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BIOINFORMATIC PREDICTION AND ANNOTATION OF APPLE MICRORNAs AND THEIR TARGETS

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Abstract

MicroRNAs are non-protein coding regulatory RNAs. These endogenously expressed RNAs range from 18-26 nucleotides in length. Their important functions have been widely reported in animals and plants during organogenesis, growth, transgene inactivation, cell signaling processes, disease development and defense against the attacking viruses and bacteria. These small molecules are evolutionarily conserved from species to species in the same kingdom and their conserved nature becomes an important logical tool for the hunt of new conserved miRNAs in other species by homology search. Apple (*Malus domestica* L.) is cultivated worldwide. It is the fourth major fruit of Pakistan and several cultivars of apple are grown in Pakistan. Bioinformatic analysis of 325,020 apple ESTs resulted in identification of sixty nine (69) new conserved miRNAs after filtration and completion of the process. The 69 potential apple miRNAs belong to 63 miRNA families (i-e. miR158, 161, 163, 165, 172, 400, 403, 472, 838, 850, 859, 866, 1120, 1170, 1310, 1313, 1426, 1427, 1428, 1438, 1509, 1510, 1512, 1513, 1514, 1518, 1533, 1847, 1861, 1863, 1873, 3629, 3630, 3633, 3635, 3694, 3699, 3706, 3707, 3711, 3954, 4354, 4407, 4412, 4413, 5138, 5142, 6260, 6261, 6271, 6275, 6280, 6290, 6295, 7516, 7520, 7521, 7526, 7528, 7532, 7536, 9672, 9776). One of the identified pre-miRNAs, i.e. mdm-mir-6271 showed 95% query coverage and 85% identity with the peach pre-miRNA. To our knowledge this is the first ever report of a plant precursor microRNA conservation and this interesting finding would open new vistas for miRNA research community. The mdm-mir 3630 pre-miRNA cluster was observed with two mature miRNA sequences. Four of the apple miRNAs (mdm-mir172, mdm-mir403, mdm-mir3635, mdm-miR6271) were found to be transcribed in sense/antisense orientation. Moreover, twelve of the newly predicted miRNAs were randomly selected for experimental validation through RT-PCR. Experimental validation of computationally predicted miRNAs endorses the powerfulness of bioinformatics prediction of miRNAs. The 69 new conserved apple miRNAs targeted a total of 84 mRNAs. These miRNA target are various proteins involved in numerous biological processes i.e. cell signaling, development, stress management and playing role as transcription factors. The results of this research would contribute in understanding the miRNA mediated life processes in apple.

Key words: Apple, MicroRNAs.

Introduction

Apple (*Malus domestica* L.) is cultivated worldwide and is a rich source of various phytochemicals including flavonoids (e.g., catechins, flavanols, and quercetin) and other phenolic compounds e.g., epicatechin and procyanidins (Ribeiro *et al*., 2014). It is the fourth major fruit tree of Pakistan and several cultivars of apple are grown in Pakistan that include Kala Kulu, Golden Delicious, Mashaday, Kashmiri Amri, Red Delicious and Sky Spur (Manzoor *et al*., 2012). MicroRNAs (miRNAs) are non-protein coding, 18-26 nucleotides long RNAs (Mica *et al*., 2006) which are major players in controling the expression of messenger RNAs (mRNAs) (Carrington & Ambros, 2003). They are generated from foldback stem-loop structures known as Precursor miRNAs (pre-miRNAs). A short double-stranded RNA (dsRNA) is created by detachment of the loop of pre-miRNAs. The mature miRNA is one of the strands of the dsRNA which later integrates into the RNA induced silencing complex (RISC) (Bai *et al*., 2012). The RISC complex containing miRNA negatively regulates the mRNA expression either by inhibiting translation process or by causing its destruction depending upon the stringency of the miRNA complementarity to its mRNA target (Tang *et al*., 2003). miRNAs are conserved in various plant and animal species (Wang *et al*., 2012). Their conserved nature can be exploited for the identification of new homolog miRNAs in other species. Although various researchers have identified miRNAs in apple (Gleave *et al* 2008; Huang *et al*., 2010; Yu *et al*., 2011) but a large number of available ESTs of apple (325,020 ESTs) provoked the idea of identifying more new conserved miRNAs in apple.

Materials and Methods

Use of the bioinformatics tools is now a routine and one of the most widely used methods for the prediction of new conserved miRNAs by comparative genomics approach (Barozai, M. Y. K. 2012; da Silva *et al.*, 2016. Zhang *et al.*, 2017).This study is also based on comparative genomics approach by applying various bioinformatics tools. The new conserved miRNAs in apple were identified and characterized by using a variety of bioinformatics tools i.e. BLASTn, BLASTx, Mfold, psRNA Target, Clustal W, Primer 3 and Weblogo. A brief description of the main steps of the methodology used is discussed in the subsequent text.

Identification of potential candidate miRNA sequences: The famous miRNA repositories i.e. miRBase (Griffiths, 2004) and PMRD (Zhang *et al*., 2010) and available miRNA literature were surveyed for the reported and non-reported miRNAs in apple, and an attempt was made to profile the new conserved miRNAs in apple. The reference miRNA sequences of the different plant species were used as query and subjected to BLAST

(Altschul *et al*., 1990) against the publicly available 325,020 ESTs of apple at National Center for Biotechnology Information (NCBI) Genbank by using BLASTn program. To find the candidate homologue sequences, the homology based search was started with the miRNA sequences of closely related plants to apple. The candidate EST sequences having maximum 4 mismatches with the mature reference sequences were saved in FASTA format.

Validation of potential candidate miRNAs as a non-protein coding sequences: In computational method of miRNA prediction, it is necessary to validate the new conserved miRNAs as non- protein coding RNAs. Therefore the predicted candidate pre-miRNAs sequences were subjected to BALST against protein database at NCBI using BLASTx (Altschul *et al*., 1997) with default parameter to validate them as non-protein coding RNAs. The protein coding pre-miRNA sequences were discarded.

Prediction of hairpin structures of potential miRNA candidate: The hairpin structure of the initial candidate sequences were generated by using the Zuker folding algorithm, MFOLD (version 3.6) (Zuker, 2003), with default parameters, publicly available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi. The predicted structures having lowest free energy were selected for physical inspection. The stem portion of the miRNAs were checked for mature sequence with either 10 base pairs or equal to the reference miRNAs involved in Watson crick and non-Watson Crick (GU, Wobble) pairing between the mature miRNA and its opposite strand (miRNA*) in the duplex. The threshold values used to select a miRNA were same as descried by Zhang *et al.,* (2006).

Conservation and Phylogenetic analysis of newly identified miRNAs: Many miRNA families are evolutionarily conserved across all major lineages of plants, including mosses, gymnosperms, monocots and eudicots (Zhang *et al*., 2006) therefore, one of the newly identified conserved miRNAs from apple (mdm-mir 400) was subjected to conservation analysis with its orthologues in different plant species. For this purpose the publically available WebLogo: a sequence logo generator http://weblogo.berkeley.edu/logo.cgi (Crooks *et al*., 2004) was used. The WebLogo result was saved and scrutinized for conservation of precursor and mature miRNA sequences. One of the newly identified miRNAs, from apple (mdm-mir472) was selected for phylogenetic studies. The cladogram was created by using the neighbor-joining clustering method. The result was saved.

RT-PCR validation: Twelve of the apple miRNAs were randomly selected for the reverse transcription polymerase chain reaction (RT-PCR) experimental validation, The Primer-3 algorithm was used to design the primers against the stem-loop sequences of the selected miRNAs from their ESTs (Table 1). Total RNA was extracted from the leaves of apple using CTAB method. cDNA was synthesized using the RevertAid™HMinus First Strand cDNA synthesis Kit (Fermentas), according to the supplier's protocol. 100 ng cDNA was used as template for the PCR. The PCR was programmed as follows: initial denaturation at 95°C for 4 min followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 60°C for 35 s, and extension at 72°C for 30s and final elongation step at 72°C for 10 min. The PCR products were separated through 1.8% (w/v) agarose gel.

Table 1. Forward and reverse primers for apple miRNAs designed against precursor sequences using primer 3.

Apple miRNAs	Tm	Primers	Product size	EST
mdm-MIR161	59.42 62.06	TCTCTCCATTCTCGGCATAAG CGAGGCTGGAATGTGGTGTA	100	DR992776.1
mdm-MIR165	59.25 58.15	TTTGTGAAAATGGAGGCAGA TCACCAATTGAGATGAAGATCA	118	CN917632.1
mdm-MIR850	59.92 59.54	GTATTGAGGACGTGTACGGTGA AATGCGCATCTCTCTCCTTC	150	EB150753.1
mdm-MIR1310	58.11 59.95	TCGGGTAAAGCCAATGATTA CACTTGGAGCTCTCGATTCC	125	GO524194.1
mdm-MIR1313	60.34 59.78	TGGCCAATCTCAGTGGGTAT CCAATGTTGATGGTGAATGC	176	CV882471.1
mdm-MIR1426	58.38 60.03	TGGCCTTTAGATCTCTATGGATAA AACAAAAGTTTGGACGCCTTT	154	CV883400.1
mdm-MIR1438	59.65 60.10	GGGGGTTACATTGTGGAGAA AGATATGGAGGCGACACCTG	185	CN867236.1
mdm-MIR1518	31.82 22.22	TGAAAATGGCTTGAAAACTTTG AACATGATAAATGATTAATTTGGAACT	190	DR995011.1
mdm-MIR1873	60.27 59.97	GGCAAGTTAGGCAAGTTAGGC CCAGCCATCTTGGCTTAGAG	182	CN921041.1
mdm-MIR3706	59.98 60.87	GATCGATTCGGAGAAATGGA GCCAAACAGGTGATCCAAAA	238	CN924246.1
mdm-MIR3707	59.87 60.35	TGTCACCGAAAGTTGACGAG GAAACCCTCTGTGGGGTCTT	198	EG631213.1
mdm-MIR9672	59.97 60.04	AAGGACTCACCCCTGGAAGT ATGGAAGCTTCAGGGGATCT	190	GO577820.1

Prediction of miRNAs targeted genes: The finding of new conserved miRNA targets is another important phase for confirmation of miRNAs identified on homology basis. To predict the miRNA targets, the newly identified apple miRNAs were subjected to RNA Hybrid (Rehmsmeier *et al*., 2004). The results were saved.

Results and Discussion

The new conserved apple miRNAs: Sixty nine new conserved miRNAs were identified in apple after filtration and completion of the process. The 69 potential apple miRNAs belong to 63 miRNA families (i-e. miR 158, 161, 163, 165, 172, 400, 403, 472, 838, 850, 859, 866, 1120, 1170, 1310, 1313, 1426, 1427, 1428, 1438, 1509, 1510, 1512, 1513, 1514, 1518, 1533, 1847, 1861, 1863, 1873, 3629, 3630, 3633, 3635, 3694, 3699, 3706, 3707, 3711, 3954, 4354, 4407, 4412, 4413, 5138, 5142, 6260, 6261, 6271, 6275, 6280, 6290, 6295, 7516, 7520, 7521, 7526, 7528, 7532, 7536, 9672, 9776). The empirical formula for biogenesis and expression of the miRNAs, suggested by Ambros *et al.*, (2003), was used as a criterion to consider the newly predicted apple miRNAs as valid candidates.

Many of the identified apple pre-miRNAs fulfilled the criteria B, C and D but all the miRNAs satisfied criterion D. According to Ambros *et al*., (2003) only the criterion D is enough for homologous sequences to be validated as new miRNAs in different species. Meyers *et al*., (2008) further confirmed it in favor of plants miRNA annotation.

Apple miRNAs characterization: The newly identified conserved apple miRNAs were characterized in terms of reference miRNAs (REF miRNAs), precursor lengths (PL), minimum free energy (MFE), mature sequences (MS), mature sequence arms (MSA), mature sequence length (ML), number of mismatches (NM), source ESTs (SE) and strand orientation (SO) (Table 2).

The long self-complementary (foldback) pre-miRNAs give rise to mature miRNAs (Bartel, 2004). Conservation of mature miRNA sequence and secondary structure is considered to be sufficient for annotation of miRNA homologs (Meyers *et al*. 2008).As compared to animal pre-miRNA, the plant pre-miRNAs are more diverse in structure and size (Zhnag *et al.,* 2006). The newly identified conserved apple pre-miRNAs lengths range from 39 to 225 nt with an average of 110 nt.

To a large degree, the function of a structural RNA molecule is determined by its structure. Free energy minimization is a long-established paradigm in computational structural biology that is based on the assumption that, at equilibrium, the solution to the underlying molecular folding problem is unique, and that the molecule folds into the lowest energy state (Ding *et al.,* 2005).The minimum free energy (MFE) of the newly identified apple pre-miRNAs is one of the key features of miRNAs characterization. As predicted by MFOLD (Zuker, 2003), the mfe of the new conserved apple miRNAs in this study have a range from -4.5 Kcal mol^{-1} to -71.7 Kcal mol^{-1} with an average -25 Kcal mol^{-1}.

The mature miRNA is the functional product that incorporates into the RNA-induced silencing complex to direct translational repression or transcriptional degradation of mRNA. Mature miRNAs are processed from one or both arms of the hairpin precursor (Griffiths *et al.,* 2011).The new conserved mature apple miRNAs were characterized for their location in pre-miRNAs. Majority (57% i.e. 40 out of 69) of apple miRNAs are located on the 5' arm and remaining (43% i.e. 30 out of 69) are on the opposite 3' arms of the pre-miRNA secondary structures as illustrated in Figure 1.

The mature miRNA of new conserved apple miRNAs were further characterized for their lengths and showed a range from 17 to 24 nt. Majority (56% i.e. 39 out of 69) of the newly predicted miRNAs have 21 nt length, followed by 22 nt (14% i.e. 10 out of 69), 20 nt (12% i.e. 8 out of 69), 19 nt (6% i.e. 4 out of 69), 23 and 24 (4% each i.e. 3 each out of 69), 18nt (3% i.e. 2 out of 69) and 17 nt (1% i.e. 1 out of 69).

Difference of 0 to 4 mismatches between the reference miRNAs and potential conserved miRNA candidates is acceptable range in case of homology based finding of new conserved miRNAs (Zhang *et al*., 2006). Maximum (38% i.e. 26 out of 69) of the apple miRNAs were observed to have 4 mismatches with their homologs, followed by 2 (33% i.e. 23 out of 69), 3 (19% i.e. 13 out of 69), 1 (9% i.e. 6 out of 69) and 0 (3% i.e. 2 out of 69) mismatches.

New conserved apple miRNAs were also characterized in terms of organ/tissue of expression. Different miRNAs were predicted in different ESTs expressed in different organs/tissues of apple i.e. in root tips, xylem and phloem tissue, shoot, leaf, flower, fruit and seeds. Most of the newly predicted miRNAs were identified in leaf and fruit (23% each i.e. 15 each out of 66), followed by flower (14% i.e. 9 out of 66), shoot (9% i.e. 6 out of 66) buds, xylem and phloem (8% each i.e 5 each out of 66), root tips (5% i.e. 3 out of 66) and seeds (3% i.e 2 out of 66).

The number of base pairing between a miRNA and its passenger strand on the opposite arm is another parameter of interest for characterization. All of the predicted apple miRNA stem-loop structures showed at least 10 nt engaged in Watson-crick or G/U base pairings between the mature miRNA and the opposite arms (miRNAs*) in the stem region and the hairpin precursors do not contain large internal loops or bulges.

Similar mfe and length ranges for pre-miRNAs and mature miRNAs, number of mismatches and strand orientations have been reported in various plants by different researchers such as flax (Barozai, 2012), *Helianthus* spp (Barozai *et al.,* 2012), switch grass (Xie *et al.,* 2014), carrot (Barozai *et al.,* 2013), tomato (Din & Barozai, 2014a) and eggplant (Din & Barozai, 2014b).The agreements of results in this study with the previously reported researches strengthens the apple miRNAs validation.

To validate the newly predicted apple miRNAs as strong candidates of miRNAs the relationship between them and known protein is very significant. The apple pre-miRNAs were subjected to Blastx against the protein database at NCBI and only those miRNA sequences were considered which indicated no homology with the known proteins. The protein coding pre-miRNA sequences were discarded but some of the miRNAs whose reference sequences showed homology with the known proteins, were also considered as new conserved miRNAs identified in apple on homology basis. This result confirmed the newly identified pre-miRNAs as strong candidate miRNAs in apple.

Table 3. Apple miRNA targets. The apple (*Malus domestica*) miRNA families and their putative targeted proteins function, Genbank Acc. and RNA-hybrid results are provided. The seed regions 2-7 and 8-13 are shown in red and blue font respectively.

Apple miRNAs	Target Acc	Target description	RNA-hybrid results
mdm-MIR158	XM_008349672.1	Serine/threonine-protein phosphatase 2A	target 5' A A 3' UUUUUCUUCUUUUG AAAA**AGAAGA**AAAC miRNA 3' AGG CCU 5'
	XM_008368513.1	ATP-dependent zinc metalloprotease	target 5' U GAAAGGUGC GU 3' UCC UCUUCUU UUGG AGG **AGAAGA**A AACC miRNA 3' AAAA CU 5'
	XM_008355810	Exportin-2-like	target 5' U A 3' UCCUUUUUCUUCUUUUGGGA AGGAAAA**AGAAGAAAACCC**U miRNA 3' 5'
	XM_008392612	Transcription factor	target 5' U A 3' UCCUUUUUCUUCUUUUGGGA AGGAAAA**AGAAGAAAACCC**U miRNA 3' 5'
	XM_008375830.1	Ethylene-responsive transcription factor ERF034	target 5' U C 3' UCCUUUUUCUUCUUUU AGGAAAAA**GAAGAA**AA miRNA 3' CCCU 5'
	XM_008356600.1	Transcription factor MYB39	target 5' A UUUUUA U 3' CCUU UUUCUUCUUUUGGG GGAA AA**AGAAGAAAACCC** miRNA 3' A U 5
mdm-MIR161	XM_008339154.1	Pentatricopeptide repeat-containing protein	target 5' A C 3' ACCCGAUGUAGUCACUUACAA UGGGCUAC**AUCAGUGAAUGU**U miRNA 3' 5'
mdm-miR163	XR_528156	Uncharacterized protein	target 5' A G 3' GGAGUUCGAACUUC CUUCAAGC**UUGAGG** miRNA 3' AGG AGAAGUU 5'
mdm-miR165	XM_008370119	Calmodulin-binding transcription activator 5-like (LOC103431946), mRNA	target 5' A C C 3' GAGCCA UAGCAUUU CUCGGU G**UUGUAA**G miRNA 3' GGAG CU 5'
mdm-miR165a	XM_008394521	*Malus x domestica* homeobox-leucine zipper protein	target 5' U GCGC CUU U 3' GGAG GAAGCCU UCC CCUU C**UUCGGA** AGG miRNA 3' C A CC CU 5'
mdm-MIR172	XM_008363616.1	Ubiquitin carboxyl-terminal hydrolase	target 5' A AU UCCAUGUAUUUG G 3' UGUGGAUC UUG GAUGUUGC ACACUUAG AAC C**UACGAC**G miRNA 3' UA 5'
mdm-MIR400	XM_008355494.1	Pentatricopeptide repeat-containing protein At3g16010-like (LOC103417302), mRNA	target 5' G A 3' GUGACUUAUAACACUGUAAUC CACUGAAU**AUUGUGACAUUA**G miRNA 3' 5'
	XM_008344417.1	*Malus x domestica* uncharacterized LOC103405417 (LOC103405417), partial mRNA	target 5' U G A 3' UGG UUAUAACACUGUAAU ACU AAU**AUUGUGACAUUA** miRNA 3' C G G 5'
mdm-MIR472	XM_008345727.1	*Malus x domestica* TMV resistance protein N-like (LOC103406735), mRNA	target 5' C A A C A 3' G GGUGGAU UGGGU AAAA C CCA**CCUA AUCCA UUU**U miRNA 3' CCCUA 5'
mdm-MIR838	XM_008375171.1	*Malus x domestica* transcription initiation factor TFIID subunit 4b (LOC103436717), mRNA	target 5' U G 3' UGUGCAAGAAGAAGAAGAGAG ACACGUUC**UUCUUCUUCUCU**C miRNA 3' 5'
mdm-MIR850	XM_008362796.1	*Malus x domestica* NADP-dependent malic enzyme-like (LOC103424699), mRNA	target 5' C N C 3' CUUUGUUGUAGUG GGAUCACG GAAACAACAUCAC C**CUAGUG**C miRNA 3' G 5'
mdm-MIR859	XM_008383859.1	*Malus x domestica* transcription factor TCP4-like (LOC103444900), mRNA	target 5' C U 3' UCUGCCUUCCACAGAGAGA AGACGG**AAGGUGUCUCUC**U miRNA 3' 5'

Table 3. (Cont'd.).

Apple miRNAs	Target Acc	Target description	RNA-hybrid results
mdm-MIR859a	XM_008352259.1	*Malus x domestica* probable alpha-amylase 2 (LOC103413819), mRNA	target 5' G A 3' UUUGACUUCACAACCAAGGGA AAACUGAA**GUGUUGGUUCCC**U miRNA 3' 5'
mdm-MIR866	XM_008359556.1	*Malus x domestica* ribosome biogenesis protein wdr12-like (LOC103421514), mRNA	target 5' A UUUCGGGUCUCUUC A 3' UCUUCAAAGGAUUU UGC AGAA**GUUUCCUAAA AC**G miRNA 3' 5'
	XM_008375208.1	*Malus x domestica* bifunctional 3-dehydroquinate dehydratase/ shikimate dehydrogenase, chloroplastic-like (LOC103436745), transcript variant X3, mRNA	target 5' C N U 3' UCUUCAAAGGAUUUU C AGAA**GUUUCC**UAAAA G miRNA 3' C 5'
mdm-MIR1120	XM_008342506.1	*Malus x domestica* TMV resistance protein N-like (LOC103403665), mRNA	target 5' A C 3' AAACUGGGACGGAGG UUUGAC**CCUGCC**UCC miRNA 3' CUCA 5'
	XM_008359389.1	*Malus x domestica* E3 ubiquitin-protein ligase RNF170-like (LOC103421363), mRNA	target 5' A CU U 3' AAACUGGGACGGAG GGA UUUG**ACCCUG**CCUC CCU miRNA 3'
mdm-MIR1170	XM_008371152.1	*Malus x domestica* probable methionine-tRNA ligase (LOC103432925), mRNA	target 5' A U 3' GGUGCCGUGUUUGGCUGAUU CCACGGC**ACAAACCGACUA**A miRNA 3' 5'
mdm-MIR1310	XR_527131.1	*Malus x domestica* uncharacterized LOC103420749 (LOC103420749), misc_RNA	target 5' A C 3' GGGCGUUGCGCCCCCGAUGCCU CCCGCAACG**CGGGGGCUACGG**A miRNA 3' 5'
mdm-MIR1313	XM_008388093.1	*Malus x domestica* tyrosine-sulfated glycopeptide receptor 1-like (LOC103448830), mRNA	target 5' G U C 3' CGG CAAUAACAUUAGUGG GCU GUUA**UUGUAA**UCACC miRNA 3' U AU 5'
mdm-MIR1426	XM_008355682.1	*Malus x domestica* G-type lectin S-receptor-like serine/ threonine-protein kinase At1g11410 (LOC103417501), mRNA	target 5' U U A 3' UUAUAUC UCAUCAAGAUUCG AAUAUAG **AGUAGUUCUAAG**C miRNA 3' U 5'
mdm-MIR1427	XM_008388668.1	*Malus x domestica* uncharacterized LOC103449358 (LOC103449358), mRNA	target 5' U A 3' UGCGCCACCCACGGUUCCGCG ACGCGGUG**GGUGCCAAGGCG**C miRNA 3' 5'
	XR_528627.1	*Malus x domestica* uncharacterized LOC103429448 (LOC103429448), misc_RNA	target 5' A A 3' UGUGCCACCCAUGGUU ACGCGGUG**GGUGCC**AA miRNA 3' GGCGC 5'
mdm-MIR1428	XM_008348462.1	*Malus x domestica* MADS-box transcription factor 23-like (LOC103409652), transcript variant X3, mRNA	target 5' A C 3' GGCCUACGAAUUUGCAAGCCA CCGGAUGC**UUAAACGUUCGG**U miRNA 3' 5'
mdm-MIR1438	XM_008375388.1	*Malus x domestica* probable methyltransferase PMT14 (LOC103436933), mRNA	target 5' A C 3' AGGAAUGAUAAAAUUGCUCU UUUUUAC**UAUUUUAAUGAG**A miRNA 3' CA 5'
mdm-MIR1509	XR_529192.1	*Malus x domestica* transcription factor PIF3 (LOC103433823), transcript variant X2, misc_RNA	target 5' C AA G 3' GUCC UGAUUAAAAGAA UAGG ACUAA**UUUUUU**U miRNA 3' CU AA 5'
	XM_008362446.1	*Malus x domestica* patatin-like protein 2 (LOC103424360), mRNA	target 5' U U 3' GAAUCCUUUGAUUAAAAAAA CUUAGGA**AACUAAUUUUUU**U miRNA 3' 5'
mdm-MIR1510	XM_008345727.1	*Malus x domestica* TMV resistance protein N-like (LOC103406735), mRNA	target 5' U C 3' GGGUGGAUUAGGUAAAACAACAG CCCACCUAAU**CCAUUUUGUUGU**C miRNA 3' 5'
mdm-MIR1512	XM_008346616.1	*Malus x domestica* telomere repeat-binding protein 5-like (LOC103407732), transcript variant X2, mRNA	target 5' C UU A 3' CAUAACUG UUCUUUAAUG GUAUUGAC AAG**AAAUUA**U miRNA 3' AC UU 5'

Table 3. (Cont'd.).

Apple miRNAs	Target Acc	Target description	RNA-hybrid results
mdm-MIR1513	XM_008346900.1	*Malus x domestica* monoglyceride lipase-like (LOC103408030), mRNA	target 5' A U G 3' AGUUAU CACAUUUAA UCAAUA GU**GUAAAU**U miRNA 3' UGUC U G 5'
mdm-MIR1514	XM_008363258.1	*Malus x domestica* peroxisome biogenesis protein 12-like (LOC103434214), mRNA	target 5' A A 3' UCAUUUUAAAAAAGAAA AGUAA**AAUUUUUUCUUU** miRNA 3' AAA A 5'
mdm-MIR1518	XM_008369073.1	*Malus x domestica* membrane steroid-binding protein 1-like (LOC103430928), mRNA	target 5' G U 3' UACUAUUCACUUACAACACA AUGAUAA**GUGAAUGUUGUG**U miRNA 3' 5'
mdo-MIR1533	XM_008358336.1	*Malus x domestica* transcription factor MYC2-like (LOC103420275), mRNA	target 5' A C 3' UUAUUAUUAUUGUUAUUA AGUAAU**AAUAACAAUAAU** miRNA 3' A 5'
mdm-MIR1847	XM_008389851.1	*Malus x domestica* random slug protein 5 (LOC103450487), mRNA	target 5' C G U 3' AGUGCA AACUGCAAACUGCG UCACGU U**UGACGUUUGACG**C miRNA 3' G 5'
mdm-MIR1861	XM_008349425.1	*Malus x domestica* probable WRKY transcription factor 17 (LOC103410758), mRNA	target 5' G GU G 3' CAA UUUCCGCCUCAGGAUCU GUU AAAG**GCGGAGUUCUAG**A miRNA 3' AA 5'
mdm-MIR1863	XM_008362694.1	*Malus x domestica* general transcription factor IIE subunit 1-like (LOC103424602), mRNA	target 5' A A U 3' AG UUAACAUGGUAUCAGAGCC UC AAUUGU**ACCAUAGUCUCG**G miRNA 3' ACA 5'
mdm-MIR1873	XR_530142.1	*Malus x domestica* uncharacterized LOC103440737 (LOC103440737), transcript variant X2, ncRNA	target 5' A UGG A 3' CCU GCUCUGAUACCAUGUUGA GGA CGAGA**CUAUGGUACAAC**U miRNA 3' CAA 5'
mdm-MIR3629	XM_008369328.1	*Malus x domestica* cyclic dof factor 3-like (LOC103431190), mRNA	target 5' U U 3' UUGGCUGCCGAGAAAAUGC AACCGAC**GGCUCUUUUACG** miRNA 3' G U 5'
mdm-MIR3630	XM_008376003.1	*Malus x domestica* squamosa promoter-binding-like protein 13A (LOC103437522), transcript variant X2, mRNA	target 5' U G 3' UGCAUCAGAGAGAUU ACGUAGUCU**CUCUAA** miRNA 3' U GGGUAAA 5'
mdm-MIR3633	XM_008392937.1	*Malus x domestica* uncharacterized LOC103453396 (LOC103453396), mRNA	target 5' U G 3' GAAGGAAUGGGAGGGG CUUCCUUAC**CCUCCC**U miRNA 3' AUCCUU 5'
mdm-MIR3635	XM_008387045.1	*Malus x domestica* ABC transporter A family member 2-like (LOC103447842), mRNA	target 5' N U 3' AUGAUGUCCCACACAUGCC UACUAC**AGGGUGUGUACG**G miRNA 3' CC 5'
mdm-MIR3694	XM_008367035.1	*Malus x domestica* uncharacterized LOC103428904 (LOC103428904), mRNA	target 5' U GUUUU G 3' AGCUG UAUUCACAACAUUAU UCGGC GU**GGGUGUUGUAAU**A miRNA 3' A 5'
mdm-MIR3699	XM_008392247.1	PREDICTED: *Malus x domestica* ABC transporter B family member 11-like (LOC103452717), transcript variant X2, mRNA	target 5' A G 3' CAAGACCUAUUUUCUGUC GUUCU**GGAUAAAAGACA**G miRNA 3' CUG 5'
mdm-MIR3706	XM_008343469.1	*Malus x domestica* pentatricopeptide repeat-containing protein At3g46610-like (LOC103404541), mRNA	target 5' A C U 3' CU UGUCCAUUUCUCCGAAU GA AUAG**GUAAAGAGGCUU**A miRNA 3' G 5'
mdm-MIR3707	XM_008342623.1	*Malus x domestica* inactive beta-amylase 9-like (LOC103403788), mRNA	target 5' U G 3' UCAAGGAUAAUGGCGGUUCAU AGUUCCUA**UUACCGCCAAGU**A miRNA 3' 5'
mdm-MIR3711	XM_008362809.1	*Malus x domestica* protein ARABIDILLO 1-like (LOC103424710), mRNA	target 5' C U 3' AGAGCCAUCCUUCUAGCGCCA UCUCGGUA**GGAAGAUCGCGG**U miRNA 3' 5'

Table 3. (Cont'd.).

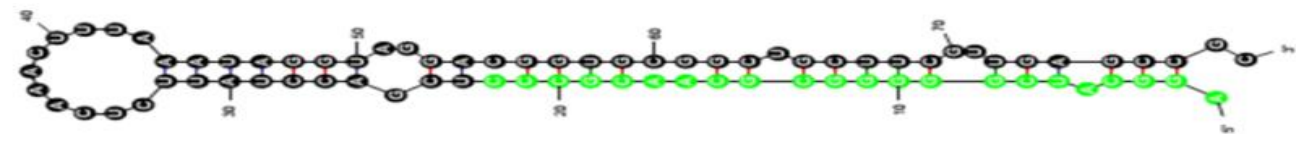
Apple miRNAs	Target Acc	Target description	RNA-hybrid results
mdm-MIR3954	XM_008374949.1	*Malus x domestica* protein IRX15-LIKE-like (LOC103436516), mRNA	target 5' C G 3' CUCCGUGAUUUCUCUGUCGC GAGGCAC**UAAAGAGACAGC**G miRNA 3' A 5'
	XM_008384672.1	*Malus x domestica* L-ascorbate oxidase-like (LOC103445655), mRNA	target 5' A AAA U 3' CCGUGAUUUCUCUGU UGC GGCAC**UAAAGA**GACA GCG miRNA 3' AGA 5'
mdm-MIR4354	XM_008388061.1	*Malus x domestica* BES1/BZR1 homolog protein 4-like (LOC103448795), mRNA	target 5' G G 3' GCCGGUUGGACCGUCGAAUUG CGGCCAAC**CUGGCAGCUUAA**C miRNA 3' 5'
	XM_008394457.1	*Malus x domestica* probable receptor-like protein kinase At1g33260 (LOC103454863), mRNA	target 5' C G G 3' G CGGUUGGACCGUCGAG C GCCAAC**CUGGCA**GCUU miRNA 3' G AAC 5'
mdm-MIR4407	XM_008355491.1	*Malus x domestica* (R)-mandelonitrile lyase 3-like (LOC103417299), transcript variant X3, mRNA	target 5' A AA C C 3' AGGUG GC UUCUACAC UUCAC CG A**AGGUGU**G miRNA 3' CUAUG GA 5'
mdm-MIR4412	XM_008353941.1	*Malus x domestica* TMV resistance protein N-like (LOC103415629), mRNA	target 5' A AGAGGAGGGA A U 3' GGGUGAAGAUGC UCG CAGC UCCGCUUCUAUG G**GC GUUG** miRNA 3' C U 5'
mdm-MIR4413	XM_008366564.1	*Malus x domestica* protein SUPPRESSOR OF npr1-1, CONSTITUTIVE 1-like (LOC103428461), mRNA	target 5' G UN A 3' GCUUUUGCAG UCUC UGGG**AAUGUU** AGAG miRNA 3' AAG A AA 5'
	XM_008366644.1	*Malus x domestica* TMV resistance protein N-like (LOC103428537), transcript variant X1, mRNA	target 5' C UG A A 3' UC U CUUACAAUUCUCUU AG G G**AAUGUUAAGAGA**A miRNA 3' A UG 5'
mdm-MIR6260	XM_008339801.1	*Malus x domestica* beta-galactosidase 3-like (LOC103401095), mRNA	target 5' G GGGUGG A AU C 3' UCCCA UCCC UC ACUCU AGGGU AGGG A**G UGAGG** miRNA 3' AAA A U 5'
mdm-MIR6261	XM_008353498.1	*Malus x domestica* pentatricopeptide repeat-containing protein At5g61990, mitochondrial-like (LOC103415144), mRNA	target 5' G UG G 3' GUU UCUCCU AAUGCU CGA AGAGGA **UUAUGA** miRNA 3' A AAA A 5'
mdm-MIR6271	XM_008346922.1	*Malus x domestica* alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1-like (LOC103408057), mRNA	target 5' G G 3' CAUUAUAUCUCUCAAUCGGAA GUAAUAUA**GAGAGUUAGCCU**U miRNA 3' 5'
	XM_008395949.1	*Malus x domestica* histone-lysine N-methyltransferase ASHH2 (LOC103456264), transcript variant X3, mRNA	target 5' U G 3' UUAUAUCUCUCAAU AAUAUA**GAGAGU**UA miRNA 3' GU GCCUU 5'
mdm-MIR6275	XM_008362344.1	*Malus x domestica* heat shock cognate 70 kDa protein-like (LOC103424258), mRNA	target 5' G N 3' CUUCCCCUUUCCAUUCCCAC GAAGGGGA**AAGGUAAGGGUG** miRNA 3' A 5'
	XM_008380381.1	*Malus x domestica* UDP-glycosyltransferase 91C1 (LOC103441681), mRNA	target 5' G A 3' CCCCUUUCCAUUCCC C GGGGA**AAGGUA**AGGG G miRNA 3' GAA U A 5'
mdm-MIR6280	XM_008374505.1	*Malus x domestica* protein fluG (LOC103436092), mRNA	target 5' A C 3' AGCCAAAAAAUCUUAUUGCCAA UCGGUUUU**UAGAAUAACGGU**U miRNA 3' 5'
	XM_008389123.1	*Malus x domestica* protein TIC 62, chloroplastic (LOC103449807), mRNA	target 5' A AGG C 3' CCAAAAAUCUUAUUG CAG GGUUUU**UAGAAU**AAC GUU miRNA 3' UC G 5'
	XM_008364696.1	*Malus x domestica* TMV resistance protein N-like (LOC103426612), transcript variant X2, mRNA	target 5' C AA A 3' AGCCAAAAAAUCUUA GU UCGGUUUU**UAGAAU** CG miRNA 3' AA GUU 5'

Table 3. (Cont'd.).

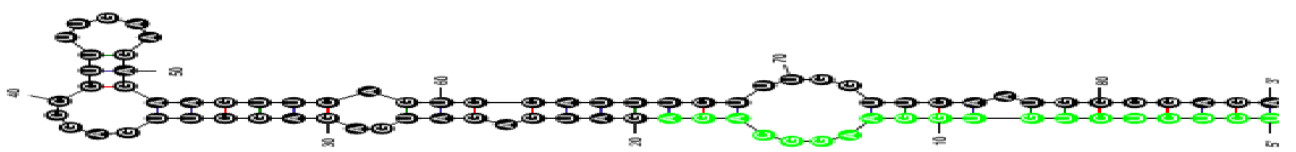
Apple miRNAs	Target Acc	Target description	RNA-hybrid results
mdm-MIR6290	XM_008363197.1	*Malus x domestica* heterogeneous nuclear ribonucleoprotein A/B-like (LOC103425126), mRNA	target 5' U G GAU G G 3' GG CAAAAA UUUUGUUG CAG UC GUUUU**U** **AGAAU**AAC GUU miRNA 3' G G 5'
	XM_008361859.1	*Malus x domestica* dentin sialophosphoprotein-like (LOC103423781), mRNA	target 5' U AAACCCUA GU U 3' AGCCAAAA AUCU UUGCCA UCGGUUUU UAGA **AACGGU** miRNA 3' AU U 5'
	XM_008360671.1	*Malus x domestica* phosphatidylinositol/ phosphatidylcholine transfer protein SFH1-like (LOC103422612), mRNA	target 5' U UG AGA UG A 3' A ACGGUUUUUGU ACUU CA U UGCUAGAG**ACA UG**AG GU miRNA 3' UG UAA 5'
	XM_008361980.1	*Malus x domestica* pentatricopeptide repeat-containing protein At4g18750, chloroplastic-like (LOC103423905), mRNA	target 5' C UCU A UCC G 3' GC CGGUCUCU GUGUUC CA UG GCUAGA**GA CAUG**AG GU miRNA 3' U U UAA 5'
mdm-MIR6295	XM_008369836.1	*Malus x domestica* ubiquitin carboxyl-terminal hydrolase 27-like (LOC103431662), mRNA	target 5' A A GCAUUUCU U 3' GC GAA UCAUCUUCUGUCCUC CGCUU **AGUAGAAGACAGGA**G miRNA 3' G 5'
	XM_008395209.1	*Malus x domestica* uncharacterized LOC103455635 (LOC103455635), mRNA	target 5' U CGGUUAA C G 3' UCGA AUCAUCUUCUGUCC C GGCU UAG**UAGAAG**ACAGG G miRNA 3' C A 5'
mdm-MIR7516	XM_008348038.1	*Malus x domestica* probable WRKY transcription factor 52 (LOC103409225), mRNA	target 5' U C 3' UCAGAGGCGAGGACACCCGC AGUCUCCGC**UUCUGU**GGGCG miRNA 3' UA 5'
mdm-MIR7520	XM_008389524.1	*Malus x domestica* TMV resistance protein N-like (LOC103450211), mRNA	target 5' C GU U 3' UCA UGGUUUUCCCUCUC AGU A**CUGAAAGGGGGA**G miRNA 3' CUAA 5'
mdm-MIR7521	XM_008342129.1	*Malus x domestica* protein IRX15-LIKE-like (LOC103403313), mRNA	target 5' A G C 3' ACACC CCGCCC UG**UGG GGU**GGG miRNA 3' UAAAAA UACU 5'
mdm-MIR7526	XM_008372628.1	*Malus x domestica* ankyrin repeat protein SKIP35-like (LOC103434290), mRNA	target 5' U A C 3' AGAAGUUGCAGCUACC GAG UCUUCAACGUCGAUGG CUC miRNA 3' AA 5'
mdm-MIR7528	XM_008365287.1	*Malus x domestica* serine/threonine-protein phosphatase 6 regulatory subunit 3-like (LOC103427218), mRNA	target 5' G C 3' AAGCUUCAGAUUUGCAAUUCGG UUCGAAGUC**UAAACGUUAAGC**C miRNA 3' 5'
mdm-MIR7532	XM_008367115.1	*Malus x domestica* WEB family protein At2g17940-like (LOC103428979), mRNA	target 5' G G 3' GCU CGAGCAGAGGCAGCUGC UGG GCUC**GUCUCCGUCGAC**G miRNA 3' U 5'
mdm-MIR7536	XM_008370075.1	*Malus x domestica* uncharacterized LOC103431900 (LOC103431900), mRNA (hypothetical protein)	target 5' C A 3' CACUCUUGAGAAUGUCUUA GUGAGA**ACUCUUACAGAA**U miRNA 3' 5'
	XM_008387456.1	*Malus x domestica* nuclear pore complex protein Nup205 (LOC103448198), mRNA	target 5' G C 3' GCUCUUGAGAAUGUU UGAGA**ACUCUU**ACAG miRNA 3' G AAU 5'
mdm-MIR9672	XM_008348225.1	*Malus x domestica* glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic (LOC103409404), mRNA	target 5' U A 3' GACACCACCACUGUCAUUAAC CUGUGGUG**GUGACAGUAAUU**G miRNA 3' 5'
mdm-MIR9776	XM_008394523.1	ABC transporter G family member	target 5' A A 3' GCACAUCCUCGUCCA CGUGUA**GGAGCA**GGU miRNA 3' GA ACAG 5'

Sense antisense miRNAs in apple: The sense/antisense miRNAs are transcribed from both sense and antisense strands of the same genomic loci. Stark *et al.*, in 2008 reported the occurance of sense/ antisense miRNAs from a single Hox locus in *Drosophila* from opposite DNA strands. In this study, four of the new conserved apple miRNAs (mdm-mir172, mdm-mir403, mdm-mir3635, mdm-miR6271) were found to be transcribed in opposite direction as depicted in Figure 2. Although mdm-mir172 , mdm-mir403 have already been reported in apple (Xia *et al.*, 2012) but here, the occurrence of these two miRNAs on both sense and antisense strands is being reported.

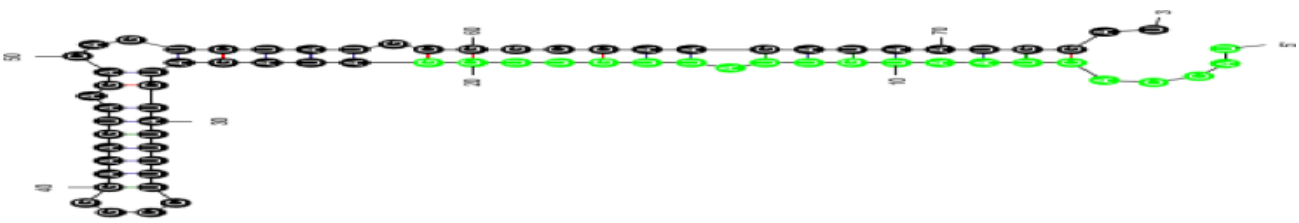
Cluster miRNA in apple: Sometimes the miRNAs are expressed in clusters. These miRNAs are expressed either as pre-miRNA clusters or non-precursor miRNAs clusters. A large number of cluster miRNAs have been detected in animals and in humans (Yu *et al.*, 2006) but miRNA clusters are rarely observed in plants. In this study mdm-mir 3630 was identified as pre-miRNA cluster (Fig. 3). The mdm-mir 3630 pre-miRNA cluster was observed with two mature miRNA sequences. The mir 3630 family is reported as cluster miRNA in many plants i.e. *Vitis vinifera* (Pantaleo *et al.*, 2010) and *Helianthus annuus* (Barozai *et al.*, 2012).



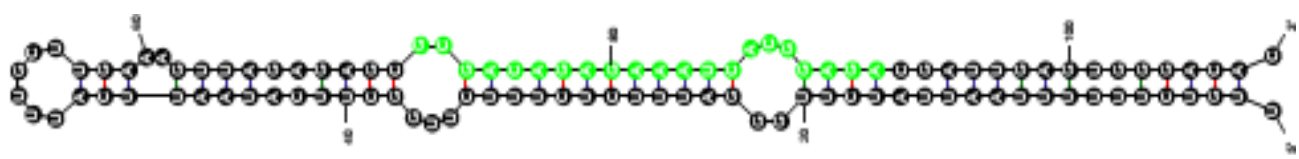
mdm-mir859



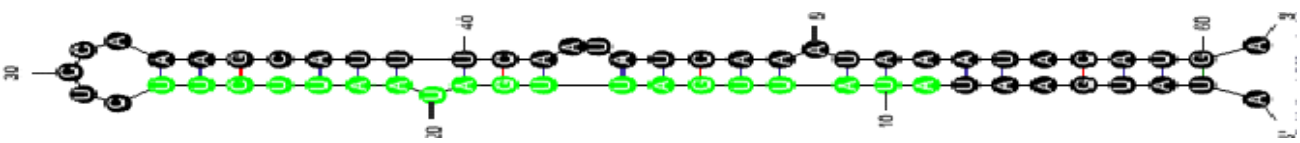
mdm-mir1310



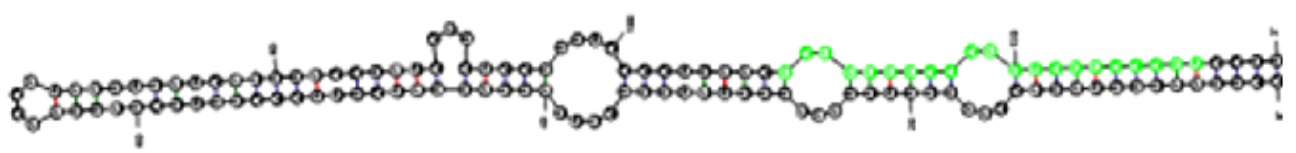
mdm-mir1313



mdm-mir3954



mdm-mir5142



mdm-mir7520

Fig. 1. The new conserved apple miRNA secondary structures.
The apple (*Malus domestica*) pre-miRNAs secondary structures are predicted by using Mfold. These structures are clearly showing the mature miRNAs in stem region of the stem-loop structures, highlighted with green.

Table 2. The newly identified conserved apple miRNAs characterization.

Apple miRNAs	Ref miRNAs	PL	MFE kcal/mol	MS with their positions in Precursor	MSA	ML	NM	SE	SO	OE
mdm-MIR158	ath-MIR158a	75	-9.70	1-TCCCAAA**A**G**A**AGA**A**AAAG**G**A-20	5'	20	4	GO554852	Minus	Leaf
mdm-MIR161	aly-miR161-5p.1	53	-13.50	4-TTG**T**AAGTGACTACATCGGG**T**-24	5'	21	2	DR992776	Minus	Leaf
mdm-miR163	ath-miR163	66	-21.10	4-TTGAAGAGGA**G**TT**C**GAACTTCG**GA**-27	5'	24	4	GO538497	Plus	Xylem tissue
mdm-miR165	aly-MIR165a	52	-12.90	7-GAATGTTGTCTGG**C**TCGAGG-26	5'	20	1	CN917632	Plus	Root tips
mdm-miR165a	aly-MIR165a	216	-48.10	3-TCGGACCAGGCTTCAT**T**CCC-22	5'	20	1	CV083323	Plus	Fruit
mdm-MIR172 Sense	ppe-MIR172a	193	-71.70	24-**G**CAGCATCATCAAGATTCAC**A**-44	5'	21	2	CV997844	Plus	Shoot internodes
mdm-MIR172 antisense	ppe-MIR172a	192	-60.55	24-**G**CAGCATCATCAAGATTC**CA**-44	5'	21	3		Minus	
mdm-MIR400	ath-MIR400	166	-18.00	6-**G**AT**TA**CAGT**G**TTATAAGTCAC-26	5'	21	4	CN914779	Minus	Fruit cells
mdm-MIR403 Sense	ptc-MIR403a	90	-30.90	68-TTAGATTCACGCACAAACTCG-88	3'	21	0	EB148641	Plus	Leaf
mdm-MIR403 antisense	ptc-MIR403a	94	-38.40	70-TT**T**GATTCA**G**GCACAAACT**G**G-90	3'	21	3		Minus	
mdm-MIR472	ptc-MIR472a	84	-13.40	53- TTTT**A**CCTA**A**TCCACCCATCCC -74	3'	22	2	EB153791	Minus	Shavings of phloem tissue
mdm-MIR838	aly-miR838-3p	62	-8.10	36-**C**T**C**TCTTCTTCTTCTTGCACA-56	3'	21	2	CN916519	Minus	Root tips
mdm-MIR850	ath-MIR850	52	-16.80	30-**CGT**GATCCG**C**ACTACAACAAAG-51	3'	22	4	EB150753	Plus	Leaf
mdm-MIR859	ath-MIR859	85	-22.10	1-TCTCTCTGT-G-GAAG**G**CA**G**A-19	5'	19	4	CV631958	Minus	Fruit
mdm-MIR859a	ath-MIR859	57	-12.20	30-TC**CCTTG**GTTGTGAAGTCAAA-50	5'	21	3	CN579779	Minus	Flower
mdm-MIR866	ath-MIR866	125	-19.78	3-**G**CAAAATCC**---**TTTGAAGA-19	5'	17	4	EB149816	Minus	Leaf
mdm-MIR1120	hpr-MIR1120	115	-21.18	2-ACTCCCTCCGTCCCA**GTTT**-20	5'	19	4	CN892050	Plus	Fruit core
mdm-MIR1170	cre-MIR1170	70	-21.60	5-AATCAGCCAAACACGGCA**CC**-24	5'	20	2	GO513097	Plus	Flower
mdm-MIR1310	han-MIR1310	79	-29.00	1-AGGCATCGGGGGCGCAACGCCC-22	5'	22	0	GO524194	Plus	Fruit tissue
mdm-MIR1313	pta-MIR1313	75	-14.40	1-TACCACT**AA**T**G**TTATTGTTCG-21	5'	21	3	CV882471	Minus	Leaf
mdm-MIR1426	osa-MIR1426	80	-13.40	1-**C**GAATCTTGATGATGAT**A**TAA-21	5'	21	2	CV883400	Minus	Leaf
mdm-MIR1427	osa-MIR1427	123	-16.49	6- **C**GCGGAACCGTG-GGTGGCGC**A** -26	5'	21	3	CO722380	Minus	Fruit
mdm-MIR1428	osa-MIR1428a	225	-50.04	203-T**GGC**TTGCAAATTCG**T**AGGCC-223	3'	21	4	GO529375	Minus	xylem tissue
mdm-MIR1438	osa-MIR1438	140	-30.14	118-AG**A**GTAATTTTATCATTTTTA**C**-139	3'	22	2	CN867236	Minus	Leaf
mdm-MIR1509	gma-MIR1509b	139	-11.85	59-T**T**TTTTTAATCAAA**GGATT**C-79	3'	21	4	GO563392	Plus	Shoot internodes
mdm-MIR1510	gma-MIR1510a	71	-10.70	41-**C**TGTTGTTTTACCTA**A**TCCACCC-63	3'	23	2	EB153526	Minus	Shavings of phloem tissue
mdm-MIR1512	gma-MIR1512a	51	-9.00	1-**TA**TTA**A**AGAATT-CAGTTATGCA-21	5'	21	4	CV997747	Plus	Shoot internodes
mdm-MIR1513	gma-MIR1513a	112	-20.40	82-**G**TTAAATGTGTATAA**C**TC-T-GT-102	3'	21	4	CO417809	Plus	Fruit
mdm-MIR1514	gma-MIR1514a	77	-13.10	43-AT**TT**CTTTTTTAAAATGAAAA-63	3'	21	2	CV986754	Minus	Shoot internodes
mdm-MIR1518	gma-MIR1518	90	-11.30	20-TGTGTTGT-AAGTGAATA**GTA**-39	5'	20	4	DR995011	Minus	Leaf
mdm-MIR1533	gma-MIR1533	157	-19.84	1-ATAATAA**C**AATAATAATGA-19	5'	19	1	GO561429	Minus	xylem tissue
mdm-MIR1847	osa-miR1847.1	65	-22.00	41-**C**GCAGTTTGCAGTTGT-GCACT-61	3'	21	2	CN934152	Plus	Vegetative bud
mdm-MIR1861	osa-MIR1861a	147	-35.04	116-**A**GATCTTGAGGC**G**GAAA**T**TGA**A**-137	3'	22	4	GO539584	Plus	xylem tissue
mdm-MIR1863	osa-MIR1863b	79	-20.10	52-**G**GCTCTGATACCATGTTAACT**ACA**-75	3'	24	4	CN489509	Minus	Flower
mdm-MIR1873	osa-MIR1873	55	-17.40	1-TCAACATGGTATCAGAGC**A**GGAA**C**-24	5'	24	2	CN921041	Minus	Leaf
mdm-MIR3629	vvi-MIR3629a	168	-30.85	148-**T**GCATTTTCTC**G**GCAGCCAAG-168	3'	21	2	CN872696	Plus	Fruit

Table 2. (Cont'd.).

Apple miRNAs	Ref miRNAs	PL	MFE kcal/mol	MS with their positions in Precursor	MSA	ML	NM	SE	SO	OE
mdm-MIR3630 Cluster	han-MIR3630	211	-55.23	1-GCAA**A**TGATGATA**AAA**CAGACA-22	5'	22	4	CN889443	Plus	Seed
				61- **AAA**TGGGAATCTCTCTGATGC**A**T -83	5'	23	4		Plus	
mdm-MIR3633	vvi-MIR3633a	155	-67.40	101-TTCCTAT**C**CC**T**CCCATTCC**TT**C-122	3'	22	4	GO520498	Plus	Fruit tissue
mdm-MIR3635 sense	vvi-MIR3635	62	-9.10	42-GGCATGTGTGGG**A**CAT**C**AT**CC**-62	3'	21	4	CO052598	Plus	Flower
mdm-MIR3635 antisense		144	-28.65	14- AT**G**ATGTCCCACACATGCCT**T**-34	5'	21	2		Minus	
mdm-MIR3694	pab-MIR3694	147	-25.42	116-AA**T**AA**T**GT**T**GT**T**GGGTGCGGCT-136	3'	21	4	GO503131	Plus	Flower
mdm-MIR3699	pab-MIR3699	39	-7.20	19-GACAGAAA**A**TAG**GTC**TTGGTC-39	3'	21	4	CN889416	Plus	Seed
mdm-MIR3706	pab-MIR3706	150	-35.04	1-**A**TTCGGAGAAATGGATAAG**G**-20	5'	20	2	CN924246	Minus	Leaf
mdm-MIR3707	pab-MIR3707	102	-21.24	71- ATGA**AC**CGCCATTATCCTTGA-91	3'	21	2	EG631213	Minus	Leaf
mdm-MIR3711	pab-MIR3711	98	-22.50	11-UGGCGCTAGAAGGA**T**GGC**T**CT-31	5'	21	2	GO513814	Plus	Flower
mdm-MIR3954	csi-MIR3954	108	-35.80	72-**GC**GACAGAGAAATCACGG**AG**A-92	3'	21	4	CN909441	Minus	Cell cultures
mdm-MIR4354	gma-MIR4354	65	-21.00	6-CAATT**C**GA-CGGTCCAACCGGC-26	5'	21	2	CN925899	Minus	Leaf
mdm-MIR4407	gma-MIR4407	63	-15.60	42-**GU**GU**U**GGAAGCAGCACUUGUA**U**C-63	3'	22	4	CN916188	Minus	Root tips
mdm-MIR4412	gma-MIR4412	102	-35.78	2- TGTTGCGGGTATCTT**C**GCCTC-22	5'	21	1	EB129971	Minus	Vegetative bud
mdm-MIR4413	gma-MIR4413a	130	-31.20	111-AAGAGAATTGTAA**GG**GT**G**A**A** -130	3'	20	4	GO503940	Minus	Flower
mdm-MIR5138	rgl-MIR5138	92	-18.93	5-AAAA**GA**CG**A**TAGGCGCTA-22	5'	18	3	GO562954	Plus	Leaf
mdm-MIR5142	rgl-MIR5142	61	-4.50	9-ATATTGATTGATAA**TT**C**T**T-27	5'	18	3	GO560249	Plus	Fruit
mdm-MIR6260	ppe-MIR6260	126	-31.25	106-TGGAGTGAG**G**GAATGGGA**AAA**-126	3'	21	4	EB119632	Minus	Phloem
mdm-MIR6261	ppe-MIR6261	114	-24.70	2-AAGT-ATTA**AA**GGAGAAGCA-22	5'	20	3	GO558206	Minus	Fruit
mdm-miR6271 Sense	ppe-MIR6271	180	-41.76	155-T**TCC**GATTGAGAGATATAATG-175	3'	21	3	CN496447	Plus	Flower
mdm-miR6271 antisense	ppe-MIR6271	156	-30.54	3- T**TCC**GATTGAGAGATATAATG -23	5'	21	3	GO565453	Minus	Bud
mdm-MIR6275	ppe-MIR6275	60	-11.90	1-AGTGG**G**AAT**G**G**A**AAGGGGAAG-21	5'	21	4	CO754720	Plus	Fruit
mdm-MIR6280	ppe-MIR6280	86	-18.40	48-TTGGCA**A**TAAGATTTTTGG**C**T-68	3'	21	2	CN881959	Plus	Young fruit
mdm-MIR6290	ppe-MIR6290	111	-31.50	3- TGAATGAGT**A**CAGAGATCGTGT**T** -25	5'	23	2	CV880972	Plus	Shoot internodes
mdm-MIR6295	ppe-MIR6295	121	-31.20	97- GAGGACAGAAGATGATTC**G**GC-117	3'	21	1	CN935798	Plus	Vegetative bud
mdm-MIR7516	lja-MIR7516	80	-24.70	19-**AT**GCGGGTGTCTTCGCCTCTGA-40	5'	22	2	EB124765	Minus	Fruit
mdm-MIR7520	lja-MIR7520	133	-36.90	109-GAGGGGGAA**A**GT**C**ATGA**A**ATC-129	3'	21	3	GO502617	Plus	Flower
mdm-MIR7521	lja-MIR7521	99	-11.30	8-TCATGGGTGGGGTGT**A**AAAA**T**-28	5'	21	3	GO538229	Minus	Fruit tissue
mdm-MIR7526	lja-MIR7526a	147	-38.00	1-**C**TCAAGGTAGCTG**C**AACTTC**T**-21	5'	21	3	CV082998	Plus	Shoot internodes
mdm-MIR7528	lja-MIR7528	175	-33.12	31-CCGAA**T**TGC**A**AATCTGAAGCTT-52	5'	22	2	CV081179	Minus	Bud
mdm-MIR7532	lja-MIR7532a	140	-38.40	21-G**C**AGCTGCCTCTG**C**TCGTGGT-41	5'	21	2	CN913715	Minus	Shavings of phloem tissue
mdm-MIR7536	lja-MIR7536b	160	-45.54	135- TAAGACAT**T**CTCAAGAGTG -153	3'	19	1	GO529844	Plus	xylem tissue
mdm-MIR9672	ata-MIR9672	114	-32.10	1-**G**TTAATGACAGT**G**GTGGTGTC-21	3'	21	2	GO577820	Plus	Leaf
mdm-MIR9776	ata-MIR9776	56	-10.80	10-**GA**C**A**TGGACGAGGATGTGCA**G**-30	5'	21	4	EB154170	Minus	Shavings of phloem tissue

The new conserved apple miRNAs were characterized in terms of Reference microRNAs (Ref miRNAs), PL=Precursor miRNA length, MFE=Minimum free energy, MS=Mature sequence, MSA=Mature sequence arm, ML=Mature sequence length, NM=Number of mismatches (shown in bold, blue and enlarged font size), SE=Source EST, Strand orientation and OE= Organ of expression

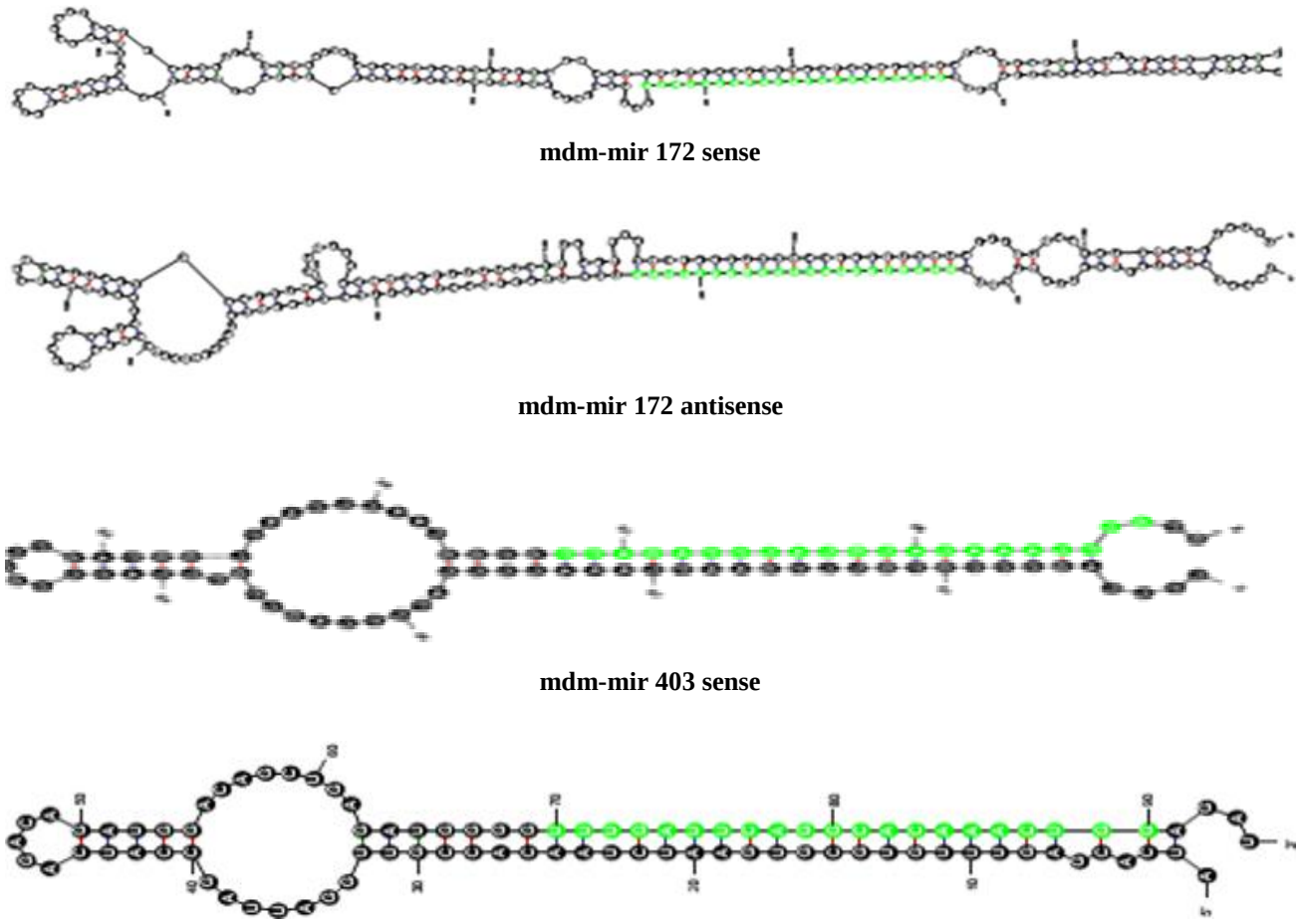


Fig. 2. Sense/antisense miRNAs predicted in apple.

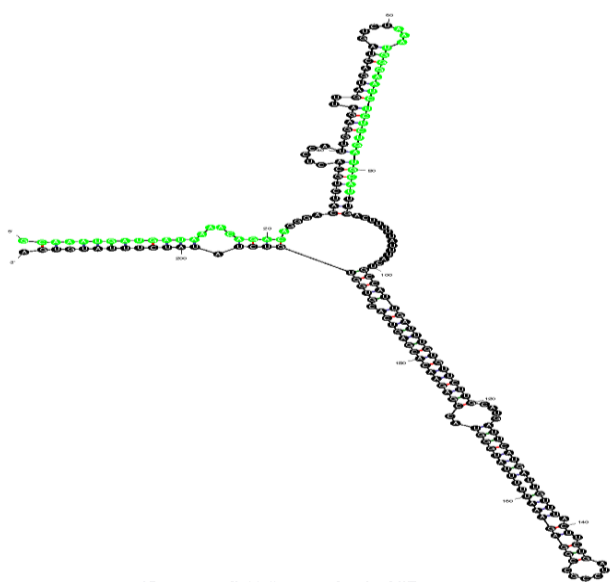


Fig. 3. The mdm-mir 3630 pre-miRNA cluster showing two mature miRNA sequences highlighted in green.

Conservation study of mature apple miRNAs: The newly identified apple miRNA (mir-400) was selected for conservation studies. The apple miRNA (mdm-mir-400) has shown conservation with the mir-400 of and *Arabidopsis thaliana* (ath) and *Brassica rapa* (bra) as shown in Figure 4.

Highly conserved pre-miRNA in apple: Usually mature sequences of miRNAs are found to be conserved among different plant families but the pre-miRNAs are not found to be conserved in plants (Bartel, 2004; Sunkar and Zhu, 2004; Axtell & Bartel, 2005). In this study a highly conserved pre-miRNA is found in apple. The apple pre-miRNA, i.e. mdm-mir-6271 has showed 95% query coverage and 85% identity with the peach pre-miRNA; ppe-mir-6271, as shown in Figure 5. To our knowledge this is the first ever report of a plant precursor microRNA conservation and this interesting finding would open new vistas for miRNA research community.

Phylogenetic study of apple miRNAs: The Phylogenetic analysis of one of the newly identified miRNAs i.e. mdm-mir-472 was done with the same miRNA family from different plants. Phylogenetic analysis suggested that on the basis of pre-miRNA sequences, the apple (*Malus domestica*) is more closed to *Arabidopsis thaliana* as compared to *Populus trichocarpa* and *Citrus sinensis* (Fig. 6).

RT-PCR validation of apple miRNAs: The RT-PCR analysis was conceded for the experimental validation of some of the new conserved apple miRNAs. The randomly selected 12 miRNAs were employed to RT-PCR validation studies. All of the selected miRNAs confirmed their experimental validation (Fig. 7).

Apple miRNA targets: The prediction of miRNAs targets is a crucial step to comprehend their regulatory functions. Total 84 miRNA targets were predicted for the 69 new conserved apple miRNAs (Table 3 for details). These miRNAs target different proteins involved in growth and development, transcription, metabolism, transport, signaling, biotic and abiotic stresses. Most (31% i.e. 26/84) of the identified miRNAs appeat to target metabolism related proteins followed by hypothetical proteins (21%, 18 out of 84), transcription factors (20%, 17 out of 84), biotic and abiotic stress related proteins (11% i.e. 9 out of 84,), signaling (6% i.e. 5 out of 84, transport (6% i.e 5 out of 84), growth and development (5%, 4 out of 84,). These proteins have been reported to be targeted by miRNAs by various researchers (Frazier *et al.*, 2010; Xie *et al.*, 2010; Bai *et al.*, 2012).

Fig. 4. The apple (*Malus domestica*) miRNA conservati on studies.
Alignment of the apple pre-miRNA (400) with *Brassica rapa* (bra) and *Arabidopsis thaliana (ath)* miRNAs using Weblogo: a sequence logo generator, showing miRNA sequences conservation. The conserved mature sequence is highlighted in a box.

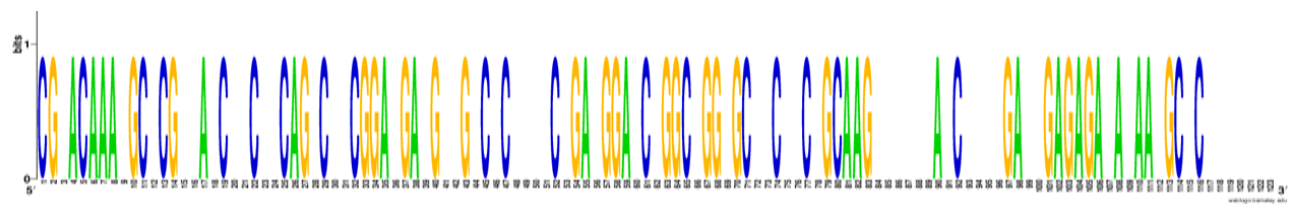


Fig. 5. The highly conserved apple (*Malus domestica*) pre-miRNA (mdm-mir 6271).
Alignment of the apple pre-miRNA mdm-mir 6271 with the reference peach pre-miRNA ppe-mir 6271 by using Weblogo: a sequence logo generator, showing pre-miRNA sequences conservation.

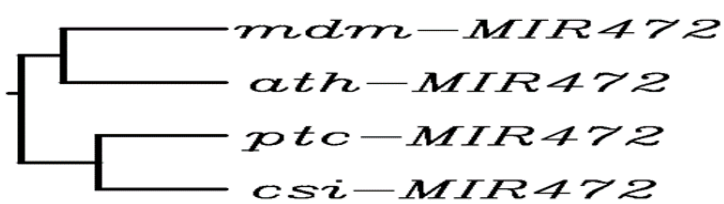


Fig. 6. Phylogenetic analysis of apple (*Malus domestica*) miRNA 472.
The Phylogenetic analysis of the apple pre-miRNAs (mdm-mir472) with *Populus trichocarpa* (ptc), *Citrus sinensis* (csi) and *Arabidopsis thaliana (ath)* miRNAs, was done with the help of ClustalW and cladogram tree was generated using neighbor joining clustering method. The phylogenetic tree shows that on the basis of pre-miRNA sequences, the *Malus domestica* is more closed to *Arabidopsis thaliana* as compared to *Populus trichocarpa* and *Citrus sinensis.*

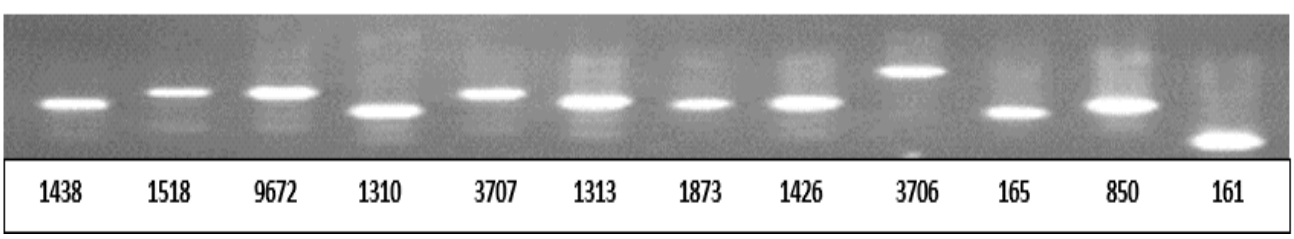


Fig. 7. RT-PCR expressional validation for apple miRNAs.
Twelve apple miRNAs were selected and subjected to RT-PCR expression analysis for the experimental validation. The product of each sample was separated on a 1.8% (w/v) agarose gel.

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