Identification and Analysis of *C. annuum* microRNAs by High-throughput Sequencing and Their Association with High Temperature and High Air Humidity Stress

Xiao-wang Xu[1,2], Tao Li[1,2*], Ying Li[1], Zhen-xing Li[1]

[1]*Vegetable Research Institute*
Guangdong Academy of Agricultural Sciences
Guangzhou 510640, China
E-mails: xxw7505@163.com, tianxing84@163.com, ly38469@163.com, lizhxgaas@163.com

[2]*Guangdong Key Laboratory for New Technology Research of Vegetables*
Guangzhou 510640, China

[*]*Corresponding author*

Received: May 13, 2015 **Accepted: November 16, 2015**

Published: December 22, 2015

Abstract: *MicroRNAs (miRNAs) play an important role in many developmental processes and stress responses in plants. In this study, tolerant hot pepper cultivar 'R597' (CaR) and sensitive cultivar 'S590' (CaS) were used to detected differentially expressed miRNAs under high temperatures and high air humidity. The length distribution of obtained small RNAs was significantly different between libraries. There were a total of 71 miRNA families identified in two genotypes, and 24 conserved miRNA families were detected in all four sRNA libraries. MIR166, MIR156/157, MIR167, MIR168, MIR2118, and MIR5301 were highly expressed in four libraries, and 93 miRNAs had a species-specific expression. Among them, 60 miRNAs were preferentially expressed in S590 leaves and 33 miRNAs were preferentially expressed in R597 leaves. Mostly miRNAs were less-conserved miRNAs. The most abundant miRNAs with different expressions between two pepper species was miR6149b, which exhibited a high level (read count 42,443) in CaSCK but no expressed in CaRCK. We found 650 (CaRCK), 1054 (CaRHH), 914 (CaSCK), 1045 (CaSHH) potential targets for 92 (CaRCK), 124 (CaRHH), 128 (CaSCK), 117 (CaSHH) hot pepper miRNAs, respectively. These findings facilitate in better understanding of the molecular mechanism underlying high temperature and high air humidity condition in different pepper genotypes.*

Keywords: *miRNAs, High temperature, High air humidity, Hot pepper.*

Introduction

As the environment stress factors in south China, such as high temperatures and high air humidity are often experienced in the summer, the heat and high air humidity of hot pepper have become the main factors disturbing its normal growth. How to improve the tolerance of these plants to high temperature and high air humidity has been one of the important research objectives in hot pepper breeding. In the past few decades, much progress has been made in unraveling the complex stress response mechanisms involved in high temperature and high air humidity stress tolerance. These are linked to different pathways and processes and lead to molecular, biochemical, cellular, physiological and morphological adaptations of the whole plant response to stress [25-31].

Recent findings have suggested new layers of regulation in the plant response to stress. MicroRNAs (miRNAs) are a new class of endogenous, non-coding RNAs that range in length

from 18 to 25 nucleotides (nt) [38]. In plants, miRNAs finely regulated gene expression by binding to targeted mRNA sequences, leading to mRNA cleavage or, in a few cases, translational repression [40]. The current knowledge of miRNA regulatory roles is spread over a large spectrum of plant developmental programs and stress responses [8, 20]. Dozens of miRNAs have been identified with altered expression profiles in plants under various abiotic stress conditions, including drought [5, 33], waterlogging [14, 36], cold [9, 17], heat [12, 19], nutrient deficiency [15,18, 35], and UV-B radiation [2, 24].

Pepper (*Capsicum* spp.) is one of the most economically and agriculturally important vegetable crops in the world, with high consumption of fresh or processed products. Although variations in this tolerance have been observed among different cultivars, no investigation into miRNAs from *C. annuum* L. under stress has been reported. The tolerant cultivar 'R597' (CaR) and the sensitive cultivar 'S590' (CaS) were used to detect miRNAs that were differentially expressed in these two cultivars. Target genes of the detected miRNAs were predicted and their expression profiles were further analyzed.

Materials and methods

Plant materials and high temperature and high air humidity stress treatment.

Two pepper genotypes, hot-and-humid susceptible *C. annuum* 'S590' (CaS) and hot-and-humid tolerant 'R597' (CaR) were obtained from Guangdong province of China and used in this study. 'R597' (CaR) was a variety selected for hot-and-humid tolerance under long-term pressures of both nature and indoor appraisement at physiological and biochemical [27, 28, 30]. Plants were grown with 27 °C day temperature and 20 °C night temperature, a 12 hour light exposure period and relative humidity of 75% in a greenhouse of Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China. Four weeks old seedlings were used in this study, and hot-and-humid treatments were carried out in a growth chamber (model PGV36, Conviron, Winnipeg, MB, Canada) with a day high temperature 37 °C for 12 hours and 27 °C night temperature, relative humidity of 90% for 4 days. Control was carried out in PGV36 with the same condition of growth. The forth leaf was collected from healthy plants and frozen in liquid nitrogen immediately and stored at –80 °C.

Small RNA library preparation and sequencing

The tissues were sampled from three biological replications of every treatment and produced an independent pool. Total RNA of every sample was extracted from the dissected tissue using the TRIzol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. RNA quality was examined using agarose gel electrophoresis (28S:18S > 1.5) and a bioanalyzer (Aglilent 2100, RNA Integrity Number ≥ 8.0). The small RNA library was then sequenced with Solexa sequencing technology of the Beijing Genomics Intitute (BGI), Shenzhen, China. In brief, small RNAs, 18-30 nt in length, were first separated from the total RNA by size fractionation [32]. After PAGE purification and ligation of a pair of Solexa adaptors to their 5' and 3' ends, the small RNA molecules were converted to cDNA by RT-PCR and then the product of RT-PCR was used directly for cluster generation and sequencing analysis. The Illumina FASTQ data generated from this study has been submitted to the NCBI Squence Read Archive (http://www.ncbi.nlm.nih.bov/sra) under accession number SRA236143.

Bioinformatics analysis for miRNA identification

The raw sequences were processed using PHRED and CROSS MATCH programs as previously reported [22]. High-quality small RNA reads were obtained from raw reads through filtering out poor quality reads and removing adaptor sequences using FAXTX toolkit [16]. These clean sequences were then queried against non-coding RNAs (rRNA, tRNA, snRNA, snoRNA) from the Rfam database (http://www.sanger.ac.uk/software/Rfam). Any small RNA read matches to these sequences were excluded from further analysis. Then, all unique sequences were used to do a BLASTN search against the know plant miRNA database (miRBase 20.0) to identify conserved miRNAs. Only the perfectly matched sequences were considered to be conserved miRNAs.

Based on transcriptome sequences of *C. annuum* (deposited in NCBI database under SRA107820 accession number), conserved and novel miRNAs precursor sequences were identified using MIREAP software (https:// sourceforge.net/projects/mireap/) developed by the BGI. The MIREAP parameters were set as follows:

(1) a characteristic stem-loop structure was formed;
(2) the length of the miRNA sequence was 20-23 nt;
(3) the maximal free energy allowed for the miRNA precursor was –20 kcal·mol^{-1};
(4) the minimal number of common base pairs between miRNA and miRNA* was 16, with no more than four bulges;
(5) the maximal asymmetry of the miRNA/miRNA* duplex was four bases. Finally, an RNA secondary structure was constructed using Mfold [41].

Identification of miRNA targets by transcriptome sequencing

Conserved and non-conserved miRNAs of *C. annuum* were used as query sequences for BLASTn searches against our *C. annuum* transcriptome sequences (deposited in NCBI database under SRA107820 accession number). The following rules were used for predicting potential miRNA targets [3, 11, 23, 37]:

(1) no more than four mismatches were allowed between miRNA and its target (G-U bases count as 0.5 mismatches) site;
(2) no more than two adjacent mismatches in the miRNA/target duplex were allowed;
(3) no consecutive mismatches were allowed at positions 2-12 of the miRNA/target duplex (5' of miRNA);
(4) no mismatches were allowed at positions 10-11 of miRNA/target duplex;
(5) no more than 2.5 mismatches were at positions 1-12 of the of the miRNA/target duplex (5' of miRNA);
(6) minimum free energy (MFE) of the miRNA/target duplex should be ≥ 75% of the MFE of the miRNA bound to its perfect complement.

Results

Analysis of sequences from libraries

Four separate cDNA libraries of small RNAs (sRNAS) were generated from pepper leaves including two from a tolerant cultivar (CaRCK: untreated plants; CaRHH: plants treated with high temperature and air humidity) and two from a sensitive cultivar (CaSCK: untreated plants; CaSHH: plants treated with high temperature and air humidity). The sRNA digitalization analysis was based on Solexa sequencing system. High-throughput sequencing generated 11,965,177 primary reads for CaRCK, 15,301,897 for CaRHH, 17,685,098 for CaSCK, and 13,994,890 for CaSHH, respectively (Table 1). After removing adaptor/acceptor sequences, filtering low-quality tags and cleaning up the contamination formed by adaptor-

adaptor ligation, a total of 11,836,214, 15,146,228, 17,455,132, and 13,844,827 clean reads, corresponding to 4,223,782, 4,709,699, 5,909,214, and 3,730,481 unique signatures, remained for the CaRCK, CaRHH, CaSCK and CaSHH libraries, respectively. The small RNA sequences were matched to the NCBI Sequence Read Archive (SRA107820). When the total reads were analyzed, 17.12-22.55% reads could be matched to the transcriptome database, respectively. A large percentage of sequences failed to map because the *C. annuum* genome has not yet been completely sequenced.

Table 1. Summary statistics of small RNAs sequenced from leaves

Category	No. of reads			
	CaRCK	CaRHH	CaSCK	CaSHH
Total reads	11,965,177	15,301,897	17,685,098	13,994,890
High quality	11,919,045	15,243,362	17,618,359	13,942,682
Clean reads	11,836,214	15,146,228	17,455,132	13,844,827
Unique sRNAs	4,223,782	4,709,699	5,909,214	3,730,481
Total miRNA reads	458,532	845,750	893,342	810,244
Total rRNA reads	1,111,872	949,612	877,667	763,618
Total tRNAreads	427,679	508,932	353,809	369,185
Transcriptome	2,445,721 (20.66%)	3,114,529 (20.56%)	2,988,924 (17.12%)	3,122,628 (22.55%)
Unann	7,383,467 (62.38%)	9,711,722 (64.12%)	12,334,447 (70.66%)	8,771,158 (63.35%)

CaR: a tolerant cultivar; CaS: a sensitive cultivar; CK: untreated plants; HH: plants treated with high temperature and air humidity.

These were screened as miRNA candidates used in subsequent analyses. The size distribution of reads is shown in Fig. 1. Approximately 70% were 20-24 nt in length with 21 or 24 nt as the major size classes, as shown in Fig. 1. The distribution of different sized sRNA was strikingly different between the CaS libraries. For the CaSCK data set, the sRNA distribution showed a major peak at 24 nt (48.58%), and another minor peak at 21 nt (19.73%). Instead, the major peak in CaSHH was at 21 nt (37.59%), and the secondary class was 24 nt (32.03%). In the CaR library, the frequency of 24 nt and 21 nt sRNAs showed a resemblance, 24 nt reduced after treating with high temperature and air humidity, but 21 nt increased. This observation suggests that expressions of small RNAs in leaves could be modulated by high temperature and air humidity treating.

The proportions of common and specific small RNAs were further analyzed between pairs of libraries. For total small RNAs in all pairs of libraries, 62.51-72.28% was common to both libraries and 9.24-24.52% was specific to one library, respectively (Fig. 2). However, for unique small RNAs, the opposite was found. There were larger proportions of specific sequences than those of common sequences. For example, analysis comparing high temperature and air humidity treatment in CaS leafs showed that more than 50% of unique small RNAs were specific to the CaSCK library, whereas only 29.48% were specific to the CaSHH library (Fig. 3). In all, these observations highlighted differences in the complexity of the four small RNA pools, and suggested different regulation underlying the response to high temperature and air humidity treating.

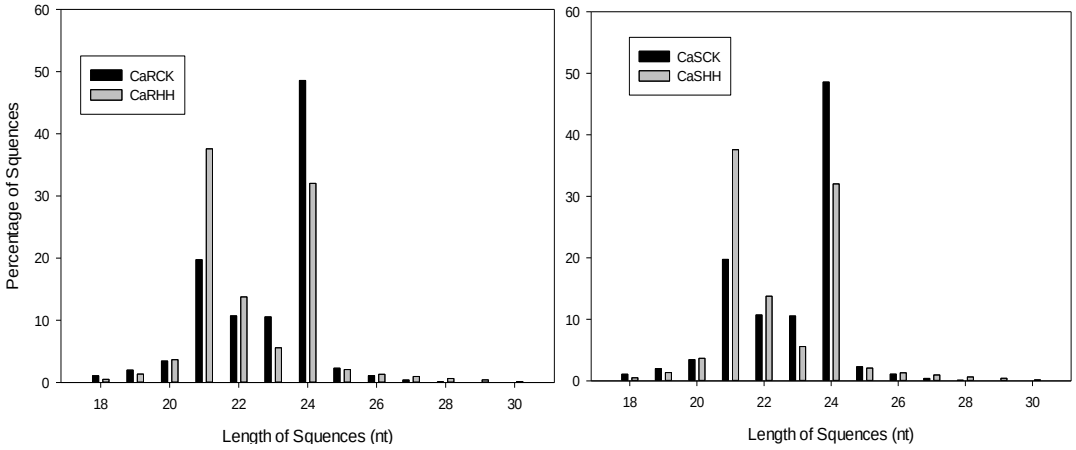

Fig. 1 The length distribution of small RNAs in two hot pepper cultivars: CaR: a tolerant cultivar; CaS: a sensitive cultivar; CK: untreated plants; HH: plants treated with high temperature and air humidity.

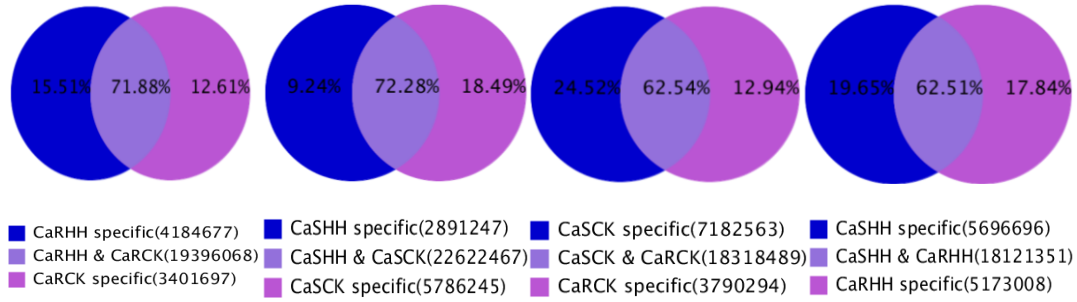

Fig. 2 The sample-specific total sequences from the four libraries

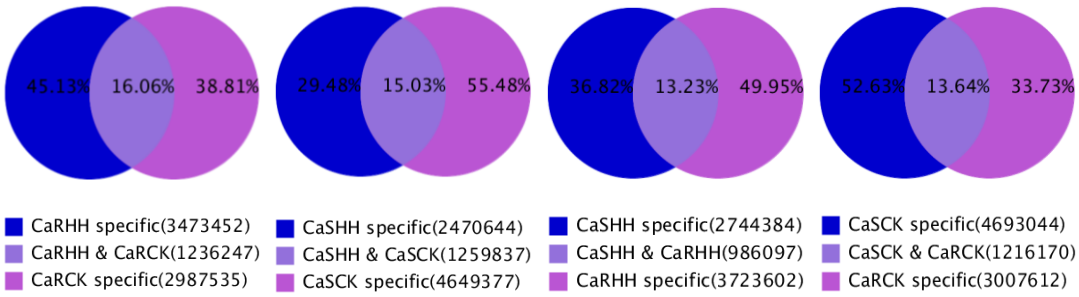

Fig. 3 The sample-specific unique sequences from the four libraries

Identification of conserved and non-conserved miRNA families

An *ad hoc* bioinformatics pipeline was used to annotate known and unknown miRNAs, both conserved and lineage-specific. In our manuscript, all miRNAs belonging to families already annotated in the miRBase registry (www.mirbase.org, release 20.0) in at least one species are defined as known. To investigate the repertoire of conserved miRNAs in CaS and CaR, all tag sequences in four sRNA libraries were aligned with all known miRNAs in the miRBase database (release 20.0). To minimize false positives, only the unique tags which were represented by more than 2 reads were considered to be true miRNAs. As a result, 315 known miRNAs were obtained in the four sRNA libraries. The BLASTN searches identified 96 conserved miRNAs corresponding to 71 miRNA families in the four sRNA libraries.

There were 41 (CaRCK), 56 (CaRHH), 52 (CaSCK), 48 (CaSHH) conserved miRNA families in the four RNA libraries, respectively. Among these conserved miRNA families, 31 conserved miRNA families were expressed in both CaRCK and CaRHH small RNA libraries, and 36 conserved miRNA families were expressed in both CaSCK and CaSHH small RNA libraries. In addition, 24 conserved miRNA families were detected in all four sRNA libraries.

Some of the miRNA families, such as MIR166, MIR156/157, MIR167, MIR168, MIR2118, and MIR5301 were highly expressed in the four libraries, whereas others had relatively low levels of expression. In the total date set, MIR166 had a dominant number of reads and were expressed more than 100,000 counts. MiRNAs expression abundance in date sets was analyzed by counting the number of transcripts per million (TPM) clean reads in libraries. Among them, fewer than 5 families had a count number higher than 600 counts per million reads, and approximately 18 families were 60-600 counts. The varied frequency of sequencing between miRNA families might suggest their distinct physiological role in leaf development.

Deep sequencing also detected 46 non-conserved miRNA families from *C. annuum*. This group of miRNAs is conserved in only a few plant species, such as miRNA1512 being conserved in *Glycine max* [10]. Also, miRNA1862 has been shown to exist only in *Oryza sativa* [1]. These miRNA families had a moderate or low abundance in the libraries. MiRNA1023, miRNA1026, miRNA3036, and miRNA5477 were preferentially expressed in *C. annuum* R597, whereas miRNA5554 and miRNA2600 were preferentially expressed in *C. annuum* S590.

Differential expression of miRNAs in response to stress

To identify the response of miRNAs to high temperature and air humidity, this study compares the abundance of miRNAs between any two libraries. We first normalized the read density measurement and then used p-value < 0.01 and the absolute value of $|\log2\text{Ratio}| \geq 1.5$ as a threshold to judge the statistical significance of miRNA expression. From the four date sets, many genes were found to be differentially expressed between libraries. We first made a comparative analysis of miRNA expression between the pepper species with control. It was shown that a total of 93 miRNAs had a species-specific expression. Among them, 60 miRNAs were preferentially expressed in *C. annuum* S590 leaves and 33 miRNAs were preferentially expressed in *C. annuum* R597 leaves. Mostly miRNAs were less-conserved miRNAs. The most abundant miRNAs with different expression between two pepper species was miR6149b, which exhibited a high level read count of 42,443 in CaSCK but no expressed in CaRCK (Table 2). This miRNAs were firstly cloned in *Nicotiana tabacum* using Illumimia's Solexa sequencing technology. The majority of these differently expressed miRNAs had low accumulation, but they might be playing a significant role in forming genotype-specific phenotype.

There were a total 86 miRNAs differently expressed between the CaSHH and CaSCK libraries. Among them, 31 miRNAs were of higher abundance and 55 miRNAs were of lower abundance in CaSHH library (Table 3), indicating that the expression levels of many miRNAs was suppressed during high temperature and air humidity treatment.

Table 2. Differentially expressed miRNAs between CaSCK and CaRCK library

miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change
miR1869	17.177	miR5666	10.552	miR2275	9.473	miR1873	7.876	miR5772	3.236	miR2118	–3.08
miR1508	14.598	miR3949	10.535	miR6435	9.233	miR5745	7.766	miR6023	3.188	miR1507	–3.65
miR5066	14.369	miR5284	10.473	miR2600	9.219	miR5296	7.688	miR6027	2.889	miR1863	–3.94
miR1436	13.295	miR1118	10.300	miR6149	9.140	miR4371	7.606	miR156	2.334	miR3637	–7.17
miR6477	13.068	miR5675	10.239	miR6196	8.893	miR843	7.606	miR479	2.320	miR2120	–7.25
miR6464	12.906	miR5721	10.219	miR5485	8.876	miR3951	7.518	miR390	1.663	miR5565	–7.40
miR5070	12.818	miR5024	10.177	miR5751	8.496	miR5169	7.518	miR5059	–1.559	miR5718	–7.60
miR5513	12.208	miR5658	10.042	miR5554	8.473	miR6458	7.425	miR6300	–1.560	miR5826	–7.66
miR2613	11.887	miR4228	10.034	miR5715	8.351	miR420	7.376	miR398	–1.734	miR5526	–8.08
miR437	11.590	miR5261	10.034	miR5557	8.273	miR5198	7.376	miR530	–1.851	miR5374	–9.01
miR1862	11.549	miR5656	10.034	miR4415	8.191	miR5673	7.219	miR397	–1.886	miR5241	–10.12
miR5758	11.428	miR5815	9.902	miR5679	8.133	miR5218	6.978	miR4408	–1.948	miR1223	–10.81
miR1134	11.122	miR5752	9.885	miR3946	8.073	miR3627	6.911	miR827	–2.08	miR6253	–14.72
miR2651	10.906	miR6203	9.804	miR3515	7.978	miR4221	6.688	miR5072	–2.11		
miR4372	10.871	miR2590	9.794	miR5176	7.978	miR1023	4.376	miR408	–2.56		
miR2083	10.584	miR5141	9.530	miR5501	7.945	miR5568	3.427	miR5750	–2.79		

Table 3. Differentially expressed miRNAs between between CaSHH and CaSCK library

miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change
miR5292	15.244	miR1065	8.376	miR2275	1.638	miR3951	–7.518	miR5141	–9.530	miR4372	–10.871
miR6170	14.968	miR5826	8.312	miR2620	–1.534	miR5169	–7.518	miR1877	–9.574	miR1134	–11.122
miR472	12.991	miR5183	7.022	miR6249	–1.537	miR4371	–7.606	miR6149	–9.791	miR2619	–11.367
miR5175	11.621	miR6191	6.940	miR5537	–1.620	miR843	–7.606	miR2590	–9.794	miR1026	–11.510
miR6475	10.685	miR1313	3.360	miR5303	–1.649	miR854	–7.606	miR6203	–9.804	miR1862	–11.549
miR1223	10.504	miR390	2.987	miR5054	–1.935	miR1074	–7.648	miR4387	–9.858	miR5207	–11.766
miR3630	10.052	miR5021	2.983	miR831	–2.028	miR5296	–7.688	miR5752	–9.885	miR4414	–12.291
miR5559	9.621	miR399	2.512	miR5024	–2.039	miR3946	–8.073	miR4228	–10.034	miR4351	–12.997
miR1160	9.567	miR5666	2.419	miR5139	–2.419	miR4415	–8.191	miR5656	–10.034	miR6477	–13.068
miR2055	9.452	miR5227	2.144	miR5077	–2.432	miR5557	–8.273	miR5675	–10.239	miR1436	–13.295
miR5562	9.344	miR5066	1.912	miR4221	–6.688	miR5715	–8.351	miR1023	–10.455	miR1312	–13.467
miR4413	9.228	miR6022	1.885	miR5218	–6.978	miR5751	–8.496	miR3949	–10.535		
miR6161	9.062	miR6464	1.719	miR5673	–7.219	miR6196	–8.893	miR2083	–10.584		
miR1037	8.467	miR5224	1.684	miR7130	–7.219	miR6435	–9.233	miR5774	–10.668		
miR447	8.407	miR2118	1.652	miR5198	–7.376	miR5830	–9.413	miR6176	–10.678		

In the treating tolerant *C. annuum* species, there were also many miRNAs with altered expression in response to stress treating (Table 4). However, the number of down-regulated miRNAs in CaRHH library was fewer than that in CaSHH library. Some of miRNAs were not detected in CaRHH library, of which miR1312 and miR5303a had a high abundance in CaRCK library, read count 1353 and 1259, respectively. With the genotype-specific expression of miRNAs under control, it was plausible to assume that some miRNAs had preferential expression in one of the two species, when both of them were treated to high temperature and humidity. Thus the expression levels of miRNAs between CaSHH and CaRHH libraries were compared. We found that 43 miRNAs were preferentially expressed in CaSHH library and 57 miRNAs were preferentially expressed in CaRHH (Table 5).

To better understand the functions of the conserved and non-conserved miRNAs, plant miRNA targets can often be predicted on the basis of sequence similarity since miRNAs usually show high sequence complementarity to their targets, although such approaches can still produce large numbers if false positive predictions. Using these criteria as described above, we found 650 (CaRCK), 1054 (CaRHH), 914 (CaSCK), 1045 (CaSHH) potential targets for 92 (CaRCK), 124 (CaRHH), 128 (CaSCK), 117 (CaSHH) hot pepper miRNAs, respectively (Table 6). The putative target genes appeared to be involved in a wide range of biological processes and most of them were classified as functional proteins, transcription factors and response to stress. Further analysis of these targets will aid in better understanding their function and their regulatory network in hot pepper.

Table 4. Differentially expressed miRNAs between between CaRHH and CaRCK library

miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-Change
miR3631	16.403	miR5261	10.027	miR447	8.581	miR2275	7.677	miR395	6.81	miR5750	–5.924
miR1508	13.99	miR5812	9.913	miR4415	8.556	miR1873	7.581	miR472	6.81	miR4351	–6.086
miR5562	13.673	miR5485	9.767	miR776	8.423	miR3627	7.581	miR5647	6.81	miR3637	–7.166
miR3520	12.27	miR846	9.689	miR3946	8.338	miR419	7.478	miR1023	3.071	miR5826	–7.664
miR812	12.143	miR5019	9.53	miR2873	8.115	miR2606	7.308	miR390	2.991	miR5537	–7.779
miR2912	11.648	miR5175	9.352	miR5752	8.045	miR5169	7.247	miR5568	2.768	miR1074	–8.033
miR6475	11.266	miR5559	9.308	miR5376	7.932	miR5253	7.247	miR156	1.944	miR5526	–8.079
miR5268	10.593	miR5721	9.063	miR5183	7.893	miR5659	7.247	miR403	1.689	miR5837	–9.454
miR2083	10.575	miR5534	9.008	miR2592	7.852	miR170	7.115	miR408	–1.628	miR5241	–10.123
miR5723	10.575	miR5141	8.971	miR1222	7.81	miR7122	6.971	miR398	–1.845	miR5207	–11.53
miR5284	10.55	miR5176	8.873	miR4342	7.81	miR3694	6.893	miR415	–2.21	miR1026	–11.618
miR5658	10.409	miR1134	8.852	miR3515	7.767	miR6191	6.893	miR1863	–2.738	miR1312	–13.481
miR5640	10.072	miR780	8.767	miR4371	7.767	miR3438	6.81	miR4414	–4.761	miR6253	–14.718

Discussion

MiRNAs, identified in plants less than a decade ago, are known to play numerous crucial roles at each major stage of development, and are involved in response to environmental stress [4]. In this study, the tolerant hot peeper cultivar CaR597 and the sensitive cultivar CaS590 were used to detect miRNAs that are differentially expressed between them. High-throughput sequencing was performed to identify small RNAs that expressed in hot

peeper leaves under high temperature and high air humidity stress. This sequencing technique provided a good chance for us to obtain a direct digital readout of small RNAs and achieved an essentially dynamic range of expression between libraries. Comparison of their sequencing data showed that the distribution of different size sRNAs was strikingly different between them. For CaSCK data set, the sRNA distribution showed a major peak at 24 nt (48.58%), and another minor peak at 21 nt (19.73%). Instead, the major peak in CaSHH was at 21 nt (37.59%), and secondary class was 24 nt (32.03%). The 21 nt and 24 nt sRNAs had a different distribution not only between the two materials, but also between different treatments with high temperature and high air humidity treating. This observation suggests that expression of small RNAs in leaves could be modulated by high temperature and air humidity treating.

Table 5. Differentially expressed miRNAs between CaSCK and CaRCK library

miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change	miRNA	Fold-change
miR1869	17.177	miR5666	10.552	miR2275	9.473	miR1873	7.876	miR5772	3.236	miR2118	–3.08
miR1508	14.598	miR3949	10.535	miR6435	9.233	miR5745	7.766	miR6023	3.188	miR1507	–3.65
miR5066	14.369	miR5284	10.473	miR2600	9.219	miR5296	7.688	miR6027	2.889	miR1863	–3.94
miR1436	13.295	miR1118	10.300	miR6149	9.140	miR4371	7.606	miR156	2.334	miR3637	–7.17
miR6477	13.068	miR5675	10.239	miR6196	8.893	miR843	7.606	miR479	2.320	miR2120	–7.25
miR6464	12.906	miR5721	10.219	miR5485	8.876	miR3951	7.518	miR390	1.663	miR5565	–7.40
miR5070	12.818	miR5024	10.177	miR5751	8.496	miR5169	7.518	miR5059	–1.559	miR5718	–7.60
miR5513	12.208	miR5658	10.042	miR5554	8.473	miR6458	7.425	miR6300	–1.560	miR5826	–7.66
miR2613	11.887	miR4228	10.034	miR5715	8.351	miR420	7.376	miR398	–1.734	miR5526	–8.08
miR437	11.590	miR5261	10.034	miR5557	8.273	miR5198	7.376	miR530	–1.851	miR5374	–9.01
miR1862	11.549	miR5656	10.034	miR4415	8.191	miR5673	7.219	miR397	–1.886	miR5241	–10.12
miR5758	11.428	miR5815	9.902	miR5679	8.133	miR5218	6.978	miR4408	–1.948	miR1223	–10.81
miR1134	11.122	miR5752	9.885	miR3946	8.073	miR3627	6.911	miR827	–2.08	miR6253	–14.72
miR2651	10.906	miR6203	9.804	miR3515	7.978	miR4221	6.688	miR5072	–2.11		
miR4372	10.871	miR2590	9.794	miR5176	7.978	miR1023	4.376	miR408	–2.56		
miR2083	10.584	miR5141	9.530	miR5501	7.945	miR5568	3.427	miR5750	–2.79		

Table 6. Summary of miRNA target prediction

Ample name	miRNA number	Target gene number	Target number
CaRCK	92	650	657
CaRHH	124	1054	1084
CaSCK	128	914	962
CaSHH	117	1045	1076

On the basis of their precursor structures and biogenesis, small RNAs can be divided into miRNAs and small interfering RNAs (siRNAs) [7], with the best-characterized class of plant sRNAs being miRNAs [6]. In our study, a total of 71 miRNA families were identified in the

two hot peeper species. To date, 21 families were found in more than 20 plant species, and they were conserved between dicots and monocots, as well as in mosses [21]. In long evolutionary timescales, well-conserved miRNAs have retained homologous target interactions and performed analogous molecular functions across phyla [34]. It is plausible to assume that the conservation is consistent with their basic function for normal growth and development of plants. Some of the miRNA families, such as MIR166, MIR156, MIR157, MIR167, MIR168, MIR2118, and MIR5301 were highly expressed in the four libraries, whereas others had relatively low levels of expression. Recently, miRNAs such as miR168, miR171, and miR396 were found to be responsive to high salinity, drought, and cold stress in Arabidopsis, thus supporting the hypothesis of a role for miRNAs in the adaptive response to abiotic stress [39]. In Arabidopsis, miR396, miR168, miR167, miR165, miR319, miR159, miR394, miR156, miR393, miR171, miR158, and miR169 were shown to be drought responsive [13]. In the total date set, MIR166 had a dominant number of reads and were expressed more than 100,000 counts. Among them, less than 5 families had the count number higher than 600 counts per million reads, approximately 18 families were 60-600 counts. The varied frequency of sequencing between miRNA families might suggest their distinct physiological role in leaf development. The read number of the dominant member may be thousands of times greater than that of other members, suggesting that the regulatory role of this family was performed by the dominant member in that specific developmental stage.

Conclusion

In summary, global transcriptional profiles of small non-coding RNAs were investigated in leaves of 'R597' (CaR) and 'S590' (CaS) with high temperature and air humidity stress, two cultured species with different tolerance. The various expression patterns of these small RNAs are a valuable resource for further study on post-transcriptional gene regulation in high temperature and air humidity response. Accordingly, further identification and detailed kinetics analysis of the target genes of these small RNAs could shed new light on their regulatory roles in this abiotic response.

Acknowledgements

This work is supported by the National Natural Science Foundation of China (No. 31301776), the China Spark Program (No. 2014GA780041), the President Foundation of Guangdong Academy of Agricultural Sciences (No. 201303), the Science and Technology Program of Guangzhou (No. 201510010119), the Science and Technology Planning Project of Guangdong Province of China (No. 2014A020208045, No. 2013B020303008, and No. 2013B050800012), the Guangdong Key Laboratory for New Technology Research of Vegetables (No. 2013112), and the China Agricultural Research System (CARS-25-G-36).

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Xiao-wang Xu, Ph.D.
E-mail: xxw7505@163.com

Xiao-wang Xu is an Associate Research Fellow in Vegetable Research Institute of Guangdong Academy of Agricultural Sciences. He received his Ph.D. in South China Agricultural University. His research interests include bioinformatics, molecular breeding and biology in pepper.

Tao Li, Ph.D.
E-mail: tianxing84@163.com

Tao Li is from Vegetable Research Institute of Guangdong Academy of Agricultural Sciences. He received his M.Sc. in South China Agricultural University and his Ph.D. in South China Normal University. His current research interest is majored in bioinformatics analysis and gene function research in Solanaceae plants.

Ying Li, B.Sc.
E-mail: ly38469@163.com

Ying Li is a Research Fellow in Vegetable Research Institute of Guangdong Academy of Agricultural Sciences. She received his B.Sc. in Hunan Agricultural University. Her research interests include bioinformatics, molecular breeding and biology in pepper.

Zhen-xing Li, B.Sc.
E-mail: lizhxgaas@163.com

Zhen-xing Li is a Research Fellow in Vegetable Research Institute of Guangdong Academy of Agricultural Sciences. He received his B.Sc. in South China Agricultural University. His research interests include bioinformatics, molecular breeding and biology in tomato and pepper.