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INVOLVEMENT OF PLANT MICRORNAS IN ABIOTIC STRESS RESPONSES

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ABSTRACT

Crop production is constrained by several biotic and abiotic stresses. Several techniques have been applied by different research groups to unravel the molecular mechanism of tolerance to these stress factors in plants. Recently micro-RNAs have contributed towards understanding the role of micro-RNAs under abiotic stress conditions. Through first reported in animals its abundance in found in plants even with some difference in biogenesis and mechanism of target gene regulation. However, the relationship between micro-RNAs and stress responses is just beginning to be explored. Micro-RNAs are about 21-24 nucleotide single stranded non coding RNA often conserved across species suggesting their evolutionary significance. MiRNAs are either up or down regulated under stress conditions suggesting their involvement in gene expression regulation by post transcriptional degradation or translational repression in plants. A major category of MiRNA target gene consists of transcription factors or other regulatory proteins that function in plant development or signal transduction. One our own research endeavour in this direction revealed the role of MiRNA towards salinity stress response in sugarcane. Several researches worldwide is leading to the identification of thousands of miRNAs, the functional validation of which will help in designing new strategies for improving tolerance to biotic and abiotic stresses. The current review gives the recent status of micro-RNA research towards its role under abiotic stress.

INTRODUCTION

In the 1990s, two independent discoveries on RNAi and miRNAs opened up new avenues in the previously unsuspected world of non-coding RNAs (ncRNAs). The phenomenon of RNA interference (RNAi) was being uncovered as co-suppression in plants (Napoli et al., 1990; Krol et al., 1990), quelling in fungi (Romano and Macino, 1992; Cogoni and Macino, 1997) and RNAi in animals (Fire et al., 1998). At the same time in 1993, Lee and colleagues discovered another curious phenomenon in nematodes, wherein they observed that product of gene lin-4 do not code for a protein (oligopeptide), instead produce a pair of short RNA transcripts (microRNA) and each regulate timing of larval development by translational repression of lin-14 that encode a nuclear protein (Lee et al., 1993). Reinhart et al., 2002 reported that miRNAs are also present in plants and suggest that miRNAs could play regulatory roles in development of plants. The two worlds of RNAi and miRNAs merged when it was observed that both RNAi and miRNAs (micro RNAs) employed the same mechanism in

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regulating the eukaryotic gene expression at post transcriptional level (Du and Zamore, 2005). The universe of small RNAs has expanded to include miRNAs, endogenous small interfering RNAs (siRNAs), 21U RNAs, and Piwi-interacting (piRNAs) RNA (Kim, 2005). However, of these small RNA classes, only miRNAs form a characteristic thermodynamically stable hairpin structure. That stable hairpin makes miRNA prediction in sequenced genomes a relatively tractable exercise.

miRNAs and short interfering RNAs (siRNAs) are small single stranded noncoding RNAs that have recently emerged as important regulators of mRNA degradation, translational repression, and chromatin modification. MicroRNAs (miRNAs) are endogenous 21–24 nucleotide, single stranded RNA product of a non-protein-coding RNA gene (Bartel, 2004). It is abundant class of tiny RNA and often found to be conserved evolutionary across species boundaries. miRNAs are highly specific regulators of target gene expression and derived from larger precursors that are transcribed from non-protein coding RNAs. A majority of miRNA precursors have considerably lower free energy (32-57 kcal/mol) than the transfer RNA or ribosomal RNA. In each species miRNAs represent 1 % of the total genome. A major category of miRNA target genes consists of transcription factors or other regulatory proteins that function in plant development or signal transduction. Though, miRNAs are known as negative regulators of target genes, Place et al., (2008) reported that miR-373 induced expression of genes, and that the promoter sequence of the induced gene was a predicted target of the miRNA. Several miRNAs are either up regulated or down regulated by abiotic stresses, suggesting that they may be involved in stress-responsive gene expression and stress adaptation (Sunkar and Zhu, 2004). The lin 4 RNA was the first discovered member of miRNAs (Lee et al., 1993). Currently, there are four approaches for identifying miRNAs viz. genetic screening, direct cloning after isolation of small RNAs, computational strategy, and expressed sequence tags (ESTs) analysis. Several hundred miRNAs have been cloned and sequenced from mouse, human, *Drosophila*, *C. elegans* and *Arabdiopsis* (Sanger Institute MirBase Sequence Database; http://microrna.sanger.ac.uk/Software/Rfam/mirna/index.html).

Plant miRNAs are potentially processed from one arm of foldback-stemloop precursors and transcribed from regions of the genome distinct from previously annotated genes. Usually only the mature miRNA are conserved in plants instead of miRNA precursors that are usually conserved in animals. Micro RNA has intriguing expression patterns. Some miRNA have spatially and others are temporally expressed. Plants also contain another type of small RNAs (known as siRNAs) that is similar in structure, biogenesis and function to miRNAs. The siRNAs originate from transcripts of transgenes, endogenous repeat sequences or transposons. One key distinction between miRNAs and siRNAs from transgenes and repeat sequences/transposons is that miRNAs target genes other than the ones that give rise to the miRNAs while siRNAs target every sequence that generate them (Bartel, 2004). However, a new class of siRNAs i.e trans-acting siRNAs (ta-siRNAs) discovered in *Arabidopsis* do not target the parental sequences. These ta-siRNAs originate from loci that give rise to non-coding transcripts that are themselves targets of miRNAs. Further, the 5' terminal phosphate and 3' terminal hydroxyl of mature miRNAs make them distinct from the most oligonucleotides and degraded fragments of functional RNAs (Bartel, 2004).

BIOGENESIS OF MICRORNA

MicroRNAs (miRNAs) are a class of gene products of 21 nucleotides in length that are derived from primary miRNAs transcribed from miRNA loci (Lee et al., 2002). Biogenesis of miRNA involves following steps.

1) First, a miRNA gene is transcribed by RNA Polymerase II enzyme to a primary miRNA (pri-miRNA), which is usually a long sequence of more than several hundred nucleotides. These transcripts of miRNA are capped and polyadenylated similar to other protein encoding genes. Some of the miRNAs are encoded by single copy genes whereas others by multicopy gene.

2) Then, pri-miRNA is cropped to a stem loop intermediate (an 60- to 300-nucleotide hairpin structure) called miRNA precursor or pre-miRNA with 2-nt 3' overhang by Pasha the RNase III endonuclease *Arabidopsis* (plants), an equivalent enzyme Drosha in animals (Lee et al., 2003).

3) In animals, pre-miRNAs are then transported by exportin 5 from the nucleus into the cytoplasm, followed by formation of miRNA: miRNA* duplex and mature miRNAs by another RNase III-like enzyme called Dicer. However, in plants the step is catalyzed possibly by Dicer like enzyme 1 (DCL1) in the nucleus rather than in the cytoplasm, then the duplex is translocated into the cytoplasm by HASTY, the plant orthologue of exportin. The Dicer like enzyme processes pre-miRNA to produce stem portion of the hairpin as an miRNA:miRNA* duplex (Knight and Bass, 2001). The duplex comprises a mature miRNA of 21 nucleotides with 2-nt overhangs at 3' end of each strand and a similarly sized miRNA* fragment on the opposing arm of the miRNA precursor, is then presumably unwound by a helicase, releasing the single-stranded mature miRNA.

4) Biogenesis of Plant miRNA consist of an additional step ie miRNAs are methylated on ribose of the last nucleotide by miRNA methyl transferase, HEN 1 (Park et al., 2002).

5) In the cytoplasm, both plant and animal mature miRNAs enter a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC) where they regulate expression of target gene. The strand in which the 5' end is less stable is selectively taken over in to RISC (Schwarz et al., 2003) and then guides the complex

to identify target messages for posttranscriptional gene silencing through direct target cleavage (Palatnik et al., 2003; Achard et al., 2004) or, in a few cases, for translational repression (Aukerman and Sakai, 2003; Chen, 2004).

Differences of Plant miRNAs from animal miRNAs

Plant miRNAs are distinct from the animal miRNAs in biogenesis and mechanism of target gene regulation (Lu and Huang, 2008). Differences between animal miRNAs and plant miRNAs are: (1) Plant miRNAs are less conserved than animal miRNAs. Usually, only the mature miRNAs are conserved in plants however the precursors as well as mature miRNAs are usually conserved in animals (Bartel, 2004). (2) As stated in biogenesis of miRNAs, in animals, the pri-miRNA is cleaved by the DICER family member Drosha to produce a 60–70 nt pre-miRNA whereas, in plants, the primary miRNAs are cleaved by Dicer-like 1 (DCL1) protein to produce a 60–300 nt miRNA precursor (Xie et al., 2004) (3) Plant miRNAs are cleaved into miRNA:miRNA* duplex possibly by DCL1 in the nucleus (Bartel, 2004). In contrast, in animals, the formation of miRNA:miRNA* duplex and mature miRNAs was controlled by Dicer in the cytoplasm (Bernstein et al., 2001; Bartel, 2004). (4) In animals, the miRNA–miRNA* duplex is transported out of the nucleus by Exportin-5 (Yi et al., 2003; Lund et al., 2004). Plants have an Exportin-5 homolog, HASTY (HST) (Bollman et al., 2003; Han et al., 2004). (5) Biogenesis of Plant miRNA consists of an additional step of methylation. Plant miRNAs are methylated on ribose of the last nucleotide by miRNA methyl transferase, HEN 1 (Park et al., 2002), whereas animal miRNAs are not methylated. (6) Animal miRNAs usually bind to target mRNAs through imperfect complementarity at multiple sites located at the 3’ untranslated regions (UTR), and repress gene expression. However, in plants, most target mRNAs only contain one single miRNA complementary site located in the open reading frame of the target, and most corresponding miRNAs typically perfectly complement to these sites and cleave the target mRNAs (Bartel, 2004; Carrington and Ambros, 2003).

Molecular mechanisms of plant gene regulation by microRNAs

In the RISC complex, miRNAs bind to target messenger RNA (mRNA) and inhibit gene expression through perfect or near-perfect complementarity between them. This causes gene silencing termed RNA interference (RNAi) in animals, quelling in fungi, and post-transcriptional gene silencing (PTGS) in plants. Plant miRNAs regulate gene expression by catalyzing the posttranscriptional gene silencing (Palatnik et al., 2003) or translation repression (Reinhart et al., 2000; Chen, 2004). Thus, miRNAs regulate gene expression at the post-transcriptional level not only by direct cleavage of mRNAs, but also by attenuating translation of target mRNAs. Plant miRNAs targets transcription factors or other regulatory proteins that have been implicated in the control of plant development and function by mediating degradation of key regulatory gene transcripts in specific daughter cell lineages. Thus, accumulation of miRNAs through induced expression of miRNAs under biotic and abiotic stresses will lead to the repression of the expression of many protein-encoding genes and affect a variety of cellular and physiological processes. In plants, most target mRNAs only contain one single miRNA complementary site and most corresponding miRNAs perfectly complement these sites and cleave target mRNAs.

Regulation of miRNA genes

Although miRNAs represent a relatively abundant class of transcripts, their expression levels vary greatly among species and tissues. Less abundant miRNAs routinely escape detection with technologies such as cloning, northern hybridization and microarray analysis (Liu et al., 2008). A typical approach for identifying miRNAs is to monitor gene expression profiles. If a miRNA is found to be differentially expressed in a certain tissue or cell type, it may be hypothesized to play a regulatory role in specifying tissue or cell identity. Similarly, if a miRNA is expressed at a specific developmental stage, then it may regulate developmental timing. Plants have highly complex small RNA fractions including both miRNAs and endogenous siRNAs as against the relatively simple small RNA populations (mainly miRNAs) found in mammals (Mineno et al., 2006). This renders under representation of miRNAs in the small RNA fraction and affects their detection using methods such as cloning and microarray hybridization. Recently, an endpoint stem-looped RT PCR method has been developed enabling highly sensitive and fast detection as well as expression profiling of miRNA. Stem-looped RT discriminates between miRNAs that differ by two bases and between the mature miRNA and its precursor. Further, spatial constraint of the stem loop structure prevents it from binding to the double stranded genomic DNA molecules and eliminates the need for RNA preparation (Varkonyi-Gasic et al., 2007).

Computational prediction of miRNA genes and their targets

A number of approaches like direct cloning and sequencing of total small RNAs with appropriate size from isolated tissues or whole organisms have enabled the identification of hundreds of miRNAs in plants and animals (Lagos-Quintana et al., 2001; Reinhart et al., 2002). Majority of recently identified miRNAs were first predicted by computational approaches and then validated by molecular techniques such as Northern blotting (Zhang et al., 2006). Apparently, computational approaches have played an increasingly important role in miRNA identification. The principles of computational approaches are based on the major characteristic features of miRNAs: hairpin-shaped stem-loop secondary structure with minimal folding free energy (Lagos-Quintana et al., 2001; Lee and Ambros, 2001) and high evolutionary conservation from species to species (Legendre et al., 2005). Accumulating evidence shows that many miRNAs are evolutionarily conserved in animals from worms to humans (Pasquinelli et al., 2000; Zhang et al., 2006), suggesting a powerful strategy to predict potential miRNAs by using homology search. In fact, homology search as a computational approach has been developed to identify miRNA genes in both plants and animals (Weber, 2005; Zhang et al., 2007; Luo and Zhang, 2009).

Prediction of miRNA targets is usually based on homology search, as all miRNAs regulate target gene expression by binding to the targeted mRNA sequence in a perfect or near perfect complementary site. Blast against particular crop genome sequence with allowing gap of 4 or less/no gap between miRNA sequence and potential mRNA target will identify the target. Sunkar and Zhu, 2004 based on sequence complementarity, predicted fifty-one potential targets with diverse function for the newly identified miRNAs in Arabidopsis. Patade et al. (2009) predicted potential targets of miRNA, miR159 using computational tool, MiRU-Plant microRNA Potential Target Finder program (Zhang, 2005) considering TIGR ATH1 (Arabidopsis mRNA) release 5 as target dataset. The prediction of potential targets was based on conditions like score allowed = 3, G: U pairs allowed = 6, indels allowed = 1, other mismatches allowed = 3. Various members of MYB transcription factor family viz. MYB 33, MYB 65, MYB 101, MYB 120 etc) were predicted as the potential targets of miR159. Further, transcript expression analysis of *MYB* transcription factor and miR159 revealed concomitant up or down regulation with down or up regulation of the miRNA, thus supported the prediction.

Functions of miRNAs in plants

In animals, miRNAs have proven to be involved in many functional processes such as development, stress response, and carcinogenesis. Also in plants, expression and function of many genes are regulated by miRNAs. A major category of miRNA target genes consists of transcription factors (TFs) or other regulatory proteins that function in plant development or signal transduction.

1) Regulation of plant development

Reduced level of mature miRNA (due to loss of function of either *DCL*I or *HASTE*Y genes) results in many developmental abnormalities, such as altered leaf shape, delayed floral transition, arrested embryos at early stages, female sterility disrupting leaf shape and flower morphology, reducing fertility, and accelerating vegetative phase change and disrupting the phyllotaxis of the inflorescence. Micro RNAs regulate plant development by controlling the levels of transcription factors that are important in development. miR165/166 and miR159/ JAW are essential for controlling the pattern and development of leaves by direct regulation of two classes of transcription factor genes (HD-ZIP and TCP), which are involved in controlling asymmetric pattern along the leaf axis. *APETALA 2 (AP2)* is one of the genes that play an important role in flowering time and flower morphology (Juarez et al., 2004; Mallory et al., 2004). miR172 regulates *AP2* gene expression through translational inhibition (See review Chen et al., 2005). Besides, miR159 and miR156, respectively are involved in flower development and plant phase transition from reproductive growth to vegetative growth includes. miR164 play an important role in controlling shoot and root development by regulating several transcription factors like NAC domain transcription factor family (Achard et al., 2004). It may be possible to increase crop yields and resistance to environmental stress by appropriately controlling miR164 expression. In addition, some miRNAs may function in the biosynthesis of cell wall metabolites or cotton fibre development.

2) miRNAs regulate its own as well as siRNAs metabolism and function. miRNAs such as miR162, miR168, miR173, miR390 and miR398 are known to regulate miRNA biogenesis or function (Xie et al., 2003).

3) miRNAs are involved in signal transduction as several miRNAs affect signal transduction, especially hormone signalling pathways (Wang et al., 2004).

4) miRNAs are involved in biotic and environmental stress responses. miRNAs are involved in pathogen induced post transcriptional gene silencing (PTGS), which ultimately enhance plant resistance to pathogens. Several of the miRNAs discovered recently are either up regulated or down regulated by abiotic environmental stresses, suggesting that they may be involved in stress-responsive gene expression and stress adaptation

Involvement of miRNAs in abiotic stress responses

Recently discovered endogenous small RNAs including miRNAs and siRNAs have emerged as important players in plant stress responses through regulation of target gene expression. Up or down regulation of some stress responsive small RNAs imply their role in stress tolerance. Stress-induced small RNAs in general down-regulate their target genes, which may encode negative regulators of stress responses. Conversely, down regulation of small RNAs in response to stress lead to the accumulation of their target mRNAs, which may contribute positively to the adaptation to stress (Shukla et al., 2008).

In order to identify novel and abiotic stress regulated miRNAs and siRNAs, Sunkar and Zhu, 2004 constructed a library of small RNAs from Arabidopsis seedlings exposed to dehydration, salinity, or cold stress or to the plant stress hormone abscisic acid (ABA). Further sequencing of the library and subsequent analysis identified 26 new miRNAs forming 15 new families. Expression of some of the miRNAs was preferentially in specific tissues, whereas several miRNAs were either up or down regulated in Arabidopsis seedlings exposed to dehydration, salinity, or cold stress. Ten of the miRNAs are highly conserved in other plant species. In order to unravel function of microRNA, Zhao et al., 2007 studied transcript expression profile of miRNAs in rice (*Oryza sativa*) under drought stress. The results validated drought-induced expression of miR-169g and miR393 based on microarray expression profiling of microRNAs under drought stress in rice. The results confirmed more prominent drought induced expression of miR-169g in roots than in shoots. Sequence analysis revealed occurrence of two proximate DREs (dehydration-responsive element) in the upstream of the MIR-169g, suggesting transcript expression regulation of miR-169g by CBF/ DREBs.

Sunkar et al., (2006) provided evidence on involvement of miRNA, miR398 in oxidative stress responses. miR398 targets two closely related Cu/ Zn cytosolic and chloroplastic superoxide dismutases that detoxify superoxide radicals. Transcript expression of miR398 in response to the oxidative stresses was down regulated, which lead to posttranscriptional accumulation of the SOD mRNA and thus oxidative stress tolerance. Further transgenic *Arabidopsis thaliana* plants over expressing a miR398-resistant form of SOD accumulated more mRNA than plants over expressing a regular CSD2 and were consequently much more tolerant to high light, heavy metals, and other oxidative stresses.

Earlier, miR159 has been isolated and cloned from *Arabidopsis* and is found to be expressed during early seed germination. miR159 is encoded by three different loci, MIR159a, MIR159b and MIR159c. Mature miR159b and miR159c differ from miR159a by one and two nucleotides, respectively at the 3' end (Reyes and Chua, 2007). Patade (2009) characterized transcript expression of mature miR159 in response to short- and long-term salt and PEG induced osmotic stress in sugarcane. Under long term (15 days) iso-osmotic (-0.7 MPa) NaCl or PEG stress, no change was detected in mature transcript level of miR159 over control. However, under the short-term (up to 24h) salt stress, transcript level of the mature miRNA increased to 112 % of the control at 16h treatment. The mature transcript level of miR159 was higher under all the PEG induced osmotic stress treatments as compared to the control, and it progressively increased with stress exposure period (1.3 fold at 8h treatment). This indicated that expression of miR159 gene was more responsive to osmotic stress than ionic stress. The early induction of the gene under the short treatments supports its involvement in the regulation of genes involved in stress perception and/or signaling. Reyes and Chua (2007) also reported induction of the miRNA under drought treatment in Arabidopsis. Achard et al., (2004) reported expression of miR159 mainly in inflorescence and floral tissues of several plants such as Arabidopsis, tobacco and barley, and induction of its expression in response to gibberellic acid (GA).

MiRNAs in cold stress responses

The involvement of miRNAs in abiotic stress has been studied in plants in response to cold, dehydration or NaCl by using expression analysis, suggesting stress specific regulation of expression of different miRNAs (Sunkar and Zhu, 2004). The results indicated strong up regulated expression of miR393 by cold, dehydration, and NaCl treatments, but only slight up regulation of miR397b and miR402 by these stress treatments. Cold stress led to up regulation of miR319c but not dehydration or NaCl stress whereas, miR389a was down regulated by all the stress treatments.

Recently, Zhou et al., (2008) developed a computational transcriptome based-approach to annotate stress inducible miRNAs in plants. Using this approach, they reported up regulation of 19 miRNA genes belonging to 11 miRNA families in *Arabidopsis thaliana* by cold stress. Further, wet lab experiments validated differential induction of eight miRNA genes and constitutive expression of three miRNA genes under low temperature. Interestingly, the promoter analysis of the miRNA genes revealed presence of many known stress-responsive cis-regulatory elements in their promoters.

Conclusions and future perspectives

As thousands of miRNAs have been identified in plants, animals, and viruses, study of its function by the suppression of expression in cells is a rapidly expanding area of research. The next step will be to understand the biological significance and roles of miRNA regulation during plant development. For this, it is important to know how the miRNAs themselves are regulated at the transcriptional and posttranscriptional levels, and the developmental consequences of disrupting miRNA regulation. Considering the important roles of miRNAs in plant responses to biotic, abiotic environmental stresses, development as well as genome maintenance, continued efforts in the future are needed to identify the complete set of miRNAs and other small RNAs. Results of the recent studies suggest that a large number of miRNAs and other small regulatory RNAs may play important roles in plant responses to environmental stresses as well as in development and genome maintenance. The identification and functional validation of stress-regulated small RNAs including miRNAs will help in the designing of new strategies for improving stress tolerance.

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