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Computational Identification of Conserved microRNAs and Their Targets in Tea (*Camellia sinensis*)

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ABSTRACT

MicroRNAs (*miRNAs*) *are a class of* ~22 *nucleotides long non coding RNA molecules which play an important role in gene regulation at the post transcriptional level. The conserved nature of miRNAs provides the basis of new miRNA identification through homology search. In an attempt to identify new conserved miRNAs in tea, previously known plant miRNAs were used for searching their homolog in a tea Expressed Sequence Tags and full length nucleotide sequence database. The sequences showing homolog no more than four mismatches were predicted for their fold back structures and passed through a series of filtration criteria, finally led us to identify* 13 *conserved miRNAs in tea belonging to* 9 *miRNA families. A total of* 37 *potential target genes in Arabidopsis were identified subsequently for* 7 *miRNA families based on their sequence complementarity which encode transcription factors* (8%), *enzymes* (30%) *and transporters* (14%) *as well as other proteins involved in physiological and metabolic processes* (48%). *Overall, our findings will accelerate the way for further researches of miRNAs and their functions in tea.*

Keywords**:** *Camellia sinensis, Computational Identification, Expressed Sequence Tags, microRNA, Targets*

1. Introduction

MicroRNAs (miRNAs) are short (~22 nt), endogenous non coding RNAs that play an important role in many biological processes [1]. They are generated from long precursor molecules which can fold into hairpin secondary structures [2]. Mature miRNAs bind to the complementary sites on target mRNAs and repress post transcriptional gene expression in both animals and plants [3, 4]. In plants, miRNAs are involved in diverse aspects of plant growth and development such as leaf morphology and polarity, root formation, transition from juvenile to adult vegetative phase and vegetative to flowering phase, flowering time, floral organ identity and reproduction [5, 6]. They are also found to be involved in response to pathogen invasion [7], hormone signaling [8,9], environmental stress [10,11] and promotion of anti-viral defence [12].

Expressed Sequence Tags (ESTs) are complementary DNA (cDNA) sequences, usually 200-500 bp in length that represents the expressed portions of genes. Therefore, ESTs can be used in gene identification, expression profiling and polymorphism analysis [13]. The EST sequencing projects have been enormously successful in the framework of many genome projects. The EST sequences are being used intensely as a source of information for the discovery of new genes whose functions can tentatively be deduced from their sequence and verified experimentally. Recently, *in silico* identification of miRNAs in various plant species have been done by EST analysis [14-17]. The biogenesis of miRNAs suggests that it is possible to find new miRNAs by homology searching of known miRNAs in ESTs. Hence, EST analysis makes it possible for studying conserved miRNAs and their functions in species whose genome sequences have not been well known [10,18,19].

There are both experimental and computational approaches for the investigation of plant miRNAs. The computational approach has been proved to be faster, affordable and more effective. Most of the miRNAs in miRBase [20] have been contributed through computational approach only. The computational approach has been developed on the principle of looking conserved sequences between different species which can fold into

hairpin secondary structures [10]. In recent years, a number of programs and bioinformatics tools have been developed and used successfully for the identification and analysis of miRNAs and their targets [21,22].

Tea is one of the most important non-alcoholic beverage drinks worldwide and gaining further popularity as an important 'health drink'. Despite the limited genome resources of tea (*Camellia sinensis*), published EST and full length nucleotide sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) has provided the scope to get more genetic information. In this study, new miRNAs were mined in local tea sequence database for the purpose of understanding their roles in regulating growth and development, metabolism and other physiological processes in tea.

2. Methods

2.1. Collection of Reference miRNAs, Full Length Nucleotides and EST Sequences

All available plant miRNAs and their fold back sequences were obtained from miRBase (http://www.mirbase.org/) on May, 2010. The homolog miRNAs were eliminated and the rest were defined as reference for searching tea miRNAs. Tea nucleotide and EST sequences (14819 as on May, 2010) were downloaded from NCBI's nucleotide and dbEST database (http://www.ncbi. nlm.nih.gov/). All redundant and poor quality sequences were eliminated and created a local nucleotide database.

2.2. Potential miRNAs and Their Precursors

The procedure for searching conserved tea miRNA homologues is summarized in **Figure 1**. The reference sequences were used as a query for homology search against our local tea nucleotide sequence database at e-value threshold < 0.01 using BLAST + 2.2.22 program [23]. The target sequences with no more than four mismatches were considered for secondary structure prediction using Mfold v 3.2 [24]. The precursor sequences were searched at 50 nucleotides upstream or downstream from the location of mature miRNAs with an increament of 10 nucleotides. While selecting a RNA sequence as a candidate miRNA precursor, following criteria were used according to Zhang *et al.* [1] with minor modifications as: 1) a RNA sequence can fold into an appropriate stemloop hairpin secondary structure, 2) a mature miRNA sequence site in one arm of the hairpin structure, 3) miRNAs

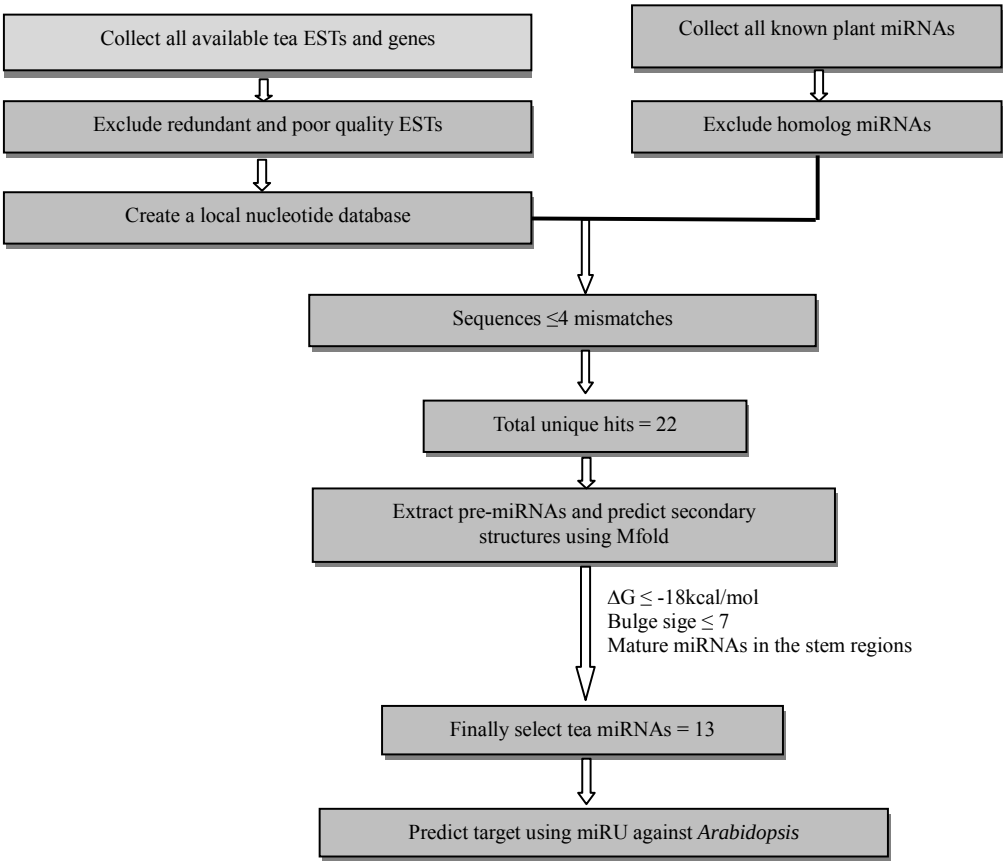


Figure 1. An overview of different steps involved in miRNA and their target prediction.

had less than seven mismatches with the opposite miRNA sequence in the other arm, 4) no loop or break in miRNA sequences, 5) predicted secondary structures had higher negative energy MFEs (≤–18 kcal/mol), and iv) 40-70% A + U contents.

2.3. Prediction of Targets

As for tea, since only few gene sequences available, we used *Arabidopsis* as a reference system for finding the targets of the candidate miRNAs. The predicted tea miRNAs were used as query against the *Arabidopsis thaliana* DFCI gene index (AGI) release 13 using miRU (http://bioinfo3.noble.org/psRNATarget/) following the criteria as 1) maximum expectation value 3; 2) multiplicity of target sites 2; 3) range of central mismatch for translational inhibition 9-11 nucleotide; 4) maximum mismatches at the complementary site ≤4 without any gaps.

2.4. Phylogenetic Analysis

Due to the conserved nature of small RNAs, orthologue discovery can be done through bioinformatics analysis. We analysed tea small RNA conservation with their orthologues. A homology search of candidate tea miRNAs was done against all plant miRNAs using NCBI standalone BLAST [23] allowing maximum of 3 mismatches and e-value <0.001. The corresponding precursor sequences of homolog small RNA's were identified and collected (Appendix). The collected sequences of diverse plant species were aligned with homolog tea miRNA using Clustal W [25].

A query of tea small RNAs against known miRNA families (miRBase, release 15, http://www.mirbase.org/) allowed us to identify 3 previously reported large families. The precursor sequences of three known family members were selected along with respective precursor sequences of tea (Appendix). Then, the maximum likelyhood trees were constructed for each family based on Tamura-Nei model [26] with default bootstrap values using MEGA 4.0 [27] to illustrate the evolutionary relationships among the members of the family.

2.5. Nomenclature of miRNAs

The predicted miRNAs were named in accordance with miRBase [28]. The mature sequences are designated 'miR', and the precursor hairpins are labeled as 'mir' with the prefix '*csi*' for *C. sinensis*, '*cas*' for *C. assamica* and '*cja*' for *C. japonica*. In the cases where distinct precursor sequences have identical miRNAs with different mismatch pattern, they were named as *csi-mir-1-a* and *csi-mir*-1-*b*.

3. Results

3.1. Prediction of miRNAs

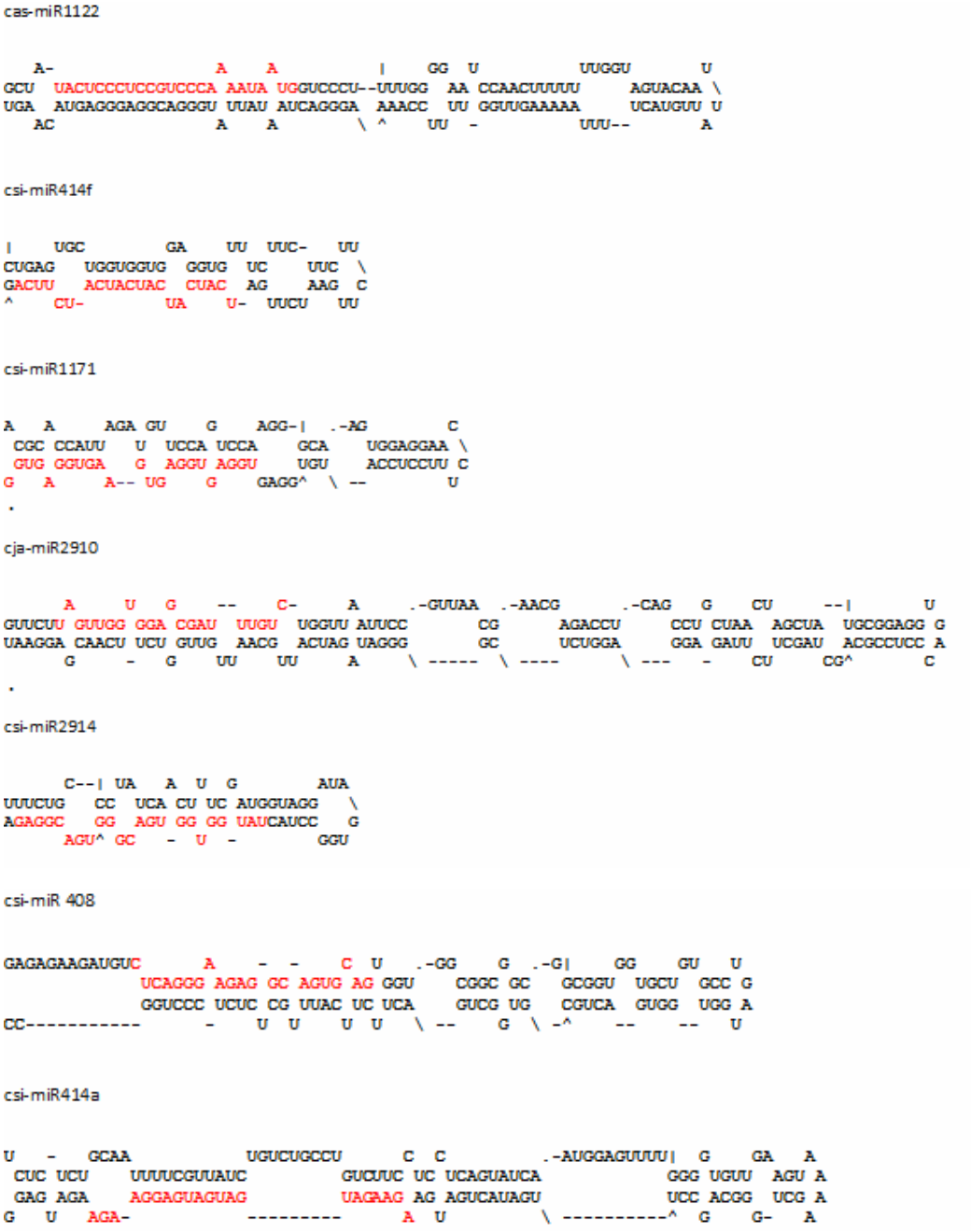
A total of 14,819 sequences containing 2,023 full length nucleotides and 12,796 ESTs were obtained from GenBank. Out of these, 22 sequences had less than five mismatches with previously known plant miRNAs. After carefully evaluating the hairpin structures using the criteria mentioned in the method, 13 small RNAs were finally identified from different species of *Camellia* namely *sinensis, japonica* and *assamica*. Details of the predicted miRNAs such as source sequences, location in the source sequences, length of precursor sequences and their minimum folding free energies and A+ U content are tabulated below (**Table 1**). A total of 9 miRNAs were

Table 1. Details of the predicted miRNAs in tea.

New MiRNAs	NS	Gene ID	Strand	SP	EP	ME	Mature MiRNAs	E Value	P L	A + U (%)	MFE
csi-miR 408	EST	206583693	3'	137	117	18/21	CUGCACUGCCUCUUCCCUGAG	0.001	336	45.24	-120.1
csi-miR1171	EST	171355265	5'	286	308	22/23	UGGAGUGGAGUGAAGUGGAGUGG	3E-04	181	56.98	-45.97
csi-miR414a	EST	206583641	3'	757	637	18/21	UCUUCCUCAUCAUCAUCUUCU	0.001	663	57.32	-63.18
csi-miR414d	EST	284026209	3'	186	166	20/21	UCAUCGUCAUCGUCAUCAUCU	0.004	193	61.14	-37.72
csi-miR414f	EST	212378632	5'	122	142	20/21	UCAUCAUCAUCAUCAUCUUCA	6E-05	68	57.35	-18.5
cas-miR1122	FL	214011104	5'	214	237	20/24	UACUCCCUCCGUCCCAAAAUAAUG	6E-05	294	69.83	-91.23
csi-miR414g	EST	51453040	3'	474	454	18/21	CCUUCCUCAUCAUCAUCGUCC	0.001	70	45.71	-25.2
csi-miRf10132-akr	EST	51453383	3'	58	34	25/25	GCGAGCUUCUCGAAGAUGUCGUUGA	9E-08	200	49.00	-69.5
cja-miR2910	FL	1777723	5'	1262	1282	21/21	UAGUUGGUGGAGCGAUUUGUC	1E-05	301	49.83	-91.0
csi-miR2914	FL	34787361	5'	345	367	22/23	UAUGGUGGUGACGGGUGACGGAG	5E-06	65	49.23	-20.9
cas-miRf10185-akr	EST	221071827	3'	232	212	17/21	GAAAGGGGAAAACAUUGUAGC	0.004	139	48.92	-51.1
cas-miR11590-akr	EST	212379609	3'	113	94	17/20	UUUUGGUGUGCCUUCAACCU	0.003	75	53.33	-23.8
csi-miR414h	EST	295345415	3'	79	58	17/21	UCAUCCUCAUCAUCGUCAGAA	0.004	644	55.36	-86.83

NS = Nucleotide Source, FL = Full-Length, SP = Start Point, EP = End Point, ME = Match Extent, PL = Pre-miRNA Length, MEF = Minimal Free Energy

predicted from ESTs whereas 4 were from full length nucleotide sequences. Five of them were located in the direct strand and the rest were in indirect strand. The newly identified precursor miRNAs have minimum folding free energies (mfe) ranging from –186.83 to –18.5 kcal/mol, with an average of about –72.69 kcal/mol and the A + U content were ranges from 45.24 to 69.83% with an average of 53.79%. The length of the precursors ranges from 65 to 663 nt with an average of 248 nt and mature sequences ranges from 20 to 25 nt. The newly predicted two tea miRNA (cja-miR2910, csi-miRf10132-akr) sequences were perfectly (100%) matched with the corresponding homologues of populus and rice, whereas the remaining 11 mature miRNA sequences differ by 1 to 4 nucleotides from their homologues. All the mature miRNAs were found in the stem portion of the hairpin structures (**Figure 2**) containing less than 7 mismatches in the other arm without break or loop inside the se- quences. It was found that tea miRNA (csi-miR408) has been conserved with diverse plant species (**Figure 3**) from monocotyledonous plants such as rice, maize to dicotyledonous plants such as populous.



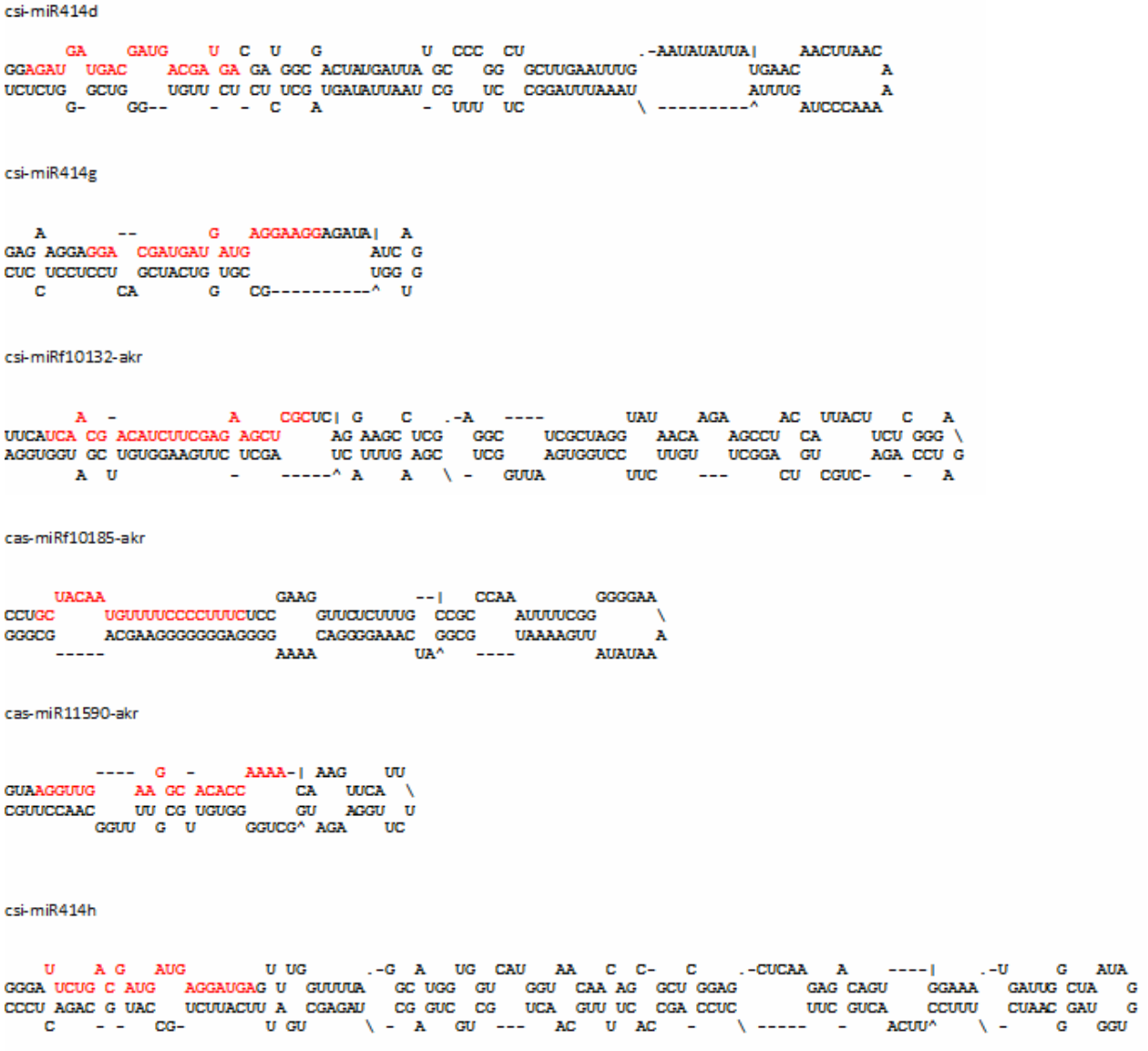


Figure 2. Predicted hairpin secondary structures of pre-miRNAs. MiRNAs are highlighted (red color) in the stem portion.

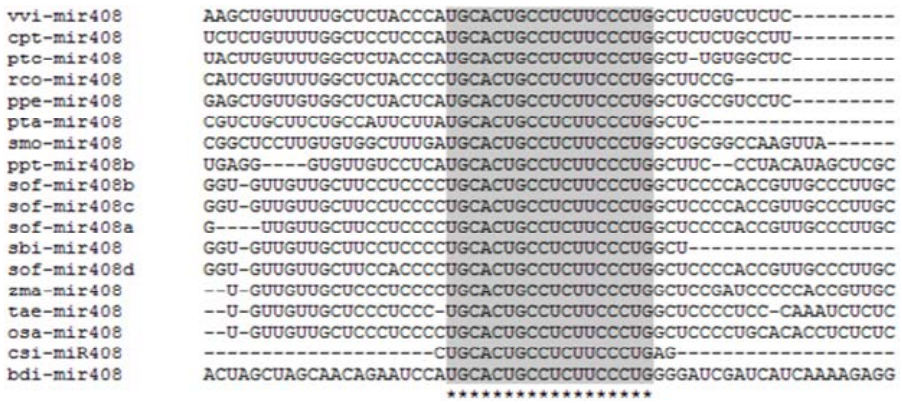


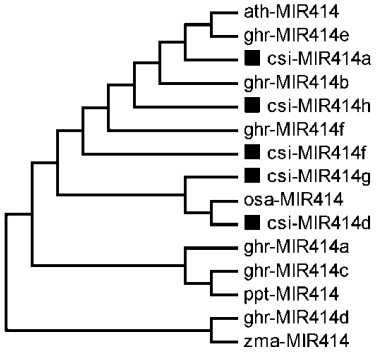
Figure 3. Conservation of tea miRNA (csi-miR408) with diverse plant species. Conserved portion is highlighted. Abbreviated names are given in full in appendix.

3.2. Phylogenetic Analysis

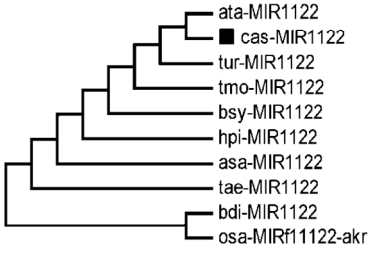
The newly identified tea miRNAs belong to 9 miRNA families including three known independent large miRNA families (mir 408, mir414 and mir1122). There are one tea miRNA namely csi-miR 408 and cas-miR1122 in each family of mir-408 and mir-1122, respectively. However, five members of family mir408 were found in tea (csi-miR414a, csi-miR414d, csi-miR414f, csi-miR414g, and csi-miR414h). The comparison of the predicted miRNA precursor sequences with other members in the same family showed that most members could be found to have a high degree of sequence similarity with others. The phylogenetic trees among the members of each family illustrated the evolutionary relationships of tea miRNAs (**Figure 4**).

3.3. Target Prediction

A total of 37 potential targets were identified for the 7 predicted miRNA families which include 11 miRNAs based on their perfect or nearly perfect complementarity with their target sequences in *Arabidopsis* (**Table 2**, **Figure 5**). For all the miRNAs, single binding site was found in the targets without any gaps in the complementtary region and expectation value ranges from 0 to 3. These potential miRNA targets were belonged to a number of gene families that involved in different biological functions such as regulation of cell cycle, metal ion transportation, starch metabolic processes etc. There were 8% of genes encoding transcription factors, 30% of genes encoding different enzymes and 14% of genes encoding transporters as well as 48% of genes encoding various proteins of physiological and metabolic processes (**Table 2**). The miRNA family 'miR414' showed the highest 30 numbers of independent target genes followed by 'miR408' family with 2 numbers of target genes. The rest miRNA families were with single target genes in *Arabidopsis* (**Table 2**). The 'miR1171' and 'miR1122' miRNA family members did not bind to any target sequences within our filtration criteria.



(a)



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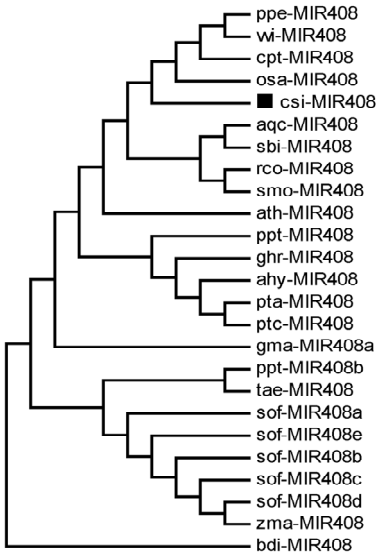

(c)

Figure 4. Phylogenetic relationships among the miRNA family members of (a) miRNA414 (b) miRNA1122 (c) miRNA408.

4. Discussion

With the availability of sequence resources in public databases, computer based miRNA identification methods have been focused more and more in the recent years due to its advantages of low cost and high efficiency. Sequence and structure homologies are the main theory behind the computer-based approach for miRNAs prediction. At present, four kinds of databases namely genome, GSS, EST and nucleotide are mainly used for plant miRNA mining. Considering the unavailability of genome and genomic survey sequences of tea, both EST and nucleotide databases were mined for miRNA identification. The number and sorts of miRNAs predicted in tea supported the fact that software-based approach is feasible and effective [3,10,14].

The idenfied new miRNAs were belonged to 9 families where miR414 family has 5 members and the rests have single member in each. This familial distribution of miRNAs was also observed in *Arabidopsis,* rice and maize

Table 2. Potential target genes of the identified miRNA families.

MiRNAs Family	Target sites	Targeted proteins	Targets involved in	EV*	Gene IDs
miR408	1	Cyclin-dependent protein kinase	regulation of cell cycle	3.0	TC284764
	1	Copper ion binding protein	metal ion transport	3.0	TC300377
miR414	1	RAN GTPase activating protein 2	cytokinesis	1.0	TC283936
	1	50S ribosomal protein L21	translation process	1.0	TC290468
	1	26S proteasome AAA-ATPase subunit RPT5a	proteasomal protein catabolic process	1.0	TC304323
	1	SEC12p-like transporter	ER to golgi vesicle mediated transpor	1.5	NP225634
	1	Nucleotide binding protein	nucleotide binding	2.0	TC280880
	1	MYB transcription factor	regulation of circadian rythm	2.0	TC283178
	1	Aldose 1-epimerase	carbohydrate metabolic process	0.5	TC285263
	1	Phosphatase 2C-like protein	protein amino acid dephosphorylation	0.5	TC285483
	1	Phosphatidylinositol phosphatase	inositol or phosphatidylinositol activity	0.5	TC312518
	1	Starch branching enzyme class II	starch metabolic process	1.0	AA586097
	1	Reproductive meristem protein 1	regulation of transcription	1.0	TC284340
	1	Calcium ion binding protein	calcium ion binding	1.0	TC299015
	1	Emb protein	RNA processing	1.0	TC294192
	1	Zinc ion binding protein	regulation of transcription	1.0	TC299076
	1	Calmodulin-4	calcium ion binding	1.5	TC294389
	1	Translation initiation factor 3 subunit 8	translation initiation	0	TC280751
	1	SMC3 protein	chromosome segregation process	0	TC281006
	1	MLO-like protein 3	cell death	0	TC293173
	1	Ubiquitin conjugating enzyme	proteolysis	0	TC299050
	1	Ubiquitin conjugating enzyme	ubiquitin dependant protein catabolic process	0.5	CA781750
	1	Zinc finger protein	regulation of transcription	0.5	NP030706
	1	Plastid protein	protein targetting to chloroplast	0.5	TC290688
	1	Ubiquitin thiolesterase	ubiquitin dependant protein catabolic process	1.5, 1.5	TC280710, TC298096
				1.5	TC305774
	1	Methionyl-tRNA synthetase	methionyl-tRNA aminoacylation	1.5	TC282196
	1	Metal ion binding protein	metal ion binding	1.5	TC305801
	1	Sfc4 protein	xylem or phloem pattern formation	2.0	TC280894
	1	Transcription factor	regulation of transcription	2.0, 2.0	TC293875, TC294793
	1	Synaptosomal-associated protein SNAP25	vesicle mediated transport	2.5	TC293303
	1	ATP binding	protein amino acid phosphorylation	2.5	TC299397
	1	ADP-ribosylation factor-like protein	intracellular protein transport	3.0	TC289959
miRf10132	1	Histone H2B like protein	nucleosome assembly	1.5, 1.5	TC297551, TC313314
				2.5, 1.5	TC313977, TC294144
miR2910	1	Extracellular matrix structural constituent	matrix organisation	0	TC310823,
miR2914	1	Glutamate semialdehyde dehydrogenase	glutamate metabolism	2.0	TC287905
miRf10185	1	Carboxylic ester hydrolase	hydrolase activity	3.0, 3.0	TC298946, TC308821
miR11590	1	FRIGIDA protein	regulation of flower development	2.0	TC309547

*EV=Expectation value

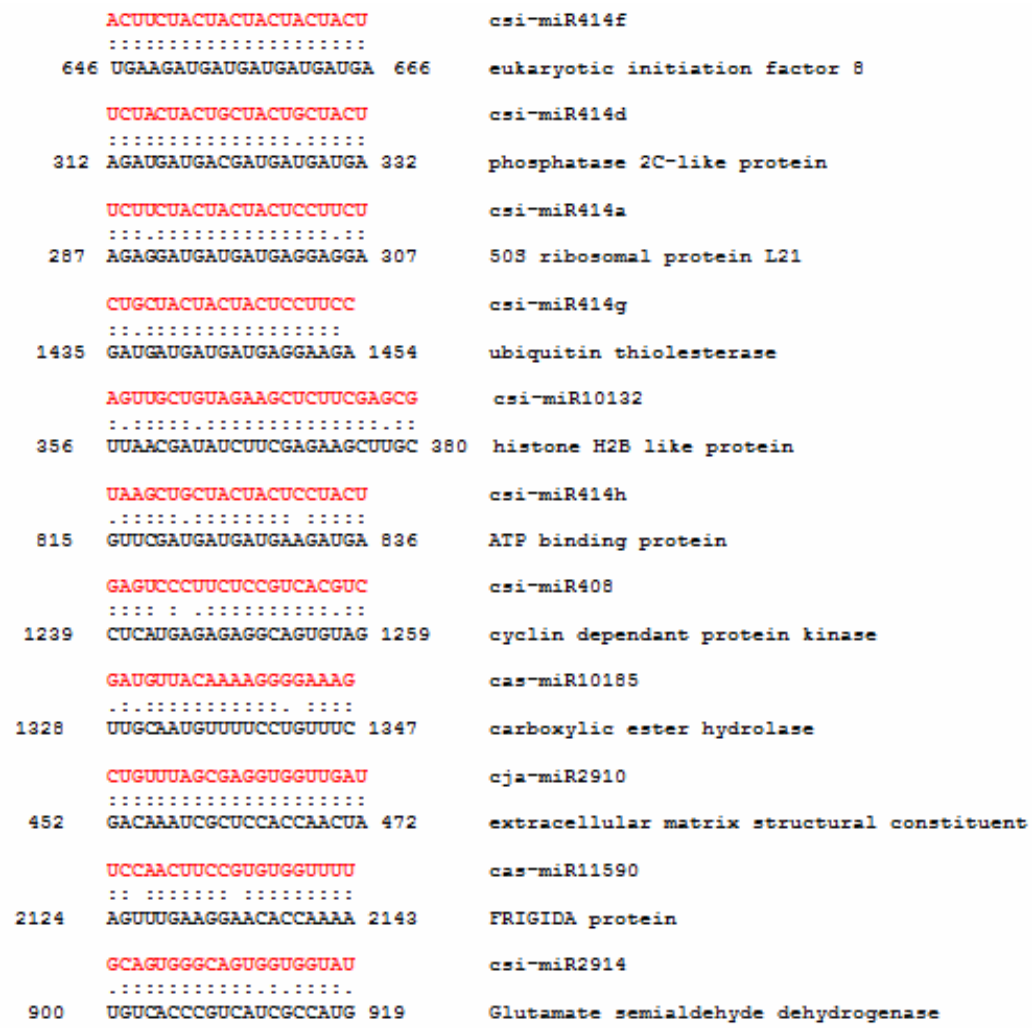

Figure 5. Predicted miRNA targets (black in colour) and their complementary sites with miRNAs (red in colour).

[29]. This may be an indicative of dominant nature of miR414 family in miRNA-mediated gene regulation in tea. The miRNAs were found diverse in nature such as location of mature miRNA sequences and length of precursor sequences. The average length of precursor sequence was 248 nucleotides; however a majority of them (62%) have 65-200 nucleotides. This finding is similar to other plants where the length of precursors varied in contrast to consistent miRNA length of animal miRNAs (70-80 nt) [30,31]. In tea miRNAs, diversity was also observed within the members of same family which was also found in maize [1]. The identified precursor miRNAs were fold into hairpin secondary structures using minimum free energies, with an average –72.69 kcal/mol which was lower than the values of *Arabidopsis thaliana* precursor miRNAs and much lower than the folding free energies of tRNA (–27.5 kcal /mol) and rRNA (–33 kcal/ mol) [32].

Out of 13 newly identified miRNAs, ten were from ESTs. There are several reports on miRNA identification from ESTs in various plant species [1,33]. The source sequences of miRNAs show a link between miRNAs and their tissues, organs, developmental stages or expression to which it belongs. On that basis, it was recognized that csi-miR414f, cas-miRf10185 and cas-miR11590-akr might be expressed in root and the rests were in leaf tissues. Moreover, csi-miR1171 and csi-miR414d were found in leaf tissue under the stress of winter dormancy and pest infestation, respectively. Three miRNAs namely cas-miR1122, cja-miR2910 and csi-miR2914 were identified from the full length nucleotides of RNA polymerase second largest subunit (intron 23) and 18S ribosomal subunit, respectively. Plant miRNAs are highly conserved among distantly related plant species, both in terms of primary and mature miRNAs [4]. This finding is also supported by our results, the identified miRNA was

found conserved in diverse plant species from monocotyledonous to dicotyledonous plants. These results suggested that different miRNAs might have evolved at different rates not only within the same plant species, but also in different ones.

The miRNA target gene identification is an important step for understanding the role of miRNAs in gene regulatory networks. Our prediction of target genes for the tea miRNAs revealed that more than one gene was regulated by individual miRNA. This result was similar to the recent findings in other plant species [1,10] which suggested that miRNA research should be focused on networks rather than individual connections between miRNA and strongly predicted targets. MiRNAs may directly target transcription factors which affect plant growth and development, and also specific genes which control metabolism [4]. In this study, we identified a total of 37 potential targets for the 7 identified miRNA families in tea. The identified target genes appeared to be associated with diverse biological functions. There were genes encoding transcription factors such as MYB, translation intiation factor such as TIF3, important proteosome degrading pathway enzyme such as ubiquitin conjugating enzyme, different ion transporters such as copper ion binding protein, carbohydrate metabolism related enzyme such as aldose 1-epimerase, glutamate metabolism related enzymes such Glutamate semialdehyde dehydrogenase, important protein for nucleosome assembly such as histone as well as ribosomal proteins. In an earlier report, it was found as 20% transcription factors and 53% proteins related to diverse physiological processes, however their investigation was limited to only four miRNAs [34]. Overall, these findings made us clear that tea miRNAs targeted both trancription factors as well as specific genes.

This findings of miRNAs in tea will pave the way for understanding the function and processing of tea small RNAs in future. Moreover, it shows a path for the prediction and analysis of miRNAs to those species whose genomes are not available through bioinformatics tools.

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Abbreviations used: BLAST, basic local alignment search tool; dbEST, database of expressed sequence tags; ESTs, expressed sequence tags; mRNA, messenger RNA; miRNA, microRNA; mfe, minimum free energy; nt, nucleotide; NCBI, national center for biotechnological information

