Computational Identification of miRNAs and Temperature Responsive lncRNAs from Mango (Mangifera indica, L.)

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Research article

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Abstract

Background

Mango is a major tropical fruit in the world and is known as the king of fruits because of its flavour, aroma, taste, and nutritional values. Moreover, various parts of mango trees have been used for medical purposes. Although various regulatory roles of miRNAs and lncRNAs have been investigated in many plants, there is yet an absence of study in mango. This is the first study to provide information on ncRNAs of mango with the aim of identifying miRNAs and lncRNAs of mango and discovering of their potential functions by the interaction prediction of the miRNAs, lncRNAs and their target genes.

Results

In this analysis, 104 miRNAs and 7,610 temperature responsive lncRNAs were identified and the target genes of these ncRNAs were characterized. By analysing the interaction of miRNAs and their target genes, it was observed that miRNAs are mainly involved in growth, development, and stress responses of mango. For the lncRNAs, cold responsive lncRNAs bound to low temperature responsive proteins expressed at low temperature stress. GO enrichment analysis of heat and cold responsive lncRNAs revealed that they involved in all three basic processes; biological process, cellular component, and molecular function. Moreover, mango lncRNAs can target miRNAs to reduce the stability of lncRNAs and can function as molecular decoys or sponges of miRNAs.

Conclusion

This paper would provide the new information about miRNAs and lncRNAs of mango and would help for the further investigation of mango ncRNAs.

Background

Non-coding RNAs (ncRNAs) are RNA molecules that have no or little protein coding potential and are not translated into proteins although they are transcribed from DNA. Small ncRNAs such as microRNA (miRNA), small interfering RNA (siRNA), small nucleolar RNA (snoRNA) and piwi-interacting RNA (piRNA) are shorter than 200 nucleotides (nt) in length and piwi-interacting RNA (lncRNAs) are longer than 200 nt.

The miRNAs are small (18–24 nt), endogenous and regulatory RNA molecules derived from their long self-complementary precursor sequences which can fold into hairpin secondary structures [1]. In plants, these long primary precursor miRNAs are transcribed by RNA polymerase II or RNA polymerase III and then processed by dicer-like 1 enzyme (DCL1) into miRNA/miRNA* duplex [1, 2]. Finally the mature miRNAs are incorporated into an RNA-induced silencing complex (RISC) [3]. The binding of miRNAs to their targeted mRNAs in a perfect or nearly perfect complementarity suggests a method for identifying

their targets by BLAST analysis or other related publicly available software [4]. Many experimental researches have proved that miRNAs involve in many important biological and metabolic processes. In plants, miRNAs play a fundamental role in almost all biological and metabolic processes including plant growth, development, signal transduction and various stress response by binding to their target genes [5].

LncRNAs are a family of regulatory RNAs having a minimal length of 200 nt and unable to encode proteins. Most lncRNAs are transcribed by RNA polymerase II although some are transcribed by RNA polymerase III [6–8]. LncRNAs can interact with ncRNAs such as miRNAs [9]. LncRNAs not only can target to miRNAs to reduce the stability of lncRNAs but also can function as molecular decoys or sponges of miRNAs [10]. Moreover, lncRNAs can compete with miRNAs to bind to their target mRNAs and are the precursors for the generation of miRNAs to silence target mRNAs [11]. Many evidence showed that plant lncRNAs play an important role in fundamental biological processes including the growth and development and abiotic stress responses [12, 13]. But, the molecular basis of how lncRNAs function and mediate gene regulation is still poorly understood [14].

The genus *Mangifera* belongs to the family Anacardiaceae and contains about 69 different species. *Mangifera indica*, L (mango) is the most common species among them [15, 16]. Mango is one of the main tropical fruits over the world and is believed to be originated from Asia [17]. The well-known countries for mango cultivation are China, India, Thailand, Pakistan, Mexico, Philippine and Myanmar. The annual production of mango is approximately 42 million tons which is second after banana production [18]. Mango is called as the king of fruits because of its special characteristic flavour, pleasant aroma, taste, and nutritional values. Both ripe and raw fruits can be used as the food products such as pickles, juice, jam, powder, sauce, cereal flakes and so on [19]. Moreover, various parts of mango trees have been used for medical purposes since long times ago, mostly in Southeast Asian and African countries [20]. *In vitro* and *in vivo* studies have been indicated the various pharmacological potentials of *M.indica* such as anticancer, anti-inflammatory, antidiabetic, antioxidant, antifungal, antibacterial, anthelmintic, gastroprotective, hepatoprotective, immunomodulatory, antiplasmodial and antihyperlipemic effects [21].

Although mango is a popular plant with many important usages, its ncRNA data is still limited. Over 10,000 miRNA data of several plants can be accessed in miRNA database, miRBase, but mango miRNAs and their functions have not yet been identified. The regulatory roles of lncRNAs and the molecular basis of lncRNA-mediated gene regulation are also still poorly understood in plants including mango. So, the aim of this research work is to identify and study about the miRNAs and lncRNAs of mango and to examine their potential functions by the interaction prediction of the miRNAs, lncRNAs and their target genes.

Results

Identification and characterization of mango miRNAs

From the mango unigene sequences, we have identified 104 miRNAs by following the identification workflow explained in Figure 1. The length of the resulting mature miRNAs is in the range of 18-22 nt. Among them, nearly 40% (41 miRNAs) of mango mature miRNAs are in the length of 18 nt and 6 miRNAs have the length of 22 nt. 32 miRNAs, 17 miRNAs and 8miRNAs are 19 nt, 20 nt and 21 nt of length respectively (Figure 2A). However, the precursor length of mango miRNAs (MmiRs) was varied significantly from 67 to 144 nt with an average length of 94 nt. The secondary structure of precursor sequences was predicted by Zuker folding algorithm in MFOLD. The hairpin structures of five miRNAs are shown in Figure 2B. Average MFE of pre-miRNAs is 29.92. The MFEI values were also calculated and were in the range of 0.7 to 1.45 with the average MFEI of 0.84 (Table S1).

Target genes analysis of miRNAs

According to the result of target gene prediction by psRNATarget server, all the newly identified mango miRNAs could bind to their targets and a total of 2,347 target genes were predicted for 104 mango miRNAs. The predicted target genes were annotated and assigned to GO term by BLAST2GO. According to the result of GO analysis, the predicted target genes of mango miRNAs involved in all three broad categories; biological processes, cellular components and molecular functions. Among 2347 target genes, 2081 target genes were enriched in 925 GO terms, and 502 in biological process, 127 in cellular component and 296 in molecular function. Highly enriched GO terms of miRNA target genes were visualized in Figure 2C. From KEGG pathway analysis, 310 targets were involved in the 103 different KEGG pathways. Purine metabolism was the pathway with the highest 136 target genes. The predicted miRNAs, their target genes, target descriptions, target GO terms and target KEGG pathways are shown in Table S2.

Identification and characterization of mango lncRNAs

For the identification of lncRNAs, a total of 277,071 RNA transcripts from Zill, Shelly and Keitt mango cultivars were used. First, the sequences less than 200 nt were removed because lncRNAs were always longer than 200 nt. Then, the coding transcripts were removed by their protein-coding potential, homology with known proteins, and potential ORFs. Finally, the house keeping RNAs and precursor of miRNAs were removed. After a series of filtering steps, a total of 31,226 candidate lncRNAs were predicted.

The temperature responsive lncRNAs were then defined by fold change value and FDR. Fold change value of <-2 or >2 and FDR adjusted p-value 0.05 were used to filter out the significantly expressed mango lncRNAs, and as the result, 24 lncRNAs were significantly expressed to heat stress (55°C hot water brushing) and 7586 lncRNAs to cold stress (5°C, 8°C or 12°C) (Figure 3A). In heat responsive lncRNAs, 18 lncRNAs were upregulated and 6 lncRNAs were downregulated. The length of heat responsive lncRNAs was ranging from 213 to 1186 nt (Table S3). Among the 7619 cold responsive lncRNAs, 4335 were upregulated and 3251 were downregulated. The length of cold responsive lncRNAs was in the range of 201 to 2746 nt (Table S4).

Conservation analysis of lncRNAs

The mango lncRNAs were searched by using BLASTn against the plant lncRNA database, CANTATAdb, with e-value cutoff 1e-20 to check their evolutionary conservation. As the result, no heat responsive lncRNAs was conserved and 22 cold responsive lncRNAs were conserved with 12 different plant species (Figure 3B).

Target gene prediction of lncRNAs

To analyse the interaction of newly identified lncRNAs of mango with protein-coding genes, the lncRNA target prediction tool LncTar was used. A total of 1998 mango mRNAs downloaded from NCBI were used for the target prediction of lncRNAs. From the resulting data, 6975 lncRNAs interacted with 1985 target mRNAs. To analyse the functional overview of identified lncRNAs, the targets of the identified lncRNAs were predicted by BLAST2GO. Among 24 heat responsive lncRNAs, 8 lncRNAs had 115 target genes (SCL14, STP13, Hsp70, At4g39970, ACO1 and so on) involved in the plant development and stress response. In cold responsive lncRNAs, 6951 lncRNAs interacted with 1985 target genes. The WRKY proteins are a large family of transcriptional regulators in higher plant and 64 cold responsive lncRNAs interact with WRKY gene family in this study (Figure 3C).

Moreover, functional prediction of the target genes of identified lncRNAs were performed by GO enrichment analysis and the resulting data showed that 11 GO terms were enriched in biological process, 8 GO terms in cellular component, and 3 GO terms in molecular function for heat responsive lncRNAs. In the biological process, metabolic process, cellular process, cellular component biogenesis and cellular metabolic process were highly enriched. In the cellular component analysis, GO terms associated with membranes and intracellular were highly enriched. Catalytic activity and binding GO terms were highly enriched in molecular function analysis (Figure 3D). For the target genes of the cold responsive lncRNAs, 40 GO terms were highly enriched; GO terms of 20 were enriched in the biological process, 7 in cellular component and 14 in molecular functions. Metabolic processes and cellular processes were highly enriched for biological processes. In the cellular component analysis, GOs related to membranes, intracellular and cytoplasm were highly enriched. For molecular function analysis, most of the enriched GO terms were related to catalytic activity and binding (Figure 3E).

From the results of the KEGG pathway analysis, heat responsive lncRNAs had target genes involved in 17 KEGG pathways (Table S5). Among these different pathways, amino sugar and nucleotide sugar metabolism was the most significant pathway and 8 target genes involved in this pathway. For cold responsive lncRNAs, 209 target genes had mapped to 87 KEGG pathways (Table S6). JK513026_1, alcohol dehydrogenase 1 (ADH1, EC:1.1.1.1) was the most enriched target gene and involved in 12 different pathways.

Prediction of lncRNAs as miRNAs targets

To analyse the direct interaction of miRNAs and lncRNAs of mango, the psRNATarget server was used to predict the target lncRNAs of miRNAs. The resulting data showed that 3 heat responsive lncRNAs

interacted with 6 miRNAs (Table S7). For cold responsive lncRNAs, 763 lncRNAs had 1203 pairs of interactions with 89 miRNAs (Table S8).

The miRNA target mimicry search was also performed by using TAPIR. No heat responsive lncRNA act as the target mimic of miRNAs. But 20 cold responsive lncRNAs were predicted as the target mimics of 20 miRNAs (Table S9). CRlnc31221 was the target mimic of MmiR5408 which targeted to 8 cold responsive lncRNAs and 47 target genes (Figure 4B). Base-pairing interaction between MmiR5408 and its target mimic cold responsive lncRNA, CRlnc31221 was shown in Figure 4C.

The interaction network of mango ncRNAs (miRNAs, lncRNAs and mimic) and their target genes was visualized by using Cytoscape contained a total of 5388 pairs of interaction among miRNAs, lncRNAs and their targets (Figure 4A). These interactions were 4155 pairs of 104 MmiRNAs and 2347 mRNAs, 1203 pairs of 89 MmiRNAs and 763 CRlncRNAs, 6 pairs of 6 MmiRNAs and 4 HRlncRNAs, and 24 pairs of 20 miRNAs and their 20 target mimics.

Discussion

Identification, characterization and target gene prediction of miRNAs

Most of the plant miRNAs are evolutionarily conserved from species to species [22, 23] and this indicates the powerful strategy for the identification of new miRNAs by using the already known miRNAs [24]. Many conserved miRNAs have been identified from the expressed sequence tag (EST) [25, 26] and genome survey sequence (GSS) [27] by using this homology search approach. For mango, there is no GSS data and the available EST data for mango is only 1709 and it was not sufficient for identification of miRNA. Hence, unigene sequences (107,744) were used for the identification of miRNAs in this study. Unigene is a unique transcript that is transcribed from a genome and many miRNAs have been identified from the unigenes of many plant species such as *Artemisia annua* [28], coconut [29], Litchi fruit [30] and black pepper [31].

The potential 104 pre-miRNAs of mango were predicted based on the parameters of Zhang[25] and the MFEI values were also calculated as the MFEI gave the best prediction of miRNAs [32]. Although the length of the predicted mature miRNAs was in the range of 18-22 nt, the length of precursor miRNAs varied significantly from 67 to144 nt with an average length of 94 nt. The predicted 104 mango miRNAs belong to 86 different families. Among them, over 70% of the miRNA families have only one family member. The highest 5 family members were found in the miR2673 family followed by miR159, with 4 family members. The remaining miRNAs have the family member of 2 or 3. Therefore, we can see that the mango miRNA distribution across various families is highly heterogeneous.

The previous studies have already proved that the plant miRNAs bind to their targets in a perfect or nearly perfect complementarity and thus the psRNATarget server was used to search the target gene of mango miRNAs in this study. Both mRNAs collected from NCBI and mRNA identified in this study were used as the target candidates of miRNAs due to the absence of *Mangifera indica* target candidates in

psRNATarget server. Some previous studies indicated that miR156 was a master regulator of the juvenile phase in plants and it targeted the squamosa promoter binding protein-Like (SPL) gene family to regulate the transition from vegetative phase to floral phase in *Arabidopsis*, maize and rice [33-39]. In mango, MmiR105772, a family of miR156, also bound to its target SPL6 and thus the predicted targets of mango miRNAs were in the agreement with the previously published papers in other plants. The resulting data from psRNATarget also showed that only one miRNA (MmiR1653) had the single target gene which was the member of miR482 family and bound to monodehydroascorbate reductase 4 enzyme, the important gene related to the nutritional quality of mango fruit [33]. All other miRNAs could target to multiple genes and some miRNAs had over two hundred target genes. For example, MmiR73030 had 230 target genes and these target genes involved in 16 KEGG pathways such as biosynthesis of antibiotics, purine metabolism, sulfur metabolism, glycerophospholipid metabolism, T cell receptor signalling pathway, steroid degradation and so on.

Sivankalyani, Sela et al. published that the mango stress-response pathways were activated by cyclic nucleotide-gated channel (CNGC) and leucine-rich repeat receptor (Lrr) [40]. In this study, we found that MmiR90392 targeted to CNGC1, and MmiR68471 and MmiR68478 targeted to Lrr2. Moreover, MmiR10167 and MmiR15558 bound to the stress WRKY transcription factor 44 which play a major role in plant defence to biotic and abiotic stresses. MmiR78769 and MmiR101928 also bound to their target genes of phospholipase A and phospholipase D which were key factors in plant responses to biotic and abiotic stresses [41]. The ethylene response could improve the tolerance of mango fruit to chilling stress [42] and ten mango miRNAs identified in this study had six ethylene responsive target genes such as ethylene-insensitive protein and ethylene-responsive transcription factor. So, these newly identified mango miRNAs have potential roles in chilling stress responsive process of mango.

Two mango miRNAs (MmiR23777 and MmiR36814) also targeted to the auxin efflux carrier which had the potential role in mango plant organ development [43]. A total of 17 miRNAs interacted with auxin-related genes. MmiR51876 was a miRNA that targeted to auxin responsive protein. The pentose and glucoronate interconversions pathway, phenlypropanoid biosynthesis pathway and alpha-linolenic acid metabolism pathway were KEGG pathway involved in the adventitious root formation of mango cotyledon segments [44]. In this study, 9 miRNAs bound to 8 target genes involved in these three pathways for mango root formation. MmiR10167 bound to target genes that involved in phenlypropanoid biosynthesis pathway and MmiR7519 bound to target genes involved in alpha-linolenic acid metabolism pathway. From these findings, it could be observed that these five mango miRNAs (MmiR23777, MmiR36814, MmiR51876, MmiR10167 and MmiR7519) involved in the developmental process of mango (Figure 2B).

Identification, characterization and target gene prediction of lncRNAs

As the genome sequence of mango is not available till now, the *de novo* assembled transcriptome sequences were used for the identification of lncRNAs in this study. A total of 277,071 RNA transcripts from Zill, Shelly and Keitt mango cultivars studied by the former researchers were used and a total of

31,226 candidate lncRNAs were predicted in this study. Among them, 24 lncRNAs were significantly expressed to heat stress and 7586 lncRNAs to cold stress. The most significantly expressed down-regulated heat responsive lncRNA was HRlnc25944 with the fold change value of -6.22. HRlnc11351 and HRlnc27371 were the mostly expressed up-regulated lncRNAs with fold change value greater than 7. For the cold responsive lncRNAs, CRlnc10871 was the mostly expressed down-regulated lncRNA (FC value -11.19), and CRlnc26299, CRlnc30496 and CRlnc36473 were the most significantly expressed lncRNAs with fold change value greater than 11.

No heat responsive lncRNAs was conserved but 0.29% of cold responsive lncRNAs were conserved with 12 different plant species. Among them, the highest conserved lncRNAs were CRlnc32663 and CRlnc47883. Each of which was conserved with 4 different lncRNAs of other plants. CRlnc32663 conserved with 4 different lncRNAs of 3 three different plant species such as *Manihot esculenta*, *Malus domestica* and *Populus trichocarpa.* CRlnc42883 also conserved with 4 lncRNAs of *Oryza rufipogon*, *Oryza barthii* and *Solanum lycopersicum* (Figure 3B).

For heat responsive lncRNAs, 8 bound to 115 target genes involved in the plant development and stress response. HRlnc11351 was the most significantly expressed up-regulated lncRNAs with fold change value of 7.55 and bound to six heat shock proteins. In cold responsive lncRNAs, CRlnc26299 was one of the most significantly expressed up-regulated lncRNAs and bound to RC12B (JK513200_1) which is the low temperature and salt responsive protein found in *Arabidopsis thaliana* [45]. The WRKY proteins are a large family of transcriptional regulators in higher plant and are exhibited the variable expression patterns in response to chilling stress in cucumber, mango and rice [40, 46, 47]. In this study, 64 cold responsive lncRNAs have interaction with WRKY gene family. So, we can observe that the cold responsive lncRNAs of mango have the interaction with the target genes that are expressed at the low temperature stress.

GO enrichment analysis and KEGG pathway analysis were performed for the better understanding of the target genes of newly identified lncRNAs. From GO enrichment analysis result, we could see that both types of heat responsive lncRNAs and cold responsive lncRNAs had interaction with the target genes involved in all three broad categories such as biological process, cellular component and molecular function. For the biological processes, both heat responsive and cold responsive lncRNAs were highly enriched in metabolic processes and cellular processes. In the cellular component analysis, GOs related to membranes, intracellular and cytoplasm were highly enriched for both type of lncRNAs. Also for molecular function analysis, most of the enriched GO terms in both type of lncRNAs were related to catalytic activity and binding. Therefore, we could see that, the GO terms highly enriched in both heat responsive and cold responsive lncRNAs were not quite different.

Among 17 KEGG pathways of the target genes of the heat responsive lncRNAs, amino sugar and nucleotide sugar metabolism was the most significant pathway and 8 target genes involved in this pathway. As mentioned above, HRlnc11351 was the most significantly expressed up-regulated lncRNAs and its target gene, JK513625_1 is 3-ketoacyl-CoA thiolase 2 (KAT2, EC:2.3.1.16) which could be mapped to 9 different pathways such as benzoate degradation, fatty acid elongation, biosynthesis of unsaturated

fatty acids, alpha-linolenic acid metabolism, fatty acid degradation, valine, leucine and isoleucine degradation, biosynthesis of antibiotics, geraniol degradation and ethylbenzene degradation according to the result of KEGG pathway analysis. In *Arabidopsis*, KAT2 is an enzyme that catalyses the β-oxidation of fatty acid and involves in abscisic acid (ABA) signal transduction [48]. The phytohormone ABA plays an important role in plant development and adaptation to diverse environmental stresses. Therefore, HRlnc11351 may involve in the important role of mango development and stress response by targeting to KAT2. For cold responsive lncRNAs, 209 target genes had mapped to 86 KEGG pathways. JK513026_1, alcohol dehydrogenase 1 (ADH1, EC:1.1.1.1) was the most enriched target gene and involved in 12 different pathways including glycolysis/gluconeogenesis, metabolism of xenobiotics by cytochrome P450, glycine, serine and threonine metabolism, methane metabolism, fatty acid degradation and so on. In plants, ADH genes are involved in mediating stress responses and developments. In mango, ADH1 has important role in the fruit ripening [49] and thus, cold responsive lncRNAs that target to ADH1 gene may play important role in mango fruit ripening process. According to the KEGG pathway analysis results, purine metabolism and biosynthesis of antibiotics were the highly enriched pathways among 86 pathways and more than 50 target genes were enriched in each pathway.

Interaction between lncRNAs and miRNAs

The interaction between the miRNAs and lncRNAs showed that the most of the miRNAs had targeted to more than one lncRNAs and only 8 miRNAs had single target lncRNAs. The number of lncRNAs targeted by a single miRNA was in the range of 1 to 90. A total of 90 target lncRNAs were found for MmiR73030 which also targeted 230 mRNAs. This miRNA had the highest target numbers in both lncRNAs and mRNAs.

LncRNAs not only can target to miRNAs to reduce the stability of lncRNAs but also can function as molecular decoys or sponges of miRNAs [[10, 50]]. So the miRNA target mimicry search was performed by using TAPIR, which is a web server for the prediction of plant miRNA targets including target mimics. Although no heat responsive lncRNA act as the target mimic of miRNAs, 20 cold responsive lncRNAs were predicted as the target mimics of 20 miRNAs. CRlnc31221 was the target mimic of MmiR5408 which targeted to 8 cold responsive lncRNAs and 47 target genes. These target genes were involved in starch and sucrose metabolism, inositol phosphate metabolism and phenylpropanoid biosynthesis pathways which pathways are important for plant growth and development, and plant response towards biotic and abiotic stress. During target mimicry, the interactions between miRNAs and their authentic targets were blocked by binding of decoy RNA to miRNAs via partially complementary sequences [51]. So, the target mimicry of CRlnc31221 had the potential regulation effect to the interaction between the target genes and MmiR5408.

Conclusion

In conclusion, this study identified the 104 miRNAs and 7610 temperature responsive lncRNAs from mango transcriptome sequences. And the interactions of these ncRNAs with their target genes were also

predicted. According to the result, newly identified mango ncRNAs, like other plant ncRNAs, have potential role in biological and metabolic pathways including plant growth and developmental process, pathogen defence mechanism, and stress responsive process. Therefore, the resulting data of this project may help for the further prediction of the specific functions of mango ncRNAs and wet lab experiments.

Methods

Data collection

A total of 10,415 plant miRNAs (release 21) were downloaded from miRBase database (http://www.mirbase.org/cgi-bin/browse.pl) and the redundancy sequences were removed. The resulting 6042 non-redundant known miRNAs were used as the reference for the prediction of conserved miRNAs.

107,744 mango unigenes collected from the mango RNA-Seq database (http://bioinfo.bti.cornell.edu/cgi-bin/mango/index.cgi) were used for the identification of mango miRNAs.

As the whole genome of mango is not available, the publically available *de novo* assembled transcripts were used for the prediction of candidate lncRNAs. A total of 277,071 mango transcripts obtained from Zill, Shelly and Keitt mango cultivars [40, 52-54] were used in this study. A total of 1998 mango mRNAs downloaded from NCBI were used for the target prediction of miRNAs and lncRNAs.

Identification of miRNAs and their precursors

First, the homology search of mango unigenes against non-redundant plant miRNAs was performed by using BLASTn with default parameters. The following criteria were used to choose the candidate miRNAs; the length of candidate miRNA should be greater than or equal to 18 nt without gap and the number of mismatches between mango sequences and plant miRNAs should not be more than 2. The sequences of 100 nt upstream and 100 nt downstream from the BLAST hit were extracted for precursor sequences. If the length of query sequence was less than 200 nt, the entire sequence was selected. BLASTx against NCBI non-redundant (Nr) protein databases was used to remove the protein-coding sequences from the extracted precursor sequences with the e-value cut off 0.01. The secondary structures of remaining precursor sequences were predicted by using the Zuker folding algorithm in MFOLD software [55] with default parameters. The workflow for the identification of miRNAs was briefly described in Figure 1. Based on the parameters of Zhang [25], the potential pre-miRNAs were predicted as follows:

1. the minimum length of precursor should be at least 60 nt;
2. the pre-miRNA sequence should be folded into an appropriate stem-loop hairpin secondary structure;
3. it should contain the mature miRNA within one arm of the hairpin;
4. the predicted mature miRNAs and its opposite miRNA* sequence in the other arm should not have more than 6 nt mismatches;
5. loop or break should not be contained between miRNA/miRNA* duplex;

6. maximum size of a bulge in the mature miRNA sequences should not be more than 3 nt;
7. the predicted secondary structures should have higher negative minimal free energies (MFE) and minimal free energy index (MFEI);
8. MFEI of pre-miRNA should be greater than 0.7;
9. A+U content should be within 30-70%.

The following equations were used to calculate the minimal free energy index (MFEI) and adjusted minimal free energy (AMFE).

AMFE = (MFE/length of pre-miRNA) x 100

MFEI = AMFE/(G+C)%

Prediction of candidate lncRNAs

To predict the lncRNAs, the transcripts smaller than 200 nt were firstly removed. The coding potential of remaining transcripts was then calculated by CPC [56] and LncFinder [57]. Only sequences with the CPC score less than -1 and LncFinder score less than 0.5 were used for further prediction. The protein coding sequences were removed by BLASTx against NCBI Nr protein databases and ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict the open reading frame (ORF) of the remaining sequences and the minimal ORF cutoff less than 102 amino acids was applied for prediction. Then, house-keeping genes were removed against Rfam database (http://rfam.xfam.org) with e-value 0.001. Finally, to remove the lncRNAs acting as precursors of known or novel miRNAs, lncRNAs were aligned with precursors of known non-redundant plant miRNAs from the miRBase database (http://www.mirbase.org/) using BLASTn with the default parameters (Figure 1).

The remaining transcriptome sequences that were not captured as lncRNAs were used as queries against the NCBI Nr protein database using BLASTx with a cutoff e-value of 1e-5. The sequences with the blast hits were then analysed to remove the house-keeping RNAs. The final sequences were identified as protein coding sequences in this study for target gene analysis.

Identification of significantly expressed temperature responsive lncRNAs

From the resulting lncRNA transcripts, the temperature responsive lncRNAs were filtered by two parameters. The mango lncRNAs with the adjusted p-value of 0.05 and the log2 fold change of greater than 2 or less than -2 were identified as the significantly expressed lncRNAs.

Target gene prediction of miRNAs and lncRNAs

Mango mRNAs downloaded from NCBI database and mango protein coding sequences previously identified were used for the target genes prediction of miRNAs. The putative target sites of miRNAs were identified by aligning the miRNA sequences using plant target prediction tool, psRNATarget server (http://plantgrn.noble.org/psRNATarget/) [58]. To reduce the number of false predictions, the maximum

expectation threshold was set to the value of 3.0. The cut-off length of nucleotides for complementarity scoring, hsp size, was set as the length of the mature miRNAs. The maximum energy of unpairing (UPE) the target site was set as 25 kcal. The flanking length around target site for target accessibility analysis was 17 bp in upstream and 13 bp in downstream. The range of central mismatch leading to translation inhibition was adjusted as 9-11 nt. No gap and no more than four mismatches between miRNA and its target (G-U pair count as 0.5 mismatch) was allowed. The target genes of mango lncRNAs were predicted by using LncTar tool [59] with the normalized binding free energy (ndG) cutoff value less than 0.1.

Prediction of lncRNAs as miRNA target or target mimic

To predict the lncRNAs as the target genes of miRNAs, psRNATarget was used as previously mentioned in the interaction prediction of miRNAs and mRNAs. For target mimic prediction, TAPIR server [60] was used in this study. TAPIR is a web server for the prediction of plant miRNA targets including target mimics,

Functional annotation and pathway analysis of target genes

The gene ontology (GO) analysis of the identified target transcripts was executed by combining both BLASTx data and interproscan analysis data by means of the BLAST2go software [61]. The GO enrichment analysis was performed by using Fisher's exact test with multiple testing correction of false discovery rate (FDR). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was also performed for better understanding of the functions of the target genes.

Conservation analysis of lncRNAs

The analysis of conservation of mango lncRNAs was detected by using BLASTn against all lncRNA sequences from the plant lncRNA database, CANTATAdb [62], with e-value cutoff 1e-20.

Interaction network of miRNAs, lncRNAs and their target genes

Finally, the interaction network of miRNAs, lncRNAs and their target genes were visualized by using Cytoscape [63].

Abbreviations

ABA
abscisic acid
ACO1
1-aminocyclopropane-1-carboxylate oxidase 1
ADH1
alcohol dehydrogenase 1
AMFE
adjusted minimal free energy
BLAST

basic local alignment search tool

bp

base pair

C

cytosine

CNGC

cyclic nucleotide-gated channel

CPC

coding potential calculator

DCL1

dicer-like 1 enzyme

EST

expressed sequence tag

FDR

false discovery rate

G

guanine

GO

gene ontology

GSS

genome survey sequence

Hsp70

heat shock protein 70

KAT2

3-ketoacyl-CoA thiolase 2

Kcal

kitocalorie

KEGG

Kyoto Encyclopedia of Genes and Genomes

lncRNA

long non-coding RNA

Lrr

leucine-rich repeat receptor

MFE

minimal free energies

MFEI

minimal free energy index

miRNA

microRNA

mRNA

messenger RNA

NCBI

national center for biotechnology information

ncRNA

non-coding RNA

ndG

normalized binding free energy

Nr

non-redundant

nt

nucleotide

ORF

open reading frame

piRNA

piwi-interacting RNA

RC12B

related cDNA 12 B

RISC

RNA-induced silencing complex

RNA

ribonucleic acid

SCL14

scarecrow-like 14

siRNA

small interfering

snoRNA

small nucleolar RNA

SPL

squamosa promoter binding protein-Like

STP13

sugar transport protein 13

UPE

maximum energy of unpairing

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The unigene sequence data are available from the mango RNA-Seq database (http://bioinfo.bti.cornell.edu/cgi-bin/mango/index.cgi) and mango transcriptome data are available from the supplementary data of the published journals [40, 52-54]. All data generated and analysed during this study are included in this published article (and its supplementary information files).

Competing interests

The authors have declared no competing interests.

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Authors' contributions

NMMM and MC designed the research. NMMM and PZ performed bioinformatics analysis.

NMMM and YC analyzed plant genes data. All authors approved the final manuscript.

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Supplementary Materials

Supplementary Table 1 miRNAs data

Supplementary Table 2 miRNAs targets data

Supplementary Table 3 Heat responsive lncRNAs data

Supplementary Table 4 Cold responsive lncRNAs data

Supplementary Table 5 KEGG pathways of the target of heat responsive lncRNAs

Supplementary Table 6 KEGG pathways of the target of cold responsive lncRNAs

Supplementary Table 7 Heat responsive lncRNAs as target of miRNAs

Supplementary Table 8 Cold responsive lncRNAs as target of miRNAs

Supplementary Table 9 Cold responsive lncRNAs as target mimic of miRNAs

Supplementary Data Set 1 Heat responsive lncRNAs sequences

Supplementary Data Set 2 Cold responsive lncRNAs sequences

Figures

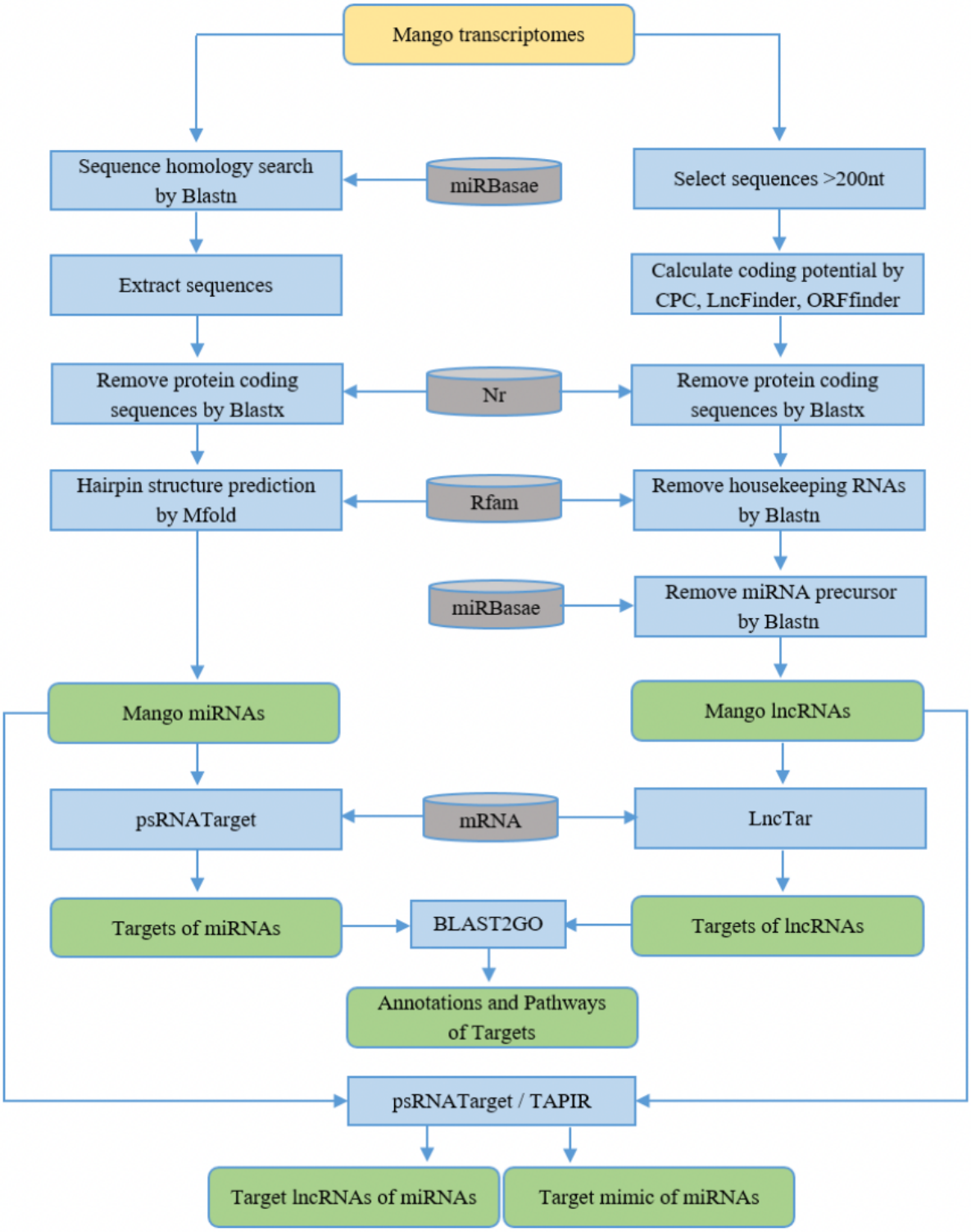

Figure 1

Workflow for identification of ncRNAs (miRNAs and LncRNAs) and their targets. The yellow rounded rectangle represents the data input and green rounded rectangles for data output. The blue rectangles are the processing steps and grey cans represent databases.

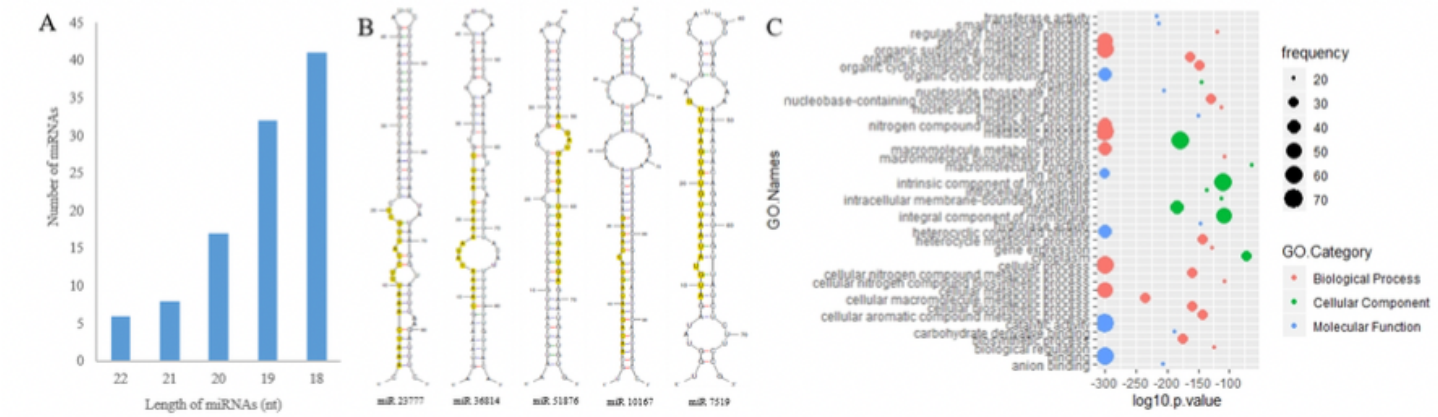

Figure 2

Mango miRNAs (A) Length distribution of miRNAs; (B) Hairpin structures of five precursor miRNAs (Mature miRNA are highlighted by yellow colour) involved in the development process of mango; (C) GO enrichment analysis of highly enriched target genes of miRNAs.

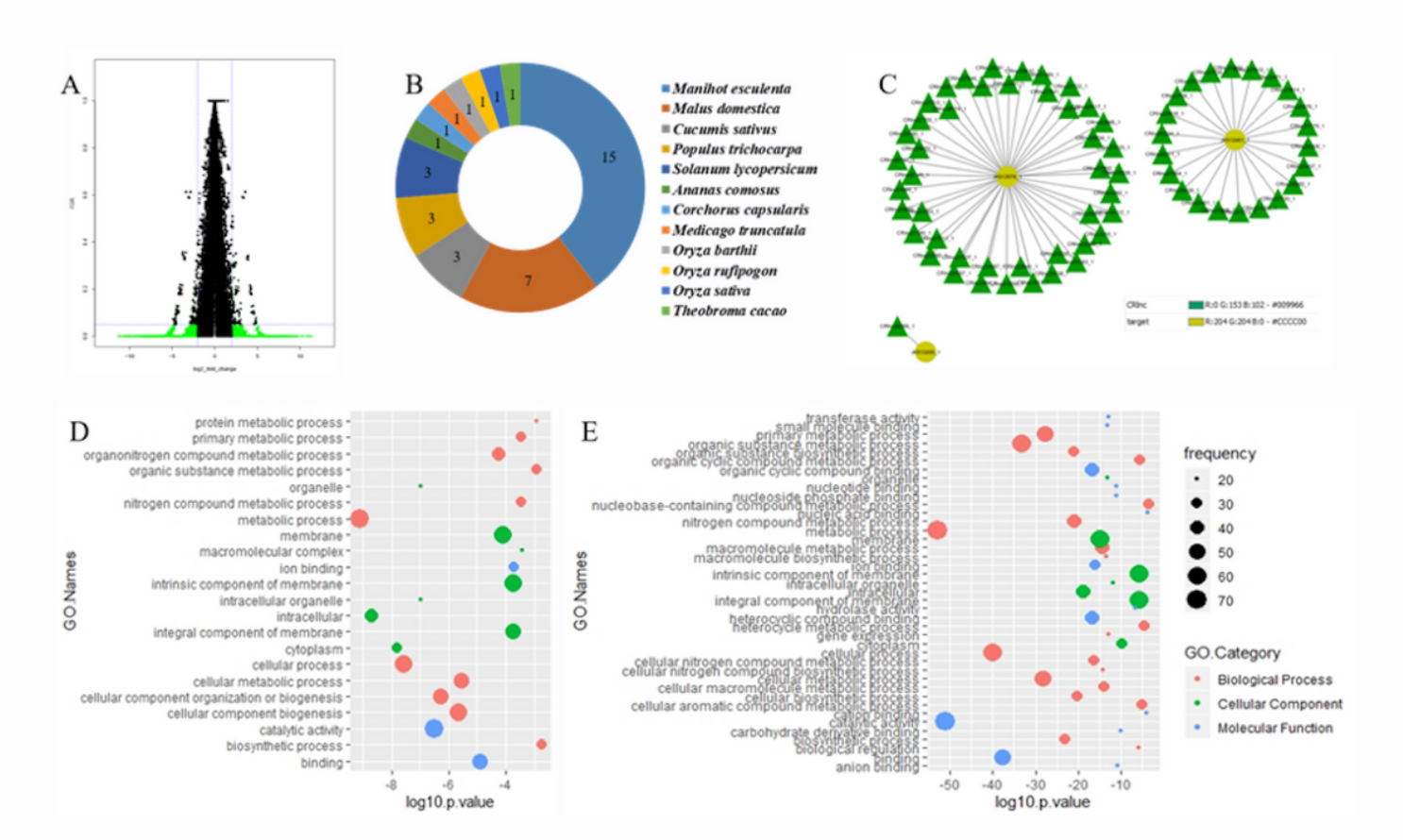

Figure 3

Temperature responsive lncRNAs of mango. (A) Volcano plot of cold responsive lncRNAs of mango; green plots represent significantly expressed cold responsive lncRNAs with false discovery rate (adjusted p-value) of less than 0.05 and log2 fold change of less than -2 or greater than 2; (B) Conserved number of mango lncRNAs in different plant species; (C) Interaction subnetwork between three low temperature responsive proteins and cold responsive lncRNAs of mango; (D) GO enrichment analysis of highly enriched genes targeted by heat responsive lncRNAs; (E) GO enrichment analysis of highly enriched target genes of cold responsive lncRNAs.

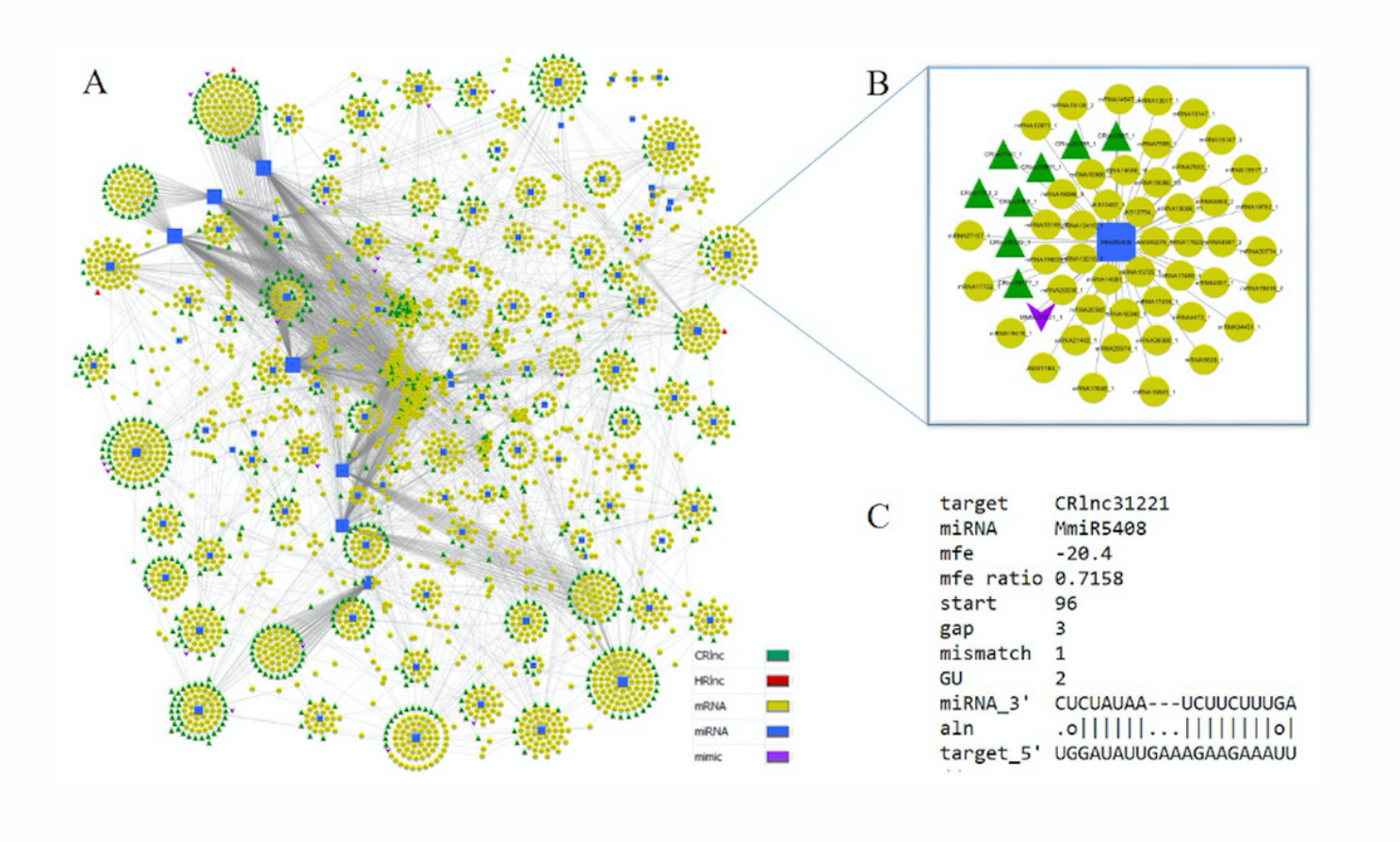

Figure 4

Interaction network among mango ncRNAs and their targets; Green triangles represent cold responsive lncRNAs, red triangles represent heat responsive lncRNAs, yellow ellipses are used for target genes, blue rectangles are for miRNAs and purple V-shapes for target mimics. (A) Network of interaction among newly identified miRNAs, newly identified lncRNAs (cold responsive lncRNAs and heat responsive lncRNAs), newly identified target mimic of miRNAs and mRNAs; (B) subnetwork of interaction among MmiR5408, its target mimic CRlnc31221, target CRlncRNAs and 47 target genes; (C) Base-pairing interaction between MmiR5408 and its target mimic cold responsive lncRNA, CRlnc31221.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable7.xlsx
- SupplementaryDataSet1.txt
- SupplementaryDataSet2.txt
- SupplementaryTable4.xlsx
- SupplementaryTable5.xlsx
- SupplementaryTable1.xlsx
- SupplementaryTable3.xlsx
- SupplementaryTable2.xlsx
- SupplementaryTable9.xlsx
- SupplementaryTable8.xlsx
- SupplementaryTable6.xlsx