Cutting-edge research on plant miRNAs

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The discovery of widespread microRNAs (miRNAs) in animals encouraged the scientists to explore miRNAs and its corresponding roles in plants, which lead to the discovery of incredible physiological functions of these novel small entities in plant systems biology. This discovery made the study on functional analysis of plant miRNA as one of the hottest research topics, globally. The year 2012 was a remarkable year of fascinating miRNA research, where numerous reports deciphering the plant miRNAs right from its origin and evolution to its diverse functions were published (Box 1). To-date the popular miRNA repository, miRBase, has about 5159 plant entries in its latest release 19 (Griffiths-Jones, pers. commun.).

Identification and characterization of stress-related miRNAs in several plant species were reported more frequently. Gébelin *et al.*[1] identified abiotic stress-related miRNAs in *Hevea brasiliensis* using deep sequencing and *in silico* analysis. The information from plant microRNA database (PMRD) and *Hevea* expressed sequence tag (EST) sequences were retrieved and combined with the LeARN pipeline to identify 10 putatively novel miRNA families and 48 conserved miRNA families. The functional duality of both the families was indicated by the presence of two miRNA class sizes, viz. 24 nt and 23–27 nt. Prediction scanning in EST databases showed that the miRNA targets were involved in the regulation of redox homeostasis[1]. In *Hordeum vulgare* L., 133 novel miRNAs belonging to 50 families and 126 highly conserved miRNAs (58 families) were identified through deep sequencing of a library created from four different stress-treated transcripts and their targets were predicted using the psRNATarget tools. The study highlighted the presence of novel candidates, miR-n026* and miR-n028 which may be putatively involved in potential regulation of abiotic stress response[2]. A genome-wide identification of heavy-metal stress-related miRNAs in *Brassica napus* was made by Zhou *et al.*[3]. In the study, four small RNA libraries and four degradome libraries were constructed from cadmium (Cd)-treated and non-treated roots and shoots of *B. napus* seedlings. Next generation sequencing identified the presence of 84 conserved and non-conserved miRNAs (belonging to 37 miRNA families), including 19 novel miRNAs. Northern hybridization analysis showed a significant differential expression of more miRNAs in shoot when compared with root during Cd exposure[3].

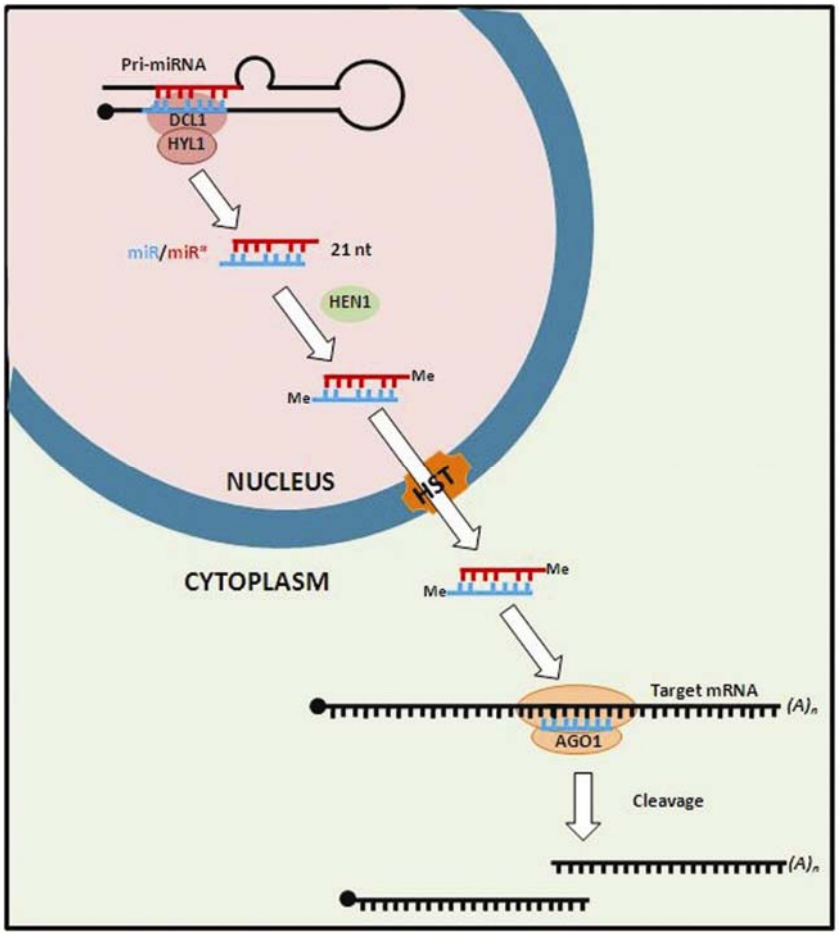
Recently, the origins and evolutionary dynamics of miRNA genes in ten plant species, with reference to animal model *Drosophila* were reported[4]. The results indicated that the plant miRNA genes have formed primarily during duplication of pre-existing miRNA genes or protein-coding genes. It was noted that the abundance of miRNA genes has significantly high inland plant lineage, but the number has altered in a lineage-specific manner following the divergence of eudicots and monocots[4]. Moreover, it was found that the transposable elements also assisted in the evolution of species-specific miRNA genes in plants. The mechanism of miRNA origin is relatively different in *Drosophila*, where the formation of hairpin structures in genomes is the predominant source of miRNA genes and this variation is plausibly due to the variation in the binding of miRNA to target mRNAs. Interestingly, it was found that the juvenile miRNA genes are less conserved than old genes in both plants and *Drosophila*. However, nearly half of the gene families in the ancestor of flowering plants have been lost in at least one species studied, thus indicating that the repertoires of miRNA genes have been adapted more dynamically than previously hypothesized during evolution[4].

As the miRNAs have extensive complementarity to their targets, the identification of miRNAs is usually made by the use of experiential factors predicted from known miRNA–target interactions, but the main drawback of this strategy is that, it could not be applied for identifying new targets. To tackle this, Chorostecki *et al.*[5] developed a strategy which is primarily based on the conservation of the potential regulation in different species. They demonstrated the feasibility of this strategy in *A. thaliana* and identified 3 novel targets (MiR159, MiR408, MiR167) containing bulged nucleotides. MiR159 controls MYB transcription factors and NOZZLE, which regulates the formation of stamen and pollen. MiR408 controls both the copper transporter PAA2 (P-type ATPase of *Arabidopsis*) and copper-binding proteins. MiR167 regulates auxin response factors (ARFs) and IAA-ALANINE RESISTANT 3 (IAR3), which are involved in regulation of auxin synthesis and activity. Moreover, this strategy could be easily customized to include data from alternate expression libraries, with/or else look for the targets mandatory present in a particular plant species[5]. Besides, Zheng *et al.*[6] had reported a novel algorithm termed SeqTar, for decoding the sRNA-induced cleavages captured in degradomes and for identifying novel sRNA targets. SeqTar comprises two statistics, one will compute the miRNA-Target alignment and the other will calculate the abundance of reads at the middle of miRNA complementary site. They proved the capability of SeqTar by applying this algorithm to *Arabidopsis* and rice degradome data sets available in public domains and identified a significant quantity of novel targets for conserved and non-conserved miRNAs besides the reported ones. Thus, SeqTar is an efficient system for deciphering miRNA targets in plants using degradome data sets[6].

Besides the role of miRNA in post-transcriptional regulation of gene expression by cleaving the mRNAs via RNA-induced silencing complexes (RISCs), they play a vital task in the production of trans-acting siRNAs (tasiRNAs) depending on the characteristics of the target transcripts, through the phenomena called transitivity[7]. Zhang *et al.* reported the consequences of target site position and sequence complementarity on tasiRNA formation through a modified synthetic tasiRNA system and examined the features of *TAS* transcripts that are imperative for tasiRNA synthesis. A synthetic sequence possessing amiR173 target site and two siRNAs targeting an endogenous mRNA encoding PHYTOENE DESATURASE3 were ligated into the coding region or 3′ UTR (upstream transcription region) of green fluorescent protein (GFP) gene and tasiRNAs were generated in the transgenic seedlings. The tasiRNAs

Box 1. miRNA biogenesis and mode of action.

MicroRNAs are of 21–22 nt size, specifically involved in regulation of gene expression through RNA interference phenomena. It forms an effector complex and degrades complementary mRNA, thus blocking the mRNA from being translated. miRNAs are denoted as 'MiR' followed by the respective number.



Based on the well-established miRNA biogenesis and mode-of-action pathway, transcription of miRNA genes in the nucleus generates pri-miRNA, which is then processed by the Dicer activity of the DCL1/HYL1 (DICER-LIKE1/HYPONASTIC LEAVES1) complex into 21 nt miRNA/miRNA* duplex. The miRNA duplex is further methylated (Me) by the methylase activity of HEN1 (HUA ENHANCER1). Methylated miRNA duplex was exported from the nucleus to cytoplasm assisted by HST (HASTY). In cytoplasm, the mature miRNA is loaded into the AGO1 (ARGONAUTE1) protein, while the miRNA* strand is degraded. The AGO1 protein induces cleavage of target mRNA strand through the 'slicer' activity of AGO1.

were found to be high efficiently formed when the miR173 target site was positioned immediately next to the stop codon. Introducing premature stop codons caused a remarkable reduction of tasiRNAs and over-accumulation of 3′ cleavage products, suggesting positive effects of translation on processing the 3′ cleavage products into tasiRNAs. Mutating the end of miR173 target site showed the essentiality of perfect complementarity between the 3′ end of miR173 and the 5′ end of the target sequence, whereas mutation at 5′ end showed insignificant effect. It provided new insights into tasiRNA biogenesis and a strategy for improving the efficiency of RNA interference (RNAi) using tasiRNAs[7]. Manavella *et al.*[8] demonstrated that the miRNA:miRNA* strand (though 22-nt length are sufficient, it is not essential for this pathway), which is not part of active RISC, and its asymmetrically positioned bulged bases in the duplex can influence miRNA triggered transitivity.

The possibility of utilizing miRNAs for the systematic silencing of plant genes was revealed by Felippes *et al.*[9] and the phenomenon is termed miRNA-induced gene silencing (MIGS). It was demonstrated in *A. thaliana* and *N. benthamiana*, exploiting the ability of non-conserved miR173 to trigger tasiRNA production from its primary target, which is provided on the MIGS vector. The

tasiRNAs can then target an endogenous mRNA and silence them. To facilitate its application, they have also developed a set of suitable vectors that can be used in different plant species. In contrast to other gene silencing strategies such as virus-induced gene silencing (VIGS), hairpin RNA interference (hpRNAi) and artificial miRNA (amiRNAs), MIGS has the benefit of generating vectors easily through a single PCR step[9]. Hence MIGS can be effectively utilized in silencing a single gene or multiple unrelated genes for functional genomics studies.

In vitro experimentations on global expression analysis of miRNA involve complementary DNA (cDNA) synthesis and microarray or quantitative real-time PCR (qRT-PCR) assisted with Northern blotting. As reliable quantification of mature miRNA levels is crucial to understand their function, the cDNA synthesis is a highly decisive step in this process. The cDNA synthesis is done either by polyadenylation or hairpin priming (stemloop RT). Long-term controversies exist in the reliability of both the methods and recently Adhikari *et al.*[10] made a comprehensive study on this aspect and reported that the hairpin priming is better suited than poly-A tailing to generate cDNA for qPCR because the latter might lead to erroneous abundance measurements, possibly depending on the methylation status of mature miRNAs[10].

In an interesting study, Zhang *et al.*[11] showed that the ability of plant small RNAs ingested through food can directly affect gene expression in animals upon getting migrated through the plasma and released to tissues. They discovered the presence of an exogenous plant miRNA, MiR168a which is abundant in rice is also present in the sera and tissues of human and mice subjects. Functional studies *in vitro* and *in vivo* revealed that MIR168a may attach to the animal low-density lipoprotein receptor adaptor protein 1 ((LDLRAP1) mRNA), hinders its expression in liver and subsequently reduce low-density lipoprotein (LDL) removal from plasma[11]. However, this report on cross-kingdom regulation by miRNA has been partially disproved by Zhang *et al.*[12]. Through computational and experimental analyses, they revealed the occurrence of plant miRNAs in animal sRNA datasets and significantly the MiR168 was exceedingly over-represented. To study the possibility of plant-derived miRNA accumulation and movement in insects, feeding studies for three insects including corn rootworm were conducted. Their analyses ultimately suggested that the observed plant miRNAs in animal sRNA databases can originate in the process of sequencing and the accumulation of plant miRNAs via dietary exposure is not widespread among animals[12].

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