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M. B. Mohamed Sathik & Linu Kuruvilla

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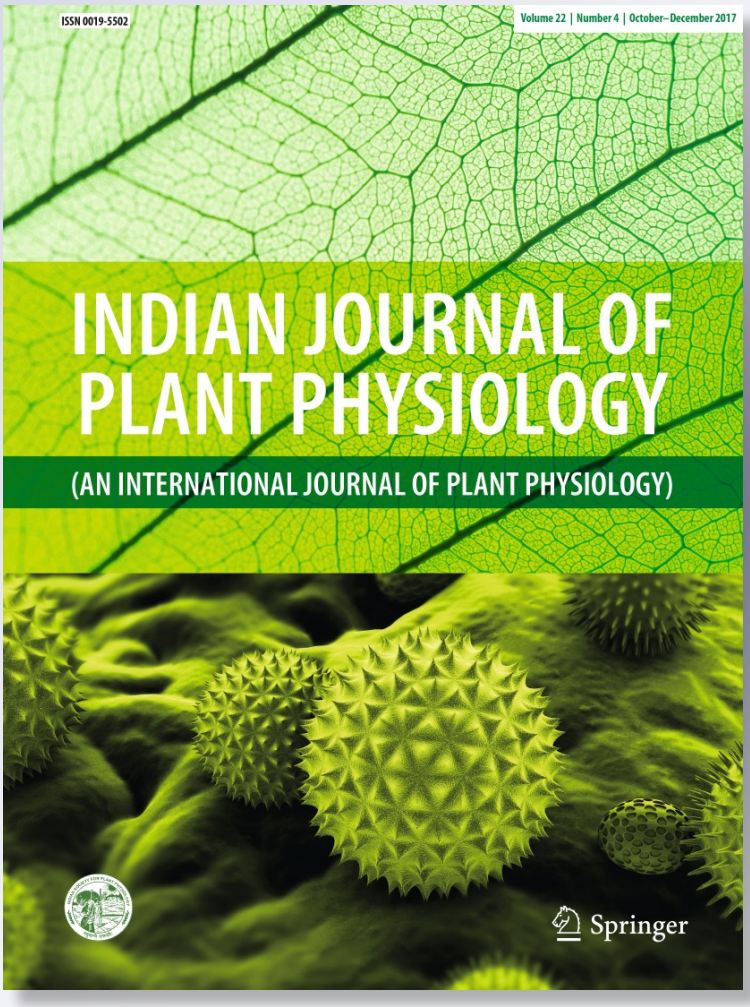

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REVIEW ARTICLE

Developments in research on abiotic stress responsive microRNAs of *Hevea brasiliensis*

M. B. Mohamed Sathik[1] · **Linu Kuruvilla**[1]

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Abstract Abiotic stresses such as drought and low temperature are the major environmental factors that restrict the expansion of *Hevea brasiliensis* (rubber tree) cultivation to non-traditional regions of India. The *H. brasiliensis* cultivars, which are proven superior in traditional regions, do not perform well in such regions and hence it is imperative to find/develop stress tolerant cultivars specifically for these regions. Marker assisted selection for stress tolerance is widely employed to minimize time required to develop such cultivars with desired traits. In this review, developments in abiotic stress responsive gene expression studies in *Hevea* with special reference to miRNA research are discussed. The recent researches in this field have revealed the nature of abiotic stress responsive miRNAs and their possible role on their corresponding target genes. These attempts indicate the possibility of employing specific stress tolerance associated miRNAs in the crop improvement programmes by the breeders to identify or develop drought/cold-tolerant cultivars of *H. brasiliensis.*

Keywords Abiotic stress · Drought · Cold stress · Gene expression · *Hevea brasiliensis* · MicroRNA

Introduction

Hevea brasiliensis, a native of the Amazonian rain forest in Brazil, is the major source of natural rubber (NR). Tropical environment with hot humid wet weather and plenty of sunshine is the ideal agro-climate for rubber cultivation. Due to non-availability of land in traditional rubber growing regions, NR cultivation is being extended to non-traditional areas of India, which are known for their adverse climatic conditions that limit the growth, development and productivity of *Hevea*. These include North Konkan region where the summer will be severe and northeastern regions of India where the temperature during winter is too low. The best performing cultivars of *Hevea* being cultivated in traditional regions do not perform well in such regions, as they are inherently sensitive to such extreme weather conditions. Though the perennial nature of *Hevea* makes developing improved varieties a tedious and time-consuming process, it is essential to identify or develop cultivars that can withstand such extreme weather factors without compromising on yield and productivity. The attempts made to screen for drought and cold tolerant cultivars, strengthened the crop improvement programmes aimed at developing varieties suitable for the non- traditional regions.

Abiotic stress responses in plants

Plant growth and development is highly dependent on a variety of environmental conditions such as temperature, light, water availability and soil conditions that strongly affect the growth and productivity of crops worldwide. Abiotic stress can be defined as the negative impact of non-living factors on the living organisms in a specific environment. Abiotic stress inflicts various deleterious effects at the molecular, biological and physiological levels (Yamaguchi-Shinozaki and Shinozaki 2006). Since abiotic stress disrupts many normal cellular functions, plants resort to quick and extensive molecular reprogramming both at

✉ M. B. Mohamed Sathik
sathik@rubberboard.org.in

[1] Rubber Research Institute of India, Rubber Board, Kottayam 686009, India

the transcriptional and post-transcriptional level in order to recover from the stress effects. Response to abiotic stress in plants depends on a number of factors including the developmental stage, severity of stress, age, plant species and the cultivar (Le Gall et al. 2015). The most studied abiotic stress conditions are cold, high temperature, salt, and drought stress. Plants exhibit a wide range of stress response mechanisms at the whole plant, tissue, cellular and molecular levels for the metabolic adjustment and gene expression regulation to enhance physiological and morphological adaptation (Fig. 1). To develop novel effective molecular strategies for enhancing stress tolerance, understanding the mechanism of stress perception and downstream gene regulatory pathways is of paramount importance.

Small RNAs as regulators of gene expression in plants

Post-transcriptional regulation of gene expression is one of the complex gene regulatory mechanisms employed by plants in response to development, biotic and abiotic stresses. Small-RNA-mediated gene expression regulation has emerged as one of the fundamental principles in cell function (Meister 2013). Small RNAs are 20–30 nucleotide (nt) non-coding RNAs, which guide several regulatory processes in a wide range of eukaryotic organisms (Shukla et al. 2008; Chen 2009; Khraiwesh et al. 2012; Jeong and Green 2013). Based on their size, biogenesis, mode of action and regulatory role, three distinctive types of small RNAs viz., microRNAs (miRNAs), short interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) have been well characterized in animals and plants. Although both miRNAs and siRNAs are products of RNA precursor transcripts by the RNase III endonuclease Dicer-like proteins, the 21–24 nt siRNAs are generated from long double-stranded RNAs, which give rise to multiple siRNA species from both strands while the 21–22 nt miRNAs are derived from single-stranded RNA precursors that form imperfect hairpin structures (Axtell and Bowman 2008).

In plants, the biogenesis and function of siRNAs and miRNAs are controlled by a group of three protein families viz., RNA-dependent RNA polymerases (RDRs), Dicer-like (DCLs) and ARGONAUTES (AGOs) proteins. The DCL RNAse III endonucleases process the hairpin RNA precursors into 20–24 short double-stranded duplexes with a two nucleotide 3′ overhangs (Margis et al. 2006) while the RDRs produce dsRNAs by synthesizing second strand from an RNA template, which is an essential step in the siRNA biogenesis pathway (Zong et al. 2009). The AGO proteins effect the downstream silencing function by forming complexes with the small RNAs to target the mRNA transcripts for slicing or translation repression

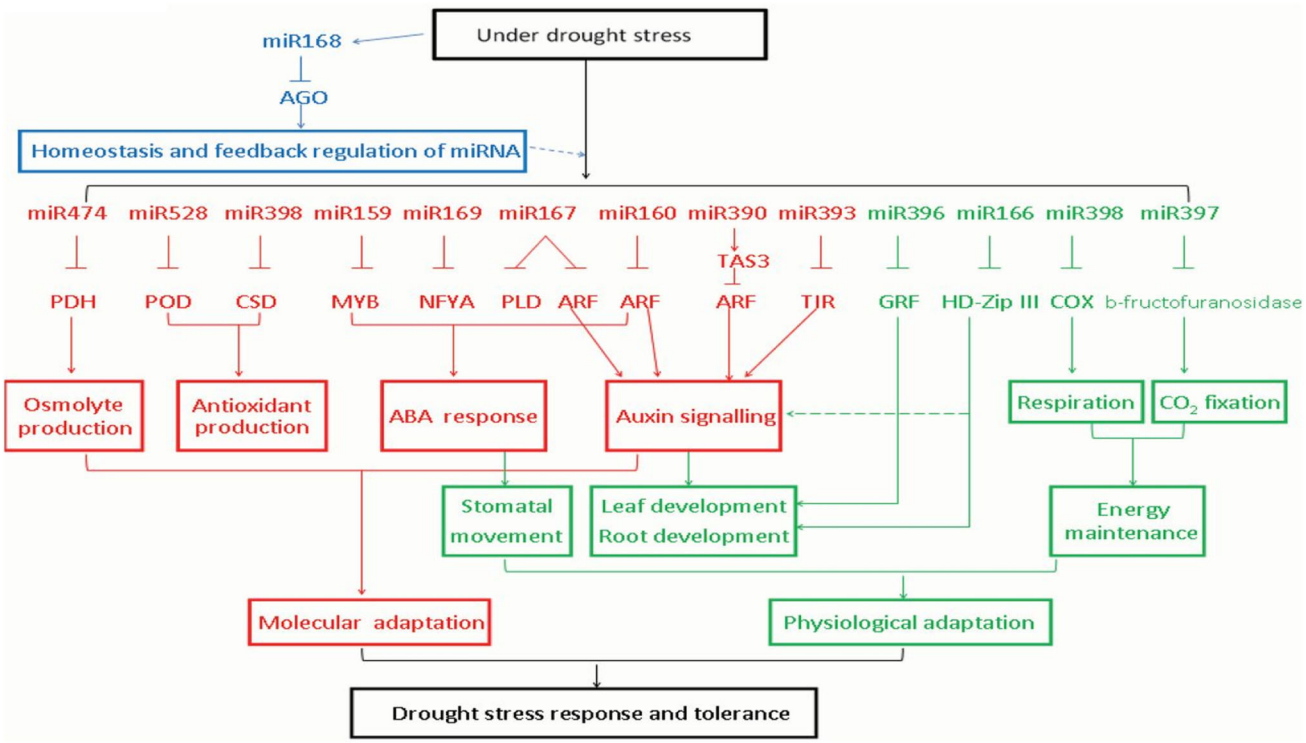

Fig. 1 Drought responsive regulatory networks involving miRNAs and their target genes in plants (Ding et al. 2013)

(Vaucheret 2008). Although biogenesis and functions of miRNAs and siRNAs are almost similar, they require distinct set of Dicer-like and AGO proteins for their biogenesis and target recognition (Jones-Rhoades et al. 2006).

Identification of miRNAs in plants

In order to identify and elucidate miRNA function in both plant and animal kingdoms, computational as well as experimental methods are widely employed. The conventional sequencing of relatively small-sized cDNA libraries of plant sRNAs from *Arabidopsis,* rice and poplar with Sanger method had led to the conclusion that plant miRNAs are highly conserved (Axtell and Bartel 2005). Several studies show that most known mature miRNAs are evolutionarily conserved within the plant kingdom, which makes performing computational search for new miRNA homologues or orthologues in other plant species much easier (Zhang et al. 2006). Although numerous miRNAs were identified by computational algorithms, they were not found appropriate for species with less annotated genomes (Chen and Xiong 2012).

Continued technical improvements and decreasing cost of next-generation sequencing technology have made RNA sequencing (RNA-seq) a popular choice for gene expression studies. Subsequently, deep sequencing approach which can generate millions of sequences per run that can be used for the genome-wide identification of all potential miRNAs and their expression levels, became the most commonly used strategy for plant miRNA study. High throughput sequencing of small RNA libraries has also revealed an unexpected diversity and greater abundance of endogenous siRNAs in plants (Rajagopalan et al. 2006). The first release of miRBase in the year 2002 included a total of l5 miRNAs from *Arabidopsis thaliana.* This was followed by *Oryza sativa* in the year 2003. There after, miRNAs were reported from *Medicago truncatula, Glycine max* and *Populus trichocarpa* in the year 2005. The current version of miRBase (release21) includes 48,496 mature plant miRNAs derived from 6992 hairpin precursors reported in 73 plant species (Tripathi et al. 2015). The number of identified plant miRNAs keeps increasing and accordingly their target genes are also being identified.

High throughput sequencing technologies have an important role in identification and characterization of miRNA targets with parallel analysis of RNA ends (PARE) or Degradome sequencing. This involves sequencing of the entire pool of cleaved targets followed by mapping of the miR-guided cleavage sites (Ding et al. 2012). High throughput sequencing and degradome analysis identified several stress induced miRNAs and their targets in maize (Liu et al. 2014), tomato (Cao et al. 2014), *Raphanus sativus* (Wang et al. 2014), Populus (Chen et al. 2015) rice (Qin et al. 2015), *Phaseolus vulgaris* (Formey et al. 2015) and barley (Hackenberg et al. 2015). Better understanding of miRNA-guided gene regulations can contribute to improving the abiotic stress tolerance in plants (Sunkar et al. 2006). On the other hand, deep sequencing approaches which generate a large number of sequences and datasets require in depth bioinformatics analysis to extract information in detail.

miRNA function in plants

Most of the miRNAs reported earlier were associated with plant growth and development that target different transcription factors and hormone related genes (Reinhart et al. 2002). Investigations on miRNAs from different plant species revealed its highly conserved nature across the plant kingdom (Willmann and Poethig 2007; Groszhans and Filipowicz 2008). Since miRNA targets the mRNA in a sequence specific manner, it can be presumed that they have similar functional role across different plant species. Most of the miRNAs target transcription factor genes are involved in leaf, shoot and root development, floral identity, flower development, flowering time, hormone signaling and vascular development (Llave et al. 2002; Palatnik et al. 2003; Achard et al. 2004; Mallory et al. 2004; Kim et al. 2005; Jones-Rhoades et al. 2006). Various researches on miRNA indicate the existence of conserved miRNAs in plant species as well as species-specific miRNAs. This suggests that conserved miRNAs may regulate common traits in plants, such as plant morphology and phase change, and that species-specific miRNAs may control unique and variable processes in individual plant species, such as fibre initiation and development in cotton (Xie et al. 2015). Both conserved and species-specific miRNAs are involved in plant's response to abiotic stress.

miRNAs and abiotic stress responses in plants

During the course of evolution, plants evolved complicated physiological and genetic mechanisms in order to cope with and adapt to the harsh environment. Most of the conserved miRNAs are known to have key roles in plant development and adaptive responses to abiotic stresses by targeting a variety of transcription factors (TFs) (Sunkar and Zhu 2004; Sunkar et al. 2006; Todesco et al. 2010). Abiotic stresses influence synthesis of new miRNAs to cope with the effects of stress. The abiotic stress responsive role of miRNAs in plants was initially suggested after obtaining data from miRNA target prediction, expression profiling studies of miRNAs during plant response to

abiotic stress, and NCBI expressed sequence tags (ESTs) surveys (Zhang 2015).

Involvement of miRNAs in plant abiotic stress came from the identification of miR398 (Sunkar et al. 2006), which targets two Cu/Zn superoxide dismutases (SODs). SODs (cytosolic-CSD1 and chloroplastic CSD2) convert reactive oxygen species (ROS) produced during regular metabolism to less toxic hydrogen peroxide. But during abiotic stress, enhanced production of ROS results in its accumulation to toxic levels (Apel and Hirt 2004; Sunkar et al. 2007) which need to be quickly scavenged. Detailed study on the expression of Cu/Zn SODs during oxidative stress conditions revealed that they are under post-transcriptional control by miR398, indicating the key role of miRNA-mediated regulation of SODs during abiotic stress. In rice, miR169 family members were induced by drought and salinity stress (Zhao et al. 2009) while miR396 was found responsive to high salinity, drought and cold stresses (Liu et al. 2008). These initial studies on the role of miRNAs in plants' response to environmental stresses started attracting attention of many researchers.

Plant miRNAs, target transcripts in a sequence-specific manner, which allowed Jones-Rhoades and Bartel (2004) to predict and validate ATP sulphurylase (APS), the enzyme that catalyses the first step of inorganic sulphate assimilation, as the target of miR395, which is responsive to sulphate levels in plants. Based on further analysis on the response of miR395 to cellular sulphate levels, they found expression of miR395 to be depending upon sulphate availability. Expression of miR399, which targets ubiquitin-conjugating enzyme (UBC), was induced during low-phosphate stress. In *Arabidopsis,* UBC mRNA accumulation is decreased during low-phosphate stress for the induction of phosphate transporter gene *AtPT1* and attenuation of primary root elongation (Chiou et al. 2006). Overexpression of miR399 even under high phosphate conditions led to the down regulation of UBC and induced accumulation of phosphate. Conversely, *mir399*-UBC mutants showed limited induction of *AtPT1* under low-phosphate conditions and showed limited attenuation of primary root elongation. Sunkar and Zhu (2004) found miR393 miR397b and miR402 to be strongly induced by various stress conditions (cold, dehydration, NaCl, and ABA treatments). In contrast, miR389a.1 was inhibited by all the stress treatments, which was found related to ta-siRNAs (trans-acting siRNA; Allen and Howell 2010). miR319 was found induced only by cold and not by salinity, dehydration, or ABA stresses.

There are many reports available on drought associated miRNAs from many plant species such as *Arabidopsis* (Sunkar and Zhu 2004; Liu et al. 2008), tobacco (Frazier et al. 2011), *Phaseolus vulgaris* (Arenas-Huertero et al. 2009), populus (Shuai et al. 2013), cowpea (Barrera-Figueroa et al. 2011), soya bean (Kulcheski et al. 2011), and rice (Zhou et al. 2010) (Table 1). In drought stressed *Arabidopsis,* miR159, miR156, miR167, miR171,miR168, miR172, miR319, miR393, miR394a, miR395c, miR395e, miR396 and miR397 were found up-regulated, while miR161, miR168a, miR168b, miR169, miR171a and miR319c were found down-regulated (Liu et al. 2008; Sunkar and Zhu 2004). In tomato, up-regulation of miR169 under drought led to the down regulation of its targets NF-YA1/2/3. In tomato, overexpression of miR169 resulted in enhanced drought tolerance with reduced stomatal opening, transpiration, and leaf water loss (Zhang et al. 2011). Contrarily, in response to drought in *Arabidopsis,* expression of NFYA5 got strongly up-regulated while miR169 got down regulated (Li et al. 2008). Generally under abiotic stress situations, MIR169 family members exhibit up-regulation in both monocots and dicots except for few cases where down regulation was also observed (Xu et al. 2014). In some plant species, members of the same miRNA families were found differently expressed under drought stress. Trindade et al. (2010) reported up-regulation of miR398a/b in *M. truncatula* under drought stress whereas in another study it was found repressed (Wang et al. 2011). Under drought conditions, these miRNAs get regulated by their corresponding regulators thus reflecting in the levels of miRNAs and their respective targets (Reyes and Chua 2007; Trindade et al. 2010). It is also possible to identify the functional role of both the conserved and specific miRNAs in each plant species by target validation.

Post-transcriptional regulation of gene expression plays an important role in response to low temperature stress (Chinnusamy et al. 2007). Previously in *Arabidopsis,* five miRNAs were reported cold responsive (Sunkar and Zhu 2004). Later in *Arabidopsis* seedlings, miR168, miR171 and miR396 were found induced by drought, cold and salt stresses (Liu et al. 2008), suggesting that miRNAs are involved in the pathways common to all these stimuli. Zhou et al. (2008) identified four cold inducible *MIR* genes (miR165, miR166, miR169 and miR172) from *Arabidopsis.* miR166 family were also found up-regulated in similar conditions in rice, while miR168, miR169 and miR171 showed opposite expression profiles (Lv et al. 2010). Interestingly, most of these conserved cold regulated miRNAs are known to target TFs with known roles in plant development (Jones-Rhoades and Bartel 2004), suggesting the miRNA-mediated responses at structural level.

Regulatory motifs associated with cold response such as W-box (TTGAC), ABRE-core (ACGTGG/TC) and LTRE-core (A/GCCGAC) were found in abundance on the promoter region of cold inducible *MIR* genes (Zhou et al. 2008) suggesting the regulation of stress-responsive miRNAs at transcriptional level. In *Brachypodium*, 25 cold stress responsive miRNAs were identified of which only

Table 1 Drought-responsive miRNAs in plants (adopted from Ferdous et al. 2015)

miRNA	Target name and function	Species	Reference
miR156	SBP family of transcription factors—promote phase transitions, flowering time	*Ath*↑, *Tdi*↑, *Hvu*↑, Rice↓ *Peu*↑, *Ppe* (slightly)↑, *Pto*↓	Eldem et al. (2012), Kantar et al. (2011), Liu et al, (2008), Ren et al, (2012) and Zhou et al. 2010
miR157	SBP family of transcription factors	*Ppe*↑↓	Eldem et al. (2012)
miR159	MYB and TCP transcription factors—ABA response, Nacl stress response, floral asymmetry and leaf development	Ath↑ Rice↓ Ppe↓Ppe↑, Pto↑, Ptc↓	Arenas-Huertero et al. (2009), Eldem et al. (2012), Jones-Rhoades and Bartel (2004), Liu et al, (2008), Reyes and Chua (2007) and Zhou et al, (2010)
miR164	NAC domain TF—lateral root development	Mtr↓, Ptc↓, Bdi↓	Shuai et al, (2013) and Wang et al, (2011)
miR160	ARF 10, ARF 16 and ARF 17—seed germination and postgermination stages	Ppe↑, Pto↑, Ptc↓	Eldem et al. (2012), Jones-Rhoades and Bartel (2004), Liu et al. (2007), Ren et al. (2012) and Shuai et al, (2013)
miR166	HD-ZIPIII transcription factor—axillary meristem initiation, leaf and vascular development	Tdi↓, Gma↑	Kantar et al. (2011), Li et al. (2011a, b), Sun et al. (2012) and Williams et al. (2005)
miR167	ARF6 and ARF8—gynoecium and stamen development	Ath↑, Ppe↓, Pto↑	Eldem et al. (2012), Liu et al. (2008), Ren et al.(2012) and Wu and Poethig (2006)
miR168	ARGONAUTE1, MAPK—miRNA biogenesis and mRNA degradation, plant development	Ath↑ Rice↓ Z. mays↓	Liu et al. (2008), Wei et al. (2009) and Zhou et al, (2010)
miR169	NF-YA transcription factor subunit A-3, NF-YA transcription factor subunit A-10, SlMRP1—Plant development and Flowering timing, response to different abiotic stresses	Ath↓, Tomato↑, Rice↑, Mtr↓, Ppe↓, Gma↑, Pto↓, Peu↑	Eldem et al. (2012), Li et al, (2008), Li et al. (2011a, b), Qin et al. (2011), Ren et al. (2012), Trindade et al. (2010), Wang et al. (2011), Zhang et al. (2011), Zhao et al. (2007) and Zhou et al. (2010)
miR171	GRAS transcription factors—response to abiotic stresses and floral development	Ath↓, Rice↓	Sun et al. (2012) and Zhou et al. (2010)
miR393	TIR1 and AFB2 and AFB3—susceptibility to virulent bacteria	Ath↑ Ppe↓	Liu et al. (2008), Navarro et al. (2006) and Eldem et al. (2012)
miR394	Dehydration-responsive protein and F-box proteins—abiotic stress-response pathway	Pto↑, Ptc↓, Gma↑	Li et al, (2011a, 2011b), Ren et al. (2012) and Shuai et al. (2013)
miR395	Sulphate transporter—response to sulphate deprivation	Rice↑, Ppe↓, Pto↓	Eldem et al. (2012), Liang et al. (2010), Ren et al. (2012) and Zhou et al. (2010)
miR398	Copper superoxide dismutases; cytochrome C oxidase subunit V—Copper homoeostasis, oxidative stress; enzyme involved in respiration	Mtr↑, Tdi↑, Mtr↓, Ppe↓	Eldem et al. (2012), Jones-Rhoades and Bartel (2004), Kantar et al. (2011), Sunkar et al., (2006), Trindade et al. (2010) and Wang et al. (2011)
miR1432	Poly (ADP-ribose) polymerase; calcium binding EF hand domains—activate in signal transduction pathways	Tdi↑	Kantar et al. (2011) and Zhang et al. (2009)

three miRNAs (miR397, miR169 and miR172) were found up-regulated (Zhang et al. 2009). As in drought stress, under cold stress also members of same miRNA family exhibited different response patterns. In cassava, differential expressions of miRNAs were observed between two cultivars (SC124 and C4) under cold stress. In SC124, most of the miRNAs were down regulated, but in cultivar C4 only four miRNAs were down regulated and 31 miRNAs were up-regulated (Zeng et al. 2010). These results indicate the regulation of miRNA at both species and variety/cultivar levels. Comparative profiles of cold stress influenced miR expression among *Arabidopsis, Brachypodium,* and *Populus trichocarpa* revealed the up-regulation of miR397 and miR169 indicating the presence of conserved cold responsive pathways in all the species.

Cultivar-dependent response of miRNAs to abiotic stress

Different cultivars of the same plant species may show differential gene expression due to difference in individual plant growth conditions and due to the human interventions in cultivated crops compared with their wild relatives. The cultivar-dependent response of miRNAs to abiotic stresses is not only different among plant species but also found

to vary among cultivars of the same species (Zhang 2015). It is well known that the varieties within a plant species may differ in their capacity to respond to abiotic stress. When the impact of drought treatment on two cowpea cultivars (drought-tolerant IT93K503-1 and drought-sensitive CB46) was investigated using deep sequencing (Barrera-Figueroa et al. 2011), 20 miRNAs were found differentially expressed. Of these, nine got highly expressed in one of the two cultivars but not in the other. Simultaneously, they also identified 11 drought-regulated miRNAs in only one cultivar while they were absent in the other. miRNA expression profiles of two cotton cultivars with varying levels of tolerance to salinity (SN-011 with high tolerance to salinity and LM-6 with sensitivity to salinity) (Yin et al. 2012) indicated the expression of 12 miRNAs in a cultivar-specific pattern. Under salinity treatment, four miRNAs (miR156, miR169, miR535, and miR827) showed significantly higher expression in LM-6 while expression of three miRNAs (miR167, miR397, and miR399) got significantly inhibited. Mondal and Ganie (2014), identified 12 polymorphic miR-SSRs (simple sequence repeats) by comparing 12 salinity-tolerant and 12 salinity-susceptible cultivars in rice which indicated lesser variability of miRNA genes in the tolerant cultivars than in the susceptible cultivars. Ma et al. 2015, also reported the opposite patterns of expression of 13 miRNAs in response to dehydration stress in two wheat cultivars viz. Hanxuan10 (drought tolerant) and Zhengyin1 (drought-susceptible).

miRNA based genetic modification for developing abiotic stress tolerant plants

The developments in miRNA research have paved way for manipulating miRNA mediated gene regulations to engineer plants for enhanced abiotic stress tolerance (Zhang and Wang 2015). Due to their vital role in complex gene regulatory networks, miRNAs may prove potent targets for improving tolerance to abiotic stresses in plants. There are several methods employed for miRNA manipulations, which include over-expression/repression of stress-responsive miRNAs and/or their target mRNAs, miRNA-resistant target genes, target-mimics and artificial miRNAs (Zhou and Luo 2013). Over-expression of *gma-miR394a* in *Arabidopsis* showed enhanced drought tolerance (Ni et al. 2012). Transgenic *Arabidopsis* over-expressing *miR394* as well as *LCR* (*LEAF CURLING RESPONSIVENESS,* a target of *iR394*) *lcr* mutants exhibited enhanced cold stress tolerance, indicating the involvement of *miR394* and its target gene LCR in low-temperature responses in plants (Song et al. 2016). Over-expression of *gma-miR172* in *Arabidopsis* revealed enhanced water deficit and salt tolerance (Li et al. 2016). *MiR156* over-expressing rice plants showed reduced cold tolerance (Cui et al. 2015). Over-expression of *osa-miR319a* in creeping bentgrass (*Agrostis stolonifera*) significantly improved the salt and drought tolerance in transgenic plants (Zhou et al. 2013).Transgenic rice over-expressing *miR319* showed enhanced cold tolerance (Yang et al. 2013).

miRNA based markers

DNA-based molecular markers have been widely employed in crop improvement programmes. Interestingly, miRNA based molecular markers are functional markers that were exploited mainly in animal sciences, but were lesser reported in plants. The higher level of conservation found in miRNA sequences provides an opportunity to develop novel molecular markers (Table 2). Yadav et al. (2014) used miRNAs as genetic markers for genotyping foxtail millet and related grass species. They could identify 66 miRNA-based markers when they retrieved and aligned pre-miRNA sequences of foxtail millet and other related crops for the identification of conserved regions. In order to understand the genetic diversity of salt responsive-miRNA genes in rice, SSR markers were mined from salt-responsive miRNA genes and validated in tolerant as well as susceptible rice cultivars (Mondal and Ganie 2014). Although 12 miR-SSRs were found polymorphic, only miR172b-SSR was able to differentiate the tolerant and susceptible cultivars in 2 different groups. miRNA based molecular markers displayed sufficient level of polymorphism in *Silybum marianum* cultivars (Ražná et al. 2015).

miRNAs identified from *Hevea brasiliensis*

The first report on miRNA from *Hevea brasiliensis* was furnished by Zeng et al. (2010) who observed conservation and diverse expression patterns of 23 miRNA families in response to development and abiotic stress in four Euphorbiaceous plants (*Ricinus communis, Manihot esculenta, Hevea brasiliensis and Jatropha curcas* L). However, this approach did not allow comprehensive identification of miRNA families from *Hevea*. This was followed by Gebelin et al. (2012) who reported 48 conserved miRNA families and 10 putative novel miRNA families from plantlets subjected to abiotic stress by deep sequencing. They also predicted miRNA targets involved in stress response, antioxidant activity and transcription regulation. Similarly, 115 miRNAs belonging to 56 families were identified and 20 novel miRNAs were predicted through high throughput sequencing in high (PB 260) and low yielding (PB 217) *Hevea* genotypes (Lertpanyasampatha et al. 2012). They also found miR159/319, miR167, and miR166 families that

Table 2 Over-expression of miRNAs in transgenic plants and their stress response

miRNA	Source of the miRNA gene	Target	Transgenic plant	Expression strategy	Response	Reference
miR156	*Oryza sativa*	SPL	*Oryza sativa*	Overexpression of OsmiR156 k	Decreased cold tolerance	Cui et al. 2015
miR172	*Glycine max*	AP2 like Tfs	*Arabidopsis*	Overexpression of gma-miR172c	Increased water deficit and salt tolerance	Li et al. 2016
miR319	*Oryza sativa*	PCF5 and PCF8	*Oryza sativa*	RNAi	Increased cold tolerance	Yang C. et al. 2013
miR319	*Oryza sativa*	TCP	*Agrostis stolonifera*	Constitutive overexpression of osa-miR319a	Increased drought and salt tolerance	Zhou et al,2013
miR390	*Oryza sativa*	SRK	*Oryza sativa*	Overexpression of miR390	Decreased Cd tolerance/enhanced Cd accumulation	Ding et al. 2016
miR394a	*G. max*	F-box protein	*Arabidopsis*	Overexpression of gma miR394a	Increased drought tolerance	Ni et al. 2012
miR394a	*Arabidopsis thaliana*	LCR	*Arabidopsis*	Overexpression of miR394a/LCR loss of function mutant	Increased cold tolerance	Song et al. 2016
miR395	*A. thaliana*	*BnSultr, BnAPS*	*Brassica napus*	Overexpression of miR395 driven by CaMV35S promoter	Shorten or no surface trichomes with delayed transition from juvenile to adult vegetative stage	Huang et al. 2010
miR398	*A. thaliana*	CSD1,CSD2,CCS	*A. thaliana*	Loss function of CSD1 and CCs, knockdown mutant of CSD2	Increased thermo tolerance	Guan et al. 2013
miR399	*A. thaliana*	IPS-1	*Solanum lycopersicum*	Overexpression of *Ath-miR399d* under control of *rd29A* promoter	Better growth performance under phosphrous deficiency and low temperature	Gao et al. 2015
miR408	*A. thaliana*	Copper related gene	*Cicer arietinum*	Overexpression of Athpre-miR408	Enhanced drought tolerance	Hajyzadeh et al. 2015

regulate MYB transcription factor, auxin responsive factor (ARF), and type III HD-Zip transcription factors, abundantly represented in leaves. This investigation predicted miRNA targets computationally and identified genes involved in various biological processes including stress responses and rubber biosynthesis.

Subsequently, Gebelin et al. (2013a) reported the regulation of microRNAs in response to different types of abiotic stress and hormone treatments in *Hevea*. A negative co-regulation of *HbMIR398b* with its chloroplastic HbCuZnSOD target messenger was observed in response to salinity in this study. Expression of *MIR159b* gene was found enhanced in response to cold in leaves and bark, as well as in response to jasmonic acid treatment in leaves of juvenile plantlets. Gebelin et al. (2013b) also identified Tapping Panel Dryness (TPD) associated miRNAs and their targets from latex cells and observed the abundance of 21nt size small RNAs in TPD trees as against 24 nt in healthy trees. They also observed a decline in small RNAs in TPD affected trees, due to both RNA degradation and a shift in miRNA biogenesis. Enhanced expression of *Hbpre-MIR159b* gene was also observed in TPD-affected trees. Later, Kuruvilla et al. (2016) demonstrated differential expression of miRNAs among various cultivars of *H. brasiliensis* under drought stress by conventional sequencing method. Four miRNAs viz. miR482, miR164, miR167 and HbmiRn_42 were found to display a definite pattern under drought. The down regulation of miR482 in tolerant cultivars indicated its association with drought tolerance. Similarly, HbmiRn_42, the novel miRNA also exhibited strong association with drought tolerance.

Eventually, Kuruvilla et al. (2017) identified cold stress responsive miRNAs from cold tolerant cultivar RRIM 600 by high throughput sequencing which revealed expression of 218 conserved miRNAs belonging to 21 conserved families and 42 novel miRNAs from the pair-end cDNA sequencing library. Further, DGE analysis revealed eight miRNAs viz. miR166, miR482, miR159, miR171, miR399, miR4995, miR535 and miR858 to be commonly

Table 3 List of abiotic stress responsive miRNA families of *H. brasiliensis* and their predicted targets

miRNA family	Predicted target	References
miR156/	Squamosa promoter-binding protein	Gebelin et al. (2012)
157	APETALA2-like protein	
	flavonoid 3′,5′-hydroxylase	
miR158	Chromosome chr19 scaffold_4	Lertpanyasampatha et al.(2012)
miR159	Serine/threonine protein kinase	Gebelin et al. (2012), Gebelin et al. (2013a, b)
	CuZnSOD peroxysomal	
	ABC transporter C family	
	HMG-CoA_reductase (HMGR)	
	Rubber elongation factor	
miR319	Ferritin putative	Gebelin et al. (2012)
miR160	ARF	Gebelin et al. (2012)
miR161	Hypotetical protein	Gebelin et al. (2012)
miR162	FAD/NAD(P)-binding oxidoreductase-like protein	Gebelin et al. (2012), Lertpanyasampatha et al.(2012)
	ABC transporter C family	
	Zinc finger family protein	
	Glutathione S-transferase GST 14	
miR163	Zinc finger CCHC domain-containing Protein	Lertpanyasampatha et al.(2012)
	Plastid ATP/ADP transport protein 1	
miR164	NAC-domain protein	Gebelin et al. (2012),
	PHAVOLUTA-like HD-ZIPIII protein	Lertpanyasampatha et al.(2012)
	DTDP-glucose 4-6-dehydratases-like protein	
miR165/	LRR protein	Gebelin et al. (2012), Gebelin et al. (2013a, b), Kuruvilla et al. (2017)
166	Malate deshydrogenase	
	Abscisic acid insensitive protein	
	Glutathione peroxidase	
	Homeobox-leucine zipper family protein	
mir167	cap binding protein	Gebelin et al. (2012)
miR168	Glyceraldehyde-3-phosphate dehydrogenase B subunit	Lertpanyasampatha et al.(2012)
miR169	Laccase	Gebelin et al. (2012),
	Aldehyde dehydrogenase	Lertpanyasampatha et al.(2012)
	Nuclear transcription factor Y subunit A	
miR170	GRAS domain-containing protein	Gebelin et al. (2012)
miR171	Ribonucleoside-diphosphate reductase	Gebelin et al. (2012), Kuruvilla et al. (2017)
	Glyceraldehyde-3-phosphate dehydrogenase	
	Oxophytodienoate reductase (OPR)	
	Beta-1,3-galactosyltransferase 2	
miR172	NAD(P)H-quinone oxidoreductase	Gebelin et al. (2012), Gebelin et al. (2013a, b)
	APETALA2-like protein	
	Squalene monooxygenase	
	Aspartate aminotransferase	
	Myb family transcription factor	
miR2111	Laccase	Gebelin et al. (2012)
miR2910	Ketol-acid reductoisomerase	Gebelin et al. (2012),
miR2914	FAD Binding domain-containing protein	Gebelin et al. (2012),
miR390	WRKY transcription factor	Gebelin et al. (2012), Lertpanyasampatha et al.(2012)
	Phosphoenolpyruvate carboxylase	
	Heat shock protein	
	NADH-ubiquinone oxidoreductase chain 1	

Table 3 continued

miRNA family	Predicted target	References
miR393	APETALA2-like protein	Lertpanyasampatha et al.(2012)
	Auxin-responsive factor TIR1-like protein	
miR395	2OG-Fe(II) -dependent-oxygenase-like protein	Gebelin et al. (2012),
	APETALA2-like protein	Lertpanyasampatha et al.(2012)
	Sulfate adenylyltransferase	
miR396	Zinc finger family protein	Gebelin et al. (2012),
	Lignin-forming anionic peroxidase	Lertpanyasampatha et al.(2012)
	Glutamyl-tRNA reductase	
	Fatty acyl-coenzyme A reductases (FAR)	
	Glutathione peroxidase	
miR397	Laccase	Gebelin et al. (2012)
miR398	Superoxide dismutase [Cu–Zn]	Gebelin et al. (2012),
	CuZnSOD chloroplastique	Lertpanyasampatha et al.(2012)
	HVA22-like protein	
	Serine/threonine protein kinase	
miR399	Phosphate transporter	Lertpanyasampatha et al.(2012)
	Disease resistance protein	
miR408	Cytochrome P450	Gebelin et al. (2012)
	Ribonucleoside-diphosphate reductase	
	Thioredoxin reductase	
miR444	WRKY	Gebelin et al. (2012)
miR447	Glycosyl transferase group 2 family protein	Lertpanyasampatha et al.(2012)
miR472	Mitogen-activated protein kinase kinase kinase	Lertpanyasampatha et al.(2012)
miR476	Heat shock protein	Gebelin et al. (2012)
	Cationic peroxidase 2 precursor	
miR482	Arsenical pump-driving ATPase, putative	Kuruvilla et al.(2017)
miR535	LRR protein	Gebelin et al. (2012)
miR827	Fructose-bisphosphate aldolase	Gebelin et al. (2012)
miR828	MYB class transcription factor	Gebelin et al. (2012)
miR858	Transcription factor Myb1	Lertpanyasampatha et al.(2012), Kuruvilla et al.(2016)
miR2911	TPD_SSH_BC127 TPD responsive transcripts of *Hevea brasiliensis*	Lertpanyasampatha et al.(2012)
miR9386	Phospholipase C 4 precursor,	Kuruvilla et al.(2017)
	Putative[*Ricinus communis*]	
	Aldehyde dehydrogenase family	
miR3630	Phospholipase d delta, putative	Kuruvilla et al.(2017)

expressed in both the control and cold stressed samples. Subsequent validation analysis by qPCR, substantiated the DGE results and revealed miR169, miR159 and miR482 to have stronger association with cold tolerance. The validation experiments demonstrated the distinct association between miR169 and cold tolerance. miR169 is known to regulate its target NF-YA which is known to play vital role in increasing the abiotic stress tolerance in many plants (Li et al. 2008). Down regulation of miR169 in tolerant genotype observed in this study indicated the possibility of up-regulation of its corresponding target mRNA (NF-YA) that can lead to improved stress tolerance (Luke et al. 2015). A similar trend was also observed for miR482 while other miRNAs (miR159, miR166, miR171 and miR858) exhibited up-regulation in tolerant genotype. A complete list of conserved miRNAs families reported so far and their predicted target is furnished in Table 3.

Several novel miRNAs were identified in *H. brasiliensis* (Gebelin et al. 2012; Gebelin et al. 2013b;

Lertpanyasampatha et al. 2012; Kuruvilla et al. 2016). The subsequent target prediction by computational approach revealed genes involved in various biological processes including stress responses and rubber biosynthesis. Gebelin et al. (2012) reported identification of four species-specific latex-specific novel miRNA families from *Hevea* plants. The gene encoding HMG-reductase, a key enzyme in rubber biosynthesis, was predicted to be targeted by miRNAs in plant tissues and not in latex. They identified 48 conserved and 10 putative novel miRNA families. Lertpanyasampatha et al. (2012) identified 115 conserved miRNAs (from leaves) representing 56 families among which miR396 was found most abundant, accounting for close to 50% of the total sequence reads. miR396 was found to target six growth-regulating factor (GRF) genes encoding putative transcription factors that influenced growth and cell proliferation in leaves of Arabidopsis (Wang et al. 2011). In addition to this, miR159/319, miR167 and miR166 families that regulate Myb tf, ARF and type III HD-Zip tf, respectively were found abundantly present in the young and mature leaves. Lertpanyasampatha et al. (2012) also predicted 20 novel miRNA targets which included metabolic enzymes, transcription factors, and protein kinases. This corroborated with the findings on cold and drought responsive transcripts (Sathik et al. 2012; Thomas et al. 2011, 2012) which demonstrated the expression of several transcription factors, protein kinases, etc. in *Hevea*. A novel gene, HbmiRn_42 identified by conventional cloning method also assumes importance as it was found highly up-regulated under drought conditions in tolerant genotypes (Kuruvilla et al. 2016) and predicted to target HMG-CoA reductase (HMGR). In mevalonate pathway, HMGR is involved in the synthesis of mevalonate which is the precursor of downstream isopentenyl pyrophosphate (IPP) and further the isoprenoid compounds that also include natural rubber (Stermer et al. 1994). Up-regulation of HbmiRn_42 in drought tolerant genotypes implied the suppression of HMGR and eventually the rubber biosynthesis. More investigations in this context only can throw more light on the relationship between the aspects of drought and yield in tolerant genotypes.

Future perspectives

Identification of drought and cold responsive miRNAs opens up the possibility of further employing them as marker in crop improvement programmes of *H. brasiliensis*. The information gathered from several reports have beyond doubt proven the importance of miRNAs as important gene regulators (riboregulators) that control the plant's response to abiotic and biotic stresses. miRNA-based gene regulation appears to be most promising towards developing superior cultivars as it regulates gene expression at transcriptional and post-transcriptional levels. This makes over-expression or repression of stress responsive miRNAs and their target mRNAs possible. It would also be possible to manipulate expression of miRNA-resistant target genes by using artificial miRNAs (amiRNAs) to bring out desired changes (Zhou and Luo 2013; Djami-Tchatchou et al. 2017). Employing artificial target mimics is a recently developed technology, which is used to repress the activity of specific miRNAs (Gupta et al. 2015). This method of gene silencing approach has been used efficiently in rice and several plant species from dicots to moss (Khraiwesh et al. 2008; Sharma et al. 2015). While miRNAs can be employed as next generation targets for genetic engineering towards crop improvement with desired traits, a deeper understanding of its potential and side effects would help design suitable strategies to achieve desired traits with lesser undesirable results in the modified crops. However, there is still a long way to go for effective use of this strategy (Shriram et al. 2016) towards producing abiotic stress tolerant plants.

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