

**PLANT miRNAs: KEY PLAYERS IN INTER-KINGDOM
AND INTRA-KINGDOM GENE REGULATION****SANGEETA SAXENA* , ASHISH KUMAR AND SUNIL G BABU***Department of Biotechnology Babasaheb Bhimrao Ambedkar University,
Vidya Vihar, Raebareli Road, Lucknow. India.***ABSTRACT**

Micro RNAs (miRNAs) are a class of 21-24 nucleotide long single stranded, endogenous, non-coding RNAs involved mainly in post transcriptional gene silencing in animals and plants. Their role in the gene regulation of plant growth and development, biotic and abiotic stress responses, methylation is revealed and much more is expected by these tiny molecules. With each passing day a new insight about miRNAs and their explicit role in signaling, plant immunity and plant pathogen interactions are accumulating to the literature. In addition, the identification of miRNAs in plants and human secretions and their role in regulating the human genes are now reported. This observation leads to the speculation of therapeutic role of secretory miRNAs of such plants which exhibits medicinal value. Further, with proven evidences of their crucial role in cross kingdom gene regulation of several human genes many diseases can be treated by mere consumption of these plant miRNAs through food. In this review we have discussed about the significant role of plant miRNA in various biological system, speculating their potential use as therapeutics giving an altogether new direction to molecular biology research on these small RNA molecules.

KEY WORDS: miRNA, plant development, stress, immunity, gene regulation, mirbase, siRNA**SANGEETA SAXENA***Department of Biotechnology Babasaheb Bhimrao Ambedkar University,
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1: INTRODUCTION

The miRNAs are quite interesting, small, endogenous, non coding RNA molecules. The first endogenous ~22-nt RNAs to be identified were *lin-4* RNA and *let-7* RNA, in the nematode (Lee *et al*; 1993, Reinhart *et al*; 2000). Further, Ambros and coworkers have also detailed miRNAs and other tiny endogenous RNAs in the nematode *C.elegans* (Ambros *et al*; 2003, Bartel; 2004). These miRNAs are 19-25 nucleotides long, derived from hair pin precursors and mediate post transcriptional regulation of mRNA expression in many multicellular organisms such as plants, insects, fish and mammals etc.(Duursma *et al*; 2008, Zhang *et al*; 2011, Amiteye *et al*; 2011, Li *et al*; 2012, Zhu *et al*; 2012). In animals, miRNAs have been shown to inhibit mRNA translation and decrease mRNA stability by binding its sequences in 3'UTR (Duursma *et al*; 2008). The binding of miRNA within first 10 bases of miRNA, especially 2-7 nucleotide specific sequence, called "seed sequence", is critical for playing key role in miRNA mediated regulation (Duursma *et al*; 2008). While in plants, most of desired miRNAs bind the protein coding sequences (CDS) of their target mRNAs with very high sequence complementarity thus inducing translational repression or RNA degradation in a way similar to RNA interference (Amiteye *et al*; 2011). The plant miRNA have more limited number of targets than animals because they require higher base pairing in comparison to animals (Pulido *et al*; 2010). Earlier, no functional miRNA binding sites were demonstrated in animals within CDS region, however in 2008, Duursma *et al*, first demonstrated the binding of mammalian miRNA in CDS region too. The miRNA in plant was first demonstrated in *Arabidopsis* in 2002 (Reinhart *et al*; 2002). The discovery of these miRNAs in both plants and animals suggests that this is a class of non-coding RNA that has been modulating gene expression since at least the last common ancestors of this lineage (Bartel; 2004). More than thousands of plant miRNAs have been identified using three primary strategies i.e direct cloning from small RNA libraries with different tissues and conditions, bioinformatics prediction from existing

database and traditional methods, that lead to first identification of small RNAs in *C.elegans* (Yang *et al*; 2007). These mi RNAs are reported in viruses also like in Epstein-Barr virus, HCMV etc. and assist them in inducing infection inside host system (Gosh *et al*; 2009, Babu *et al*,2011). According to miRNA registry database Release19, 5159 miRNAs have been identified in various plants (<http://www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl>). This number has now increased considerably registry database Release20. The different types of miRNAs of different plant species registered in miRBase are listed in table 1. The text color highlight indicates some of the plants which are eaten raw, and the miRNAs thus consumed by means of these plants could play a role in regulation of genes involved in several pathways directly or indirectly related to human health if cross kingdom gene regulation is prevalent as has been shown in a few recent papers to be described later in this review. As we have emphasized in this review many Indian complementary or alternative medicines and therapies involve the use of medicinally important plants or their specific parts to be consumed in certain diseased state. These plants may have a medicinal effect not only due to the phytochemical constituents that have been variously characterized, but also due to its miRNA playing a role in cross kingdom gene regulation targeted to the genes having a role in disease manifestation. Recently an appealing possibility that food-derived miRNAs may offer us another means to deliver necessary nutrients or therapeutics to our bodies has been reported by Jiang *et al*; 2012. These miRNA genes are mostly reported from intronic regions however some miRNA genes in plant are reported in exonic regions also. In plants a few example of intronic miRNAs have been found in *Arabidopsis* and Rice (Colaiacovo, et al; 2012). In *Arabidopsis*, miR402 and in rice miR1429.2 are identified within the intronic site and are referred as "mirtrons" (Colaiacovo, et al; 2012). The rice miR3981 was detected in the exonic region of a putative glyoxylase gene and its biogenesis pathway is

thought to be involved in regulation of glyoxylase expression (Colaiacovo, et al; 2012). We are here presenting a list of total number of mature and precursor plant miRNAs registered in miRBase as per miRNA registry database release 20 (table 1). Plant miRNAs were found to be highly conserved among either plants or animals but not conserved in between plants and animals (Yang *et al*; 2007). Approximately 21 miRNA families are conserved in angiosperms and few in others. This evolutionary conservation is one of the defining characteristics of the plant miRNA and many plant miRNAs remained essentially unchanged before the emergence of angiosperms (Axtell *et al*; 2005). However, it also became apparent that evolution of miRNA did not stop after speciation, therefore in addition to conserved miRNAs there are another class of putative miRNAs, so called “ young miRNAs” (Dalmay; 2010, Jagdeeswaran *et al*; 2012). The conserved miRNAs regulate the expression of genes involved in basic development process and non conserved miRNAs may be involved in development of traits that are specific for certain taxonomic groups (Dalmay; 2010). Moreover it was also found that the evolution of species specific miRNA genes in plants is assisted by transposons and further incorporation of transposable elements (TEs) into CDS of protein coding genes may lead to their integration into miRNA regulation network (Li *et al*; 2011).

The plant miRNAs are very significant for the plant development process and are involved in variety of functions in plants like in organ development (leaf morphogenesis, floral organ identity and root development), feedback regulation, in directing siRNA biogenesis and even in stress responses (oxidative, mineral, nutrient, dehydration and mechanical stress) (Axtell *et al*; 2006, Yang *et al*; 2007, Gielen *et al*; 2012). In addition to above reported diverse intra-kingdom gene regulation by plant miRNAs, inter-kingdom gene regulation has also been reported for plant miRNAs. In 2012 Zhang *et al*, have reported involvement of exogenous plant MIR168a, in regulation of LDLRAP1 (Low Density Lipoprotein Receptor Adapter Protein 1) gene of mammals. This is the first ever reported evidence of cross-kingdom regulation

by miRNA (Zhang *et al*; 2012). In this review we will focus on updated reports in biogenesis of plant miRNAs, highlighting their multifunctional role in plants as well as the cross-kingdom regulation of gene expression by them.

2: BIOGENESIS OF PLANT miRNA

The biogenesis of plant miRNA begins with the transcription of MIRNA genes by RNA polymerase II enzyme (Lee *et al*; 2004, Kim *et al*; 2008, Jagdeeswaran *et al*; 2012). Like animal miRNAs, the primary transcript so formed is called pri-miRNA (Liu *et al*; 2012). This primary transcript so formed contains an imperfect hairpin structure that is sequentially cleaved to form miRNA/ miRNA* duplex by a series of family of RNase III family enzymes (Xie *et al*; 2010). Unlike animals, these pri-miRNAs are processed entirely in nucleus in case of plants (Liu *et al*; 2012). These miRNAs accumulates inside plant cell and then matures to become functional. The key enzyme involved in processing of pri miRNA is Dicer like 1(DCL1) enzyme, a ds-RNA specific RNase family member (Lobbes *et al*; 2006, Kim *et al*; 2008, Jagdeeswaran *et al*; 2012). Besides DCL1, several other enzymes are also involved in plant miRNA biogenesis, including HYL1, Serrate(SE), Dawdle(DDL), CBP-20 and CBP80, HEN1and Hasty protein (Lobbes *et al*; 2006, Kim *et al*;2008, Yu *et al*;2008, Dong *et al*;2008, Song *et al*; 2011, Liu *et al*;2012). Hyponastic leaves 1(HYL1) is a dsRNA binding protein and interacts with DCL1. Both DCL1 and HYL1 co-localizes in small nuclear bodies containing pri-miRNA (Dong *et al*; 2008, Song *et al*; 2011, Liu *et al*; 2012). Now, an another RNA binding protein Serrate (SE), a C₂H₂ Zinc finger protein complexes with HYL1 and DCL1 and enhances the processing of pri-miRNAs to pre miRNAs (Dong *et al*; 2008, Song *et al*; 2011, Liu *et al*; 2012). The serrate is found to be responsible for accumulation of miRNAs and is localized in the nucleus as are DCL1and HYL 1 (Lobbes *et al*; 2006). The SE interacts with HYL 1 in the DCL1-SE-HYL 1 complexes, as shown by Lobbes *et al*; in 2006. Another protein DDL, fork head associated domain protein, FHA is also involved in processing of pri-miRNA by facilitating the access of DCL1

to pri-miRNA (Yu *et al*; 2008, Dong *et al*; 2008, Song *et al*; 2011, Liu *et al*; 2012). Further two more proteins CBP-20 and CBP-80 are also found to be involved in biogenesis of miRNA. Both these proteins forms a complex with the 5' cap structure of primary transcript and performs three important functions- transcript stability, splicing efficiency and 3'end formation. Some pri-miRNAs are capped and are also found to be spliced, hence these proteins are found to be involved in splicing and stability of pri-miRNA (Kim *et al*; 2008, Yu *et al*; 2008, Dong *et al*; 2008, Song *et al*; 2011, Liu *et al*; 2012). These proteins form a heterodimeric complex to bind to the 5' structure of nascent mRNA transcribed by RNA pol II stimulating its splicing and so, they are also involved in miRNA splicing (Kim *et al*; 2008). On processing of pri-miRNA the mature miRNA thus generated is methylated by HUA ENHANCER 1 (HEN 1), a methylase (Kim *et al*; 2008, Yu *et al*; 2008, Dong *et al*; 2008). This generates a methylated 2' OH overhang at 3' end (Dong *et al*; 2008). This mature miRNA is finally transported to cytoplasm by a

exportin-5 homolog, HASTY protein (Kim *et al*; 2008, Yu *et al*; 2008, Dong *et al*; 2008). The structures and steps involved in miRNA biogenesis are given in fig: 1. Although the detailed mechanism of miRNA biogenesis is still unknown, DCL 1 is found to be efficient in carrying out cleavage of pri-miRNA to pre-miRNA and then finally release of mature miRNA (Dong *et al*; 2008). The DCL 1 contains two ds RNA binding domain (RBD) and release of mature miRNA from pre miRNA, may not require both ds RBD (Dong *et al*; 2008). Finally, the ds RNA binding proteins TRBP and PACT are required for miRNA mediated gene silencing and are believed to help DICER load mature miRNA in to RISC complex (Farlane *et al*; 2010). The mature miRNAs also display some variants called iso-miRNAs and isoforms of miRNAs, differing in sequence length. These iso-miRNAs are identified in both plants and animals (Kulcheski *et al*; 2011). The functions of the above discussed enzymes/proteins involved in miRNA biogenesis are summarized in table 2.

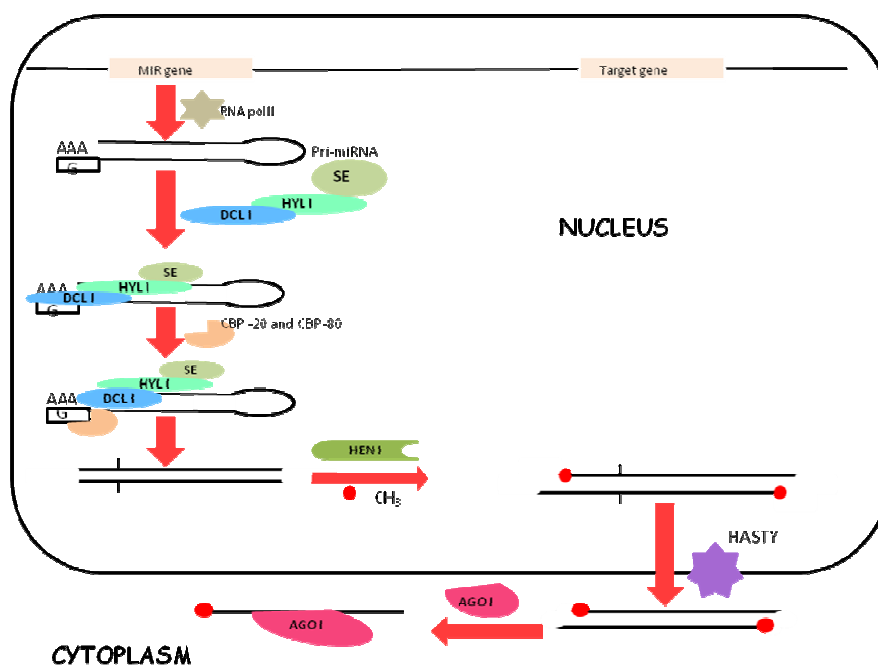


Figure 1

Steps involved in miRNA biogenesis in plants. The 20-24 base pair long MIR gene is transcribed by RNA Pol II in nucleus and forms pri-miRNA. A ribonuclease III like nuclease, DCL I, processes pri miRNA into pre miRNA by the assistance of several other enzymes like SE, HYL I, etc. finally HEN I further processes this pre miRNA and a methyl group is added to produce miRNA: miRNA* duplex, which is further exported outside by exportin -5 like transporter, HASTY. This further associates with AGO complex to process into functional miRNA.

Table 1

List of plant miRNAs present in miRNA registry database Release 20 (<http://www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl>) Color bars represent plants or their parts consumed as such by humans, ensuring straight away intake of miRNA by humans.

FAMILY	PLANT	MATURE miRNA SEQUENCE	PRECURSOR miRNAS
Araliaceae	<i>Panax ginseng</i>	32 mature	29 precursors
Asteraceae	<i>Cynara cardunculus</i>	57 mature	48 precursors
	<i>Helianthus annuus</i>	8 mature	7 precursor
	<i>Helianthus argophyllus aargophiarophyllus</i>	3 mature	3 precursors
	<i>Helianthus ciliaris</i>	3 mature	3 precursors
	<i>Helianthus exilis</i>	2 mature	2 precursors
	<i>Helianthus paradoxus</i>	3 mature	3 precursors
	<i>Helianthus petiolaris</i>	3 mature	3 precursors
	<i>Helianthus tuberosus</i>	16 mature	16 precursors
Brassicaceae	<i>Arabidopsis lyrata</i>	384 mature	205 precursors
	<i>Arabidopsis thaliana</i>	337 mature	298 precursors
	<i>Brassica napus</i>	92 mature	90 precursors
	<i>Brassica oleracea</i>	7 mature	6 precursors
	<i>Brassica rapa</i>	43 mature	39 precursors
Caricaceae	<i>Carica papaya</i>	81 mature	79 precursors
Cucurbitaceae	<i>Cucumis melo</i>	120 mature	120 precursors
Euphorbiaceae	<i>Hevea brasiliensis</i>	28 mature	28 precursors
	<i>Manihot esculenta</i>	153 mature	153 precursors
	<i>Ricinus communis</i>	63 mature	63 precursors
Fabaceae	<i>Acacia auriculiformis</i>	7 mature	7 precursors
	<i>Arachis hypogaea</i>	32 mature	23 precursors
	<i>Acacia mangium</i>	3 mature	3 precursors
	<i>Glycine max</i>	554 mature	505 precursors
	<i>Glycine soja</i>	13 mature	13 precursors
	<i>Lotus japonicus</i>	67 mature	67 precursors
	<i>Medicago truncatula</i>	756 mature	671 precursors
	<i>Phaseolus vulgaris</i>	10 mature	8 precursors
	<i>Vigna unguiculata</i>	18 mature	18 precursors
Lamiales	<i>Avicennia marina</i>	3 mature	2 precursors
	<i>Digitalis purpurea</i>	13 mature	13 precursors
	<i>Rehmannia glutinosa</i>	13 mature	13 precursors
	<i>Salvia sclarea</i>	18 mature	18 precursors
Linaceae	<i>Linum usitatissimum</i>	124 mature	124 precursors
Malvaceae	<i>Gossypium arboreum</i>	1 mature	1 precursors
	<i>Gossypium herbaceum</i>	1 mature	1 precursors
	<i>Gossypium hirsutum</i>	80 mature	78 precursors
	<i>Gossypium raimondii</i>	4 mature	4 precursors
	<i>Theobroma cacao</i>	82 mature	82 precursors
Ranunculaceae	<i>Aquilegia caerulea</i>	45 mature	45 precursors
Rhizophoraceae	<i>Bruquiera cylindrica</i>	4 mature	4 precursors
	<i>Bruquiera gymnorhiza</i>	4 mature	4 precursors
Rosaceae	<i>Malus domestica</i>	207 mature	206 precursors
	<i>Prunus persica</i>	214 mature	180 precursors
Rutaceae	<i>Citrus clementine</i>	5 mature	5 precursors
	<i>Citrus reticulata</i>	4 mature	4 precursors
	<i>Citrus sinensis</i>	64 mature	60 precursors
	<i>Citrus trifoliata</i>	6 mature	6 precursors
Salicaceae	<i>Populus euphratica</i>	4 mature	4 precursors
	<i>Populus trichocarpa</i>	401 mature	352 precursors
Solanaceae	<i>Nicotiana tabacum</i>	164 mature	162 precursors
	<i>Solanum lycopersicum</i>	48 mature	46 precursors
	<i>Solanum tuberosum</i>	343 mature	224 precursors
Vitaceae	<i>Vitis vinifera</i>	186 mature	163 precursors
Monocotyledons	<i>Aegilops tauschii</i>	2 mature	2 precursors
	<i>Brachypodium distachyon</i>	464 mature	258 precursors
	<i>Elaeis guineensis</i>	6 mature	6 precursors
	<i>Festuca arundinacea</i>	15 mature	15 precursors
	<i>Hordeum vulgare</i>	69 mature	67 precursors
	<i>Oryza sativa</i>	713 mature	590 precursors
	<i>Sorghum bicolor</i>	241 mature	204 precursors
	<i>Saccharum officinarum</i>	16 mature	16 precursors
	<i>Saccharum ssp.</i>	20 mature	19 precursors
	<i>Triticum aestivum</i>	42 mature	42 precursors
	<i>Triticum turaidum</i>	1 mature	1 precursors
	<i>Zea mays</i>	321 mature	172 precursors

Table 2
Proteins/enzymes involved in miRNA biogenesis and their function.

proteins/enzymes involved in miRNA biogenesis	Function
HYL 1	Interacts with DCL-1
SE	Accumulation of miRNAs, interacts with HYL 1 in hetrocomplex of DCL 1 and HYL-1
CBP-20 and CBP-80	Pri miRNA splicing and stability; binds at 5' cap end of pri-miRNA
HEN 1	A methylase, generates methylated 2' OH overhang at 3' end of mature miRNA, proper accumulation of miRNA
DDL	Processing of pri-miRNA, and acts in multiple development process like growth, fertility, root and shoot morphogenesis
HASTY	Exportin -5 homolog, transport miRNA to cytoplasm
DCL 1	Homolog of DICER, cleaves ds pri-miRNA or ds pre-miRNA to produce mature miRNA

3: INTRA-KINGDOM GENE REGULATION BY PLANT miRNAs

The miRNAs are found to regulate several genes within the plant itself. With each new day several new miRNAs are being discovered and new functions are being assigned to them exploring several important genes which may be controlled by them. The tissue specific expression of miRNAs is known to be involved in carrying out various developmental processes by regulating several genes. *In silico* studies of miRNA has been done in *Glycine max* and *Brassica napus* to predict several role and targets of miRNA. (Dubey, 2011). The role of miRNA is reported in plant embryogenesis, floral development, leaf morphogenesis, hormone regulation, stress conditions, siRNA biogenesis, plant immunity etc. (Willmann *et al*; 2011, Gielen *et al*; 2012, Kruszka *et al*; 2012, Huang *et al*; 2012, Pulido *et al*; 2010, Chen *et al*; 2010, Staiger *et al*; 2012). Recently miRNAs are also reported to play role during UV B damage (Zhou *et al*; 2007). Also it is suggested that they may play a role in nutrient stress and other biotic stress like pathogens and viral infections etc.

3.1: miRNAs IN PLANT DEVELOPMENTAL PROCESS

Development is an important phenomenon in life cycle of any organism. It can be defined as sum of all the changes that progressively elaborates an organism's body. The developmental process in plants like formation of leaves and flowers, flowering time, formation of embryo etc. are complex processes regulated through an array of proteins and environmental factors. The developmental pathways in plants include the passage from seed germination to an adult

stage, marked initially by formation of vegetative structures, different floral and reproductive organs once it enters in the reproductive phase. As for example, in *Arabidopsis*, the onset of adult phase is marked by the production of trichomes in abaxial surface of leaves with concomitant decrease in cell size and in *Zea mays* the transition to adult stage is marked by changes in cell shape, production of epidermal wax deposits and specialized cell types like leaf, hairs and change in identity of organs that grows from axillary meristem. An extensive network of miRNAs is also found to be involved in regulating the developmental timings in plants. Two antagonistic miRNAs miR156 and miR172 are involved in regulating the developmental timings in plants and the interaction between these two miRNA is found to play a highly conserved role in promoting progression through different developmental phases in both monocots and dicots. miR156 expression level is found to be decreasing with the age of leaves while increase has been found in case of miR172 (Rubio - Somoza and Weigel; 2011). In *Arabidopsis*, miR156 targets SPL (Squamosa Promoter Binding Protein Like) also modulates phase changing through its temporal expression in the shoot and miR172 acts on several proteins known to play role in floral repression (Wu *et al*; 2009, Zhu *et al*; 2011, Xie *et al*; 2012). Thus due to increase in action of miR172 the floral repressors are reduced and transition occurs to floral developmental phase. The flowering time in angiosperms is one of the most critical stages for reproductive success in them. The flowering commences when there is a switch from vegetative to reproductive phase in response to certain environmental signals. The flowering time is

controlled by several endogenous and environmental pathways, with about 180 genes responsible for controlling flowering time is shown in *Arabidopsis* (Zhu *et al*; 2011). miR319 is also found to play a significant role in flowering in addition to leaf morphogenesis and is thus one of the critical conserved miRNA for development, growth, morphogenesis, and reproduction in plants (Nag *et al*; 2009, Schommer *et al*; 2012). The MIR172 gene generates miR172 that regulates the translation of plant specific transcription factor gene, e.g. APETALA 2 (AP2) and some AP2 like genes in *Arabidopsis* (Zhu *et al*; 2011, Aukerman *et al*; 2003). In *Arabidopsis* flower, three miRNA 164 gene (MIR 164a, b and c) regulates the expression of CUP- shaped cotyledon 1 (CUC1) and cotyledon 2 (CUC2), leaf development is also an important process during plant development during which small group of undifferentiated cells recruited in the meristem gives rise to flat structure organized into different cell types (Pulido *et al*; 2010). The regulatory network of leaf development is also by miRNA network. These miRNAs are involved in regulating both position and time of organ primordia emergence and in their very early development. The miRNA miR156 is found to be involved in regulating the time between the initiation of two successive leaves i.e, plastochron length and its over expression accelerate the rate of leaf initiation (Pulido *et al*; 2010).

Many transcription factors play critical role in the establishment and maintenance of organ boundaries. The formation of these organ boundaries between different cell populations is crucial for development. CUC 1 and CUC 2 are two NAC domain genes which precisely regulates organ number and boundary formation through vegetative and reproductive development. These are also actively involved in shoot apical meristem formation (Huang *et al*; 2012). MIR164 genes are also one of the important genes in developmental regulation of plants and found to act in redundant manner. A loss in MIR164 gene in mutants during experiments resulted in severe disruption of shoot development also if there is a loss of function of the MIR164C gene, also known as EARLY EXTRA PETALS 1 (EEP1), then it results in repression of CUC1 and

CUC2 in the second whorl (Huang *et al*; 2012). It is also involved in organ boundary formation (Xie *et al*; 2012). The expression of the three miR 164 genes were known to be regulated by C₂H₂ Zn finger transcriptional repressor encoded by RABBM EARS (RBE) (Huang *et al*; 2012). The transcriptional regulation of the miR164 genes by RBE appears to be important in defining both localization and timing of mature miR164 accumulation that in turn, impacts CUC-dependant boundary specification and concomitant organogenesis. In 2011, Amiteye *et al* reported 15 conserved miRNA families in floral tissue of sexual and apomictic in *Boechera spp.* by Microassay technique. This kind of study may lead to development of better understanding in role of miRNA in apomictic gene regulation and other floral tissues (Amiteye *et al*; 2011).

The other miRNAs like miR160 and miR164 are involved in regulating leaf initiation (Xie *et al*; 2012), miR390, miR165/166 is found to control leaf polarity while miR164, miR319, miR396, miR159 and miR324 were reported to regulate leaf growth, shape and differentiation (Vaucheret; 2006, Sakaguchi *et al*; 2012). The reproductive development is also guided by several miRNA, like miR156 and miR172 which plays prominent role in guiding reproductive development. Even the control of senescence is also found to be regulated by two miRNA-miR164 and miR319 (Rubio -Somoza and Weigel; 2011). It has been found that *Arabidopsis* embryo lacking DCL-1, which is required for miRNA during biogenesis, arrests early in development. Later investigations suggest that there is a requirement of miRNAs for proper embryonic patterning and cell differentiation in embryo. miR156 is an important miRNA which is involved in this regulation and it has been experimentally shown that, by preventing precocious expression of differentiation-promoting transcription factors, miRNA enables proper embryonic patterning (Nordine *et al*; 2010).

3:2: miRNAs IN HORMONE SIGNALING AND HOMEOSTASIS

The growth and other developmental processes in plant are under the control of several growth regulators, commonly called

PHYTOHORMONES. These phytohormones are classified into the five classes: Auxins; Gibberellins (GAs); Cytokinins; Abscisic acid (ABA) and Ethylene. Some recently added class of these growth regulators includes Jasmonic acid (JA) and Brassinosteroids (BRs) (Liu, *et al*; 2009). Recently several miRNAs are reported to regulate the hormone signaling pathway in plants. The first report linking miRNAs and hormone signaling was that the *hyl1-1* mutant displayed impaired responses to auxin, ABA and cytokinin (Liu, *et al*; 2009). Also, the disturbance in these small RNA pathways are found to enhance abscisic acid response in *Arabidopsis* (Liu, *et al*; 2009) and disruption of AGO1 or miRNA mediated cleavage of NAC1 target mRNA resulted in dramatic effects on auxin mediated induction of adventitious and lateral root formation, respectively. Second, the GH3 genes that are putative targets of ARF17 encode auxin-conjugating proteins (Staswick *et al*; 2005). Auxin is an important phytohormone that plays a crucial role in growth and development of plants and their parts. It performs a variety of functions including apical dominance, shoot elongation, phototropic movements, lateral and adventitious root formation etc. through F-box receptors (Eckardt; 2005, Si-Ammour *et al*; 2011). The variety of function that auxin performs is regulated by four partially redundant auxin receptors- TRANSPORT INHIBITOR RESPONSE 1 (TIR 1), AUXIN SIGNALING F-BOX 1 (AFB 1), AFB 2, and AFB 3. These proteins are the members of the TIR 1/AFB 2 clade of auxin receptors (TAARs) in AFB family of plant F-box proteins (Si-Ammour *et al*; 2011). TAARs are believed to function as component of F-box-ubiquitin ligase complex that targets the members of AUX/IAA transcriptional repressor protein family to proteasome-dependent degradation. Degradation of these AUX/IAA transcriptional repressor function allows specific ARF to act at the promoter of primary-auxin responsive genes to activate their transcription. ARFs are the transcription factors that bind to TGTCTC auxin response elements in promoters of early auxin response genes, contain an N-terminal DNA Binding Domain (DBD) and a middle region MR, that is proposed to function as either activation or

repression domain (Tiwari *et al*; 2003, Si-Ammour *et al*; 2011).

Recently miR393 is found to down regulate all the 4 TAAR genes (Windels, *et al*; 2011, Si-Ammour *et al*; 2011), also it has been found to be significant in triggering the secondary siRNA biogenesis termed siTAARs (Si-Ammour, *et al*; 2011). Thus, miR 393 is found to be significant miRNA in homeostasis of auxin signaling (Eckardt; 2005, Windels, *et al*; 2011). In addition to this miR 393 is also found to play diverse role in nitrate response, defense against bacterial pathogen and in plant development (Si-Ammour *et al*; 2011). Studies of *Arabidopsis* mutants with altered adventitious rooting parameters have allowed to identify several candidate regulatory genes; out of which ARF 6, ARF 8 and ARF 17 are found to be involved in formation of adventitious roots (Sorin *et al*; 2005, Gutierrez *et al*; 2009). The fine tuning in regulation of all these genes is found to be done by miRNAs. ARF 6 and ARF 8 are targeted by miR 167 and ARF 10, 16 and 17 are found to be regulated by miR160. The ARF- 6 and ARF-8 are demonstrated as positive regulator of adventitious root formation by and ARF 17 was previously reported as negative regulator of adventitious root formation (Gutierrez *et al*; 2009). ; Liu, *et al*; 2009 have found twenty-two conserved miRNAs and investigated the expression pattern in response to phytohormone treatments in *Oryza sativa*. Their results showed that 11 miRNAs deregulated by one or more phytohormone treatments. The target genes of these miRNAs were validated *in vivo* and their expression profiling were revealed. They also analyzed the promoter region of the 22 conserved miRNAs for phytohormone-responsive elements and found the existence of these elements further supporting their result. miR159 and miR394 expression was reported to be regulated by ethylene and miR167 and miR413 are regulated by ABA (Liu, *et al*; 2009). Gibberellic acid (GA) has also be shown to modulate miR159 levels during anther development (Reyes *et al*; 2007). Cytokinins, also reported to be regulated by miRNA are the class of plant hormones that affects cell proliferation, elongation, cell differentiation or senescence etc. In legume crops, this phytohormone is necessary and

sufficient for symbiotic nodule organogenesis, allowing them to fix atmospheric nitrogen (Ariel *et al*; 2012). Reyes *et al*; 2007, have shown that MYB33 and MYB101 are positive regulators of ABA response during germination and are subjected to regulation by miR159.

3.3: miRNAS IN STRESS RESPONSE

The plants due to their sessile nature are continuously and constantly exposed to several biotic and abiotic stress conditions. The several abiotic stresses faced by the plants include the stress conditions like metal toxicity, nutrient stress like phosphorus, sulphur and copper etc, water related stress, temperature and salinity related stress and also stress due to radiations. However, the biotic stress includes the stress due to various biological agents like virus, bacteria, fungi and other pathogens. Due to various anthropogenic activities, the concentration of various toxic heavy metals is continuously and tremendously increasing in our environment. The metal toxicity, a major stress affecting crop production and includes metals that are essential for plants (Cu, Fe, Zn, Mn) along with non-essential (Cd, Al, Co, Hg) (Mendoza-Soto *et al*; 2012). These metals are found to alter several physiological processes like water balance, mineral nutrition and photosynthesis etc. in plants (Gielen *et al*; 2012). The metal toxicity triggers the accumulation of ROS leading to damage of lipids, proteins and DNA (Sharma *et al*; 2012, Kruszka *et al*; 2012) and plants use a variety of mechanisms to prevent itself from such abiotic stress. Various proteins are synthesized by plants in response to metal toxicity such as phytochelatins, glutathiones and methallothioneins (Cobbett and Goldsbrough; 2002, Gielen *et al*; 2012). The plants overcome this stress by production of several acids such as citric acid, oxalic acid, malic acid and amino acids such as histidine by the root rhizosphere, that can form complexes with heavy metals which leads to their detoxification. Several metal transporters are also significant in overcoming metal toxicity like ABC transporters and NRAMP a Zn transporter (Mendoza-Soto *et al*; 2012). Thus the various plants in which the studies regarding involvement of miRNAs in response

to different metal toxicity have been done includes *Arabidopsis thaliana*, *Medicago truncatula*, *Brassica napus*, *Oryza sativa*, *Nicotiana tabacum*, and *Phaseolus vulgaris* (Ding *et al*; 2011, Gielen *et al*; 2012). Along with this conventional internal mechanism several microRNAs are also found to be involved in regulation/signaling of metal toxicity response. Some of the important miRNAs involved in overcoming this stress are miR319, miR171, miR390, miR393, miR396, miR164, miR167, miR156, miR160 etc. acting on various transcription factor receptors (Mendoza-Soto *et al*; 2012). The conserved miRNAs that respond to cytotoxicity are miR160, miR164 and miR167 (Soto *et al*; 2012). Except miR172 and miR397 all the above miRNAs are involved in Cd toxicity and they target various transcription factors (TFs) like TCP transcription factor by miR319, GRF transcription factors by miR396, SCL transcription factor by miR170NAC, CUP TFs by miR160 etc. Conserved 5' TGCGCNC 3' sequence is found to be present in promoter region of most of Cd responsive miRNA genes (Ding *et al*; 2011, Mendoza-Soto *et al*; 2012).

Ding *et al*; 2011, has demonstrated microarray based analysis of Cd- responsive miRNAs in rice. They identified total 19 Cd responsive miRNAs and validated 6 of them experimentally. Among the 19-Cd responsive miRNAs only miR528 was found to be significantly up regulated and 18 other miRNAs (miR162a, miR168a, miR168b, miR166m, miR166i, miR166e, miR166e, miR166k, miR166g, miR171b, miR171a, miR171g, miR396d, miR390, miR1561, miR156k, miR156a, miR1432, and miR444b.1) were down regulated in expression being confirmed by qRT PCR analysis. The plants require 14 essential mineral elements for their growth and survival. The role of miRNAs in the nutrient homeostasis has also been demonstrated. Some of the important macronutrients like phosphate, sulphur, copper homeostasis are being maintained by miRNA (Kruszka K, *et al*; 2012). miR399 is found to be elevated in *Arabidopsis* during phosphate starvation. It is found to guide the cleavage of PHO2 mRNAs, a protein involved in regulation of cellular phosphate content (Kruszaka K, *et al*; 2012). Similarly miR395 is involved in regulation of

low affinity sulfate transporter SULTR2; 1 and of ATP sulfurylase gene APS1, APS3, and APS4 in *Arabidopsis* (Gielen *et al*; 2012). The miRNA like miR398 is involved in oxidative stress is down regulated, resulting in accumulation of CSD-1 and CSD-2 mRNA leading to increased tolerance to oxidative stress (Sunkar *et al*; 2006). Kulcheski *et al*; 2011, has reported 24 families of novel miRNAs, six families of conserved miRNAs and 22 families of previously reported miRNAs in soybean (*Glycine max*) regulated by water deficit and rust stress using RT qPCR. miRNA targets have also been identified in soybean in response to aluminum stress (Zeng *et al*; 2012). In addition to these wide arrays of miRNAs regulating various stress condition some more are also found in controlling cold stress and UV B stress. Sunkar and Zhu (2004) originally reported miR 393, miR 397b, miR 402 and miR 319c up regulation during cold stress in *Arabidopsis* and miR156, miR159, miR160, miR165/166, miR167, miR169, miR170, miR172, miR393, miR398 and miR401 in *Arabidopsis* in case of UV B stress. In addition to involvement of these miRNAs in various abiotic stress condition several miRNAs and siRNAs have also been reported to be involved in biotic stress like in viral, bacterial, fungal and nematode infection. The role of gene silencing has also been widely evolved in plant immunity (Kang *et al*; 2012). The antiviral and antibacterial mechanism of miRNAs has been well described in animal system and now recently this has been established in plants also. Recently, the miRNA pathway has also been shown to be implicated in impairing the replication of engineered potyvirus bearing a miRNA target (Qu *et al*; 2007). In 2007, Qu *et al* have shown that miRNA-mediated viral silencing approach is an effective approach against CMV infection and there by supporting the role of miRNA during biotic stress. The miRNAs could serve as likely indicator of viral infection and could be potentially employed to develop viral resistance strategies. This has been demonstrated by Naqvi *et al*; 2010 by microarray analysis of miRNAs isolated from both healthy and Tomato Leaf Curl New Delhi Virus (*ToLCNDV*) agroinfected tomato cv Pusa Ruby. They reported that ToLCNDV agroinfection can significantly deregulate the

host miRNA expression and corresponding targets as well. They observed that the expression level of miR 159/319 and miR172 were associated with disease progression.

The first miRNA that was found to be involved in plant defense was miR393, discovered in *Arabidopsis*. The expression level of this miRNA was found to be increased against microbes such as flagellin22 and pseudomonas antigens. This suggests the role of miRNAs in plants during bacterial infection also. Some more miRNAs are also reported during several biotic stress like miR160-3, miR156, miR160 and miR167 etc. The miRNAs are also found to be orchestrating hormone signaling pathways for defense response in plants against bacterial infections. A group of miRNAs have been found to be expressed after infection of bacterial pathogen *Pseudomonas syringae* pv. Tomato (Pst). Many of these manipulate the components of plant hormone signaling pathways like auxin, ABA and jasmonic acid (JA) signaling pathways. Recently it has been discovered that several miRNA families targets genes encoding nucleotide binding site – leucine –rich repeat (NBS-LRR) plant innate immune receptors in legumes and in solanaceae (Eckardt; 2012). All these reports are speculating the involvement of miRNAs in various biotic stresses and providing an opportunity to reveal their role in stress conditions, especially in biotic stress.

3.4: miRNAs IN PLANT IMMUNITY

The plants are susceptible to infection by a wide variety of pathogens like viruses, bacteria, fungi, nematodes and some insects. The attack through these agents significantly affects the crop yield and thus they possess a sort of biotic stress to the plants. Plants have however shown to achieve immunity against these organisms. Although the plants do not show a well defined immune system like jawed vertebrates i.e showing three important characteristics, high specificity, self tolerance and immune memory and having circulating immune cells- B cells and T cells still they are capable of establishing a highly specific, restricted self reactivity and often generates life long memory (Spoel *et al*; 2012). The first line of defense is provided by the identification of microorganism associated molecular

pattern (MAMPs) by pattern recognition receptors (PRPs) (Li *et al*; 2010, Li *et al*; 2012, Spoel *et al*; 2012). There are two major classes of plant innate immune receptors-PRPs and resistance (R) proteins. PRPs recognizes conserved pathogens associated molecular patterns and activates PAMP triggered immunity (PTI) whereas R proteins are involved in recognition of divergent pathogen effectors and activates hypersensitive cell death response in response to this (Li *et al*; 2012). This local hypersensitive response can also immunize plants against future infection. This phenomenon was called systemic acquired resistance (SAR) by Frank Ross. This similarity suggest that these component have arisen through convergent evolution (Spoel and Dong; 2012). In addition to the conventional methods of generating immunity in host plants, now some small RNAs specifically miRNAs have been reported to trigger host defense mechanism against pathogens. The involvement of siRNA mediated gene silencing in defense against virus have already been reported in plants (Saxena *et al*; 2011, Saxena *et al*; 2013) but the miRNA guided regulation have been identified as one of the new strategy used by plants against viral and other pathogen infection.

The dcl 1-9 mutant were found to be defective in miRNA biogenesis and Ago 1-25 and Ago 1-27 mutants were found to be defective in miRNA activity in PTI response in plants. This study demonstrated the role of miRNA in plant immunity (Staiger *et al*; 2012). The first miRNA directly shown to be involved in plant immunity was miR393 (Staiger *et al*; 2012). The flg 22 is found to induce the accumulation of miR393, which contributes to plant resistance against bacteria by negatively regulating the mRNA of F box auxin receptors (TIR1, AFB2 and AFB3) (Li *et al*; 2010). The *Arabidopsis* contains two genes, MIR393a and MIR393b that are processed into an identical mature miR 393. A recent study has demonstrated the role of miR393b* in plant immunity, which was initially considered nonfunctional (Staiger *et al*; 2012). This miRNA was shown to target MEMB12 encoding golgi SNARE protein which is involved in vesicle trafficking and protein

sorting. These miRNAs are also involved in regulating the signaling pathways involved in plant immunity generation. miR160, miR167, miR393 and miR159 were found to target ARF8, ARF10, ARF16, ARF17, TIR 1, AFB2, AFB3, MYB33 and MYB65 genes consequent to *Pseudomonas* infection (Kruszka *et al*; 2012). Li *et al*; 2012, has proposed that there is a conserved role for miRNA and secondary siRNAs in regulating NB-LRR and LRR innate immune receptor gene expression and pathogen resistance in plants. They have also identified two miRNAs, nta-miR6019 and nta-miR 6020, that guides sequence specific cleavage of transcripts of TIR-NB-LRR immune receptor N that confers resistance to TMV in *Nicotiana banthamiana* (Li *et al*; 2011). AGO 1 is significant component in miRNA induced gene silencing (Poulsen *et al*; 2013), thus this indicates the role of miRNA in plant immunity. The initial obstacle that a phytopathogen encounters is the plant cell wall, which can be reinforced by the deposition of callose, following the activation of host defense pathway (Spoel and Dong; 2012). AGO1 is found to positively regulate PAMP induced callose deposition, defense gene expression, seedling growth inhibition and also contributes to PAMP- induced disease resistance to *P. syringae* (Li *et al*; 2011). Furthermore, in wheat 24 miRNAs have been identified in defense against powdery mildew infection, some of them are miR156, miR164, miR167 and miR393 (Kruszka *et al*; 2012).

3.5: miRNAs in siRNA Biogenesis

siRNAs are a class of double stranded RNA molecules, predominantly 22 nucleotide in size and plays important role in both plants and animals by RNAi pathway and found to play important role in development and in response to stress and pathogens like viruses. (Chen *et al*; 2010). These siRNAs are also a type of non-coding RNA, like miRNA and cannot be easily discriminated from miRNA by either their chemical composition or mechanism of action. However, these siRNAs can be distinguished from miRNA by their origin, evolutionary conservation and the types of gene that they silence (Kulcheski *et al*; 2011). One difference in their origin is that they arise from dsRNA precursor and

sometimes requires RNA dependent RNA Polymerase (RDR) for their biogenesis. In *Arabidopsis* DCL 2, DCL 3, RDR1, RDR2, and RDR6 have their roles in siRNA biogenesis (Allen *et al*; 2005). In some instances the biogenesis of these siRNA is found to be mediated by miRNA. The miRNA mediated cleavage of an RNA trigger the production of siRNA and this phenomenon in which miRNA directs the biogenesis of siRNA is called transitivity (Manavella *et al*; 2011). After this cleavage step the cleaved target transcript is converted into dsRNA by specialized RNA polymerase RDR6 and certain co factors. The newly synthesized dsRNA molecule is then further processed by DCL4 into Trans acting siRNA (tasiRNA). The role of few miRNAs like miR390, miR173 and miR828 has been found in formation of these tasiRNA from a single target site (Manavella *et al*; 2012). The role of several other miRNAs has been elucidated in tasiRNA biogenesis, like miR167 a,b,c, miR168 a,b, miR173a, miR393a,b, etc. (Manavella *et al*; 2012). A family of 22 nucleotide miRNAs is also reported to target *Solanaceae* R genes and triggers siRNA production (Li *et al*; 2012). Another 22 nucleotide miR472 in *Arabidopsis* is reported to trigger secondary siRNA biogenesis (Chen *et al*; 2010). Thus formation of siRNA by miRNAs is one of the novel roles of miRNA out of its diverse function.

4: INTER-KINGDOM GENE REGULATION BY PLANT miRNAs

Chen *et al*; 2008 were the first to systematically characterize miRNAs in serum and demonstrated that serum miRNAs are stable and can be detected directly in serum. It was reported that miRNAs have an unusual high stability in formalin- fixed tissue (Mitchell *et al*; 2008). This surprising result leads the researchers to speculate the stability of miRNAs in serum and plasma and thus these miRNA's are proved to be a promising biomarker for detection of prostate cancer (Mitchell *et al*; 2008) and also in case of lung cancer, colorectal cancer and diabetes (Chen, *et al*; 2008). The human miRNA let-7a, miR192, miR21, miR451 and miR221 were proved to be present in both normal human serum and plasma (Chen *et al*; 2008) and

miR141 in case of prostate cancer is also found to be expressed in human serum (Mitchell *et al*; 2008). Not only this, these miRNAs are even found to be present in Tear, Urine, Ascetic fluid and Amniotic fluid (Chen *et al*; 2008). This shows that probably the miRNAs present in these body fluids and secretions may help in fighting against foreign pathogens and thus serve as one of the component of innate immune system of the body. Zhang *et al*; 2012 has demonstrated the presence of exogenous plant MIR168a (Rice) in human blood serum and proved that this miRNA is regulating the expression of mammalian LDLRAP1 gene. This is the first ever evidence of cross-kingdom gene regulation by miRNA which supports the therapeutic role of plant miRNAs. In this way plant miRNA may prove to play a role as one of the essential novel molecules for health benefit and cure of many diseases. Here we would like to speculate that many medicinal plant, ayurvedic preparations and unani medicines prescribed and prevalent in India may actually work by possessing some specific miRNAs which play a positive role in humans. Also in many Eastasian countries generally people use various herbs and kitchen garden plants to cure common diseases like cold, sore throat, infection etc for effective and quick relief rather than antibiotics both in ancient times and even now, which hints us in speculating the role of specific plant miRNA associated with respective plant species. In table1 we have highlighted certain plants which themselves or their products are consumed as such without any processing thereby meaning that the miRNAs thus consumed may have a role to play in certain diseases in which they are prescribed or suggested by controlling the gene expression. We are also working in the direction to correlate certain diseases for which a specific fruit or vegetable etc is prescribed and linked by working on miRNA and mRNA interaction studies for e.g. pomegranate in cancer (unpublished work). Recent research has been reviewed and it is suggested that genetic material in plant foods may survive digestion, circulate through our bodies and modulate our gene expression. (Hirschi, 2012) Much work is still being done by various research groups on the therapeutic role miRNA from plants

exhibiting medicinal value. It has also been reported by the Zhang *et al*; 2012, that miRNAs could be selectively packaged into micro vesicles and actively delivered into recipient cells where the exogenous miRNAs can regulate target gene expression and recipient cell function. Thus, in addition to serve as biomarkers, these novel classes of miRNAs may also serve as signaling molecule for intercellular communications. The miRNAs are proved to be highly stable to RNase A digestion, boiling, low/high pH, extended storage, and freeze-thaw cycles and so on (Chen *et al*; 2008). Thus, the exogenous miRNAs could easily survive in body when taken through diet and may regulate the gene expression or may serve as triggers for various genes thereby up regulating or down regulating them during various biological processes.

5: CONCLUSION

The miRNAs are involved in variety of physiological and pathological processes in animals, such as cell development to differentiation, apoptosis and cancer. The wide spread discovery of miRNAs in animals and their contribution in significant processes

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inside animal system has drawn the attraction of plant biologists to identify and explore the role of novel miRNAs inside the plant system and even in cross kingdom gene regulation.. The studies demonstrating the role of miRNAs is now being done by making the MIR gene knockouts and the use of bioinformatics tools have also made the task easy. They could also serve as novel biomarkers in plant as well as animal system when monitored for their presence or absence quantitatively. . There may be chances that miRNAs are present in edible portion of several medically beneficial edible crops and when taken they may help in curing a particular disease thereby reducing the load of drugs and side effects caused by them. This opens a new side of plant miRNA research, for validating the medical importance and target of plant miRNAs in animal system..

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