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Identification of miRNAs and their targets in tea (*Camellia sinensis*)[#]

Quan-wu ZHU, Yao-ping LUO[†‡]

(*Institute of Tea Science, Zhejiang University, Hangzhou 310058, China*)

[†]E-mail: ypluo@zju.edu.cn

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Abstract: MicroRNAs (miRNAs) are endogenous small RNAs playing a crucial role in plant growth and development, as well as stress responses. Among them, some are highly evolutionally conserved in the plant kingdom, this provide a powerful strategy for identifying miRNAs in a new species. Tea (*Camellia sinensis*) is one of the most important commercial beverage crops in the world, but only a limited number of miRNAs have been identified. In the present study, a total of 14 new *C. sinensis* miRNAs were identified by expressed sequence tag (EST) analysis from 47 452 available *C. sinensis* ESTs. These miRNAs potentially target 51 mRNAs, which can act as transcription factors, and participate in stress response, transmembrane transport, and signal transduction. Analysis of gene ontology (GO), based on these targets, suggested that 37 biological processes were involved, such as oxidation-reduction process, stress response, and transport. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis inferred that the identified miRNAs took part in 13 metabolic networks. Our study will help further understanding of the essential roles of miRNAs in *C. sinensis* growth and development, and stress response.

Key words: MicroRNA (miRNA), *Camellia sinensis*, Tea, Gene ontology, Pathway
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1 Introduction

MicroRNAs (miRNAs) are a class of endogenous non-coding small RNAs (about 21 nucleotides (nt)), which negatively regulate the expression of genes by targeting mRNA for cleavage or translational repression in a sequence-complementary dependent manner (Bartel, 2004; He and Hannon, 2004). They play crucial roles in plant growth and development, including flower development (Chen, 2004), leaf organ morphogenesis and polarity (Palatnik *et al*., 2003; Juarez *et al*., 2004; Mallory *et al*., 2004), root development (Guo *et al*., 2005; Williams *et al*., 2005), and fruit ripening (Moxon *et al*., 2008; Carra *et al*., 2009). Additionally, plant miRNAs also respond to drought, cold, salt, and other abiotic stress, as well as biotic stress (Sunkar *et al*., 2012).

The first plant miRNA was discovered in *Arabidopsis* in 2002 by small RNAs cloning (Reinhart *et al*., 2002). Subsequently, a large number of plant miRNAs were identified in a wide range of plant species. Currently, a total of 5940 plant miRNAs from 67 species are published in the miRBase database (http://www.mirbase.org/, Release 19: August 2012) (Kozomara and Griffiths-Jones, 2011). miRNA-related research is steadily growing, with researchers identifying miRNAs and studying their functions using a series of computational tools and/or experimental methods including small RNAs cloning, high-throughput sequencing, and degradome sequencing. Comparison of miRNAs in different plant species by expressed sequence tag (EST) analysis had shown that some miRNAs were highly evolutionary conserved among species (Zhang *et al*., 2006a); this provided a powerful strategy for identifying miRNAs in a new species. Identification of miRNAs using EST

‡ Corresponding author

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analysis has two significant advantages (Frazier and Zhang, 2011): (1) There is no specialized software required and it can be used to identify miRNAs in any species if they are previously registered EST sequences; (2) Since EST are derived from transcribed sequences, EST analysis also provided direct evidence for miRNA expression. In view of these advantages, EST analysis had been used to identify conserved miRNAs in *Brassica napus* (Xie *et al.*, 2007), *Medicago truncatula* (Zhou *et al.*, 2008), *Lycopersicon esculentum* (Yin *et al.*, 2008), *Glycine max* (Zhang *et al.*, 2008), citrus (Song *et al.*, 2010), *Nicotiana tabacum* (Frazier *et al.*, 2010), *Panicum virgatum* (Xie *et al.*, 2010), *Solanum tuberosum* (Xie *et al.*, 2011), *Malus domestica* (Yu *et al.*, 2011), strawberry (Dong *et al.*, 2012), etc.

Tea (*Camellia sinensis*) is an important commercial beverage crop grown in different agro-climatic zones in the world. Because of its extensive secondary metabolites in leaves, including theanine, polyphenols, caffeine, and volatile oils, the tea beverage possesses many health benefits to humans (Rogers *et al.*, 2008; Prabu and Mandal, 2010; Shi *et al.*, 2011). In addition to its health benefits and economic value, *C. sinensis* is also a wonderful source of experimental material to expound gene expression and regulation because of the availability of a mass of ESTs. Though numbers of miRNAs were identified from a wide range of species, there was no registered *C. sinensis* miRNA in miRBase (http://www.mirbase.org/, Release 19: August 2012). Recently, Das and Mondal (2010) and Prabu and Mandal (2010) identified several miRNAs from *C. sinensis* using computational methods, and Mohanpuria and Yadav (2012) discovered six tea-specific miRNAs using a direct cloning approach. However, compared to *Arabidopsis* (703) or rice (708) (http://www.mirbase.org/, Release 19: August 2012), more miRNA genes still remain to be discovered in *C. sinensis*. Furthermore, little attention has been focused on the function of *C. sinensis* miRNAs. In this study, we aim to identify miRNAs and their potential targets in *C. sinensis* and study their functions. To achieve this goal, EST analysis was performed to discover miRNAs and potential targets in *C. sinensis*, and Blast2GO (Conesa *et al.*, 2005; Conesa and Götz, 2008) was employed to further understand their functions.

2 Methods

2.1 Sequence sources

The test sequences were obtained from the miRBase database and the National Center for Biotechnology Information (NCBI). Currently, a total of 5940 known plant miRNAs were available in the miRBase database, and 47452 ESTs and 154468 mRNAs sequences were available for *C. sinensis* in the NCBI by October 2012. To identify more potential *C. sinensis* miRNAs, all of these sequences were downloaded for identifying miRNAs.

2.2 Identification of potential miRNAs in *C. sinensis* using EST analysis

The prediction of potential miRNA adopted a previously reported method (Zhang *et al.*, 2005; Frazier *et al.*, 2010). There were two crucial filter conditions in EST analysis: one is the conservation of mature miRNA sequences, another is the secondary structure of the pre-miRNAs (Zhang *et al.*, 2008). Briefly, the mature sequences of all known plant miRNAs were used as a query for homologous search against *C. sinensis* EST database using BLAST+ 2.2.25 program (Altschul *et al.*, 1997). The parameters used in the BLASTn were adjusted as follows: E value cutoff of 0.01; the word size was set at seven; and all other parameters used default settings. After removing the repeated ones, the rest of the ESTs with no more than 3 nt mismatches were used for additional analysis of secondary structure, based on the following criteria (Frazier *et al.*, 2010) using MFOLD V3.2 (Zuker, 2003) (http://mfold.rit.albany.edu/?q=mfold): (1) pre-miRNA could fold into a typical hairpin secondary structure and the mature miRNA was located in one stem; (2) the length of the pre-miRNA was no less than 50 nt; (3) pre-miRNA had a high minimal folding free energy (MFE) and MFE index (MFEI), which was calculated by

$$\text{MFEI}=\text{MFE}\times 100/[\text{length}\times(\text{G+C})\%],$$

where length is the length of RNA sequence and MFE is the negative folding free energy ($-\Delta G$) (Zhang *et al.*, 2006b); (4) the maximum number of nucleotides mismatches between the mature miRNA and its opposite miRNA* sequnence was six; and, (5) no loops or breaks in the miRNA/miRNA* duplex was allowed.

2.3 Prediction of miRNA targets in *C. sinensis*

In brief, we used the potential *C. sinensis* miRNAs blast against the *C. sinensis* mRNA database to search sequences conforming to the following standards as the *C. sinensis* candidate target gene: (1) the maximum number of mismatched nucleotides between the mature miRNA and its potential target genes was four; (2) the maximum number of mismatched nucleotides at positions 1–9 was one; (3) no mismatches were allowed at positions 10–11; (4) more than two continuous mismatches at any position were not allowed (Xie *et al.*, 2010).

2.4 Analysis of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

To better understand the function of *C. sinensis* miRNAs, Blast2GO (Conesa *et al.*, 2005; Conesa and Götz, 2008) was employed to investigate the predicted target genes. First, the identified miRNA targeted mRNAs were used to BLASTX against NR database with an E value of 10^{-25}. Second, the best hits identified by BLASTX were further searched against the GO and KEGG databases using default settings.

3 Results and discussion

3.1 Potential miRNAs in *C. sinensis*

In this study, we identified 14 potential miRNAs from a total of 47452 available *C. sinensis* ESTs by homologous search. This indicates that about 0.0295% of *C. sinensis* ESTs contain potential miRNAs. The ratio is as high as the previously reported about 0.0277% for switchgrass (Xie *et al.*, 2010).

The 14 new *C. sinensis* miRNAs belong to nine families, of which miR171, miR2911, miR5021, miR5368, and miR6483 have two members, while miR156, miR397, miR399, and miR2863 have only one member. Mature miRNAs have been observed to be located on the arm of pre-miRNA, but the located positions were found either on the 5′ arm of the stem (50%) or on the 3′ arm (50%). The length of mature miRNAs varies from 18 to 22 nt and 42.86% (6 out of 14) of them are 21 nt in length. The length of *C. sinensis* pre-miRNAs also varies from 85 to 201 nt with an average of (122.79±38.13) nt (Table 1). Fig. 1 showed predicted pre-miRNAs of miR171a in *C. sinensis*, and others were given in Data S1.

Table 1 *C. sinensis* miRNA identification by homolog search

New miRNA	Query miRNA	GenBank acc. No.	Tissue type	Arm	SP	EP	ME	Mature sequence*	E value	PL	A+U (%)	MFE (kcal/mol)	MFEI
miR156	aly-miR157a-5p	HS396956.1	Leaf	5′	212	232	20/21	UUGACAGAAGAU-AGAGAGCAu	1.00E−04	100	54.00	45.80	1.00
miR171a	ptc-miR171k	FS948108.1	Roots	3′	273	293	20/21	GGAUUGAGCCGC-GCCAAUAUu	1.00E−04	100	65.00	39.20	1.12
miR171b	ptc-miR171k	FS948109.1	Roots	5′	163	143	20/21	GGAUUGAGCCGC-GCCAAUAUu	1.00E−04	97	63.92	40.40	1.15
miR397	osa-miR397b	CV699725.1	Leaf	5′	99	79	21/21	UUAUUGAGUGCA-GCGUUGAUG	3.00E−05	126	59.52	39.20	0.77
miR399	osa-miR399j	FS958856.1	Young leaves	3′	109	129	20/21	UGCCAAaGGAGA-GUUGCCCUA	8.00E−03	103	51.46	52.80	1.06
miR2863	osa-miR2863a	FS950435.1	Roots	5’	37	57	18/21	UauaUAUUGUUG-AAAUGGCUU	8.00E−03	85	64.71	21.50	0.72
miR2911a	nta-miR2911	JK476023.1	Leaf	3′	364	383	20/20	GGCCGGGGGACG-GACUGGGA	1.00E−04	97	22.68	65.40	0.87
miR2911b	nta-miR2911	FS953337.1	Shoot stems	3′	177	196	20/20	GGCCGGGGGACG-GACUGGGA	1.00E−04	97	23.71	69.20	0.94
miR5021a	ath-miR5021	GW690847.1	Bud	3′	256	237	18/20	aGAGAAGAAGAA-GAAGAAAg	2.00E−03	195	55.90	71.90	0.84
miR5021b	ath-miR5021	GE651759.1	Tender root	5′	73	92	18/20	aGAGAAGAAGAA-GAAGAAAg	2.00E−03	201	56.22	72.20	0.82
miR5368a	gma-miR5368	GE653011.1	Tender root	3'	505	523	19/19	GGACAGUCUCAG-GUAGACA	4.00E-04	163	42.33	76.10	0.81
miR5368b	gma-miR5368	FS945766.1	Mature leaves	3′	196	214	19/19	GGACAGUCUCAG-GUAGACA	4.00E-04	144	42.36	68.50	0.83
miR6483a	hbr-miR6483	HS398296.1	Leaf	5′	253	232	21/22	UAUUGUAGAAAU-UUUCgGGAUC	2.00E−03	101	62.38	29.80	0.78
miR6483b	hbr-miR6483	JK714410.1	Leaf	5′	303	282	21/22	UAUUGUAGAAAU-UUUCgGGAUC	2.00E−03	110	63.64	30.00	0.75

* Lowercase letters in mature sequence mean mismatch. SP: start point; EP: end point; ME: match extent; PL: pre-miRNA length; MFE: minimal folding free energy; MFEI: MFE index

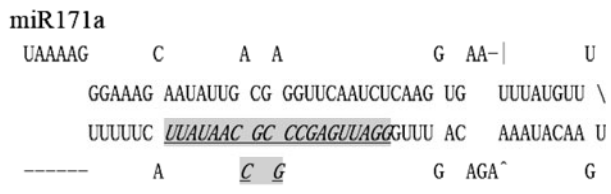

Fig. 1 Predicted pre-miRNAs of miR171a in *C. sinensis*

MFE is an important parameter for RNA folding into their secondary structures. Usually, the stability of the secondary structure of an RNA sequence increases with the reduction of the MFE. The MFEI was a sufficient criterion for distinguishing miRNAs from other RNAs. Previous research also suggested that it is more likely to be a potential miRNA if the pre-miRNA met the following criteria: MFEI>0.85 (Zhang *et al.*, 2006b). All predicted *C. sinensis* pre-miRNAs have a typical stem-loop secondary structure, pairing diversity depends on the length of precursor, and we only select the most stable one as the candidate pre-miRNA. Namely, they have a higher MFE, as well as MFEI. The MFE of new identification *C. sinensis* miRNAs ranges from 21.50 to 76.10 kcal/mol (1 kcal=4.184 kJ) with an average of (51.57±18.68) kcal/mol and the MFEI ranges from 0.72 to 1.15 with an average of 0.89±0.14 (Table 1).

Furthermore, the expression of *C. sinensis* miRNAs, according to the tissue type reported for each EST in the NCBI database, may be observed in leaf, root, stem, and bud (Table 1).

3.2 *C. sinensis* miRNA targets and their functions

Increasing evidences have demonstrated that most plant miRNAs bind to their target mRNA sequences with perfect or near-perfect sequence complementarity (Wang *et al.*, 2004; Schwab *et al.*, 2005). This provides a powerful strategy for discovering potential miRNA targets by comparing and aligning miRNAs with mRNAs sequences. Here, we performed more stringent criterion (Schwab *et al.*, 2005) to identify potential *C. sinensis* targets. After a set of screening criteria as described in the method, we achieved 51 target genes. Among the 51 predicted targets, 17 mRNAs encoded transcriptional factors, 14 mRNAs were stress responsive genes, and others were involved in transmembrane transport, signal transduction and transcription regulation. Unfortunately, 15 out of 51 targets' function remain unknown (Table 2). The results imply that miRNAs may play an important role in *C. sinensis* growth and development, as well as environmental stress.

Many miRNA targets identified by bioinformatics and/or experimental methods were transcription factors that help control plant growth and development. Here, we also found this type of targets. SQUAMOSA promoter binding protein-like (SPL) transcription factors, a class targets of miR156, play an important role in controlling flowering time, regulating plant transition from vegetative phase to reproductive phase, while overexpression of miR156 delays flowering and extends the vegetative stage (Wang *et al.*, 2009; Wu *et al.*, 2009). Furthermore, SPL is also involved in leaf development (Chen *et al.*, 2010) and anthocyanin biosynthesis (Gou *et al.*, 2011). SCARECROW-LIKE (SCL) transcription factor, miR171 target gene, was reported to act as a positive regulator in root development by integrating and maintaining a functional gibberellic acid (GA) pathway (Heo *et al.*, 2011).

Recent studies have shown that miRNAs are also involved in plant adaptation to environmental stresses, such as cold (Zhang *et al.*, 2009; Thiebaut *et al.*, 2012), salt (Ding *et al.*, 2009), drought (Li *et al.*, 2011), and nutrient deficiency (Sunkar *et al.*, 2007; Zhao *et al.*, 2012). Interestingly, we identified 14 potential targets of miR397, miR399, miR2911, miR5021, and miR5368 that were responses to stress. Further analysis of GO suggested that miR397 and miR399 play essential roles in copper ion and phosphate starvation.

To further understand the function of *C. sinensis* miRNAs, the predicted target mRNAs were subjected to analysis by GO and KEGG, a database for analyzing gene functions systematically (Kanehisa and Goto, 2000), using Blast2GO. The result suggested that *C. sinensis* miRNAs were involved in 37 biological processes. Among them, 9 targets of miR397, miR2911, and miR5021 took part in oxidation-reduction process, 3 targets of miR397 and miR399 responded to stress, and others were related to regulation of transcription, transport, growth and development, metabolism and translation (Table 3). Pathway enrichment analysis, based on the KEGG database, demonstrates that the identified miRNAs participated in 13 metabolism networks. These networks were involved in caffeine metabolism, ascorbate and

Table 2 Potential targets of the identified miRNAs in *C. sinensis*

miRNA family	Accession ID for targets	Target description	Function
miR156	KA284177, KA295488, HP757423, KA282627, KA285159, HP745756, HP751450, KA284930, KA293068, GAAC01043871, GAAC01052380	Squamosa promoter-binding-like protein	TF
miR171	HP735040, HP713619, GAAC01007557	Gras family transcription factor	TF
	HP757272, KA297400, GAAC01010861	Scarecrow-like protein	TF
miR397	HP737460	Laccase precursor	SR
	HP763272, GAAC01026665, GAAC01009301, KA285173	Laccase	SR
miR399	HP729908	Probable ubiquitin-conjugating enzyme e2 24-like	SR
miR2911	KA283566	Cytochrome p450 like_tbp	SR
	KA280075, KA285244, KA300874, KA279444, KA281442, KA287941, KA296981, KA300579, KA298382	Hypothetical protein MTR	Unknown
miR5021	KA279939, KA291019, KA303064	60s ribosomal protein	Unknown
	HP701326	Transcription activator glk1-like	TR
	KA281241	Conserved hypothetical protein	Unknown
	GAAC01052403	Probable LRR receptor-like serine threonine-protein kinase at1g14390	ST
	GAAC01011182	PREDICTED: uncharacterized protein LOC100266927	Unknown
	HP701293	Uncharacterized F-box/LRR-repeat protein C02F5.7-like	Unknown
	HP748043	Monocopper oxidase-like protein sku5-like	SR
	KA281010	Serine/threonine-protein phosphatase PP2A catalytic subunit	ST
	GAAC01045495	Erd6-like transporter	TMT
miR5368	KA283870	Metallocarboxypeptidase inhibitor	SR
	KA279481, KA283770, KA303168, KA303031, GAAC01046756	Cell wall-associated hydrolase, partial	SR
miR6483	HP736555	Envelope membrane protein	TMT

TF: transcription factor; SR: stress response; ST: signal transduction;TMT: transmembrane transport; TR: transcriptional regulation

Table 3 GO analysis of miRNA targets in *C. sinensis*

miRNA	Biological process	Accession ID for targets	GO
397, 2911, 5021	Oxidation-reduction process	HP748043; GAAC01045495; KA287941; KA283566; KA285173; GAAC01009301; HP763272; HP737460; GAAC01026665	GO:0055114
156, 171	Regulation of transcription	KA284177; GAAC01007557; KA295488; KA282627; KA285159; HP735040; HP713619; HP757272; KA297400	GO:0006351; GO:0006355
399, 5021, 6483	Transport	GAAC01045495; HP736555; HP729908	GO:0006817; GO:0015992; GO:0055085; GO:0008643
397, 399	Stress response	HP729908; HP737460; HP729908	GO:0016036; GO:0046688; GO:0055062
397	Metabolic	GAAC01009301; GAAC01026665; KA285173; HP763272; HP737460	GO:0046274; GO:0010413; GO:0009809; GO:0045492
397	Growth and development	HP737460; HP763272; GAAC01026665	GO:0010228; GO:0009834; GO:0009832
5021	Translation	KA279939; KA291019	GO:0006412

aldarate metabolism, fatty acid metabolism, T cell receptor signaling pathway, and other secondary metabolites process (Table 4). Interestingly, miR2911 was demonstrated to participate in the caffeine metabolism. Obviously, our study will help further understanding of the important regulation roles of miRNAs in *C. sinensis* growth and development, stress response, and likewise in research and development of low-caffeine tea.

4 Conclusions

In this study, we identified 14 new *C. sinensis* miRNAs by EST analysis, which belong to 9 families. These *C. sinensis* miRNAs potentially target 51 mRNAs, which can act as transcription factors, and participate in stress response, transmembrane transport, and signal transduction. GO analysis suggested that 37 biological processes were involved, such as oxidation-reduction process, stress response, and transport. KEGG pathway enrichment analysis inferred that the identified miRNAs took part in 13 metabolism networks. Interestingly, miR2911 was demonstrated to participate in caffeine metabolism. Our study will help further understanding of the essential roles of miRNAs in *C. sinensis* growth and development, stress response, as well as in research and development of low-caffeine tea.

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Compliance with ethics guidelines

Quan-wu ZHU and Yao-ping LUO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Table 4 KEGG analysis of miRNA targets in *C. sinensis*

miRNA	Accession ID for targets	Target description	Enzyme	Pathway
397	HP737460	Laccase precursor	EC:1.10.3.3	Ascorbate and aldarate metabolism
	GAAC01009301	Laccase	EC:1.10.3.3	
2911	KA283566 KA287941	Cytochrome p450 like_tbp	EC:1.14.14.1	Fatty acid metabolism, caffeine metabolism, aminobenzoate degradation, metabolism of xenobiotics by cytochrome P450, drug metabolism-cytochrome P450, drug metabolism-other enzymes, arachidonic acid metabolism, linoleic acid metabolism, tryptophan metabolism, steroid hormone biosynthesis, retinol metabolism
5021	KA281010	Serine/threonine-protein phosphatase PP2A catalytic subunit	EC:3.1.3.16	T cell receptor signaling pathway

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List of electronic supplementary materials

Data S1 The pre-miRNAs of potential miRNAs in *C. sinensis*