novel microRNAs from coffee using ESTs. A total of 174,275 ESTs, expressed at various stages of development, were downloaded from the NCBI database. All reported plant miRNAs, belonging to different plant families were retrieved from miRBase. Redundancy among the miRNAs and ESTs was excluded manually by sequence identity. The premiRNA sequences were submitted to Mfold to predict their secondary structures. The newly-predicted miRNAs were uploaded into psRNATarget for target prediction. Target predictions were carried out using C. canephora unigene sequences. These predictions resulted in identification of 18 novel miRNAs and 41 potential mRNA
targets. Functional characterisation of the 18 newly-identified miRNAs revealed that the majority were involved in transcriptional regulation and signal transduction pathways.

Vishwakarma and Jadeja, (2013) identified miRNA encoded by *Jatropha curcas* from EST and GSS. miRNA data set was retrieved from miRBase database. Jatropha EST and GSS were retrieved from NCBI. Local similarity searches were performed by Blast program downloaded from the NCBI. miRNA precursor folding was performed by MFOLD Web server. The predicted *J. curcas* miRNAs were used as query against the *Arabidopsis thaliana* by using psRNATarget. 24 new potential miRNAs were identified using this approach. 78 potential target genes were identified for 3 miRNA families. Most of the miRNA targeted genes were predicted to encode transcription factors that regulate cell growth and development, signaling, and metabolism.

Ye et al., (2013) carried out computational identification of microRNAs and their targets in apple (*Malus domestica*). Based on the conservation of miRNAs in many plant species and the currently available genome sequence, 154 individual conserved miRNAs have been predicted in apple and found that these miRNAs belong to 26 families. 424 miRNA:target pairs between 150 miRNAs and 235mRNAs have been predicted in apple, and these miRNA targets are involved in development, response to biotic and abiotic stresses, and other cellular processes.

Bioinformatics prediction of miRNAs in the Peach (*Prunus persica*) genome was carried out by Zhang et al., (2013). All known plant miRNAs were downloaded from the miRNA registry database. The peach genome sequence and peach predicted CDS were downloaded from the Peach Genome. The Patscan program was used to identify matches to known miRNAs in the target genomic sequence. The miRcheck algorithm, was used to evaluate the local secondary structure of the miRNA to identify homology miRNA candidates. All the miRNA targets were predicted through the psRNATarget web server. 262 potential miRNAs belonging to 70 miRNA families were predicted.
Wu et al., (2014), carried out in silico identification and characterization of conserved plant microRNAs in barley. The locations of miRNAs on the barley genome assembly was investigated and annotation of the functions of their predicted target genes were provided. The results were compared to previous miRNA studies and publicly available barley small RNA libraries. The result showed that 116 mature miRNA sequences from 60 miRNA families have been found in the barley genome assembly by their miRNA identification pipeline. Closely related cereal crops contain most of the miRNA families that were found in the barley genome assembly. Most miRNA genes were located in intergenic regions or introns. Among the 116 mature miRNAs predicted, 80 have been reported in previous barley miRNA studies. Eight mature miRNA sequences have never reported in the previous barley miRNA studies.

Cardoso et al., (2015) identified miRNAs by genome-wide methods and computationally characterized them and their targets in Phaseolus vulgaris L. The mature miRNA sequences and precursor miRNA sequences were retrieved and downloaded from miRBAse. The latest P. vulgaris genome data and information were accessed and downloaded from Phytozome. The putative proteins involved in the miRNA-processing pathway were identified and selected by mining P. vulgaris sequences in the NCBI database using BLAST. Prediction of the secondary structure of pre-miRNA, and estimation of diversity, MFE, frequency ensemble and MFE secondary structures were performed using RNAfold. Analyses of protein families, domains and active sites were performed using the PFAM and Conserved Domains Database. Targets of conserved miRNAs were predicted using the Webtool psRNATarget. The ontology of miRNA target genes was categorized based on their Gene Ontology (GO) terms. 221 mature miRNAs and 136 precursor miRNAs distributed across 52 different miRNA families were predicted. 483 miRNA targets were identified, including many which corroborate results from other species.

Chai et al., (2015) bioinformatically identified microRNAs and their targets in banana. Previously known plant miRNAs were BLASTed against the Expressed Sequence Tag (EST) and Genomic Survey Sequence (GSS). A total of 32 potential miRNAs belonging to 13 miRNAs families were detected using a range of filtering
Review of Literature

criteria. 244 miRNA-target pairs were subsequently predicted, most of which encode transcription factors or enzymes that participate in the regulation of development, growth, metabolism, and other physiological processes.

*Cannabis sativa* L. is an annual herb and economically important as a source of fiber, oil, food and for its medicinal and intoxicating properties. *In silico* analysis of the publically available Transcript Sequence Assemblies (TSA) and Expressed Sequence Tags (ESTs) of *C. sativa* was carried out by Das et al., (2015). Homology search of reference miRNAs against the local nucleotide sequence database of *C. sativa* was carried out using Standalone BLAST+ 2.2.29 program. The fold-back secondary structures of pre-miRNAs were predicted using Mfold. For finding the conservation of miRNA of *C. sativa* with other plant species, pre-miRNA sequences of miR172 family from 9 different species, were downloaded from miRBase and aligned by using ClustalX. The potential target genes of the identified miRNAs were predicted using the plant miRNA target finder program psRNATarget. The identified mature miRNAs were used as query for finding the complementary sequences in *A. thaliana* unigenes. As a result, total of 18 conserved miRNAs belonging to 9 independent families were identified.

Computational identification, target prediction, and validation of conserved miRNAs in Insulin plant *Costus pictus* was carried out by Das et al., (2015a). All known plant miRNAs were downloaded from miRBase. TSA sequences of *C. pictus* were downloaded from the TSA database of GenBank, NCBI. The reference miRNAs were BLASTed against the non-redundant set of *C. pictus* TSA sequences using the BLAST+ program. The secondary structures of pre-miRNA were predicted using the online program Mfold. The target genes for the predicted miRNAs were recognized using the online program, Plant miRNA Target Finder. By these methods, 42 miRNAs of 13 different families were identified. A total of 109 potential target genes of the identified miRNAs were predicted. They encode transcription factors, enzymes, and various functional proteins involved in the regulation of several metabolic pathways.
Computational prediction and experimental validation of a novel miRNA in a halophyte, *Suaeda maritima* was carried out by Gharat and Shaw, (2015). To identify potential salt-responsive miRNAs in a halophyte, all plant miRNAs were downloaded from the miRBase database. EST sequences of *S. salsa* and *S. glauca* were retrieved from the NCBI database. All non-redundant plant miRNAs were subjected to a BLASTn search. The mfold software with default parameters was used to predict hairpin structure. Target prediction was carried out using psRNATarget tools considering the EST sequences of the halophytes *S. salsa* and *S. glauca* that were available at the NCBI database with default parameters. Computational analysis predicted three miRNA sequences. One predicted miRNA sma-miR1867 was predicted to target ferredoxin-thioredoxin reductase (FTR), cell division control protein 6 (CDC6), and ubiquitin-protein ligase (UPL). It was concluded that it could be an essential component of salt resistance in halophytes.

Computational prediction and characterization of miRNA from coconut leaf transcriptome was carried out by Naganeeswaran *et al.*, (2015). A database of coconut ESTs and homology searches with non-redundant miRNA reference dataset using stand-alone BLASTN program were created for identifying coconut miRNA candidates. BLASTX) was performed using the selected sequences to remove protein coding sequences. RNA secondary structure was predicted using MFOLD program. Target prediction of the identified miRNA was done using psRNATarget tool by selecting *Arabidopsis thaliana* as reference. As a result, 16 miRNAs, which belongs to 11 miRNA families, and also targets for seven potential miRNAs in coconut leaf transcriptome, majority of these seem to encode transcription factors.

In *Phaseolus vulgaris* miRNAs and their targets were computationally predicted by Nithin *et al.*, (2015). The Viridiplantae pre-miRs were downloaded from the miRBAse. The small RNAs belonging to different families were downloaded from Rfam. Expressed Sequence Tags (ESTs) and Genomic Survey Sequences (GSSs) of *P. vulgaris* were downloaded from the GenBank. BLAST search was performed using the non-redundant dataset of Viridiplantae pre-miRs as query and non-redundant dataset of EST and GSS sequences of *P. vulgaris* as subject. The
structures with the minimum folding energy was generated using RNAfold. The targets for mature miRNAs were predicted using psRNATarget server by submitting the mature miRNAs as query and the EST sequences of *P. vulgaris* as subject. 208 mature miRNAs belonging to 118 families were identified. Out of which 201 miRNAs were novel. A total of 1305 target sequences were identified for 130 predicted miRNAs.

Santhi and Sheeja, (2015) contributed to computational prediction of miRNA function and activity in turmeric. Eight conserver miRNAs representing two different families were identified by *in silico* analysis of ESTs. The prediction was based on conservation of sequences, the stem loop secondary hairpin-loop structure and a series of filtering criteria. The targets were identified using psRNATargets, MicroPC and Target align with default parameters. Most of the targets appeared conserved and classified as proteins involved in stress response, development and metabolism.

Computational identification of miRNAs was done in 6 transcriptomes of a medicinal herb, *Picrorhiza kurroa* by Vashisht *et al.*, (2015). They retrieved all available plant miRNA entries from miRBase and used as backend datasets to computationally identify conserved miRNAs in transcriptome data sets. Total 18 conserved miRNAs were detected in *P. kurroa* followed by target prediction and functional annotation which suggested their possible role in controlling various biological processes.

Pan *et al.*, (2016) carried out bioinformatic identification and expression analysis of *Nelumbo nucifera* microRNA and their targets. miRNAs were downloaded from the miRBase database. The assembly genome contig sequences of the *N. nucifera* from both NCBI database and RNA-seq data were used for miRNA mining. The genomic and RNA-seq contigs were aligned with known mature plant miRNAs using a BLASTn algorithm. The secondary structures were predicted using MFOLD software. psRNATarget was used to predict the targets of identified miRNAs. 106 conserved miRNAs, belonging to 40 families were found and 456 of their miRNA targets were annotated by this approach.
Computational identification and functional annotation of microRNAs and their targets from ESTs and GSSs of coffee was carried out by Devi et al., (2016). A total of previously identified plant mature miRNA sequences from Viridiplantae group were retrieved from the miRNA database miRBase. Publicly available coffee EST and GSS were downloaded from NCBI. For homology searches BLAST-2.2.27+ program was used. Secondary structure analysis of miRNA precursors was performed by online version of MFOLD. psRNATarget: a plant small RNA Target Analysis Server was used for predicting the targets of the newly identified miRNA. A total of 20 new potential miRNAs belonging to 13 different miRNA families were identified through homology search. The psRNATarget server predicted 142 potential target genes for 17 miRNAs and their probable functions were illustrated. Most of the predicted target genes encoded transcription factors and genes involved in plant growth and development, signal transduction, metabolism, defense and stress responses.

Diler et al., (2016) identified miRNAs from peach using various bioinformatics tools. Previously generated peach transcriptome libraries were utilized. Databases containing pre-miRNAs and mature miRNAs were acquired from miRBase. Blast-search was performed with default parameters. ViennaRNA Package with default parameters was used to estimate the secondary structures of the all the filtered transcripts. Target transcripts were identified via the web-based psRNATarget server. Using these tools, 24 known and 3 novel potential miRNAs were characterized. The miRNA-target transcript analyses indicated that transport, plant cuticle development, intracellular part, and stress response are regulated by miRNAs. It also suggested that some of the microRNAs might play critical regulatory roles in hyper-hydricity regarding miRNA-based response to stress.

In silico microRNA identification from Stevia rebaudiana transcriptome assembly was carried out by Mehta et al., (2016). A total of 1,418,58 unigenes from Stevia transcriptome data were used for homology search against known plant miRNA database miRBase. The functionally annotated unigenes were excluded from the studies. Total 381 non-protein coding unigenes were considered for candidates of
miRNA precursor in *Stevia*. One potential miRNA from miR168 family with secondary structure was identified through the sequel of stringent filtering criteria. The target prediction of novel miRNA was carried out for using psRNATarget program based on their sequence complementarities. A total of 31 potential gene targets were predicted for identified novel miRNA, which playing crucial role in various biological processes like development of plant, DNA repair, splicing, post-translational gene silencing, plant defense response, cell growth and proliferation.

Paola *et al.*, (2016) identified conserved and novel miRNAs in wheat. Unique reads of small RNAs were subjected to similarity search using all plant mature miRNAs from miRBase as reference database. For this, BLASTN was used. All identified sequences were subjected to UNAfold to predict their secondary structure. Novel and conserved durum wheat miRNAs were used as query in the online tool psRNATarget for their target prediction. Blast2GO was used to obtain GO annotation of the wheat unigenes. As a result, total of 167 conserved and 98 potential novel miRNAs were identified in the two libraries and three novel miRNAs were found to be derived from ribosomal RNA.

*In silico* identification of candidate microRNAs and their targets in potato somatic hybrid was carried out for late blight resistance by Singh *et al.*, (2016). The study was performed with the microarray data of known genes obtained from gene expression profiles for late blight resistance. Plant miRNAs were obtained from the miRBase database. The tool C-mii software was used to identify miRNAs. To identify the potential conserved miRNAs in potato somatic hybrid a BLASTn search was performed. The software MFOLD was used to analyze the secondary structure of RNAs, whereas RNAfold tool was used to improve the efficiency of prediction of miRNAs. The potential targets of the identified conserved miRNAs were detected using the web-server psRNATarget. The phylogenetic analysis of the miRNAs was carried out using the software program Molecular Evolutionary Genetics Analysis 6 (MEGA6). Using these computational tools, eight miRNAs families to the nine potato genes were identified. These novel miRNAs were identified for pathogen-induced late blight resistance mechanism in interspecific potato somatic hybrid. Majority of the
predicted target genes of these miRNAs are involved in different biological functions, including disease resistance proteins and transcription factors families.

Vivek and Moossa, (2016) identified novel micro RNAs and their targets in *Cocos nucifera*, coconut by bioinformatics approach. miRNAs belonging to *Viridiplantae* were downloaded from miRBase. Publically available ESTs were downloaded from EST database, NCBI. The alignment tool NCBI Blast+ was used for conserved miRNA prediction. The secondary structures were performed through online version of MFOLD. The putative target genes for the predicted miRNA of coconut were identified using plant small RNA psRNATarget Web Server. These methods lead to the identification of one novel miRNA from mir2673 family. The gene targets predicted miRNA shows crucial role in regulation of auxin signaling pathway, transcription factors, abiotic stress, retrotransposons etc.

Xie *et al.*, (2016) identified and characterized microRNAs from *Ribes nigrum* ESTs. A total of 8,496 known plant miRNAs were downloaded from the publicly available miRBase. The unique mature miRNAs were subjected to a BLAST search against the publicly available EST database of *Ribes* genus. The secondary structures were assessed using RNA fold program. Phylogenetic analysis was performed using MEGA 5.2 software. The putative targets of miRNAs were predicted using the psRNATarget program against A. *thaliana* DFCI Gene Index. As a result, 2 miRNAs were firstly identified in *R. nigrum*.

In silico identification of miRNAs and their target genes and analysis of gene co-expression network in saffron (*Crocus sativus L.*) by Zinati *et al.*, (2016). Publicly available EST library of saffron stigma was retrieved from NCBI. Mature miRNAs were downloaded from miRBase. The putative miRNAs were identified using C-mii software. The mature miRNA sequences were queried against unigenes obtained from analysis of EST library of saffron stigma and unigenes of *Arabidopsis* to search for putative target mRNAs. Gene ontology (GO) annotation of identified target genes was performed using the DAVID Bioinformatics Resources and KEGG pathway database. This EST analysis led to the identification of two putative miRNAs (miR414 and
miR837-5p) along with the corresponding stem-looped precursors. These two play roles in metabolic pathways.

Ling et al., (2017) identified microRNAs by high-throughput sequencing analysis and carried out bioinformatics analysis of sRNAs in *Paris polyphylla*, an important traditional Chinese medicine. The conserved miRNAs were identified and annotated by BLASTn search against miRBase without any mismatches. The novel mature miRNAs were identified using the Mireap program. Then their precursor sequences were obtained by perfect mapping into transcript sequence data to predict the hairpin-like secondary structure. Target genes of both conserved and novel miRNAs were predicted by using the online psRNATarget server. In this study, 263 conserved miRNAs and 768 novel miRNAs in seeds and seed coats were identified by high-throughput sequencing technologies. The targets of the conserved and novel miRNAs were predicted and functionally annotated, suggesting that these miRNAs were mainly involved in the cell, metabolism and genetic information processing by direct and indirect regulation patterns in dormant seeds.

Bibi et al., (2017) provided bioinformatics profiling and characterization of potential microRNAs and their targets in the genus *Coffea*. All known plant miRNAs were downloaded from miRBAse database and used as a reference miRNA set for identifying conserved miRNAs in the genus *Coffea*. The genus *Coffea* ESTs and protein databases were obtained from the NCBI. The similarity search tool BLASTn and BLASTx programs were employed to identify potential conserved miRNAs. Mfold was obtained for the prediction of secondary structures. Conservation and phylogenetic analyses were done by the publicly available WebLogo, a sequence logo generator and ClustalW to generate a cladogram tree. For data mining in the identification of miRNAs’ targets, psRNATarget-a plant small RNA target analysis server and RNAhybrid-a miRNA target prediction tool were used. Using these a well-defined comparative genome-based computational approach, 51 potential Coffee miRNAs, belonging to 51 families were identified. These identified miRNAs potentially target 150 protein-coding genes, which can act as transcription factors and take part in multiple biological and metabolic processes, hypothetical proteins, signal
transduction, transporters, growth and development, stress-related processes, structural constituents, and disease-related processes.

In-silico based identification and functional analyses of miRNAs and their targets in Cowpea *Vigna unguiculata* was carried out by Gul *et al.*, (2017). As reference miRNAs, known plant miRNA sequences were downloaded from the microRNA registry database and then subjected to BLAST for alignment against ESTs of cowpea from NCBI, using BLASTn program. MFOLD was used to predict the secondary structures. The newly identified cowpea miRNAs were subjected to psRNATarget to predict the potential targets. A total of 46 new miRNAs belonging to 45 families were identified by these methods. A set of 138 protein targets were also identified for these newly identified 46 cowpea miRNAs. These targets have significant role in various biological processes, like metabolism, transcription regulation as transcription factor, cell transport, signal transduction, growth & development and structural proteins.

Avsar and Aliabadi, (2017) identified microRNA elements from genomic data of European hazelnut (*Corylus avellana* L.) and its close relatives. The available mature miRNA sequences were downloaded from miRBase. They were used as a query in homology-based in silico miRNA identification. *C. avellana* genome. BLAST+ stand-alone toolkit was used for detection of database sequences with homology. UNAFold was to include all possible stem-loops generated for each miRNA query to obtain secondary structures of predicted miRNAs. By using online web tool, psRNATarget, mature miRNA sequences were blasted to hazelnut transcripts. The results file was downloaded and then used as an input file for Blast2Go software to analyze gene ontologies. Using these methods, 57 putative miRNAs were predicted and identified through *C. avellana* genomic data.

Hajieghrari *et al.*, (2017) computationally identified microRNAs and their transcript target(s) in field mustard, *Brassica rapa*. Plant mature miRNA sequences were searched in non-protein coding ESTs registered in NCBI EST database. Zuker RNA folding algorithm was used to generate the secondary structures of the ESTs. Potential sequences were candidate as miRNA genes and characterized evolutionarily
only and if only they fit some described criteria. Also, the web tool psRNATarget was applied to predict candidate \textit{B. rapa} miRNA targets. 10 novel miRNAs belonging to 6 miRNA families were identified using EST-based homology analysis. Several potential targets with known/unknown functions for these novel miRNAs were identified. The target genes mainly encode transcription factors, enzymes, DNA binding proteins, disease resistance proteins, carrier proteins and other biological processes.

Computational identification of miRNAs and their targets from Niger \textit{Guizotia abyssinica} was carried out by Prathiba \textit{et al.}, (2017). EST sequences of Niger (were downloaded from NCBI and plant miRNAs from miRBase were used as reference for predicting the conserved miRNAs. The secondary structures of putative pre-miRNAs were predicted by Mfold. The \textit{Brassica rapa} transcripts, downloaded from phytozome was used to determine the potential target mRNA candidates for miRNAs using psRNATarget. Functional annotations of predicted targets were analysed using BGI WEGO platform. The KEGG (Kyoto Encyclopedia of Genes and Genomes) database was used to identify the significantly enriched pathways of miRNA target genes. As results, two potent miRNAs targeting 49 genes were identified. The newly identified miRNAs belongs to miR2592 and miR396 family. Targets recognized were F-box proteins, leucine zipper, DEAD box RNA helicase, disease resistant proteins. Gene annotations revealed miRNAs were involved in growth and development.

Subburaj \textit{et al.}, (2017) identified γ-radiation-responsive microRNAs and their target Genes in \textit{Tradescantia}, an ornamental plant. Mature miRNA sequences of different plants species were collected from miRBase and PMRD. A total of 77,236 unique ESTs of \textit{Tradescantia} were obtained from a flower transcriptome available at NABIC. Alignment tool bowtie1 was used to blast search of non-repeat miRNAs against \textit{Tradescantia} EST database. Hairpin structures of pre-miRNA sequences were generated using Mfold web server. psRNATarget web server was used to identify potential targets. Gene Ontology (GO) analysis to determine corresponding target genes GO terms such as cellular components, biological processes, and molecular functions. 37 miRNAs belonged to 36 different miRNA families were identified using
these methods. Target prediction revealed that 37 miRNAs targeted 149 genes involved in stress tolerance regulation, light response, redox systems, signaling pathways, DNA repair, and transcription factors.

miRNA and their expressed targets from *Carica papaya* were computationally identified by Jha *et al.*, (2017). The identification of Known miRNA sequences was searched through against the miRBase and sequences with less than 2 mismatches with known miRNAs in miRBase was considered. 1724 known miRNA were depicted. Computational algorithms predicted miRNA targets on the basis of the presumed mode of miRNA–mRNA interactions and also depend on the conservation of their binding sites. The mirDeep prediction software is used to predict new miRNAs where 11 novel miRNAs are estimated.

Akula and More, (2017) identified and characterized miRNAs and their targets in Pigeon pea (*Cajanus cajan* L.) EST by *in silico* methods. The processed unique miRNA reference set was used for homology search in pigeon pea contigs and singletons using BLASTn option in BioEdit software. The prediction of precursor miRNA was performed using Zuker folding algorithm in MFOLD software. The newly identified miRNA sequences were submitted to psRNATarget tool for target prediction by specifying search on *Glycine max* (soybean) unigene. The genome annotation of the identified targets was done by using QickGo. The results upon stringent selection found five conserved miRNAs belonging to five different families. The target analysis through psRNATarget server found 27 mRNA targets which code for protein that are important in growth, metabolism or in biotic and abiotic stress management.

### 2.3 REAL-TIME PCR

#### 2.3.1 Theory

There are several methodological approaches to enrich, label, amplify and profile mature miRNAs are available at present, including northern blotting with radio-labelled probes, oligonucleotide microarrays, qPCR-based detection of mature miRNAs, single molecule detection in liquid phase, oligonucleotide microarrays, *in*
situ hybridization and by using massively parallel sequencing (Benes and Castoldi, 2010).

qPCR is used to validate observations determined by genome wide profiling of miRNA expression. The successful outcome of qPCR analysis depends upon a number of interconnected steps that require individual optimization. To perform qPCR that provides meaningful and reproducible results, several parameters such as RNA extraction, RNA integrity control, cDNA synthesis, primer design, amplicon detection, and data normalization must be taken into account.

There are several steps in qPCR of miRNAs.

i. cDNA synthesis
ii. miRNA-specific primer design
iii. Detection of qPCR products
iv. Selection of reference genes and data normalization

The first step in qPCR of miRNAs is the accurate and complete conversion of RNA into complementary DNA (cDNA) by reverse transcription. To date, two different approaches to reverse transcribe miRNAs have been utilized. In the first approach, miRNAs are reverse transcribed individually by using miRNAs-specific reverse transcription primers. In the second approach, miRNAs are first tailed with a common sequence and then reverse transcribed by using a universal primer. Reverse transcription methodologies to generate cDNA are depicted in figure 2.3. (Chen et al., 2005).

The specificity and sensitivity of qPCR assays are dependent upon primer design. The design of miRNA Specific Primers is linked to both the type of cDNA synthesized and to the method used to detect the amplicon. Detection of amplicon using fluorescent molecules are depicted in figure 2.4. (Kuimelis et al., 1997).

The principle of qPCR is based on the detection, in real-time, of a fluorescent reporter molecule whose signal intensity correlates with amount of DNA present in each cycle of amplification. A number of fluorescent technologies exist for
performing qPCR SYBR Green I, TaqMan probes, Molecular Beacons, Light Upon eXtension (LUX) and HybProbes (LightCycler). However, to date only two of these technologies have found application to miRNA detection; SYBR Green I and TaqMan probes.

The estimation of the amount of Reference Genes (RG) across samples is useful to correct for sample-to-sample variation. Ideally, the identification of RG-miRNAs, analogous to Glyceraldehyde-3-phosphate-dehydrogenase dehydrogenase (GAPDH), b-Actin (ACTB) or a-Tubulin (TUBA1) that are used in expression analysis, would be useful to normalize qPCR data for miRNA expression (Wei et al., 2013).
Figure 2.3: Schematic representation of alternative reverse transcription methodologies to generate cDNA. (A) Reverse transcription of individual mature miRNAs using stem-loop or (B) linear MSPs. (C) enzymatic tailing of the miRNAs by using Poly(A) Polymerase or (D) T4 RNA Ligase.
2.3.2 Expression analysis of miRNAs by Real time PCR

Zhou et al., (2008) computationally identified 38 potential miRNAs in *Medicago truncatula*. For expression analysis, total RNAs were extracted using TRIzol reagent from roots, stems, leaves and flowers. RNAs were polyadenylated and reverse transcription was performed. The expression of 14 miRNAs was analyzed by qRT-PCR. The miRNAs showed different patterns of expression in root, stem, leaf or floral organs, except one, which appeared not to be expressed in any tissues under the normal conditions. The down-regulation of one of the miRNAs under the heavy metal stress implies that the development of leaf might be disturbed. These results implied that some of the miRNAs are involved in the regulation of development and plant response to heavy metal stress.

Xie et al., (2011) identified 202 miRNAs and their targets in potato. Total RNA was extracted from young leaves, immature flowers, and mature flowers for
validation and expression analysis using the mirVana miRNA isolation kit. TaqMan microRNA assays were used to detect and quantify potato miRNAs. A single-stranded miRNA cDNA was generated using TaqMan microRNA Reverse Transcription Kit and miRNA specific stem–loop primers. The expression levels of 12 potato miRNAs were analyzed in all three tissue types using qRT-PCR and miRNA specific primers. Analysing the results, it was concluded that conserved miRNAs are expressed in potato and that these miRNAs are expressed in a tissue-specific manner.

Identification of microRNAs in strawberry was carried out using computational methods by Dong et al., (2012). Five miRNAs were predicted using this approach. For expression analysis, total RNA was isolated using Trizol reagent from roots, leaves, sepals, flower buds, flowers, and fruits. Small RNAs were polyadenylated and reverse transcription was performed. qRT-PCR analysis confirmed the existence and sizes of the 5 identified miRNAs in strawberry. Their expression patterns indicate the aspects of tissue-species-, and/or growth-stage specificity as reflected by different expression levels.

Patanun et al., (2012) identified 169 potential miRNAs in cassava by computational methods. For expression analysis, Total RNA was isolated from fibrous roots and storage roots. The qPCR was carried out using SYBR Green technology. The qRT-PCR assay showed that one of the miRNA was down-regulated in fibrous root while it was up-regulated in storage root. The existence of some candidate miRNAs by using experimental approaches was preliminary confirmed.

Thiebaut et al., (2012) computationally identified miRNAs in sugarcane. Total RNA was isolated from ten samples of fresh root, leaves and whole plants materials using Trizol method. The expression profiles of nine sugarcane’s new mature miRNAs were assayed by stem–loop reverse transcription-PCR. RNA was reverse transcribed into cDNA. To analyze the expression profile of mature miRNA and MADS2 target, qRT-PCR was used with SYBR Green PCR Master Mix. As a result, novel miRNAs gave detectable expression levels in qRT-PCR analysis using controls.
samples of biotic and abiotic assays. The high abundance of two miRNAs was observed.

Meng et al., (2013) identified 268 development-associated microRNAs in grains of wheat (*Triticum aestivum* L.) via high-throughput sequencing. They conducted quantitative real time PCR (qRT-PCR) to validate the expression patterns. They selected eight miRNAs for examination. Seven of them were predicted to be induced while one was expected to be repressed. Their expression profiles were quite similar to those determined by high-throughput sequencing indicating that it is possible to create a set of grain filling-associated miRNAs through deep-sequencing of wheat.

Yanik et al., (2013) performed genome wide identification of Olive miRNAs associated with alternate bearing. After finding thirty-eight putative novel miRNAs they selected nine miRNA target gene pairs for further analysis by qRT-PCR in order to confirm the results of the Illumina sequencing and quantify the expression patterns of both the miRNA and their target transcripts. The expression of the miRNA varied greatly between the six libraries, indicating the contribution of diverse miRNAs in the balancing between the reproductive and the vegetative developments.

Khan et al., (2014) identified three hundred 55 mature miRNAs along with their secondary structure as well as corresponding targets in foxtail millet (*Setaria italica* (L.)) in response to abiotic stress. Expression profiling of eight candidate miRNAs under abiotic stress conditions were performed using northern hybridization and quantitative stem-loop RT-PCR. This unraveled the putative involvement of these miRNAs in stress tolerance. They have constructed a foxtail millet MiRNA database (FmMiRNADb) with an aim of providing the generated miRNA marker information to the global scientific community.

Vashisht et al., (2014) identified 18 conserved miRNAs in medicinal herb, *Picrorhiza kurroa* by computational methods. For validation of miRNA profile by qRT-PCR expression analysis, total RNA was isolated from shoot, root, and stolon using TRIzol Reagent. cDNA was synthesized by selective poly-A tailing of miRNA. miRNA-specific primers were designed to analyze expression levels and amplicons.
Almost all miRNAs showed presence in all organs. Stolon had a higher expression profile for maximum (39 %) miRNAs and field-grown shoot had lowest expression for almost all miRNAs. qRT-PCR analysis of the target transcript of one miRNA was done to evaluate for any downregulation and the results showed ~10-fold higher expression of the target transcript in field grown shoot than tissue cultured one. These experiments identified 18 conserved miRNAs which have their targets in primary and secondary metabolic pathways and the expression of miRNA variants was confirmed along with certain important targets.

Chai et al., (2015) computationally identified 32 potential miRNAs in banana. In order to examine the expression of these miRNAs, stem-loop RT-PCR were performed using a total RNA isolated from roots, leaves, flowers, and fruits. 12 miRNAs from 11 families were chosen randomly. Using this approach, they detected a positive signal of the expected size. 12 potential miRNAs were expressed and exhibited different expression patterns in four tissues indicating that the conserved miRNAs were expressed in a tissue-specific manner.

Das et al., (2015) computationally identified 18 conserved miRNAs in Cannabis sativa L. For expression analysis of six predicted miRNAs, total RNA was isolated from young and mature leaf samples by Trizol method. The isolated RNAs were polyadenylated and reverse transcribed. Quantitative real-time PCR (qPCR) experiment was carried out following the protocol of Mir-X miRNA SYBR qPCR Kit. Validation of the predicted miRNAs showed differential expression pattern in different tissue types. Compared to the young leaf, three miRNAs were highly expressed in mature leaf. The high expression may be for the change of leaf architecture due to aging. This qPCR based verification of the predicted miRNAs proved the reliability of in silico approach of miRNA identification.

Das et al., (2015a) identified 42 miRNAs using computational methods in insulin plant. For validation of predicted miRNAs, total RNA was isolated from leaf samples using Trizol reagent. The isolated RNAs were polyadenylated and reverse-transcribed. Expression analysis of mature miRNAs was done using SYBR dye in a qRT-PCR. Differential expression patterns of the validated miRNAs were observed in
these tissues. In between young and mature leaf tissues, three miRNAs were found abundant in mature leaf. It may be due to response of age-effected leaf curling and multiple abiotic stresses. Expression analysis of the identified miRNAs proved the authentication of computational method of miRNA prediction.

Pan et al., (2016) bioinformatically identified 106 miRNAs in Sacred lotus. For validation of miRNA and the targets by qRT-PCR, ten miRNAs were selected. Total RNA from young leaves, stems, and flowers was extracted. Each sample was reverse transcribed to stem-loop reverse transcription using specific RT primer and PrimeScript Reverse Transcriptase for miRNAs. RT-PCR was performed using SYBR Green technology. Negative correlation of the expression levels between five miRNAs and their target genes were observed in leaves, stems, and flowers. Computational prediction of miRNAs and their targets in Phaseolus vulgaris was done by Nithin et al., (2015). Using this approach 208 mature miRNAs were predicted. For experimental validation, small RNA was isolated from leaves of 10 days old seedlings using mirPremier microRNA isolation kit. Small RNA was reverse transcribed to cDNA using stem-loop reverse transcription primers for miRNAs. qRT-PCR reactions were carried out for the five randomly selected miRNAs using SYBR green techniques. The expression profiles obtained by qRT-PCR analysis mostly agreed with the expression values obtained from the sequencing data of these 5 miRNAs. This qRT-PCR showed experimental validation of computational method.

Paola et al., (2016) isolated and characterized 167 conserved and 98 potential novel miRNAs from durum wheat. For the qPCR validation of selected miRNAs, Small RNAs were isolated using the mirPremier microRNA Isolation Kit and miRNAs were detected using stem-loop RT-PCR method. Quantitative qPCR analyses were performed using the Universal Probe Library probe assay specific for mature miRNA expression. The expression of miRNA putative target genes was analysed using the SYBR Green dye. Results of miRNA expression analysis showed that six conserved miRNAs were significantly downregulated in leaves at the mature stage compared to the leaves at the early stage. Additionally, two miRNAs were found to be significantly upregulated in leaves at the adult stage compared to the early
stage. Eleven predicted target genes of seven miRNAs were differentially expressed between the two developmental stages and possibly involved in plant development.

Xie et al., (2016) identified and characterized two microRNAs from *Ribes nigrum* ESTs. For expression analysis, Total RNA was isolated from leaves, buds, flowers and fruits. miRNA qRT-PCR was performed with SYBR PrimeScript miRNA RT-PCR Kit using *ACTIN* as internal control. Out of two miRNAs, MIR5021 gene was highly expressed in leaves, while MIR5185 gene displayed higher expression in buds. Both the miRNAs showed higher accumulation in fruit as compared with other tissues, suggesting that they might play important roles in fruit development.

Differential expression of microRNAs and potential targets under drought stress in barley was carried out by Ferdous et al., (2017). For qRT-PCR of 11 miRNAs, total RNA was extracted from leaves using TRIzol reagent. Total RNA was reverse transcribed to cDNA using stem-loop primers and gene specific primers. qRT-PCR assays were carried out using SYBR Green technology. Among the 11 miRNAs, four miRNAs showed differential expression under drought.