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MicroRNAs in tomato plants

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MicroRNAs (miRNAs) are a specialized class of small silencing RNAs that regulate gene expression in eukaryotes. In plants, miRNAs negatively regulate target mRNAs containing a highly complementary sequence by either mRNA cleavage or translational repression. As a model plant to study fleshy fruit ripening, miRNA studies in tomato have made great progress recently. MiRNAs were predicted to be involved in nearly all biological processes in tomato, particularly development, differentiation, and biotic and abiotic stress responses. Surprisingly, several miRNAs were verified to be involved in tomato fruit ripening and senescence. Recent studies suggest that miRNAs are related to host–virus interactions, which raises the possibility that miRNAs can be used as diagnostic markers for response to virus infection in tomato plants. In this review, we summarize our current knowledge systematically and advance future directions for miRNA research in tomato.

microRNAs, tomato, research methods, target gene prediction, functional analysis

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MicroRNAs (miRNAs) are a class of small endogenous non-coding RNAs that modulate gene expression in both plants and animals. A large number of miRNAs are evolutionarily conserved across species boundaries [1–3]. Mature miRNAs are derived from single-stranded RNA transcripts that possess an imperfect stem-loop secondary structure [4]. These hairpins are processed by DCL1 into the miRNA/miRNA* duplex in the nucleus and are transported to the cytoplasm in plants [5,6]. The miRNAs are incorporated into the RNA induced silencing complex (RISC), which use them as guides to recognize target complementary mRNAs and negatively regulate their expression by degradation [7] or repression of productive translation [8]. Plant miRNAs play vital roles in multiple essential biological processes, such as leaf morphogenesis and polarity [9], floral organ identity [10], and stress responses [11,12].

Tomato (*Solanum lycopersicum*) is a model plant for the study of fleshy fruit ripening and senescence owing to its genetic and molecular tractability [13]. Tomato fruit development goes through distinct stages [14,15]: (i) floral development and fruit set, (ii) cell division after anthesis and fertilization, (iii) cell expansion, and (iv) fruit ripening.

Tomato fruit development and ripening is an orderly, highly harmonious genetic process, and the substantial changes in cellular and biochemical events during this process allow integrative analyses of many aspects of plant biology [16–18]. Although the tomato genome database is not complete, great progress has been made in tomato miRNA research recently [19–21]. In this review, we summarize our current knowledge and advance future directions for microRNA research in tomato.

1 Research methods

1.1 Bioinformatic analysis

The high degree of sequence conservation of miRNAs [22] provides the opportunity to identify conserved miRNAs

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from all plant species [23]. The homology search approach was adopted to identify conserved miRNAs in tomato [24,25]. However, this method cannot be used to explore novel and species-specific miRNAs.

By searching known miRNAs identified from plant species against tomato nucleotide sequences, Zhang *et al*. [24] predicted 13 miRNA candidates distributed in nine miRNA families (miR157, miR159, miR162, miR167, miR171, miR172, miR319, miR395 and miR399) from over 578000 tomato sequences. In addition, mature miRNAs and the precursors of miR319, miR171 and miR162 have been cloned [15,19,26].

Using the same filtering criteria, Yin *et al*. [25] detected 21 conserved miRNAs that belong to 14 miRNA families (miR156/157, miR159, miR160, miR162, miR167, miR168, miR169, miR172, miR399, miR403, miR437, miR830, miR869.1 and miR1030), of which seven were identified in the EST database and 14 in the GSS database, but some of them were not confirmed in tomato.

1.2 Direct cloning

Direct cloning is a straightforward and basic means to identify miRNAs in tomato. Several conserved and novel miRNAs can be found by this method, but the quantity is far from enough. Pilcher *et al.* [26] cloned 4018 sRNAs from tomato fruit tissue at the mature green stage and detected tomato homologs of nine known miRNAs (miR159, miR160, miR162, miR164, miR166, miR168, miR171, miR408 and miR482), particularly miR482, a poplar miRNA not conserved in *Arabidopsis* or rice. These authors also identified three novel putative miRNAs (Put-miRNA1, 2 and 3), of which Put-miRNA3 exhibited significantly higher expression in fruit compared with leaf tissues, indicating a specific role in fruit development processes [26].

In contrast to Pilcher *et al.* [26], Itaya *et al*. [15] cloned and sequenced RNAs of 15–30 nt from tomato mature leaves as well as fruit at three successive stages of development: flower bud, young fruit at 10 days post-anthesis (equivalent to the early cell expansion stage), and mature ripe fruit. A total of 1210 non-redundant sequences were obtained. Additional miRNAs (miR167, miR169, miR172, miR390, miR424, and miR472) were identified, but miR424 has not been confirmed experimentally. Itaya *et al.* identified 6 putative miRNAs whose target genes are unknown, and also found that SlsmR-596 (23 nt) contains the sequence of the putative miRNA2 (18 nt) reported previously [26]. However, these authors did not recover miR319 reported by Ori *et al*. [19]. Most important of all, they established a database for tomato sRNAs (http://ted.bti.cornell.edu/cgi-bin/TFGD/sRNA/home.cgi) that is updated regularly and which can provide ultramodern and overall knowledge.

1.3 High-throughput sequencing

The 454 sequencing platform was used to sequence tomato sRNAs from young leaves and a mixture of young green fruits of the Micro-Tom cultivar [27]. Most known conserved miRNAs were found in their sRNA libraries, and many of them showed differential expression levels between leaves and fruit. A total of 7912 redundant sequences were found to match 20 known miRNA families and 25436 sequences identified that were either shorter/longer or contained up to two mismatches to another 10 known miRNA families. However, the expressions of two miRNAs in tomato were confirmed by Northern blot analysis that had previously been thought specific to *Arabidopsis* [23] and *Physcomitrella patens* [28].

Moxon Simon *et al*. [27] analyzed the expression levels of 13 additional known miRNAs that were present in their libraries and that had not been examined in their previous study using Northern blot [26]. All tested miRNAs showed differential expression patterns in these tissues except for miR165/166, miR403, and miR472. Several miRNAs (miR156/157, miR164, miR408, miR858 and miR894) were more abundant in leaves and closed flowers than in fruits. In contrast, miR169 was expressed at a higher level in all fruit stages than in closed flowers, and it was almost undetectable in leaves. Two known miRNAs (miR171 and miR390) showed higher accumulation in very small fruits but accumulated at a very low level in more mature fruits, which suggests a specific role in early fruit formation. Interestingly, one of the target genes of miR156/157 is *CNR*, which implies miRNA involvement in the fruit maturation process. This result opens a new avenue in the field of gene expression regulation during fruit development and ripening.

These authors found four new tomato miRNAs (miR1916, miR1917, miR1918 and miR1919). MiR1916 is expressed at a similar level in all analyzed tissues. MiR1917 produced a consistently weak signal, and it was necessary to use an LNA probe to reveal stronger accumulation in more mature fruit. Accumulation of the new miRNAs was analyzed by Northern blotting, and miR1918 and miR1919 showed significantly stronger expression in fruit than in the leaf or flower bud. In fact, these two miRNAs accumulated at a higher level in more mature fruit than in very young fruit. Intriguingly, the target genes of miR1917 are a member of the *CTR* family that suppresses ethylene response and is involved in fruit ripening [29].

2 Target gene prediction of conserved and novel miRNAs in tomato

Obtaining insight into the miRNA targets will help us to understand the functional importance of miRNAs. According to information provided in National Center for Biotechnology Information (NCBI) databases, the identified

mRNA targets could be mainly separated into three groups. The first and largest group contained targets thought to encode transcription factors, which are known to be involved mainly in plant growth and developmental patterning. The second group contained targets encoding a range of different proteins implicated in a variety of metabolic processes, while the third group was involved in functions such as hormone responses, stress defense and signaling [25,30].

In the present study, we presented global predictions of the targets (Table 1) of all the conserved and novel tomato miRNAs obtained mainly from three databases (http://www.mirbase.org/, http://ted.bti.cornell.edu/cgi-bin/TFGD/sRNA/miRNA.cgi and http://bioinfo3.noble.org/psRNATarget/). This is only a computational approach, which can never replace biological verification and can be used only to guide experimental design. At present, several target genes are confirmed [20,21], but most have not been verified yet and so experimental analysis of the target genes will be an important focus of future research.

3 Functional analyses

Delicate molecular mechanisms are required for plants to accomplish physiological and developmental processes, as well as responses to environmental stimuli. Increasing evidence indicates miRNAs are one essential member of these mechanisms [4,31–33]. Determining how miRNAs play a role in plant adaptation to environmental stimuli is very important. The first step is identification of miRNAs that are differentially expressed in response to these stimuli. Several studies on this topic have been published recently [34,35].

Table 1 The targets of conserved miRNAs and novel miRNAs in tomato

MiRNA	Targeted protein	Target function
miR156/157	Squamosa promoter-binding protein	Transcription factor
miR159	GAMyb-like1/2	Transcription factor
	ACC synthase	Metabolic process
miR160	Auxin response factor 10	Hormone response
miR162	MYB-like DNA-binding protein	Transcription factor
miR164	NO APICAL MERISTEM family protein	Transcription factor
miR165/166	DNA-binding protein	Transcription factor
	PHAVOLUTA-like HD-ZIPIII protein	
miR167	Auxin response factor	Hormone response
miR168	Argonaute protein (AGO1)	Metabolic process
miR169	CCAAT-binding transcription factor	Transcription factor
miR170/171	Scarecrow transcription factor family protein	Transcription factor
miR172	APETALA2 (AP2)-like protein	Transcription factor
miR319	GAMYB	Transcription factor
miR390	Protein phosphatase 2C-related	Transcription factor
miR394	F-box family protein	Transcription factor
miR395	Sulfate adenylyltransferase 1	Metabolic process
	ATP-sulfurylase	
miR396	GRAS family transcription factor	Transcription factor
	MADS-box protein	Transcription factor
	Auxin response factor 8	Hormone response
miR397	Laccase	Metabolic process
	GTP-binding-like protein	
miR399	Oxoglutarate/malate translocator	Metabolic process
	Glucose-1-phosphate adenylyltransferase	
miR403	Argonaute protein	Metabolic process
miR408	Cyclin A3	Metabolic process
miR472	Disease resistance protein	Transcription factor
miR482	Disease resistance protein	Transcription factor
miR827	SPX domain-containing protein	Transcription factor
miR828	Myb family transcription factor	Transcription factor
	Ethylene-insensitive 2 (EIN2)	
miR858	Myb family transcription factor	Transcription factor
	Ripening-regulated protein DDTFR18	Metabolic process
miR1916	Mitochondrial glycoprotein family protein	Transcription factor
miR1917	Serine/threonine protein kinase (CTR1)	Transcription factor
	Transcription regulatory protein SNF5	Metabolic process
	2OG-Fe(II) oxygenase family protein	
miR1918	AMP-binding protein	Transcription factor
	Protochlorophyllide reductase B gibberellin-regulated protein 4	Metabolic process
miR1919	Unknown	Unknown

3.1 Phosphate stress-related miRNAs

Recently, miRNA395, miRNA398, and miRNA399 have been well characterized and linked with nutrient deficiency-induced stresses [36–38], of which miRNA399 is specifically induced by phosphate (Pi) starvation.

Gu *et al*. [35] identified a total of 16 miRNAs in tomato that were differentially regulated by either P nutrition or arbuscular mycorrhizal (AM) colonization or both. These authors found that miRNA319, miRNA394 and miR399 were differentially regulated under three different treatments in roots, of which miR319 and miR399 were also responsive to the treatments in leaves. This indicates that miRNAs are probably a component for P nutrition and AM symbiosis signaling.

Plants have evolved a set of strategies to adjust to an environment with limited Pi, which involves alteration of root architecture, enhanced excretion of organic acid and acid phosphatase, and formation of symbiotic associations with AM fungi [39]. Alterations caused by Pi deprivation are finely controlled by diverse molecular mechanisms, in which miRNAs besides miR399 might also display an important regulatory role. On the other hand, AM colonization in turn can help plants with exploration for P away from the rhizosphere, transform the inaccessible forms of P into Pi, and thus improve the P nutrition of plants [40].

3.2 Viral stress-related miRNAs

Viral infections of plants can result in disease symptoms that range from mild discoloration to severe developmental defects and death [41,42]. In recent years, it has been demonstrated that small interfering RNAs (siRNAs) and miRNAs play important roles in host–pathogen interactions [43,44].

Cucumber mosaic virus (CMV) and *Tomato aspermy virus* (TAV) are species of the genus *Cucumovirus*, within the family *Bromoviridae* [45]. The two viruses share very similar genomic structures, but they differ in both host range and symptomatology. CMV-Fny causes systemic mosaic with mild leaf distortion, whereas TAV-Bj induces stunting, reduced internodal distances, mosaic and pronounced lobbing of leaves. After CMV and TAV infection in tomato, expression levels of seven miRNAs were elevated. Among these miRNAs, miR159, miR162, miR168 and miR171 showed significant expression level changes, while expression of miR164, miR165/166 and miR167 were less affected. The significant increase in expression levels of miR159, miR162 and miR168 after CMV-Fny infection was concordant with those reported by Zhang *et al*. [46] in *Arabidopsis*, and the lowest change of miR171 expression in CMV-2b transgenic plants was also reported previously [47], which may correlate with their biological function. However, elucidation of the mechanism underlying TAV and CMV interference of plant miRNA pathways is required to support this hypothesis.

Tomato leaf curl New Delhi virus (ToLCNDV) is a member of the *Begomovirus* genus infecting tomato with a hallmark disease symptom of upward leaf curling. Since miRNAs are known to control plant developmental processes, Naqvi *et al*. evaluated the roles of miRNAs in ToLCNDV-induced leaf curling [48]. These authors found that ToLCNDV infection significantly deregulated numerous miRNAs representing 13 different conserved families. The precursors of these miRNAs showed similar deregulated patterns, indicating that the transcriptional regulation of the respective miRNA genes was perhaps the cause of deregulation. The expression levels of the miRNA-targeted genes were antagonistic with respect to the amount of corresponding miRNA.

MiRNA profiling is a good indicator of many diseases, especially cancers [49]. MiRNAs can also serve as an ideal biomarker for discriminating poor-quality or ‘manipulated’ milk from pure raw milk, as well as for quality control of commercial milk products, such as fluid milk and powdered formula milk [50]. However, use of plant miRNAs as biomarkers of disease is still at an exploratory stage. The research of Naqvi *et al*. [48] raises the possibility of using miRNA(s) as potential signature molecules for ToLCNDV infection. Thus, we can deduce certain host miRs are likely indicators of viral infection and potentially could be employed to develop viral resistance strategies.

MiRNA suppression is likely to lead to developmental defects in infected plants [51], some of which might resemble symptoms of virus-infected plants [41]. To obtain high P19 expression and study its effects on host plant development in the absence of virus infection, HA-tagged P19 (P19HA)-transgenic tomato reporter plants using the pOp/LhG4 transactivation system were generated [52]. The transactivated F_1 plants expressed high levels of a functional P19HA protein and displayed multiple abnormal phenotypes. Phenotype severity correlated with P19HA expression level, amount of bound miRNA/miRNA* duplexes, and accumulation of miRNA target transcripts. These results demonstrated that the tomato miRNA pathway is markedly compromised by P19, in particular when this protein is relatively abundant, as occurs during natural infection [52]. P19 is a high-affinity short (19–21 nt) double-stranded RNA-binding protein and that dimers of P19 bind siRNA duplexes in a 1∶1 stoichiometric ratio [53–56]. This binding prohibits bound duplexes from unwinding and programming the RISC [57]. As a consequence, RISC-mediated degradation of cognate viral RNAs is repressed [53].

3.3 Transgenic analysis in exploring miRNAs function

MiRNAs regulate diverse plant growth and developmental processes. Furthermore, plant miRNAs work cooperatively or antagonistically to establish a balanced regulation [58]. To date, the roles of miRNAs are mostly deduced from ob-

taining miRNA-overexpressing transgenic plants or gain-of-function mutants in which miRNA-resistant target genes are ectopically expressed.

The biological function of miR156 was investigated by overexpression in tomato. Transgenic tomato plants exhibited a drastically altered phenotype, such as dwarfism, a 'bush-like' structure, more abundant leaves, shorter plastochron, later flowering, smaller and fewer fruits, and produced numerous adventitious roots [59]. The inflorescence structure of miR156-overexpressing plants phenocopied the *sft* mutant [60]. Tomato sympodial shoot development is regulated by the SFT/SP balance [61], so the aberrant vegetative inflorescence shoots of the transgenic plants may be attributed to the decreased SFT/SP ratio. The targets of miR156 included six (SBP)-box transcription factor genes. These target genes, as well as the tomato FLOWERING LOCUST (FT) ortholog SFT, were significantly down-regulated in the miR156-overexpressing plants [59]. An epigenetic mutation in a tomato SBP-box gene (*COLORLESS NON-RIPENING*; *CNR*) resulted in colorless non-ripening fruits [62]. Cleavage of *CNR* by miR156 was also demonstrated by 5′-RACE analysis [27], but in the transgenic plants, the fruit color was slightly lighter red than that of the wild type. This could be because overexpression of miR156 down-regulated, but did not eliminate expression of *CNR* genes. The transgenic tomato plants showed not only a reduced fruit number, but also a decreased fruit weight, implying that miR156 plays an important role in fresh fruit development [59].

MiR159 is a highly conserved miRNA with roles in flowering under short days, anther development and seed germination via repression of *GAMYB-Like* genes [63]. Accordingly, the function of miR159 in tomato is currently poorly understood mainly because the corresponding target mRNAs have not been identified nor their biological roles elucidated. To date, only MYB-related transcription factors, most of which belong to the GAMYB family, have been experimentally validated as targets of miR159 in *Arabidopsis* [9,64–66] and rice [67], establishing miR159 as a major GAMYB post-transcriptional regulator in plants. But Buxdorf *et al*. identified and characterized a new miR159 target gene (*SGN-U567133*) in tomato that is not related to MYB [21]. These authors found that miR159 also functions as a posttranscriptional regulator of *SGN-U567133*, which encodes a novel tomato protein that is dissimilar to the GAMYB family of transcription factors. This target defines an as yet unknown novel function for this miRNA in tomato.

Several NAC-domain genes, including *CUC1* and *CUC2* in *Arabidopsis*, are subject to negative control by miR164. Analysis of miR164 mutations and overexpression, as well as of miR164-insensitive CUC forms, has further revealed the importance of these genes for proper plant development [68–70]. A NAC-domain transcription factor encoded by the *GOB* gene is also a target gene in tomato. Berger *et al*. [20] found that overexpression of miR164 and mutations in GOB lead to loss of secondary-leaflet initiation and to smooth leaflet margins. MiR164 also affects leaflet separation in *Cardamine hirsuta*, a Brassicaceae species with complex leaves [71,72].

LA encodes a transcription factor from the TCP family that contains a miR319-binding site [9,73]. Ori *et al*. [19] found that the reduced sensitivity to miR319 resulted in elevated *LA* expression in very young leaf primordia and accelerated differentiation of leaf margins. On the other hand, increased expression of miR319 led to larger leaflets and continuous growth of leaf margins. These results explain why a higher level of *LA* causes simple leaf formation and overexpression of mir319 leads to enlarged leaflets with highly lobed margins.

4 Conclusion and perspectives

Research on miRNAs in tomato has passed through several stages: classical miRNA cloning including bioinformatics prediction based on the criteria for plant miRNA definition [74], and subsequent experimental validation. With emergence of deep sequencing, 454 pyrosequencing was first employed by Moxon *et al*. [27] in tomato, and is the only deep sequencing study so far proved to be unsaturated. More effective platforms such as Solexa can be used to explore a greater number of miRNAs, but the lack of a complete genome sequence is a limiting factor for miRNA research in tomato.

Plant growth and developmental processes regulated by miRNAs are quite diverse. Several miRNAs are differentially expressed in different tissues. The miRNAs in tomato also show different expression levels, which has been validated by Northern blotting. In addition, quantitative expression differences can be obtained by deep sequencing of different tissues and confirmed by qRT-PCR, particularly for fruit at different stages of maturity, and specific analysis of these can help us understand the regulatory mechanism of miRNAs.

The all-important step to understand the biological functions of miRNAs is the search for target genes. There are several websites for target prediction (e.g. http://bioinfo3.noble.org/psRNATarget/ and http://ted.bti.cornell.edu/cgi-bin/TFGD/sRNA/miRNA.cgi). Several miRNAs have multitarget genes involved in a range of transcription factors, metabolic processes, stress defense and signaling. Validation of miRNA targets by 5′-RACE is achieved by miRNA-guided cleavage at the transcriptome level [75]. Plant miRNAs regulate numerous target genes and several miRNAs share the downstream targets. For example, miR160 and miR167 signals converge at the *GH3* genes; miR172 and miR156 also seem to share targets, such as the *SPL3/4/5* genes, to modulate vegetative phase transition. These studies indicate that the network of miRNAs is quite complicated, and elucidation of the molecular mechanisms underlying the interplay between miRNAs within the plant

cell requires further study.

The expression level of miRNAs varies in response to environmental changes, and especially under different kinds of stresses and virus infection the expression level of specific miRNAs change. This may offer a rationale for further study of miRNA regulatory mechanisms. In addition, especially after microbe or virus infection, the use of specific miRNAs as a biomarker is still under exploration.

There are several approaches to study the functions of miRNAs, such as overexpression of the target miRNAs, expression of mutant target genes [9,76], and expression of miRNA target mimicries [77], and these methods are usually tedious and time consuming for generation of stable transgenic plants. Surprisingly, a viral miRNA expression system could be used to study the function of endogenous miRNA genes by agro-infiltration in *Nicotiana benthamiana* plants [78]. The modified CbLCV vector may be useful in high-throughput screening of miRNAs in *N. benthamiana.* Whether a microRNA–virus-induced gene silencing system is feasible in tomato requires exploration, and we believe it will be an important topic in future research.

Recently, an artificial miRNA (amiRNA) approach has emerged and can be used as a highly specific, high-throughput silencing system. An amiRNA system has been developed in several species [79–81]. A specialized website exists for amiRNAs research (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). Artificial microRNAs have been used already in tomato [82]. Recently, Zhang *et al.* also found that expression of amiRNAs in tomato can target and degrade the invading viral RNA, consequently conferring virus resistance. Their study provides new evidence for the use of amiRNAs as an effective approach to engineer viral resistance in tomato and possibly other crops.

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