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Genome-wide analyses of abiotic stress-related microRNAs and their targets in *Arabidopsis thaliana*

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Abstract

MicroRNAs (miRNAs) are known to regulate plant growth and development via regulating gene expression at both transcriptional and post-transcriptional levels. Although several miRNAs have been reported to be associated with abiotic stress responses in plant, systematic investigation of stress-related miRNAs and their targets in plants is limited. In this study, we systematically investigated stress-related miRNAs and their targets in *Arabidopsis thaliana*. We identified 94 putative stress-related miRNA genes, in which 8 miRNAs were new identified with stress-related response function based on targets prediction. Sequence analysis of these miRNA genes showed that most stress-related miRNAs possess TATA boxes in their promoters, and more than half contain at least two promoters. We also demonstrated that most stress-related miRNA genes contain stress-related elements in their promoters. Furthermore, conservation analysis showed that many stress-related miRNAs are species/family-specific and a subset of stress-related miRNAs may be derived from repeat sequences. Finally, we found that the stress-related miRNAs target 374 genes with 1,153 predicted target sites, of which 87.2% are targeted for gene cleavage and 12.8% affect protein translation. In conclusion, our findings provide an insight into both the function and evolution of stress-related miRNAs.

Keywords: Abiotic stress; *Arabidopsis thaliana*; miRNA gene; evolution; target gene.
Abbreviations: ath_*Arabidopsis thaliana*; CDS_coding sequence; C_cellular component; CpG _cytosine-phosphate-guanine; F_molecular function; GO_gene ontology;miRNA_microRNA; mRNA_message RNA; pri-miRNA_primary miRNA; pre-miRNA_precursor miRNA; P_biological process; TFs_transcription factors; TSS_transcription start site.

Introduction

MicroRNAs (miRNAs) are endogenous non-coding RNAs, which are present in all eukaryotes and are evolutionary conserved among species (Bartel, 2004). Although few miRNAs are shared between plants and animals (Arteaga-Vazquez et al., 2006), many basic mechanisms are common between plants and animals, such as the mechanisms of miRNA biogenesis and function (Bartel, 2004). MiRNAs are first transcribed to primary miRNA (pri-miRNA) with variable length from several hundred to a few thousand bases by RNA polymerase II (Zhang et al., 2006). The pri-miRNAs are further cleaved to pre-miRNAs, which are exported to the cytoplasm and processed to mature miRNAs (Bernstein et al., 2001; Tang et al., 2003; Yi et al., 2003; Lee et al., 2004).

Compared to animals in which 13,931 miRNAs have been deposited in the miRBase database (release 18.0), only 4014 miRNAs have been reported in plants and are less understood (Arteaga-Vazquez, et al., 2006; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011). Plant miRNAs function in gene regulation via binding to the protein-coding region of their target genes to initiate degradation or translational repression of the target gene transcripts (Palatnik et al., 2003; Brodersen et al., 2008), and play important roles in diverse biological processes, including leaf development, auxin signaling, phase transition, flowering and genome maintenance (Aukerman and Sakai, 2003; Palatnik et al., 2003; Vaucheret et al., 2004). Plant abiotic stresses, including drought, high-salinity and extreme temperature, are environmental conditions that have significant impacts on plant growth and development. Recently, several miRNAs were reported to be sensitive to abiotic stresses (Song et al., 2013; Sunkar and Zhu 2004; Lu et al., 2005; Liu et al., 2008). For examples, *A. thaliana* miR398 is involved in oxidative stress tolerance (Sunkar et al., 2006) , miR394a/b and its target gene *LCR* are involved in regulation of *Arabidopsis* abiotic stress (Song et al., 2013), miR395 and miR399 are related to sulfate and inorganic phosphate starvation responses (Jones-Rhoades and Bartel, 2004; Fujii et al., 2005; Bari et al., 2006), and 21 miRNA gene expressions are upregulated in response to UV-B exposure (Zhou et al., 2007).

In the present work, we systematically investigated the stress-related miRNAs in *A. thaliana* to provide valuable information on the conservation and expression of stress-related miRNAs in plant and improve our understanding of the regulatory roles of the stress-related miRNAs in stress tolerance of plant.

Results and Discussion

Identification of stress-related miRNAs

Based on PMRD database annotation and literature survey, 86 stress-related miRNA genes in *A. thaliana* were identified (Fujii et al., 2005; Zhou et al., 2007; Liu et al., 2008). Additional 8 miRNA candidates (ath-miR414, ath-miR5021, ath-miR5658, and ath-miR854a/b/c/d/e) were newly predicted to be involved

in stress response via analyzing the 92 reported stress/stress-related protein-coding genes (Supplementary Table 1 and Table 2). In total, 94 stress-related miRNA genes of 23 miRNA families were found in *A. thaliana*.

Clustering of miRNA genes

We examined the potential clustering of miRNAs on the *A. thaliana* genome and analyzed the genomic organization of these stress-related miRNA genes within 10kb. The pairwise distance of miRNAs less than 10kb were considered as clustered miRNAs (Cui et al., 2009). Our results revealed that 25 out of 94 miRNAs formed 9 clusters, out of which 5 clusters contain two miRNA genes per cluster, 3 clusters contain three miRNA genes per cluster and one cluster contains 6 miRNA genes (Fig. 1). These nine clusters were found in chromosome 1, 2, 3 and 5 but not 4 (Fig. 1). MiRNA genes from different clusters vary in length and structure, suggesting that miRNAs in different clusters may be differentially transcribed and may offer unique functions for regulating expression of miRNAs (Cui et al., 2009). Consistent with the feature of the rice miR395 cluster, the majority of stress-related miRNAs from the same cluster in *A. thaliana* are derived from the same miRNA family (Guddeti et al., 2005).

Conservation and evolutionary analysis of miRNAs

The identification of conserved and non-conserved miRNA homologs within different species would be helpful for analysis of miRNA evolution, and also be informative for discovery species-specific miRNAs. As expected, sequence similarity searches against the annotated miRNAs from miRBase showed that most *A. thaliana* miRNAs were conserved across plant species. Eighty six, 85, 50, 85, 109 homologs of the *A. thaliana* stress-related miRNAs were found in *Arabidopsis lyrata*, *Oryza sativa*, *Theobroma cacao*, *Zea mays*, and *Populus trichocarpa*, respectively. Only 7 homologs were found in *Pinus taeda*, which might be due to the limited miRNAs numbers in the current database (Fig. 2A). With the discovery of new miRNAs in plants, there will be more *A. thaliana* homologous miRNAs were found. The 94 stress-related miRNAs can be grouped into 23 families, of which 18 (78.26%) contain more than one miRNA gene per family, and 9 (39.13%) contain five or more miRNA genes per family (Supplementary Table 3). For examples, there are 12 and 9 miRNA genes in the miR156 and miR166 family respectively. The average miRNA gene copy number per family in *A. thaliana* is 3.91 and different from those in other plant species, such as *O. sativa* (7.18 copies/family) and *Selaginella moellendorffii* (3.00 copies/family). Obvious expansions of stress-related miRNA genes were found in some plants, such as *Glycine max* (the average copy number = 7.00), *Zea mays* (the average copy number = 7.18). MiRNA gene expansions are obvious in several miRNA families, such as the miR399 family that underwent gene expansion from only 6 copies in *A. thaliana* to 18 gene copies in *M. truncatula*, 11 copies in *O. sativa*, and 12 copies in *P. trichocarpa*. In the previous study, miRNAs derived from miRNA families and repetitive elements can expanded through genomic duplication events in animals and plants (Piriyapongsa et al., 2007). Similar to the amplification and diversification of protein-coding genes, the miRNA gene expansion in a given family might have been generated via gene duplication or tandem and segmental duplication event (Li and Mao, 2007; Sun et al., 2012). The 23 conserved miRNA families can be further sorted into different species/family-specific groups (Fig. 2). The miR854 family was only found in *A. thaliana*. The miR414 family only existed in *A. thaliana* and *P. patens*. MiR400, miR402 and miR158 families were present in *A. thaliana* and *A. lyrata*. The miR159 family was present in all the investigated plant species except *T. cacao*. The evolving of species-specific miRNA families may contribute to genome diversity of plant during evolution. Six long terminal repeat (LTR) elements, 5 simple repeats and 2 low complexities were found in 12 *A. thaliana* stress-related miRNA precursors, including 7 new stress-related miRNAs (ath-miR414, ath-miR5658, and ath-miR854a/b/c/d/e) and 5 known stress-related miRNAs (ath-miR156b, ath-miR157c, ath-miR167c, ath-miR169a and ath-miR401) (Supplementary Table 4). Most of these miRNAs are species-specific. For examples, ath-miR854a/b/c/d/e and ath-miR401 were only found in *A. thaliana*, ath-miR414 was found in *A. thaliana* and *P. patens*, and ath-miR167c was found in *A. thaliana*, *A. lyrata* and *Z. mays*. Besides supporting the argument which claims that some plant stress-related miRNAs are originated from transposable elements (TEs) or other miniature inverted repeat transposable elements (MITEs) during evolution (Piriyapongsa and Jordan, 2008), our findings suggest that stress-related miRNAs have undergone dynamic evolutionary changes and are likely to be evolutionarily active and undergoing a rapid "birth-and-death" process within species.

Promoter and CpG island analysis of stress-related miRNA genes

One hundred and fifty three putative promoters were predicted from 73 *A. thaliana* stress-related pre-miRNA upstream sequences, of which, 21 (28.8%) have only one promoter, 29 (39.7%) contain two promoters, and 23 (31.5%) contain at least three promoters. In these promoters, 19.6% of putative promoter/transcription start sites (TSSs) located within 200 bp upstream of stress-related pre-miRNAs in *A. thaliana* (Fig. 3) and 118 (77.1%) have a putative TATA-box. This finding indicates that the majority of *A. thaliana* stress-related miRNA genes could be transcribed by RNA polymerase II (Bartel, 2004). There are 5 TATA box-null stress-related miRNA genes (ath-miR156a, ath-miIR168a, ath-miR393a, ath-miR399c, and ath-miR167b) each of which has one promoter without any TATA-box-like sequences defined (Xie et al., 2005; Zuo et al., 2011). CpG islands are genomic regions that contain a high frequency of CpG sites (Gardiner-Garden and Frommer, 1987). Sixteen *A. thaliana* stress-related miRNA genes contained 17 CpG islands, of which 41.2% existed in the downstream region of the TSS sites, one existed in the upstream sequence of the TSS, and three encompass the TSS (Supplementary Table 5). CpG islands are frequently associated with ubiquitously expressed housekeeping genes and typically occur at or near the TSS of genes (Saxonov et al., 2006). CpG islands are often associated with promoter regions, and some are hyper-methylated in cancer (Beier et al., 2007). Recently studies showed that CpG islands were implicated as a putative source for animal miRNAs evolution and function (Dahary et al., 2011). However, the research of miRNAs with CpG islands in plants were limited (Zhou et al., 2007). Our results indicate that similar to housekeeping genes, miRNAs as tissue-specific genes also contain CpG islands in their promoters (Larsen et al., 1992; Blackledge and Klose, 2011). These miRNA genes are accociated with CpG islands and promoter regions suggesting a potential role for these miRNAs in CpG-island methylation and gene regulation (Ng et al.,2010; Dahary et al., 2011).

Analysis of conserved cis- regulatory elements

To explore the putative stress-related elements in the *A. thaliana* stress-related miRNA genes, the 1,000 bp DNA sequence upstream of each miRNA gene was analyzed using the Plant-

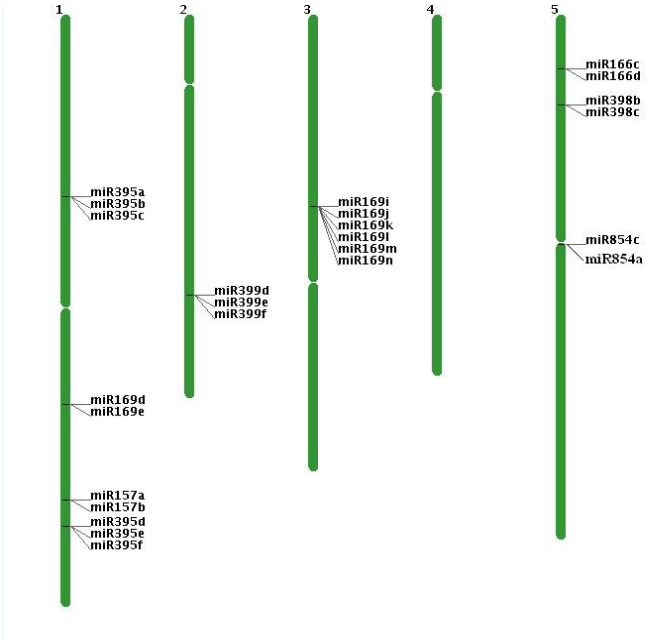

Fig 1. Cluster analysis of potential stress-related miRNAs in the genome of *Arabidopsis thaliana*. The relative locations of cluster miRNAs are shown across 5 *Arabidopsis thaliana* chromosomes except for chromosome 4, where no *Arabidopsis* miRNA clusters are detected.

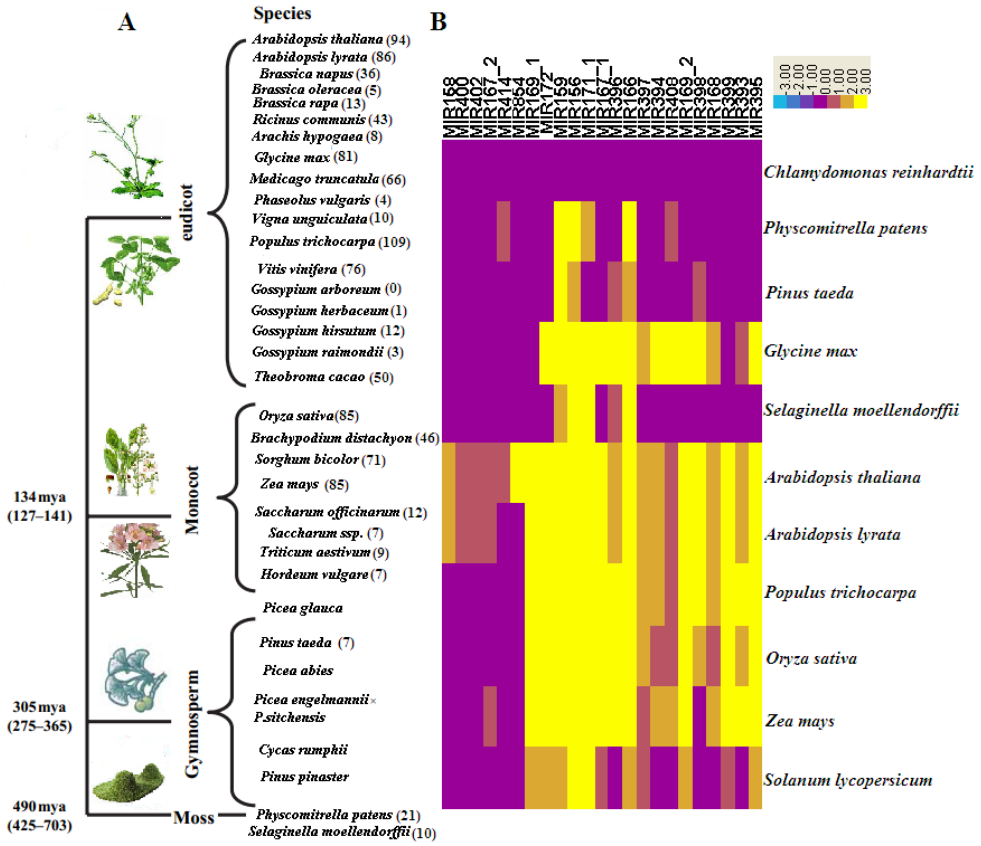

Fig 2. Conservation analysis of *Arabidopsis thaliana* miRNAs. A, The distribution of conserved *Arabidopsis* miRNAs across other species. The number in the brackets shows that the *Arabidopsis*'s homologous miRNAs are found in other plants. The numbers indicated at selected nodes show the estimated divergence times (million years ago) (Sanderson et al., 2004). B, The miRNA family conservation within species. Homologous *Arabidopsis* miRNA families were searched within other 16 plants ranging from moss to eudicot. The copy numbers of each family were counted and clustered using Cluster software. The hierarchical clustering diagram was generated using Java TreeView software. The different colors represent the different family copies within different plants.

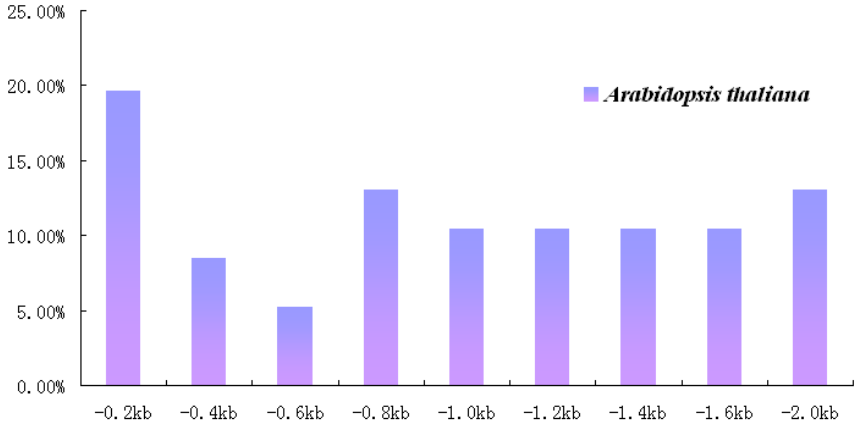

Fig 3. Genomic distribution of TSS sites from the 5′ end of the pre-miRNA sequences. The percentage of the predicted TSS site numbers are marked in Y-axis.

PlantCARE database (Supplementary Figure 1 and Supplementary Table 6). Several known stress-responsive elements were identified, including ABA-response elements (ABREs), anaerobic induction elements (AREs), cis-elements involved in salicylic acid responsiveness (TCA-elements), a MYB binding site involved in light responsiveness (MBS), a MYB binding site involved in flavonoid biosynthetic genes regulation (MBSI), defense-and stress-responsive elements (TC-rich repeats), low-temperature -responsive elements (LTRs), heat-stress-responsive elements (HSEs), elements in response to Gibberellic acid (GA). The stress-related elements are very important for genes to respond to abiotic stress in *A. thaliana* (Xu et al., 1996). Among the *A. thaliana* stress-related miRNA genes, 26 miRNA genes with ABREs might be involved in the abscisic acid responsiveness, and 69 miRNA genes with ARE motifs and 23 with LTR elements might be responsive to low-temperature, hypoxic and dehydration stresses (Dolferus et al., 1994). Cis- and trans- regulatory elements are extensively involved in stress-induced gene expression (Jaglo-Ottosen et al., 1998; Zhang et al., 2005). The presence of ABREs and AREs in the *A. thaliana* stress-related miRNA genes, such as the presence of 7 ABRE and 17 ARE elements in the promoters of 14 MIR169 family members, suggests that these miRNA genes might be regulated by stress conditions.

Prediction, gene ontology (GO) and pathway annotation of miRNA targets

In comparison with animal miRNAs, plant miRNAs usually bind to the protein-coding region of their target genes with nearly perfect sequence complementarity that result in either degradation of their target mRNAs or translation inhibition of the target mRNAs (Rhoades et al., 2002). The 94 *A. thaliana* stress-related miRNAs have 374 target genes with 1,153 predicted target sites, of which 1005 and 148 belong to the cleavage and translation inhibition categories, respectively. The number of predicted target genes per *A. thaliana* stress-related miRNA varies from 1 to 25. Most predicted target genes are transcription factors involved in development. For example, the auxin response factor (ARF) required for expression of auxin responsive genes is a predicted target gene of miR167c, and the SQUAMOSA promoter-binding protein (SBP) involved in regulating flower and fruit development as well as other physiological processes is a predicted target gene of miR156 (Moreno et al., 1997). Interestingly, different miRNAs in the same cluster were found to target genes from a same gene family. For instance, 14 miRNAs of the MIR156 family target 9 members of the SBP transcription factor family. The target genes of *A. thaliana* stress-related miRNAs were found to function as signal transduction factors, disease resistance proteins, and enzymes involved in metabolism, cell differentiation and growth, and biotic and abiotic stress responsive genes using annotation of GO terms (Ashburner et al., 2000) (Supplementary Figure 2). The pathway analysis of the target genes showed that 14 out of 284 genes participate in 26 pathways (Supplementary Table 7), including biosynthesis of plant hormones, nitrogen metabolism, Biosynthesis of alkaloids derived from shikimate pathway, and proteasome. And the 14 genes were targeted by 24 miRNAs including ath-miR159a/b/c, ath-miR414, ath-miR854a/b/c/d/e and ath-miR395a/b/c/d/e/f etc. For example, ACA5 (AT1G08065) gene targeted by ath-miR854 gene was found to take part in the nitrogen metabolism pathway, the target gene AT5G57580 of ath-miR414 was identified to take part in the pathway of Phosphatidylinositol signaling system, the target gene CKB3 (AT3G60250) of ath-miR397b was predicted to be involved in circadian rhythm pathway (Supplementary Figure 3). Expression and function analyses of these predicted miRNA target genes are warranted to decipher the complex genetic networks utilized by plants to cope with drastic environmental conditions.

Materials and Methods

Data collection

A. thaliana stress-related miRNAs and upstream sequences were downloaded from the PMRD web site (http://bioinformatics.cau.edu.cn/PMRD/). The known miRNA sequences and the genome positions of these miRNAs were obtained from the miRBase database (http://microrna.sanger.ac.uk/sequences/, release 18.0). The stress-related genes were retrieved from the published literatures and the CDS sequences of these genes were downloaded from the NCBI database.

Promoter sequence analysis and conserved cis- regulatory element and CpG island prediction

The transcription start site (TSS) and TATA-box of a pre-miRNA were predicted using TSSP program (http://mendel.cs.rhul.ac.uk/mendel.php?topic=gen). CpG islands were predicted using EMBOSS CpGReport tools (http://www.ebi.ac.uk/Tools/ emboss/cpgplot/). The PlantCARE

software was used to predict plant cis- regulatory DNA elements related to stress responses.

MiRNA family conservation, miRNA clustering and repeat sequences analysis

BioEdit freeware was used to create a local blast database containing 21,643 known mature miRNAs downloaded from the miRBase database (release 18.0). The high homologous miRNAs cross plant species were identified using *A. thaliana* stress-related miRNAs as query sequences for a BLASTN search (E-value 1.0E-5) against the local database. The numbers of homologous miRNAs from different species were shown in the tree. The numbers of miRNA families from 16 plants were counted, clustered and visualized using software Cluster 3.0 and Java TreeView (release 1.1.6). The potential repeat sequences were predicted using the RepeatMasker Web Server (current version: open-3.3.0).

MiRNA target gene prediction and function annotation

The potential target genes of stress-related miRNAs were predicted using the psRNATarget software and were annotated using gene ontology (GO) annotation in TAIR database (TAIR 10, released 12/14/2010). Pathway analysis of the potential target genes was performed using the DAVID database (http://david.abcc.ncifcrf.gov/summary.jsp).

Conclusion

In this study, we systematically investigated the abiotic stress-related miRNAs in *A. thaliana* by using silico methods. In addition, we extensively analyzed these miRNAs, including their genome location and cluster pattern, phylogenetic comparison of cross-species conservation miRNA families, promoter sequences and conserved cis-acting regulatory elements, CpG islands, target gene function annotations and pathway analysis. In conclusion, genome-wide analysis of abiotic stress-related miRNAs and their targets in *A. thaliana* provide a significant insight into both the function and evolution of stress-related miRNAs.

Conflict of interest statement

We declare that we have no conflict of interest.

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