Pak. J. Bot., 45(S1): 353-358, January 2013.

PROFILING THE CARROT (*DAUCUS CAROTA* L.) MICRORNAS AND THEIR TARGETS

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Abstract

MicroRNAs (miRNAs) are small, non-protein coding and negative regulatory RNAs approximately 18-26 nucleotides in length. The comparative genomic methodology due to their conserved nature is a rational approach for the novel miRNAs discovery. In this study, total 17 novel miRNAs from 12 families were identified in an important vegetable carrot (*Daucus carota*). All the miRNA families (dca-mir-156, 160, 167, 172, 774, 778, 854, 1310, 5015, 5030, 5658 and 5664) are found for the first time in carrot. All 17 miRNA precursors form stable minimum free energy secondary structures and the mature miRNAs reside in the stem region of the secondary structures. A total of 24 putative targets were also identified. These findings will be useful to understand the complicated negative gene regulation in the important plant carrot.

Introduction

MicroRNAs (miRNAs) are an important type of small, non-coding RNAs. They are endogenous in nature and about 18-26 nucleotides (nt) in length (Tang *et al.,* 2003). Gene regulation is an important process in plant cell growth, development and stress resistant (Wang *et al.,* 2012). They have negative role in the gene regulation of an organism at post transcriptional stage (Ambros *et al.,* 2003; Carrington & Ambros, 2003). The functional mature miRNAs generate from a special self folded stable stem-loop structures known as precursor-miRNAs (pre-miRNAs). A complex enzymatic pathway is involved in the biogenesis of the mature miRNA from the pre-miRNA. Later RNA induced silencing complex (RISC) takes the charge by integrating the mature miRNA in itself (Hammond *et al.,* 2000). The RISC complex with integrated mature miRNA destructively controls gene expression either by obstructing translation elongation or by triggering messenger RNA (mRNA) degradation depending on the degree of complementation of miRNA with its target (Aukerman & Sakai, 2003; Tang *et al.,* 2003). For fractional miRNAs complementarity with mRNA target, leads to the gene suppression (Carrington & Ambros, 2003). On the other hand perfect or nearly perfect miRNA complementarity with the mRNA target leads to the mRNA degradation (Kidner & Martienssen, 2005). The miRNAs perform different role in organisms such as; development and growth (Chen, 2003), transformed genes suppression (Allen *et al.,* 2005), cell signaling pathways (Hagen & Lai, 2008), abiotic stresses (Sunker *et al.,* 2004; Lu *et al.,* 2005) and defense against the viruses (Tagami *et al.,* 2007).

Majority of the miRNAs are conserved in the plant and animal organisms (Reinhart *et al.,* 2002; Barozai *et al.,* 2008; Rhoades *et al.,* 2011; Barozai, 2012a). The convergence property of the miRNA became a sensible logic for the identification of new orthologues by comparative genomics. Carrot (*Daucus carota* L.) is an important agricultural plant member and a well-known vegetable. It is a winter season crop grown for its edible storage taproots throughout the world. According to the latest microRNA Registry Database (Version Rfam 18.0 released November, 2011) (Griffiths-Jones, 2004), no single miRNA is reported in this important plant. This creates an idea to focus and identify miRNAs in Carrot (*Daucus carota*).

Through this approach 17 novel miRNAs from 12 families were identified in the important vegetable carrot (*Daucus carota*). All the miRNA families (dca-mir-156, 160, 167, 172, 774, 778, 854, 1310, 5015, 5030, 5658 and 5664) are reported here for the first time in carrot. Their Pre-miRNAs form stable minimum free energy (mfe) stem loop structures, as their orthologues form and the mature miRNAs reside in the stem portion of the stem loop structures.

Materials and Methods

Prediction of candidate's pre-miRNAs: Nearly similar procedure with little adjustment as reported earlier (Barozai *et al.,* 2011a) was used to predict the candidate's pre-miRNAs from carrot Expressed Sequence Tags (ESTs). Total known plant miRNA sequences (3964) were downloaded and saved from the microRNA Registry Database (Version Rfam 18.0 released November 2011). These sequences, one at a time, searched using Basic Local Alignment Search Tool (BLAST) against publicly available 18,044 carrot (*Daucus carota* subsp. *sativus*) ESTs from the database, i.e., dbEST release 040112 at http://blast.ncbi.nlm.nih.gov/Blast.cgi using BLASTN (Altschul *et al.,* 1990). The FASTA formats of the initial candidate sequences showing 0-4 mismatches in the mature sequences were saved. The same ESTs from a single gene were removed and created a single representation for the candidate sequence using the BLAST program with default parameters.

Removal of the protein coding sequences: The carrot initial candidate miRNA sequences, predicted through the mature reference miRNAs were further analyzed in term of protein homology. Simply, the FASTA format sequences were BALST against protein database at NCBI using BLASTX with default parameter (Stephen *et al.,* 1997) and the protein coding sequences were excluded.

Generation of stem-loop secondary structures: The stem-loop secondary structures prediction is the most important criterion for the validation of candidate miRNA sequence. So, the initial candidate's sequences were subjected to Zuker folding algorithm, MFOLD (version 3.2) (Zuker, 2003), publicly available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi. to produce the folded structure. Similar

considerations as previously reported were used (Barozai *et al.,* 2011a). The secondary structures either having lowest free energy ≤-18Kcal/mol or ≤ lowest free energy of the reference miRNAs were selected for manual inspection. The threshold values used to select a miRNA were same as described by Ambros *et al.,* (2003). The stem portion of the hairpin was inspected for the mature sequences either with at least 16 or equal to the reference miRNAs base pairs involved in Watson-Crick or G/U base pairing between the mature miRNA and the opposite strand (miRNA*).

Conservation and phylogenetic analyses: The miRNA, mir-160 due to its conserved nature among the plants (Rhoades *et al.,* 2011), was selected and analyzed for conservation and phylogenetic studies. The carrot miRNA, dca-mir-160 with *Arabidopsis thaliana*, *Oryza sativa*, *Medicago truncatula* and *Populus trichocarpa* orthologues were analyzed through publically available weblogo: a sequence logo generator (Crooks *et al.,* 2004) and ClustalW (Larkin *et al.,* 2007) to generate cladogram tree using neighbor joining clustering method respectively. The results were saved.

Potential miRNA targets prediction: The carrot miRNA targets were identified by applying the same strategy (Barozai, 2012b) with more stringent criteria. Briefly, the carrot mature miRNA sequences were used as queries in the NCBI BLASTN program (Altschul *et al.,* 1990). The parameters were adjusted as, Database; nucleotide collection (nr/nt), organism; *Daucus carota* (taxid: 4039) and Program Selection; highly similar sequences (megablast). The mRNA sequences showing 60% query coverage were selected and subjected to RNAhybrid, a miRNA target prediction tool (Kruger & Rehmsmeier, 2006) for the confirmation of the targets. Only targets having stringent seed site located at either positions 2-7 or/and 8-13 from the 5' end of the miRNA along with the supplementary site and the minimum free energy (MFE) of the hybridization was -20 kcal/mol were selected. The Gene Ontology analysis was conducted on AmiGO website.

Results and Discussion

The novel Carrot miRNAs: The homology based research through comparative genetics is an advance and logical approach to find interesting outcomes (Barozai & Husnain, 2011; Barozai & Wahid, 2012; Barozai *et al.,* 2012). A total of 17 miRNAs from 12 families were identified in carrot applying homology based approach. These 17 new potential miRNAs are predicted in 16 Pre-miRNAs from the analyses of 18044 carrot ESTs. The 17 novel miRNAs belong to 12 families (dca-mir-156, 160, 167, 172, 774, 778, 854, 1310, 5015, 5030, 5658 and 5664). For the first time in carrot, all these miRNAs were identified. The dca-miR-156 family was observed with maximum six members, followed by the rest of families with one member in each. The miR-156d is identified as pre-miRNA cluster with two miRNAs (Fig. 1). All the identified putative carrot miRNAs were considered as a valid candidate after satisfying the empirical formula for biogenesis and expression of the miRNAs, suggested by Ambros *et al.,* (2003). The criteria B, C and D were meet by the novel identified carrot's miRNAs. According to Ambros *et al.,* (2003) only the criterion D is enough for homologous sequences to validate as new miRNAs in different species.

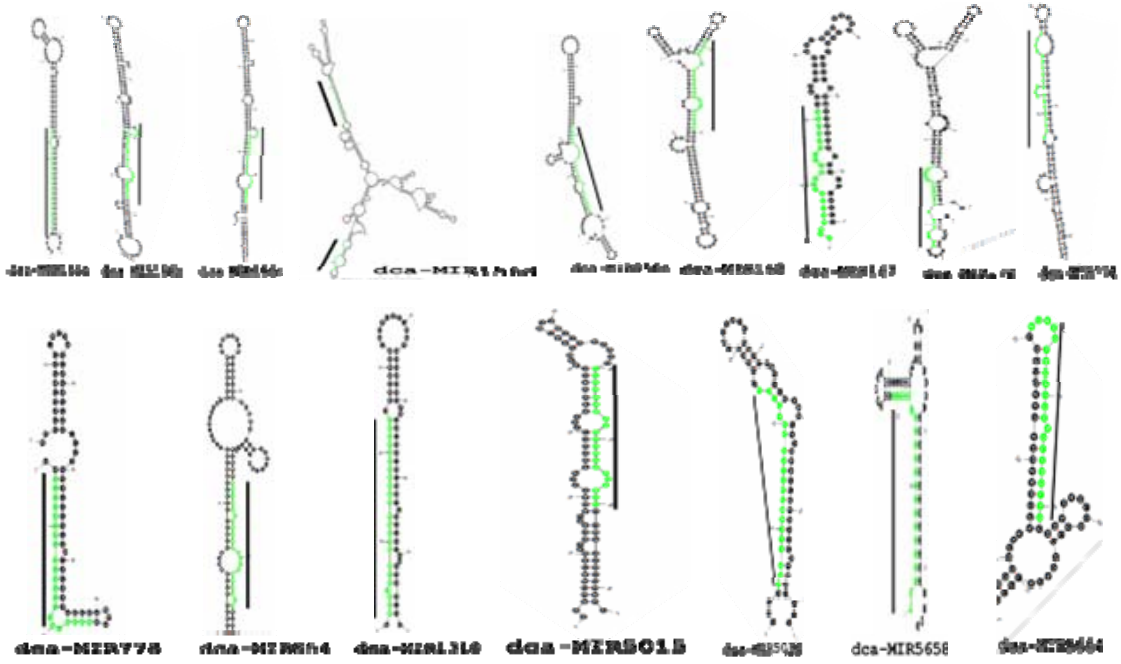

Fig. 1. The novel carrot miRNA secondary structures
The Carrot pre-miRNAs secondary structures are predicted using Mfold algorithm. These structures are clearly showing that mature miRNAs in stem region of the stem-loop structures, highlighted with parallel lines.

The carrot miRNAs characterizations: The minimum folding free energies (mfe) of the putative carrot's pre-miRNAs are range from -15 to -98 Kcal mol^{-1} with an average of -32 Kcal mol^{-1}. All the novel carrot's miRNAs mature sequences are observed in the stem region of the stem-loop structures, as shown in Fig. 1. Many researchers have reported similar findings in different organisms (Mica *et al.,* 2006; Barozai *et al.,* 2011b). The (Table 1) summarizes the novel identified carrot miRNAs, reference miRNAs, pre- miRNAs length, the minimum free folding energies (MFE), mature miRNAs, mature sequence arm, mature sequence length, number of nucleotides difference and source EST.

The newly identified carrot's pre-miRNAs length ranges from 63-456 nt with an average of 124 nt and mature sequences range from 20 nt to 23 nt. Majority (53%, i-e, 9 out of 17) of the miRNAs are 20 nt in length, followed by 21 nt (41%) and 23 nt (6%). The lengths of the mature and pre-miRNAs are similar to previous reports in other plant species (Barozai *et al.,* 2011b, 2011c; Mica *et al.,* 2006). The novel identified carrot's miRNA sequences have a difference of 1-4 nucleotides with the corresponding homologous of reference miRNAs. The results are in agreement with the previously reported works, where the mature sequences have a difference of up to 4 nucleotides (Fuliang *et al.,* 2010; Barozai *et al.,* 2011a). The 53% (9 off 17) carrot's miRNAs sequences are located at 5' arm, while the remaining 47% are at 3' arm as illustrated in Fig. 1. At least 18 nucleotides engaged in Watson-crick or G/U base pairings between the mature miRNA and the opposite arms (miRNAs*) in the stem region except few, where the reference miRNAs have also less base pairings showed by the novel carrot's miRNA secondary stem-loop structures. Furthermore, the hairpin precursors do not contain large internal loops or bulges. Many researcher groups have reported similar findings in plants and animals (Fuliang *et al.,* 2010; Barozai, 2012a; 2012c). No significant protein homology with known proteins observed for the putative carrot miRNAs predicted through mature reference sequences. This confirms the novel carrot's miRNAs as non-coding RNAs.

Table 1. The novel identified carrot miRNAs characterization.

miRNAs	Source miRNAs	PL	MFE	MS	NM	ML	SE	MSA
dca-MIR 156a	ath-MIR 156b	103	-21	UGACAGAAGAGAG**A**GAGC**GU**	3	20	JG770897.1	5`
dca-MIR 156b	ath-MIR 156e	127	-33	UGA-AG**U**AGAGAGUGAGCA**A**	3	20	JG758943.1	3'
dca-MIR 156c	ath-MIR 156f	200	-51	UGA-AG**U**AGAGAGUGAGCA**A**	3	20	JG759560.1	3'
dca-MIR 156d	ath-MIR 156h	456	-98	UGACAGAAGA**C**AGAGAGCA**U** UGACAGAAGA**C**AGAGAGC**GA**	2 3	20 20	JG754788.1	5' 3'
dca-MIR 156e	ath-MIR 156j	118	-30	U**U**ACAGAAGAGAGA**A**A**C**CAC	3	20	JG758272.1	3'
dca-MIR 160	ath-MIR 160a	100	-44	UGCUCGGCUCCCUG**C**AUGCCA	1	21	JG755156.1	5`
dca-MIR 167	ath-MIR 167a	67	-15	**AC**AAGCUGCCAGC**C**UGAUCU**U**	4	21	JG755429.1	5`
dca-MIR 172	ath-MIR 172b	140	-30	GCAGCACCAU**A**AAGAUUCAC	1	20	JG759780.1	5`
dca-MIR774	ath-MIR774	96	-26	UUGGU**C**ACCCAUAUGGCC-U**G**	3	20	JG757910.1	5`
dca-MIR778	ath-MIR778	77	-21	U**U**GCUUGGUUU**G**UGUACACC**U**	3	21	JG754937.1	3`
dca-MIR854	ath-MIR854d	113	-30	GAUGA**A**GA**C**AGGGAGGAGGAG	1	21	JG770343.1	3`
dca-MIR1310	pta-MIR1310	78	-31	AGGCAUCGGGGGCG**C**AACGCCC-U	2	23	FJ150018.1	5`
dca-MIR5015	ath-MIR5015b	93	-23	**G**CUGUUGUUGUUG**C**UGUUAU**C**	3	21	JG761729.1	3`
dca-MIR5030	ath-MIR5030b	72	-21	**UU**GA**A**AUCUUGGCCUUG**AC**AUU	4	21	JG769007.1	5'
dca-MIR5658	ath-MIR5658	63	-18	AUGAUGAUGAUGAUGAUGAA**G**	1	21	JG766306.1	5'
dca-MIR5664	ath-MIR5664	74	-17	AUAGUCA**U**UUUU**G**AUCGGUC-U	3	20	JG753234.1	3'

The novel identified carrot miRNAs were characterized in terms of PL=Precursor miRNA Length, MFE=Minimum Free Energy, MS=Mature Sequence, NM= Number of Mismatches (represented in bold & enlarged font size), ML=Mature sequence Length, SE=Source EST and MSA=Mature Sequence Arm

Conservation and phylogenetic studies of carrot miRNA: The newly identified carrot miRNA (dca-mir-160) were additionally interpreted in terms of conservation and phylogenetic studies. The carrot miRNA, dca-miR-160 showed conservation with *Arabidopsis thaliana* (ath), *Oryza sativa (*osa*)*, *Medicago truncatula* (mtr) and *Populus trichocarpa* (ptc) miRNAs as shown in Fig. 2. Similar findings were reported for different plant's miRNAs (Barozai *et al.,* 2008; 2011a; 2011b). The Phylogenetic analysis of the same miRNA (mir-160) sequences have showed that the carrot is relatively nearby to *Oryza sativa (osa)* and *Populus trichocarpa* (ptc) than *Arabidopsis thaliana* (ath) and *Medicago truncatula (mtr)* as illustrated in Fig. 3.

Potential targets for carrot miRNAs: The prediction of the targets is an essential requirement for authentication of computationally predicted miRNAs. A total of 24 targets were predicted for the newly identified carrot miRNAs, as shown in Table 2. Almost all of these targets have already reported as miRNA targets in other plants (Barozai *et al.,* 2008; Fuilang *et al.,* 2010; Barozai *et al.,* 2011a; 2011b).

The plant miRNAs mainly target the transcription factors (Fuilang *et al.,* 2010; Oswaldo *et al.,* 2010). The carrot miRNAs also target the transcription factors. The novel carrot miRNAs 156, 172, 774, 778 and 5015 target CCAAT-box binding factor HAP3, DNA-binding protein, G-box binding factor, ABA response element-binding factor 1, Embryonic element binding Factor 7, Homeobox and leucine zipper respectively.

The proteins engaged in growth & development and metabolism are also reported as a class targeted by plant miRNAs (Fuilang *et al.,* 2010; Barozai *et al.,* 2011a; 2011b). The metabolomics proteins like; Lycopene beta

cyclase, Ubiquitin-carboxyl extension, Dihydrofolate reductase-thymidylate synthetase, ATP synthase b subunit and Extensin-like protein are predicted as targets for the carrot miRNAs. Furthermore, the Lateral suppressor region D protein, Leucine-rich receptor-like protein kinase, Somatic embryogenesis receptor-like kinase and Lateral suppressor-like protein are identified as carrot's miRNA targets, involved in growth and development.

Other carrot miRNAs targets are hypothetical proteins, Heat shock protein 70, Symbiosis-related disease resistance protein, Met1-type cytosine DNA-methyltransferase, Dehydrin protein and Cysteine protease. Similar findings were reported for various plants (Barozai *et al.,* 2008; Fuilang *et al.,* 2010; Oswaldo *et al.,* 2010; Barozai 2012d).

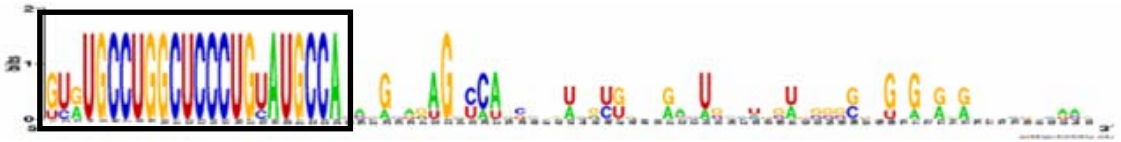

Fig. 2. The carrot miRNA conservation studies.
Alignment of the carrot pre-miRNAs (160) with *Arabidopsis thaliana* (ath), *Oryza sativa* (osa), *Medicago truncatula* (mtr) and *Populus trichocarpa* (ptc) miRNAs, using Weblogo: a sequence logo generator, showing miRNA sequences conservation. The conserved mature sequence is highlighted in a box.

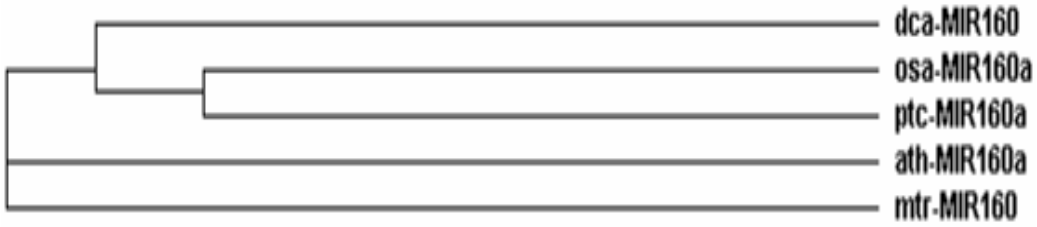

Fig. 3. The Carrot miRNA phylogenetic analysis.
The Phylogenetic analysis of the carrot (dca) pre-miRNA (160) with *Arabidopsis thaliana* (ath), *Oryza sativa (osa)*, *Medicago truncatula* (mtr) and *Populus trichocarpa* (ptc) miRNAs, was done with the help of ClustalW and cladogram tree was generated using neighbor joining clustering method. The Phylogenetic tree showed that on the basis of pre-miRNA sequences, the carrot (dca) is more closed to *Populus trichocarpa* (ptc), *Oryza sativa (osa)* than *Arabidopsis thaliana* (ath) and *Medicago truncatula* (mtr).

Table 2. Putative carrot miRNA targets List of the potential targets of the novel identified carrot miRNAs

Carrot Micro RNA family	Targets			
	Name	**Function**	**Genbank Acc.#**	**RNA-Hybrid Result**
156	CCAAT-box binding factor HAP3	Transcription Factor	AB273720.1	target 5' C C U U 3' CUCUCUCUCU UCU UC GAGAGAGAGA AGA AG miRNA 3' UGC C U 5'
156	DNA-binding protein (transcriptional regulator)	Transcription Factor	D26573.1	target 5' U GGUGGUA G 3' AUGU CUUCUGUU UGCG GAAGACAG miRNA 3' AGAGAGA U 5'
156	Cysteine protease	Stress related	AB098630.1	target 5' A AAC A 3'GCU UGU UCUGUCG CGA ACA AGACAGU miRNA 3' UA GAG GA 5'
160	Extensin-like protein	Metabolism	AJ297423.1	target 5' G UGAGGGGA GU A U 3' UGGC G G GGGGCUGGGC ACCG C C CCUCGGCUCG miRNA 3' UA GU U 5'
160	Transposon PIF-like DcMaster-a	Stress related	DQ250806.1	target 5' G A AAACA G 3' GGCA G GAGCCGAGCA CCGU C CUCGGCUCGU miRNA 3' A A GUCC 5'
167	Dehydrin protein	Stress related	AB105039.1	target 5' A GU GACCACCG C 3' AUCA GGU GGCAGCUUGU UAGU CCG CCGUCGAACA miRNA 3' UUC A 5'

167	ATP synthase b subunit	Metabolism	X60303.1	target 5' C A 3' GAGAUCAGGCUGGU GCUUG UUCUAGUCCGACCG CGAAC miRNA 3' U A 5'
172	G-box binding factor	Transcription Factor	AB334113.1	target 5' A G 3' UAUGGUGCUG AAUACCACGAC miRNA 3' CACUUAGA G 5'
172	ABA response element-binding factor 1	Transcription Factor	EU433292.1	target 5' A G 3' UAUGGUGCUG AAUACCACGAC miRNA 3' CACUUAGA G 5'
774	Embryonic element binding Factor 7	Transcription Factor	AB188295.1	target 5' U UUUUGC AUG A 3' CGGG CCAUAUGGGU GACCAG GUCC GUAUACCCA CUGGUU miRNA 3'5'
774	Met1-type cytosine DNA-methyltransferase	Stress related	AF007807.1	target 5' C A A C 3'GGCCAUGU GG GGCUAA CCGGUAUA CC CUGGUU miRNA 3' GU C A 5'
778	DNA-binding protein	Transcription Factor	D26574.1	target 5' U AACU U 3' GGUGUAUA AUUGAGUGA CCACAUGU UGGUUCGUU miRNA 3' U GUU 5'
778	Homeobox and leucine zipper	Transcription Factor	D26577.1	target 5' C GA U 3' AGGUG AAACCAAGCAA UCCAC UUUGGUUCGUU miRNA 3' AUGUG 5'
778	Lateral suppressor region D protein	Growth & Development	AB205182.1	target 5' A A 3' ACACAAACCAAGCAA UGUGUUUGGUUCGUU miRNA 3' UCCACA 5'
854	DHFR-TS gene for dihydrofolate reductase-thymidylate synthetase	Metabolism	AJ003139.1	target 5' U G G U 3' U UUCUUCCCUGUUUU GUC A GAGGAGGGACAGAA UAG miRNA 3' G G G 5'
854	Symbiosis-related disease resistance protein	Stress related	AY081212.1	target 5' A GGU CAA U 3' CUCC UCCUCCCUGUC CAUC GAGG AGGAGGGACAG GUAG miRNA 3' AA 5'
1310	Leucine-rich receptor-like protein kinase	Growth & Development	AY081216.1	target 5' C UU A U A 3' AG CG UU CGUCUUCGGUGUC UC GC AA GCGGGGGCUACGG miRNA 3' CC C A 5'
5015	Somatic embryogenesis receptor-like kinase	Growth & Development	U93048.1	target 5' A UCUU UCU C 3' GAU GCG CAACAACAACAGC CUA UGU GUUGUUGUUGUCG miRNA 3' U C 5'
5015	C-HAP5B mRNA for CCAAT-box binding factor	Transcription Factor	AB104613.1	target 5' C C U A 3' UA CAGCAACAGCAGC GC AU GUCGUUGUUGUUG CG miRNA 3' CU U U 5'
5015	Heat shock protein 70	Stress related	X60088.1	target 5' C UCA A 3' GACGGC GACGACGAUAG UUGUCG UUGUUGUUGUC miRNA 3' CUA G 5'
5030	Ubiquitin-carboxyl extension	Metabolism	U68751.1	target 5' C A G 3' AAUGUCAA GCCAAGAUUCAA UUACAGUU CGGUUCUAAGUU miRNA 3' C 5'
5030	Hypothetical protein	Unknown	Z17398.1	target 5' U CC G G 3' UGUUG GUCGAGAUU CAG ACAGU CGGUUCUAA GUU miRNA 3' UU UC 5'
5658	Lateral suppressor-like protein	Growth & Development	AB189844.1	target 5' C A G 3' CG UAUCAUCG CAUCA GU GUAGUAGU GUAGU miRNA 3' GAA A A A 5'
5664	Lycopene beta cyclase	Metabolism	DQ192190.1	target 5' A U 3' CAAAAUGACUAU GUUUUACUGAUA miRNA 3' UCUGGCUA 5'

Conclusion

This research produced 17 novel miRNAs and their 24 targets for the first time in the carrot plant. These new miRNAs will be valuable in the forthcoming era of the scientific community for the improvement of this important vegetable plant. Additionally these findings will also be worthy functional genomic resources to understand the gene regulatory mechanism in carrot.

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(Received for publication 1 September 2012)