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The functions of microRNAs in plants

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1. ABSTRACT

MicroRNAs (miRNAs) are a class of small regulatory RNAs, which repress gene expression at the posttranscriptional levels through binding to target mRNAs for directly cleaving mRNAs or inhibiting protein translation. Mature miRNAs are produced from miRNA genes by multiple biological processes, in which several important enzymes are involved in. To date, several hundreds of miRNAs have been identified in plants using a various computational and/or experimental approaches. These miRNAs regulate plant tissue differentiation, development and growth, control auxin signal transduction, involve in plant response to a variety of abiotic and biotic environmental stresses.

2. INTRODUCTION

Regulation of gene expression is one of the hottest research fields in biology and medicine. Although, lots of scientists have attempted to solve this mechanism and great progresses have been made in this field including finding a variety of transcriptional factors, lots of questions remain in mystery. More and more evidences demonstrated that recently discovered microRNAs might be the most important gene regulator in multiple biological and metabolic processes (1-6).

MicroRNAs (miRNAs) are one class of single-stranded small regulatory RNAs with 20-24 nucleotide lengths, which negatively regulate gene expression at the posttranscriptional levels. MiRNAs regulate gene expression by targeting mRNAs for direct cleavage or the inhibition of protein translation. Due to their regulatory role in gene expression and following gene functions, miRNAs have attracted the attentions of many scientists from a various fields since they were recognized in the early of 2000s (7-9). Since 2001, the number of literatures on miRNA-related research is dramatically increased. Based on the ISI web of science database, only 7 publications were related to miRNA research in 2001, but the number was increased to 694 in 2006 (Figure 1). At the same time, more and more miRNAs were identified in animals, plants and viruses and deposited in miRNA databases. According to miRBase website (http://microrna.sanger.ac.uk/, one of the most important miRNA databases in the miRNA-research field), only 218 miRNAs were deposited in miRBase in 2002, but this number was increased to 4361 in October, 2006 (10, 11) (Figure 2). As time going, more and more miRNAs will be identified and deposited in a variety of miRNA databases.

Currently, several hundreds of miRNAs (863) have been identified in plant species and deposited in the miRNA database. Table 1 lists the plant species and the

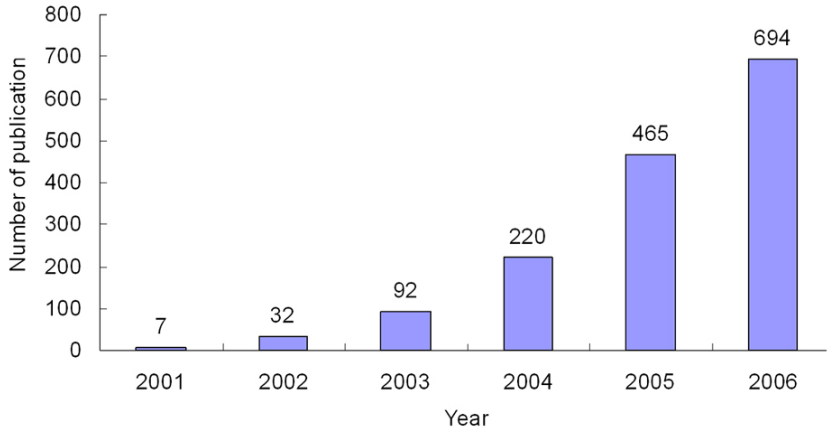

Figure 1. Number of microRNA-related publication (2001-2006) based on ISI web of science database.

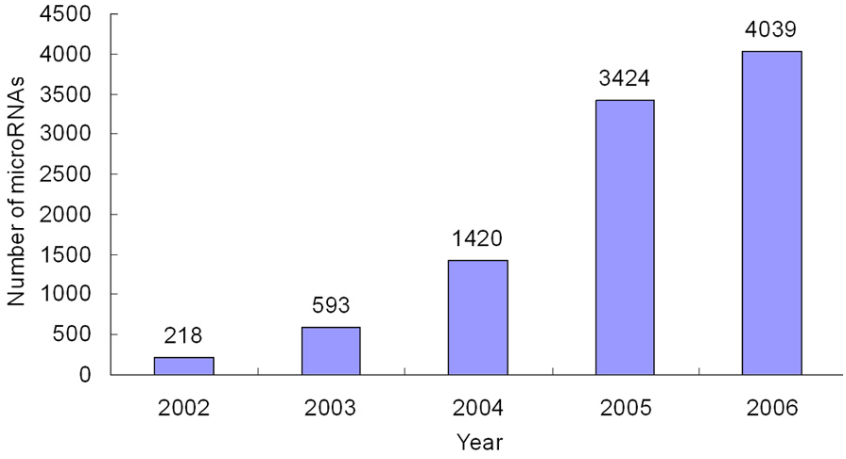

Figure 2. Number of microRNAs deposited in miRBase database.

number of miRNAs exposited in miRBase database. These species include dicots, moncots and even mosses. Of the 863 miRNAs, 242, 215, and 131 were identified from rice, black cottonwood, and *Arabidopsis*, respectively.

In this paper, we highlight the research progresses on three topics of miRNA-related research: miRNA biogenesis, methods for identifying miRNAs, and the functions of miRNAs in plants.

3. MicroRNA BIOGENESIS

A majority of miRNA genes are transcribed by RNA polymerase II into primary miRNAs (pri-miRNAs) with a long nucleotide sequences (12, 13). However, a recent study indicated that RNA polymerase III is also used to transcribe some miRNA genes (14). Then, pri-miRNAs are cleaved into miRNA precursors (pre-miRNA), which can form a secondary stem-loop hairpin structure with high negative minimal free folding energy and a high minimal folding free energy index (MFEI) (15), by dicer-like enzyme 1 (DCL1) (16). Pre-miRNAs are further cut into miRNA:miRNA* (miRNA* is the sequence at the miRNA opposite side of the pre-miRNA stem loop secondary structure) duplex in nucleus by the same enzyme DCL1 with the help of other several proteins (for example HEN and HYL) (1, 17). miRNA:miRNA* duplex is unwind into two separated single strand RNAs following it is translocated into cytoplasm by HASTY protein in plants (18). The miRNA* is cleaved by an unknown biological process and the miRNA mature sequence is moved into RNA induced silenced complex (RISC) and regulate gene expression by directly binding to specific sites of targeted mRNAs. The miRNA biogenesis in animals is followed the similar pathway with some difference, for example pre-miRNAs are transported into cytoplasm before they are cleaved into miRNA:miRNA* duplex (1).

4. APPROACHES FOR IDENTIFYING miRNAS

Since miRNA lin-4 was identified in 1993 (19), several approaches have been employed to identify miRNAs in animals, plants and viruses. These approaches can be classified into four categories.

4.1. Genetic screening approach

Genetics screening technology is the first method for identifying miRNAs, including two found

Table 1. Plant species and the number of miRNAs deposited in miRBase database

Species	Common name	Group	Number of miRNAs
Arabidopsis thaliana		Eudicots	131
Glycine max	Soybean	Eudicots	22
Medicago truncatula		Eudicots	30
Populus trichocarpa	Black cottonwood	Eudicots	215
Oryza sativa	Rice	Monocots	242
Saccharum officinarum	Sugarcane	Monocots	16
Sorghum bicolor	Sorghum	Monocots	72
Zea mays	Corn	Monocots	96
Physcomitrella patens		Mosses	39

miRNA members (lin 4 and let 7) (19); and it was also employed to identify miRNAs in early 2000s (7-9). However, this technology needs to isolate the total RNA and sequencing each RNA, so it is time-consuming, complicated and expensive, and recently this method was not employed to identify miRNAs (6).

4.2. Direct cloning after isolation of small RNAs

Direct cloning technology after isolation of small RNAs is the modified method of genetic screening technology. In this technology, small RNAs are first isolated from tissues or cells what we are interested. Then the small RNAs are separated by gel electrophoresis. Usually, a 5' and/or 3' adapter is added to the small RNA for easily operating the small RNAs. Finally, the small RNAs are sequenced and the miRNAs are identified from the isolated small RNAs. This method has been widely employed to identify miRNAs from different species in plants and animals (20, 21).

4.3. Computational approach

Based on the major characteristics of miRNAs and miRNA precursors, several computational programs have designed by different laboratories. All these software have been employed to identify miRNAs in different animal and plant species, including human, mouse, rat, *C. elegans*, and fry in animals, *Arabidopsis*, rice, and sorghum in plants. Several good examples for these computational software are miRseeker (22), miRscan (23, 24), miRscan (25), MIRcheck (26), findMiRNA (27), and miRAlign (28). Some of these softwares were also employed to predict the how much miRNA genes exist in animal genomes.

However, a majority of computational methods are based on genome sequences, it means these softwares can only been employed to identify miRNAs in model species, in which their genome sequences are already known. Another potential problem for computational approach is that the computationally predicted miRNAs need to be confirmed by experimental approaches, such as directly cloning or Northern blotting (6, 29).

4.4. Expressed sequence tag (EST) analysis

To predict and identify miRNAs from different species, particular for non-model species, Zhang and colleagues (2005) recently developed a new approach, called expressed sequence tag (EST) analysis (30). EST analysis is based on two important characteristics of miRNAs; 1) highly evolutionary conserved from species to species. In both animal and plant system, some miRNAs are highly evolutionary conserved, some from worm to human in animals (31), some from moss to high flowering plants in plants (32, 33). This means we can use the BLASTn search to find the homologues of the previously known miRNAs in the high number of EST databases. 2) the secondary stem-loop hairpin structure with high negative folding free energy and high MFEI (15). The EST analysis have been widely used to identify miRNAs in plants and animals. Zhang and colleagues (2005) used this approaches identified more than 500 miRNAs in 68 plants species; Weber (2005) used this principle to find 35 new human and 45 new mouse miRNAs (34).

EST analysis also employed to study the diversity and conservation of miRNAs in plants. A recent study by Zhang and colleagues (2006) found that many miRNA families were evolutionarily conserved across all major lineages of plants, including mosses, gymnosperms, monocots and eudicots. Based on their EST analysis, they concluded that regulation of gene expression by plant miRNAs existed at the earliest stages of plant evolution at 425 million year ago (32).

More recently, Zhang and colleagues (2006) further developed the EST analysis to genome survey sequence (GSS) analysis, and successfully identified 188 miRNAs in maize (35) and 20 miRNAs in viruses (36).

The big advantage for EST analysis is to predict miRNAs in multiple species, especially in species whose genomes are unknown, and to study the evolution of specific miRNAs (6, 29, 30, 32). The disadvantage is that EST analysis is limited by the availability of EST database and that EST analysis only can be used to identify conserved miRNAs (6, 29, 30, 32).

5. FUNCTIONS OF MICRORNAS IN PLANTS

Although they were only identified several years ago, miRNAs have become one of the most important gene regulators in both animals and plants. More and more evidences demonstrated that miRNAs get involved in almost all biological and metabolic processes in both animals and plants (4).

5.1. miRNAs control plant tissue and organ development

The easy way to study the function of miRNAs in plant development is to study the mutants of specific

enzymes which are involved in miRNA biogenesis. Currently, several important enzymes, such as DCL1, AGO1, HEN, HYL1, and HASTY, have been well demonstrated to be play very important role in plant miRNA biogenesis and functions (1, 37). Loss-of-function of any single one of these enzymes caused significantly developmental abnormalities. For example, loss-of-function of the *dcl1* gene show a variety of development abnormalities, including arrested embryos at early developmental stage, altered leaf shape, delayed floral development and caused female sterility (38-40). Loss-of-function of hasty gene blocked miRNA-miRNA* duplex transported from nucleus to cytoplasm and further effected the mature miRNA production, and caused several pleiotropic abnormal phenotypes. For example, loss-of-function of hasty gene changed leaf and flower morphology, accelerated plant phase change from vegetative growth to reproductive growth (41). However, loss-of-function of hasty mutant disrupted the phyllotaxis of inflorescence and reduced the fertility of plant (41).

More and more experimental studies demonstrated that almost all plant tissue and organ development are controlled by specific miRNAs. These tissues and organs include leaf, flower, stem, root, and shoot (6).

5.1.1. Leaf development

miRNAs controlled leaf development and leaf morphology. In 2003, Palatnik and colleagues experimentally found the first evidence that miRNAs controlled leaf development. Using a genetic screening technology, they identified one miRNA called miRNA jaw (this jaw miRNA is also called miRNA 159) (42). In their study, Palatnik and his colleagues found miRNA 159/jaw controlled leaf morphology by targeting a subset of TCP transcription factor genes. Overexpression of miRNA 159/jaw resulted low levels of TCP transcript factors and caused a jaw-D phenotypes, which included leaf curvature and uneven leaf. In contrast, overexpression of miRNA 157/jaw-resistant TCP mutants demonstrated that miRNA 159/jaw-mediated mRNA cleavage was sufficient to restrict TCP function to its normal domain of activity (42). Currently, miRNA 159/jaw gene and its target TCP gene have been identified in a wide range of plant species, including dicots, monocots and gymnosperm, which suggests that miRNA 159/jaw-mediated control of leaf development and leaf morphogenesis is highly evolutionary conserved in plant kingdom with very different leaf forms (32, 42).

Another good example for miRNAs which control leaf development is miRNA 165/166 family (43). miRNA 165/166 mediated leaf morphogenesis by targeting class-III homodomain leucine zipper (HD-ZIP) transcription factor gene family (43-45). PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) are three important HD-ZIP transcription factors in plants, and dominant mutations in each of the three transcription factor genes (*phb*, *phv,* and *rev*) caused radialization and adaxialization of leaf and vascular bundles in the stem (46, 47). Overexpression of miRNA 165/166-resistant PHB mutants caused leaf developmental abnormity (43).

miRNA 156 is also involved in leaf development and morphology. Overexpression of miRNA 156 decreased apical dominance and increased leaf initiation, and finally caused bushier plants (48).

5.1.2. Flower development

Several miRNAs controlled floral development and flower morphology. One well-studied example is miRNA 172 (49, 50). miRNA 172 controlled flower development by targeted apetal 2 (ap2) transcription factor. AP2 is one important gene of the class A genes in plant flower development, and AP2 and AP2-like proteins are required for plant flowering and flower development (49, 50). Overexpression of miRNA 172 caused developmental abnormity in floral organ and caused the disappearance of petal tissues, which is same phenotype as loss-of-function of ap2 mutants (49, 50).

Several other miRNAs ware also involved in floral morphogenesis. Mallory and colleagues demonstrated that overexpression of miRNA 164 caused the flower stamens fused together in *Arabidposis*, which suggests that miRNA 164 may control the stamen development (51). miRNA 159 controlled anther development by targeting the expression of two MYB domain transcription factor genes, MYB 33 and MYB 65 (52). One study also indicates that miRNA 171 is predominantly expressed in flowers (53), suggesting that miRNA 171 may function in floral development.

5.1.3. Shoot and root development

miRNA 160 regulated root development by targeting several members of auxin response factors (ARFs). Overexpression of miRNA 160 repressed the expression of ARF 10 and ARF 16 and further affected root development, particularly for root cap formation, in *Arabidopsis* (54).

CUP-SHAPED COTYLEDON 1 (CUC1), CUC2 and NAC1 are three important transcription factors of the NAM/ATAF/CUC (NAC)-domain transcription factor family, which control in both embryogenic and floral development (55, 56). Several studied indicated that missexpression of these three genes caused developmental abnormity in shoot and other organs (51, 55, 56). Recent studies demonstrated that CUC1, CUC2 and NAC1 are the three targets of miRNA 164 (51, 57-59). Overexpression of miRNA 164 resulted in decreased numbers of lateral roots (51, 57-59). In contrast, downregulated miRNA 164 expression caused more lateral root emergence (57).

5.1.4. Stem and vascular development

Overexpression of miRNA 166 resulted in vascular cell differentiation and producing more vascular system with expanded xylem tissue in *Arabidopsis* (60).

5.2. miRNAs control phase change and developmental timing

Developmental timing and phase change is one of the most important biological progresses in plants. Without phase change, plant would not make switch from vegetative growth to reproductive growth, and further can not produce

seeds for next generation. Several studies demonstrated that miRNAs also control phase change and developmental timing in plants.

miRNA 172 regulate phase change by targeting AP2 transcription factor gene, which control flower development. In addition, miRNA 172 also targets several AP2-like genes, such as TOE1, TOE2, TOE3, SNZ and SMZ. Overexpression of miRNA 172 caused early flowering and overcome the later flowering phenotype caused by toe1-1D mutant (49, 50, 61).

miRNA 156 is also involved in phase change and developmental timing by targeting Squamosa promoter binding protein like (SPL) transcription factors. Overexpression of miRNA 156 resulted in quick initiation of rosette leaves, decreasing the apical dominance and a moderate delay in flowering, suggesting that miRNA 156 affected plant phase change from vegetative growth to reproductive growth (48).

miRNA 159 targets the expression of GSMYP. Overexpression of miRNA 159 resulted in a decreased expression of LEAFY, and further affected anther development and delayed flowering in short-day photoperiod (62).

5.3. miRNAs control auxin signaling pathways

Auxin signaling pathway is an important signaling pathways in plant development and plant response to different abiotic and biotic stresses. Recent studies demonstrated that a number of genes in auxin signaling pathway are the targets of several miRNAs. A majority of these target genes belong to auxin response factors (ARF). The potential miRNAs are miRNA 160, miRNA 167 and miRNA 393. Of them, miRNA 160 targets ARF10, ARF16 and ARF17 (59); miRNA 167 targets ARF6 and ARF8 (59, 63). miRNA160-resistant *ARF17* mutant increased *ARF17* mRNA expression levels and altered accumulation of auxin-inducible *GH3*-like mRNAs, *YDK1/GH3.2*, *GH3.3*, *GH3.5*, and *DFL1/GH3.6*, which encode auxin-conjugating proteins, and further caused dramatic developmental defects, including embryo and emerging leaf symmetry anomalies, leaf shape defects, premature inflorescence development, altered phyllotaxy along the stem, reduced petal size, abnormal stamens, sterility, and root growth defects (64). A recent study demonstrated that a flagellin-derived peptide induces miRNA 393 expression, which negatively regulates mRNAs for the F-box auxin receptors TIR1, AFB2, and AFB3, and the repression of auxin signaling restricts bacterial growth, suggesting that auxin in disease susceptibility and miRNA-mediated suppression of auxin signaling in resistance (65).

5.4. miRNAs are involved in plant response to different stresses

Environmental biotic and abiotic stress is one of the biggest issues which affect plant growth and development. For example, hundreds of pests have been reported on cotton and caused more than 30% loss of cotton yield annually (66). In the long-term evolution, plant evolved different mechanisms resistant to different stress (67). More interestingly, recent studies demonstrated that miRNAs may have important functions in plant response to different biotic and abiotic stress. After analyzing millions of ESTs, Zhang and colleagues (2005) found that out of the 476 identified EST contigs containing miRNAs, 123, or 25.84 %, were obtained from stress-induced plant tissues. These tissues were obtained from different biotic and abiotic stresses, such as drought, heat, cold, salinity, pathogen infection, or pests (30). They also found that 36 (29%) EST contigs were associated with pathogen infection; 28 (22%) were associated with water stress; and 25 (19%) were associated with temperature stress. Other stresses, such as nutrition deficiency, salinity, and oxidative stress produced 4%, 3%, and 3% EST contigs, respectively (30). Signal molecules, such as abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) may also relate to miRNA expression (30). These results suggest that miRNAs may play some role in plant responses to environmental stress (30). This conclusion was confirmed by several recent studies. Jones-Rhoades and Bartel (2004) found that sulfate starvation induced miRNA 395 overexpression (26). Two studies demonstrated that the ATP sulfurylase APS4 and the sulfate transporter AST68 are two targets of miRNA 395, and both the ATP sulfurylase APS4 and the sulfate transporter AST68 are accumulated at low-sulfur conditions (26, 68). Another study demonstrated miRNA 399 is involved in plant response to phosphate nutrient deficiency (69). Fujii and colleagues (2005) found that miRNA 399 was overexpressed in *Arabidopsis* treated with phosphate deficiency. Recently, Sunkar and Zhu (2004) found miRNA 402 was strongly induced by different environmental stresses, including drought, cold and salinity. However, several other miRNAs are induced by either cold stress or drought condition (70). More interestingly, mechanical stress also induced the overexpression of several miRNAs (for example miRNA 473 and miRNA 477) in *Populus trichocarpa* species, suggesting that miRNAs may function in critical defense systems for structural and mechanical fitness (71).

miRNAs are also involved in plant response to pathogen infection (72), small RNA biogenesis (73) and other several biological and metabolic processes (4).

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