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RESEARCH ARTICLE

Identification of microRNAs in two species of tomato, *Solanum lycopersicum* and *Solanum habrochaites*, by deep sequencing

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Abstract

MicroRNAs (miRNAs) are ~21 nucleotide (nt), endogenous RNAs that regulate gene expression in plants. Increasing evidence suggests that miRNAs play an important role in species-specific development in plants. However, the detailed miRNA profile divergence has not been performed among tomato species. In this study, the small RNA (sRNA) profiles of *Solanum lycopersicum* cultivar 9706 and *Solanum habrochaites* species PI 134417 were obtained by deep sequencing. Sixty-three known miRNA families were identified from these two species, of which 39 were common. Further miRNA profile comparison showed that 24 known non-conserved miRNA families were species-specific between these two tomato species. In addition, six conserved miRNA families displayed an apparent divergent expression pattern between the two tomato species. Our results suggested that species-specific, non-conserved miRNAs and divergent expression of conserved miRNAs might contribute to developmental changes and phenotypic variation between the two tomato species. Twenty new miRNAs were also identified in *S. lycopersicum*. This research significantly increases the number of known miRNA families in tomato and provides the first set of small RNAs in *S. habrochaites*. It also suggests that miRNAs have an important role in species-specific plant developmental regulation.

Keywords: microRNAs, *Solanum lycopersicum*, *Solanum habrochaites*, deep sequencing

1. Introduction

miRNAs are small, endogenous RNAs that play crucial roles as regulators of gene expression in plants. The biogenesis of these ~21 nt small RNA starts with perfect, or near-perfect, double-stranded RNA precursor transcripts. Long precursors are processed by a Dicer-like enzyme that generates an miRNA:miRNA* duplex. The single-stranded mature miRNAs then recognize target genes based on sequence complementarity and repress expression of the target gene through cleavage or translational repression of mRNA in silencing

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complexes (Bartel 2004; Chen *et al*. 2004; Jones-Rhoades *et al*. 2006; Brodersen *et al*. 2008). Increasing evidence indicates that miRNAs play an important role in many biological processes, like signal transduction, stress response and plant development (Palatni *et al*. 2003; Sunkar and Zhu 2004; Jones *et al*. 2006; Liu *et al*. 2008; Gutierrez *et al*. 2009; Naqvi *et al*. 2010; Rodriguez *et al*. 2010).

Currently, computational prediction and deep sequencing are the two main approaches for miRNAs identification (Jones-Rhoades *et al*. 2006). The former relies on analysis of sRNA homologs or secondary structural characteristics. However, many recently evolved species-specific miRNAs could elude this comparative methodology detection. Compared with the computational prediction, the availability of deep sequencing technology provides a powerful tool to discover new miRNAs. Many studies have used this approach to clone and identify miRNAs (Fahlgren *et al*. 2007; Moxon *et al*. 2008; Sunkar *et al*. 2008; Zhang *et al*. 2009).

According to FAO (Food and Agriculture Orgnization of the Untied Nations), the production of tomato in the world is more than 160 million t, that is just behind potato which production is about 360 million t. The cultivated tomato (*Solanum lycopersicum*) has been the subject of numerous studies for genetic improvement (Cagas *et al*. 2008). *Solanum habrochaites,* which is considered to be the most important gene sources of tomato, has been used for improving cultivated tomato growth at suboptimal temperature, insect resistance and fruit sucrose accumulation (Goodstal *et al*. 2005; Spooner *et al*. 2005; Hanson *et al*. 2007). To date, a great deal of effort has been made into genomic and transcriptional divergence analysis between these two tomato species (Goodstal *et al*. 2005; Spooner *et al*. 2005; Hanson *et al*. 2007; McDowell *et al*. 2011).

Small RNA investigation in tomato has a relatively short history (Dalmay 2010). Only limited information has been obtained on the existence and function of miRNAs from tomato (Itaya *et al*. 2008; Moxon *et al*. 2008; Mohorianu *et al*. 2011), and no miRNA information in wild tomato has been reported.

In this research, we investigated the sRNA profiles of *S. lycopersicum* cultivar 9706 and *S. habrochaites* wide species PI 134417, using deep sequencing technology. The goal of this study is to analyze the expression divergence of known miRNAs and identify the new miRNAs in tomato. These findings provide a firm basis for further functional characterization of miRNAs in cultivated and wild tomato.

2. Results

2.1. Deep sequencing of small RNAs from *S. lycopersicum* and *S. habrochaites*

A total of 17 077 223 and 17 834 355 primary reads in *S. lycopersicum* and *S. habrochaites* libraries, respectively, were generated by deep sequencing. After removing the low quality tags and cleaning up the contaminating reads, 16 282 007 and 17 115 875 clean reads were obtained from the SL and SH libraries, respectively. These reads represented around 3 265 881 and 3 561 510 unique sRNA sequences, respectively (Table 1).

Table 1 Statistics of small RNA sequences for SL and SH libraries

	Number of reads	Number of unique sequences
SL		
Primary reads	17 077 223	
Clean reads	16 282 007	3 265 881
Match known miRNAs	3 410 905	218
SH		
Primary reads	17 834 355	
Clean reads	17 115 875	3 561 510
Match known miRNAs	3 060 404	236

The length distribution of sRNAs showed that the majority of the reads were 18–25 nt in size (Fig. 1). However, the length distribution of two libraries was different, with 21 nt sRNAs being the most abundant in SL and the 24 nt class being the most abundant in the SH library. This implies that the mechanism of sRNA regulation in the two tomato species is different (Moxon *et al*. 2008). The 21 nt classes are usually miRNAs and trans-acting siRNAs (ta-siRNAs), but the 24 nt classes are mainly heterogeneous sRNA populations, such as those found associated with RNA polymerase IV-dependent pathways in *Arabidopsis* that produce heterochromatin-related siRNAs (Zheng *et al*. 2007). There are lots of researches about miRNAs related to plants development and resistance, but only few papers are about the heterochromatin-related siRNAs thus its function are not clearly yet (Borsani *et al*. 2005; Zheng *et al*. 2007).

2.2. Identification of known miRNAs in two libraries

The known miRNA homologs were identified from the two tomato libraries; sequence counts lower than 10 were removed. In summary, 39 miRNA families are common in this two libraries, 21 are conserved families and 18 are non-conserved miRNA families. 218 and 236 known miRNAs were identified from SL and SH libraries, respectively (Table 2). The detailed sequence information is shown in Appendix A. A total of 163 and 179 miRNAs belonging to 21 conserved miRNA families were identified from the SL and SH libraries, respectively. In addition to the conserved miRNAs, 55 and 57 non-conserved miRNAs belonging to 30 miRNA families were indentified from the SL and SH libraries, respectively.

Among these conserved miRNA families that we found,

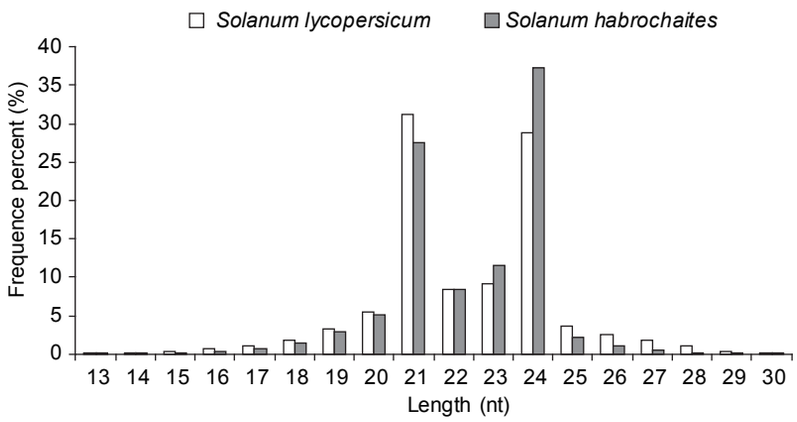

Fig. 1 The length distribution of small RNAs from SL and SH libraries.

miR162, miR168, miR394, miR395, miR396, miR403, and miR408 have no records in the miRBase for tomato. And some miRNAs that have been reported specific in other plants are also found in tomato, like miR1863, miR1873 which are specific in rice (Zhu *et al*. 2008; Wu *et al*. 2009).

2.3. Expression divergence of conserved miRNA families in *S. lycopersicum* and *S. habrochaites*

The normalized expression of conserved miRNA families in the two libraries (SL and SH) were compared (Fig. 2 and Appendix B). MiRNA families with sequencing counts greater than 2-fold difference between the two tomato species were chosen for further analysis. Six conserved miRNA families were identified, of which four families (miR167, miR171, miR394, miR396) had higher expression levels in the SL library and two families (miR397, miR408) had higher expression levels in SH library. Of these miRNAs, miR156 had the highest expression level in both libraries. The known targets of these miRNAs and their functional annotations are summarized in Table 3.

Four miRNA families have higher expression levels in the SL library. The identified target of miR167 in tomato is ARF6 and ARF8 (auxin response factor)(Allen *et al*. 2005; Yin *et al*. 2008), auxin plays critical roles during plant growth, many of which are mediated by members of the ARF family (Guilfoyle *et al*.1998). miR171 acts to negatively regulate shoot branching through targeting GRAS gene family members SCARECROW-LIKE6-II (SCL6) (Wang *et al*. 2010), and target gene SCL6 has been identified in tomato, too (Lopez-Gomollon *et al*. 2012). In tomato miR167 and miR171 expressed highly in the flowers but dropped very quickly after fruit formation (Lopez-Gomollon *et al*. 2012). There are some evidence show that miR167 and miR171 are related to pant stress response, like after the hibiscus chlorotic ringspot virus (HCRSV) infection in kenaf, the expression of miR171, miR167 and their target genes have changed (Gao *et al*. 2013). Also after the infection of CMV (*Cucumber mosaic virus*), TAV (*Tomato aspermy virus*) or ToLCNDV (*Tomato leaf curl New Delhi virus*) in tomato, the expression of miR171 has all changed in these three different conditions (Li *et al*. 2012; Jin *et al*. 2012). One identified target of the two miRNAs (miR394 and miR396) is LCR (LEAF CURLING RESPONSIVENESS), which encodes an F-box protein (SKP1-Cullin/CDC53-F-box). Many publication papers have showed that LCR expresses at all development stages (Song *et al*. 2013). Also, miR394 has been identified as a mobile signal (the protoderm) that confers stem cell competence to the distal meristem by repressing the F-box protein LCR (Knauer *et al*. 2013). Furthermore, miR394 was also reported to be induced by abiotic stresses in plants (Jones-Rhoades *et al*. 2006). The *Arabidopsis* plants which over-expression of miR394 show highly tolerant to severe drought stress compared

Table 2 miRNA family classes and miRNAs in SL and SH libraries

	SL	SH	Common miRNA family	Specific miRNA family in SL	Specific miRNA family in SH
miRNA family class	51	51	39	12	12
Conserved miRNAs family class	21	21	21	–	–
Non-conserved miRNA family class	30	30	18	12	12
miRNA class	218	236	158	12	14
Conserved miRNA class	163	179	137	–	–
Non-conserved miRNA class	55	57	43	12	14

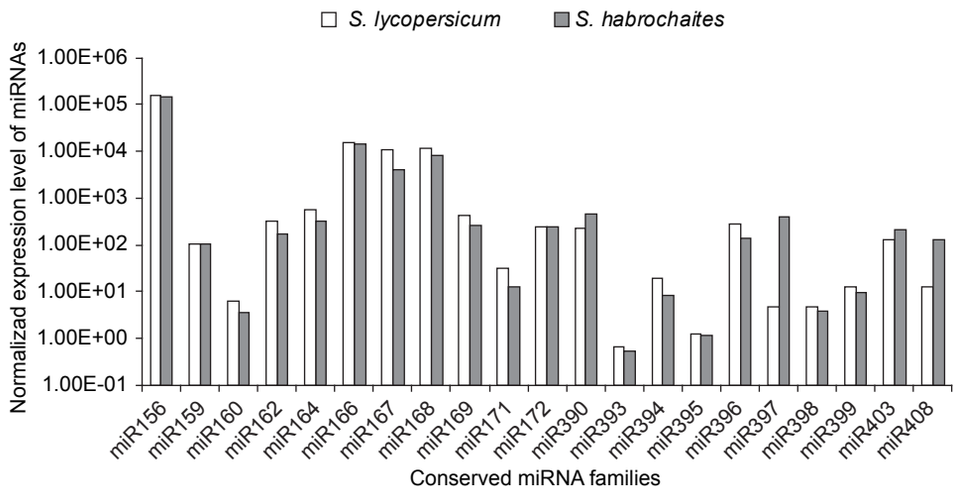

Fig. 2 Normalized sequencing frequency of conserved miRNA families from SL and SH libraries.

Table 3 The targets of differentially expressed conserved miRNA families and their function annotations

	Targets	Functions and responsiveness	References	Confirmed targets in *S. lycopersicum*[1)]
miR166	Auxin response factors ARF6, ARF8	Lateral root development	Gutierrez *et al*. (2009)	Y
miR171	GRAS transcription factors	Root and shoot development	Wang *et al*. (2010)	Y
miR394	F-box	Various developmental processes and stress responses	Yan *et al*. (2011)	N
miR396	Growth regulating factor (GRF)	Leaf and cotyledon	Rodriguez *et al*. (2010) Kim *et al*. (2003)	N
miR397	Laccase	Lignin synthesis, detoxification and so on	Cai *et al*. (2006)	N
miR408	Plantacyanin	Abiotic stress	Sunkar and Zhu (2004)	N

[1)] Y, target has been confirmed in *Solanum lycopersicum* in the research; N, target hasn't been confirmed in *S. lycopersicum.*

with wild-type (Song *et al*. 2013). Therefore, the different expression of these four miRNAs may be one reason of the different phenotype and also the different stress response mechanisms between this two tomato species.

miR397 and miR408 have higher expression levels in SH library. miR397 is slightly up-regulated by the stress treatments like cold, dehydration, NaCl, and ABA. The identified target gene in *Arabidopsis* is laccases (Sunkar and Zhu 2004), higher expression levels of miR397 in *S. habrochaites* may contribute to a lower laccase expression in this species, which may have relationships with *S. habrochaites* against various herbivorous insects (Yu *et al*. 2010). One target of miR408 is TaCLP1 which has identified in wheat and *Arabidopsis*, overexpression TaCLP1 in yeast (*Schizosacharomyces pombe*) can significantly increase cell growth under high salinity and Cu^{2+} stresses, this research shows that miR408 and its target TaCLP1 plays an important role in regulating resistance of host plants to abiotic stresses and stripe rust, and such interactions can be a valuable resource for investigating stress tolerance in wheat (Feng *et al*. 2013). *S. habrochaites* is a wild tomato species that have resistance to at least 18 kinds of insects (Labate *et al*. 2007), but if these miRNAs are involved in tomato resietance still not clear and further research is necessary.

2.4. Specific miRNA families between the two libraries

Twenty-four known non-conserved miRNA families were specific to the two tomato species, 12 were specific to the SL library and 12 were specific to the SH library. But they also have 18 common to both libraries. And the targets of these miRNAs were predicted. Transcription factors, calmodulin-binding transcription activators, protein kinases, stress response proteins, and other protein families were predicted to be potential targets of these miRNAs (Appendix C).

The predict target genes of miRNAs that specific express in *S. lycopersicum*, include transcription factors, calmodulin-binding transcription activators, protein kinases, and so on. These genes may contribute to the different phenotypes between these two species. miRNAs that specific expression in SH library contains two resistance related genes, miR2118 and miR417, the predicted target genes

is Cc-nbs-lrr resistance protein and pentatricopeptide repeated-containing protein, respectively (Shivaprasad *et al*. 2012). As known to all, *S*. *habrochaites* have resistance to many insects, so resistance genes may have contribute to that. miR858, specific expression in SH library, one predict target is MYB-related transcription factor. Noda (1994) have proved that it related to the flower color intensity. So this may cause the different color intensities between them. But all these are hypothesis, the predict target genes still need to be identified and analysed for their functions.

2.5. Identification of new miRNAs

The formation of a stable hairpin structure was one of the essential features for the identification of new miRNAs (Ambros *et al*. 2003). A miRNA* strand was also considered as strong supporting evidence for miRNA identification (Meyersa *et al*. 2008). We predicted potential new miRNA precursors from the SL library. miRNAs with counts fewer than 20 and miRNA* counts fewer than 10 were discarded. Eighteen new miRNAs were identified, as shown in Table 4. The secondary structures of the predicted precursors are given in Appendix D.

3. Discussion

3.1. Deep sequencing of tomato small RNAs

There have been several reports on the identification of miRNAs in *S*. *lycopersicum* (Itaya *et al*. 2008; Moxon *et al*. 2008; Mohorianu *et al*. 2011). Most of the sRNA profiles reported in tomato focused on fruit development and leaf development (Itaya *et al*. 2008; Moxon *et al*. 2008; Mohorianu *et al*. 2011). In our research, sRNA profiles of leaves from *S*. *lycopersicum* and *S*. *habrochaites* were obtained, with more sRNAs sequenced compared with previous studies (Itaya *et al*. 2008; Moxon *et al*. 2008; Mohorianu *et al*. 2011). Sixty-three known miRNA families were identified in our research, of which 39 were common in both species. Some of these miRNA families may be conserved in tomato. In addition, we also reported 20 new miRNAs in *S*. *lycopersicum*.

3.2. Divergence of miRNA profiles in two species

In our research, 24 known miRNA families were specific to *S*. *lycopersicum* and *S*. *habrochaites*. It is generally believed that miRNAs play an important role in species-specific gene expression regulation (Ha *et al*. 2008). The targets of these non-conserved miRNAs displayed their potential function in cellular and physiological processes regulation in the plant (Appendix B). These miRNAs evolved recently and their targets were not conserved among species; therefore, further experiments are necessary to complete their functional annotation.

In addition, six conserved miRNA families in the two libraries also showed distinct divergent miRNA expression profiles between the two tomato species.

Four miRNA families showed a higher expression level in the SL library, some of which have been reported to be

Table 4 The information of predicted new miRNAs

miRNA	Sequence (5´→3´)	Length (nt)	Counts of miRNAs/ miRNA*s	Location	Arm	Length of precursors (nt)	MFE (kcal mol^{-1})[1)]
miR–m9007	AAUACAACUAUAGCCAAGACAA	22	1211/172	SL2.31Ch01:832334:832581: +	5´	248	–107.7
miR-m9051	GCCAAGGAUGACUUGCCGACUU	22	25/11	SL2.31Ch01:41799042:41799204: –	3´	163	–77
miR-m9063	AGAAACAACACUUGCUAAAGG	21	2114/22	SL2.31Ch01:75000152:75000268: –	3´	117	–44.8
miR-m9121	UUCUCUGAUCAAGCAACGUGG	21	85/13	SL2.31Ch02:41923335:41923454: –	3´	120	–66.1
miR-m9319	GGAGUGGGUGGGAUGGAAAAA	21	128/56	SL2.31Ch06:33869422:33869550: –	3´	129	–48.3
miR-m9320	AGUGGGUGGUGUGGUAAGAUU	21	168/43	SL2.31Ch06:33877495:33877605: –	5´	111	–51.7
miR-m9376	CAACGUACGUAGGGUAAGUGG	21	1237/40	SL2.31Ch08:395902:396150: +	3´	249	–83.2
miR-m9397	UUCCGGUAGUCCUGUCGCAGAUG	23	262/85	SL2.31Ch08:53820948:53821105: +	3´	158	–76.1
miR-m9398	UAGCCAAGGAUGACUUGCCU	20	7413/10	SL2.31Ch08:54794098:54794243: +	3´	146	–51.5
miR-m9459	UCUAGACCUACGUUGCUCGGA	21	55/10	SL2.31Ch09:16023750:16023924: –	3´	175	–81.5
miR-m0468	GUGUGCACAAGUAGACACUUAAA	23	19/13	SL2.31Ch09:61842484:61842675: –	3´	192	–63.1
miR-m9488	AUGGGUAGCACAAGGAUUAAUG	22	12084/1190	SL2.31Ch10:62181313:62181475: +	3´	163	–58
miR-m9552	ACGGUGAUAAUGGUAUUCUAA	21	10782/1362	SL2.31Ch11:36952281:36952464: –	5´	184	–83.4
miR-m9596	AUUUCUCUGGUGCUUACUCAAC	22	99/42	SL2.31Ch12:1977607:1977745: –	5´	139	–53
miR-m9597	GAGGUGCUCACUCAGCUAAUA	21	6085/36	SL2.31Ch12:1980580:1980727: –	3´	148	–65.2
miR-m9614	UUCCAUGAGACUGUUUUUGGGU	22	41014/96	SL2.31Ch12:64810260:64810539: –	5´	280	–127.2
miR-m9615	ACGUGGGGGCAUGUGAUUGAA	21	21446/61	SL2.31Ch12:64810533:64810846: –	5´	314	–125.6
miR-m9616	AAGAUCUUUUACCGUAGUAAUC	22	7574/22	SL2.31Ch12:64810881:64811053: –	3´	173	–67.8

[1)] MFE, minimum free energy.

involved in root development. For example, miR171 and miR167 have been reported to negatively regulate SCL6 and ARF6 and ARF8, respectively. Overexpressing MIR171c transgenic plants showed decreased primary root elongation and other pleiotropic phenotypes (Wang *et al*. 2010). miR167 negatively regulates adventitious root formation in *Arabidopsis thaliana* (Gutierrez *et al*. 2009). Lower expression levels of these miRNAs in the SH library may contribute to the higher root-top ratio in *S. habrochaites* compared with that in *S*. *lycopersicum* (data not shown). In addition, miR394 and miR396, which target the F-box and GRF (growth-regulating factor) family, respectively, were reported to be involved in plant leaf development in *A. thaliana* (Woo *et al*. 2001; Rodriguez *et al*. 2010).

miR408 and miR397 showed higher expression in the SH library. The mechanism of different miR408 expression levels between two tomato species is unknown, because miR408 is mainly induced at low copper concentration (Burkhead *et al*. 2009). miR397 is predicted to cleave laccase in tomato (Ranocha *et al*. 2002). Higher expression levels of miR397 in *S*. *habrochaites* may contribute to a lower laccase expression in this species. This wild tomato species depends on the special secretion of 2-tridecanone and 2-undecanone, which protect the plants against various herbivorous insects (Yu *et al*. 2010); therefore, laccase might be less important in *S*. *habrochaites*. The different miR397 expression levels in the two tomato species might reflect their different chemical metabolic mechanisms.

4. Conclusion

In this research we obtain 16 282 007 and 17 115 875 clean reads from the SL and SH libraries. 21 conserved miRNA families are found, but seven families have no records in the miRBase for tomato. There are six conserved miRNA families whose expression are obvious different between this two libraries, of which four families (miR167, miR171, miR394, miR396) had higher expression levels in the SL library and two families (miR397 and miR408) had higher expression levels in SH library. Some of these miRNAs are resistance-related, like miR167, miR171, which implies they may play an important role in plant stress response. After analyzing the deep sequence data we found 18 new miRNAs, and we have predicted their secondary structures of predicted precursors.

5. Materials and methods

5.1. Plant materials and small RNA library construction

S. *lycopersicum cv*. 9706 (an advanced selfing line of cultivated tomato) and *S*. *habrochaites* species PI 134417 (from the United States Department of Agriculture) were planted in the greenhouse, the temperature was about 24°C in the day and 18°C at night, 12 h photoperiod. Mature tomato leaves were harvested 1 mon after germination, immediately frozen in liquid nitrogen, and stored at –70°C.

Following the manufacturer's instructions, total RNA of *S*. *lycopersicum cv*. 9706 (abbreviated as SL) and *S*. *habrochaites* species PI 134417 (abbreviated as SH) was isolated from tomato leaves using the RNAiso reagent (TaKaRa, Dalian, China). 18-28 nt small RNA (sRNA) fragments were then isolated from 15% denaturing polyacrylamide gels and ligated to a 5′ adaptor and a 3′ adaptor, sequentially. The RNA was subsequently converted to DNA by RT-PCR. Finally, the purified DNA products were sequenced on a SOLEXA sequencer (Illumina, San Diego, USA), according to the manufacturer's instructions, in the Beijing Genomics Institute (Guangdong, China).

5.2. Bioinformatics analysis

After removing the adaptor/acceptor sequences, filtering the low quality tags (i.e., tags less than 18 nt and tags whose adaptors were null) and cleaning up the contamination (adaptors and polyA), clean reads were harvested and the sRNA sequence length of two libraries (SL and SH) was analyzed.

rRNAs, tRNAs, snRNAs, and snoRNAs were eliminated by comparing clean reads with the sequences of non-coding RNAs available in Rfam (http://www.sanger.ac.uk/resources/software) and the GenBank noncoding RNA database (http://www.ncbi.nlm.nih.gov). The remaining unique sRNA sequences were used in a Blastn search against miRBase 17.0 (released in April 26, 2011) (Kozomara and Griffiths-Jones 2011) to identify the known miRNAs homolog in the two tomato libraries; two mismatches (G-U pairs are treated as 0.5 mismatch) and one gap were allowed. Sequences in the SL library, which did not match miRBase 17.0, were mapped to the *S*. *lycopersicum* Build2.31 genome downloaded from the SOL Genomics Network (http://solgenomics.net/) for new miRNA prediction. All the potential candidate miRNAs were identified by folding the flanking genome sequences of unique sRNAs using MIREAP ps://sourceforge.net/projects/mireap/). Related parameters were set based on the criteria for annotation of plant miRNAs (Meyersa *et al*. 2008). New miRNAs in the SH library were not predicted because of the lack of related *S. habrochaites* genome information.

Target predictions were performed using the Tomato Functional Genetics Database (http://ted.bti.cornell.edu/), with criteria set as described previously (Meyersa *et al*. 2008).

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Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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