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PROFILING MICRO RNAs AND THEIR TARGETS IN RADISH (*RAPHANUS SATIVUS* L.)

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Abstract

MicroRNAs (miRNAs) are tiny, non-protein coding and negative regulatory RNAs approximately 21 nucleotides in length. The comparative genomic methodology due to their conserved nature is a reasonable approach for the novel miRNAs discovery. In this research, total 25 novel miRNAs from 18 families (ras-miR-156, 160, 162, 163, 164, 167, 168, 319, 399, 408, 413, 414, 841, 1310, 2936, 5030 and 5661) are identified in an important vegetable radish (*Raphanus sativus* L.). The 25 miRNA precursor sequences showed secondary structures with the mature miRNAs in the stem region. Total 42 putative targets are also identified for the novel 25 radish miRNAs. These findings suggest that more thorough understanding of the function of such miRNAs will help to unravel the mysteries role in plant biology.

Key words: Blast; Comparative Genomic; MicroRNAs; Radish (*Raphanus sativus*).

Introduction

MicroRNAs (miRNAs) are an important group of small RNAs. The miRNAs are endogenous, non-coding and approximately ≈ 21 nucleotides (nt) in length (Tang *et al*., 2003; Baloch & Din, 2014). They play negative regulatory role in post transcriptional gene regulation (Ambros *et al*., 2003). The active mature miRNAs generate from folded stable hair-pin / stem-loop structures known as precursor-miRNAs (pre-miRNAs). A single strand, from the double strand RNA stem of the pre-miRNA, is created through a complex enzymatic pathway that acts as mature miRNA. RNA induced silencing complex (RISC) plays important role in the processing of mature miRNA (Hammond *et al*., 2000; Baloch *et al.,* 2013). The mature miRNA with the help of RISC complex negatively regulates protein synthesis either by stopping the process of translation or by degrading the complementary messenger RNA (mRNA). This depends on the scale of complementarily between miRNA and its targeted mRNA (Tang *et al*., 2003). For partial miRNAs complementary sites in the mRNA target, miRNAs poorly hybridized and cause gene suppression. On the other hand when the mRNA target has perfect or nearly perfect miRNA complementarity site, then miRNAs perfectly hybridized and causes the mRNA degradation (Kidner & Martienssen, 2005). The miRNAs in various living organisms are indulged in multiple biological processes such as; growth and development (Kozak, 2007), transformed genes inactivation (Gao *et al*., 2011), cell signaling pathways (Barozai *et al*., 2011a), environmental stresses (Gao *et al*., 2011; Lv *et al*., 2010) and in defense against viruses (Feng *et al*., 2009).

Majority of the miRNAs are conserved in the plant kingdom (Barozai *et al*., 2008, 2011b; Ghani *et al.,* 2013). The conserved nature of the miRNAs provides a rational logic for the identification of new orthologues by comparative genomics.

Radish (*Raphanus sativus*) is an important agricultural plant member and well known vegetable. 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 Radish (*Raphanus sativus*).

This effort resulted 25 putative miRNAs from 18 families in the important vegetable, radish (*Raphanus sativus*), using the approach described by Barozai *et al*. (2011c). These miRNAs will be remarkable source for the engineering of radish for desirable traits.

Materials and Methods

Prediction of candidate's pre-miRNAs: Almost similar methodology with little modification as reported earlier (Barozai *et al*., 2011c) was applied to predict the candidate's pre-miRNAs from radish Expressed Sequence Tags (ESTs). Total 8557 known plant miRNA sequences, both precursors and matures were downloaded from the microRNA Registry Database (Version Rfam 18.0 released November 2011), and subjected to Basic Local Alignment Search Tool (BLAST) search against publicly available 110,006 radish (*Raphanus sativus*) ESTs from the database, i.e., dbEST release 010112 at http://blast.ncbi.nlm.nih.gov/Blast.cgi using BLASTN (Altschul *et al*., 1990). The first batch generated data was saved. The frequent ESTs for one gene were discarded by applying BLASTn against the radish (*Raphanus sativus*) ESTs database.

The radish (*Raphanus sativus*) initial candidate miRNAs with 0-4 mismatches in the mature sequences was identified using Clustal W (1.83), a multiple sequence alignment tool with default parameters, publicly available at http://www.ebi.ac.uk/clustalw/ (Larkin *et al*., 2007).

Removal of the protein coding sequences: The protein homology search was preformed for the radish first batch generated candidate miRNA sequences. For this purpose BLASTX (Stephen *et al*., 1997) available at NCBI was used for homology search in protein database. The sequences showing significant homology with known proteins were discarded.

Generation of stem-loop secondary structures: The stem-loop secondary structures for the initial candidate's sequences were generated through Zuker folding algorithm,

MFOLD (version 3.2) (Zuker, 2003), publicly available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi. The same parameters as reported earlier were used (Barozai, 2012a). For manual check, the folded stem-loop structures were subjected to minimum free energy (mfe) threshold level ≤-18Kcal/mol or ≤ lowest free energy of the reference miRNAs. The folded structures stems were examined for the mature sequences either with minimum 16 or equivalent to the source miRNAs base pairs indulged in Watson-Crick or G/U base pairing as provided by Ambros *et al*. (2003).

Conservation and phylogenetic analyses: The miRNA, mir-156 due to its conserved nature among the plants was selected and subjected for conservation and phylogenetic analyses. The analyses of the radish miRNA, ras-mir-156 with *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays* and *Populus trichocarpa* orthologues were done by the 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 convergence and similarity search approaches makes possible the enlisting of identified miRNAs targets. The psRNATarget (http://bioinfo3.noble.org/psRNATarget) prediction tool with default parameters (Dai & Zhou, 2011) was applied to find the radish miRNAs targets.

Results and Discussion

The Radish miRNAs: The homology search through comparative genetics is a new and rational approach to find interesting findings (Barozai & Husnain, 2011; Barozai & Wahid, 2012; Barozai *et al.,* 2012). Interesting findings are reported in various organisms by applying molecular and bioinformatics approaches (Aziz, 2014a, 2014b; Barozai *et al.,* 2014; Mahmood-ur-Rahman *et al.,* 2013; Ray & Bagchi, 2013). The homology based search and applying Insilco approach resulted 25 novel miRNAs in radish. These 25 novel miRNAs are predicted in 25 Pre-miRNAs from the analyses of 110,006 radish ESTs. The 25 putative miRNAs belong to 18 families (ras-mir-156, 160, 162, 163, 164, 167, 168, 319, 399, 408, 413, 414, 841, 1310, 2936, 5030 and 5661). All these miRNAs are found for the first time in radish. The ras-miR-160 family was observed with maximum three members, followed by ras-miR-156, 162, 167, 168 and 5030 with two members in each. The Ambros *et al*. (2003) empirical formula with criteria B, C and D were applied on the predicted radish miRNAs. All the predicted novel radish miRNAs fulfill these criteria and validated as miRNAs. The Ambros *et al*. (2003) demands merely the criterion D is sufficient for homologous sequences to validate as new miRNAs in different species.

The radish miRNAs Characterizations: The newly identified radish's pre-miRNAs have minimum folding free energies (mfe) in a range from -13 to -86 Kcal mol^{-1} with an average of -41 Kcal mol^{-1}. All the new radish's miRNAs mature sequences are observed in the stem region of the stem-loop structures, as shown in Fig. 1. Similar findings were reported by many researchers in different organisms (Gao *et al*., 2011; Barozai, 2012b). The characteristics of the novel identified radish miRNAs were annotated in Table 1.

Table 1. Characterization of the novel identified Radish miRNAs.

Radish (*Raphanus sativus*) miRNAs	Reference miRNAs	PL	MFE	MS	NM	ML	SE	MSA
ras-miR 156a	ath-MIR 156a	120	-45	UGACAGAAGAGAGUGAGCAC	0	20	FY451477.1	5`
ras-miR 156b	ath-MIR 156j	71	-19.5	UGACAGAAGAGAGUGAGCAC	0	20	FY438496.1	5`
ras-miR 160a	ath-MIR 160a	84	-45	UGCUCGGCUCCCUGUAUGCCA	0	21	FD941248.1	5`
ras-miR 160b	ath-MIR 160c	131	-48	UGCCUGGCUCCCUGUAUGCCA	0	21	FD936933.1	5`
ras-miR 160c	ath-MIR 160c	93	-46	UGCCUGGCUCCCUGUAUGCCA	0	21	EY928785.1	5`
ras-miR 162a	ath-MIR 162a	130	-46	UCGAUAAACCUCUGCAUCCAG	0	21	FD938184.1	3`
ras-miR 162b	ath-MIR 162b	118	-42	UCGAUAAACCUCUGCAUCCAG	0	21	FD947716.1	3`
ras-miR 163	ath-MIR 163	140	-37	UUGAAGAGGACUUGGAAC-UCGAU	1	23	FD946215.1	5`
ras-miR 164a	ath-MIR 164b	112	-42	UGGAGAAGCAGGGCACGUGCA	0	21	FY441517.1	5`
ras-miR 167a	ath-MIR 167a	133	-55	UGAAGCUGCCAGCAUGAUCUA	0	21	EX892880.1	5`
ras-miR 167b	ath-MIR 167b	109	-54	UGAAGCUGCCAGCAUGAUCUA	0	21	FD948645.1	5`
ras-miR 168a	ath-MIR 168a	123	-58	UCGCUUGGUGCAG**U**UCGGGA-	2	20	FD955742.1	5`
ras-miR 168b	ath-MIR 168b	119	-47	UCGCUUG**A**UGCAGGUCGGG-	2	20	FY447875.1	5`
ras-miR 170	ath-MIR 170	117	-42	UGAUUGAGCCG**C**G**C**CAAUAUC	2	21	FD564703.1	3`
ras-miR 319	ath-MIR 319	199	-86	UUGGACUGAAGGGAGCUCCUU	0	21	FY434434.1	3`
ras-miR 399	ath-MIR 399a	106	-45	UGCCAAAGGAGAUUUGCCC**G**G	1	21	FD580445.1	3`
ras-miR 408	ath-MIR 408	152	-52	AUGCACUGCCUCUUCCCUGGC	0	21	EW713948.1	3`
ras-miR 414	ath-MIR 414	94	-15	UCAUC**A**UCAUCAUCAUCGUCA	1	21	EX899124.1	5`
ras-miR413	osa-MIR413	64	-13	CUAGUUUCACUUGUUCU**UGGG**	4	21	FD949974.1	5`
ras-miR841	ath-MIR841b	211	-50	UACGA**C**CCACUGGAAACUGAA	1	21	FY435967.1	3`
ras-miR1310	pta-MIR1310	77	-27	AGGCAUCGGGGGCG**C**AACGCCC-U	2	23	FY449265.1	5`
ras-miR2936	ath-MIR2936	114	-25	**U**UUGAGAGAGAGAA**U**ACAGACG	2	21	FD958019.1	5`
ras-miR5030a	ath-MIR5030a	72	-21	GCUAAGAGCGGUUCUGAUGGA	0	21	EX909780.1	5'
ras-miR5030b	ath-MIR5030b	72	-21	GCUAAGAGCGGUUCUGAUGGA	0	21	EX906182.1	5'
ras-miR5661	ath-MIR5661	83	-20	**G**GAGGUACAUCAUGUAGUC**G**G	2	21	FD576306.1	5'

The new identified Radish 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

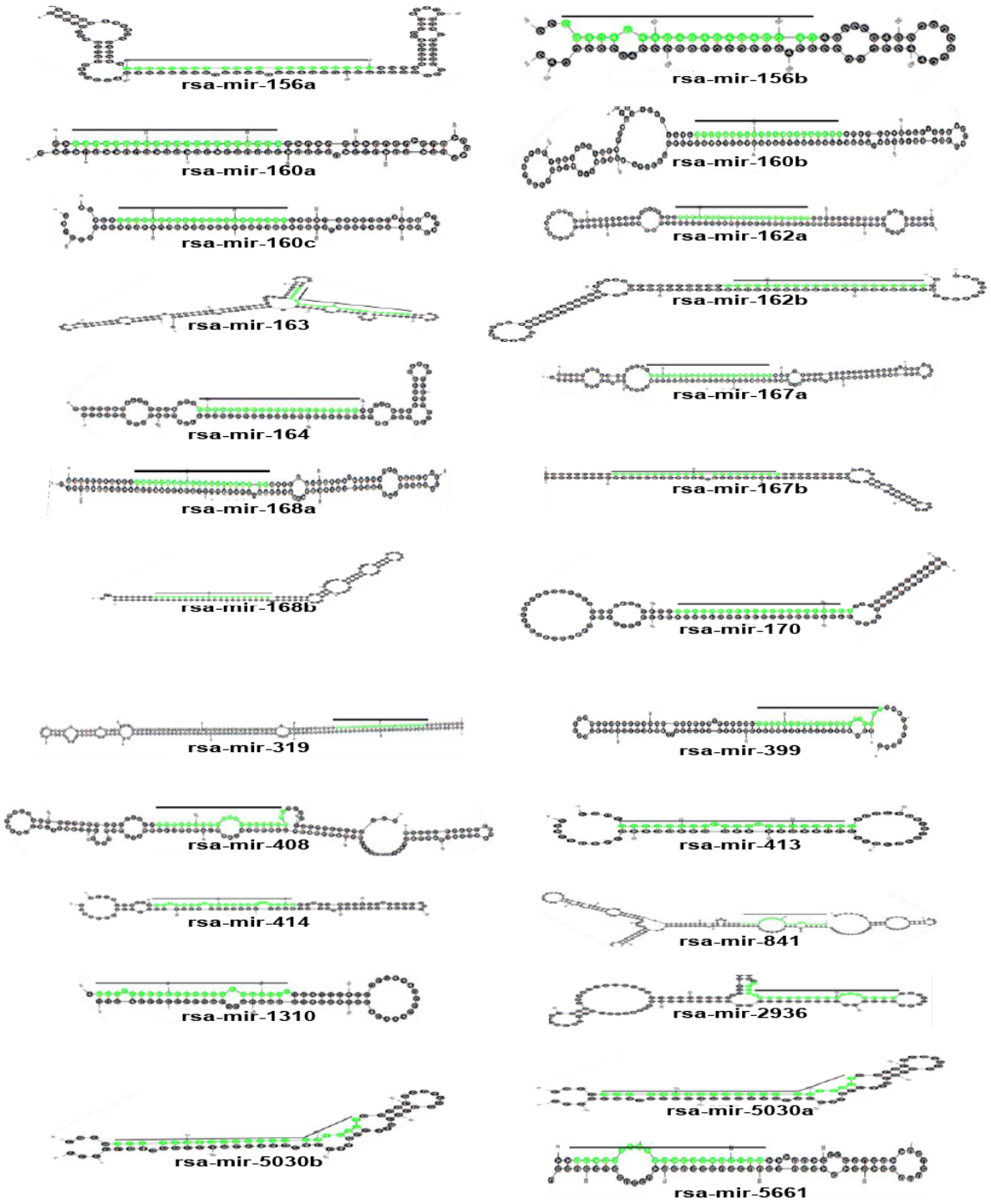

Fig. 1. The new Radish miRNA secondary structures.
The Radish pre-miRNAs secondary structures are predicted using Mfold algorithm. These structures are clearly showing that mature miRNAs are in stem region of the stem-loop structures, highlighted with parallel lines.

The newly conserved radish's pre-miRNAs length ranges from 64-211 nt with an average of 116 nt and mature sequences range from 20 nt to 23 nt. Majority (76%, i.e., 19 out of 25) of the miRNAs are 21 nt in length, followed by 22 nt (16%) and 23 nt (8%). The mature and pre-miRNAs lengths are similar to previous reports in other plant species (Lv *et al*., 2010; Barozai *et al*., 2011b, 2011c). The 56% (14 out of 25) novel conserved identified radish's miRNA sequences are perfectly (100%) matched, 16% have a difference of 1, 24% have 2 and 4% have a difference of 4 nucleotides with the related reference miRNAs. Almost similar findings are reported in other organisms (Feng *et al*., 2009; Barozai, 2012c). The 72% (18 off 25) radish's

miRNAs sequences are located at 5' arm, while the remaining 28% are at 3' arm as illustrated in Fig. 1. The identified radish's miRNA stem regions in folded structures were observed with 16 nucleotides involved in Watson-crick or G/U base. For few structures the base pairing showed less than 16 nt but their reference miRNAs have also similar properties. No big internal loops were observed in these folded structures. Almost same results were presented by various researchers (Feng *et al*., 2009; Barozai, 2012a). The newly identified radish miRNAs predicted through mature reference sequences have showed no similarity with known proteins. This confirms them as non-coding RNAs.

Conservation and Phylogenetic analyses of Radish miRNA: The newly identified radish miRNA (ras-mir-156) were further annotated in terms of conservation and phylogenetic analysis. The radish miRNA, ras-miR-156 showed conservation with *Arabidopsis thaliana* (ath), *Oryza sativa (osa)*, *Zea mays (zma)* and *Populus trichocarpa* (ptc) miRNAs as shown in Fig. 2. Similar findings were reported for cotton, *Helianthus* and *Mimulus* plants (Barozai *et al*., 2008; 2011a, 2011b). The Phylogenetic analysis of the same miRNA (mir-156) sequences have showed that the radish is more closed to *Arabidopsis thaliana* (ath) than the *Oryza sativa (osa)*, *Zea mays (zma)* and *Populus trichocarpa* (ptc) as illustrated in Fig. 3.

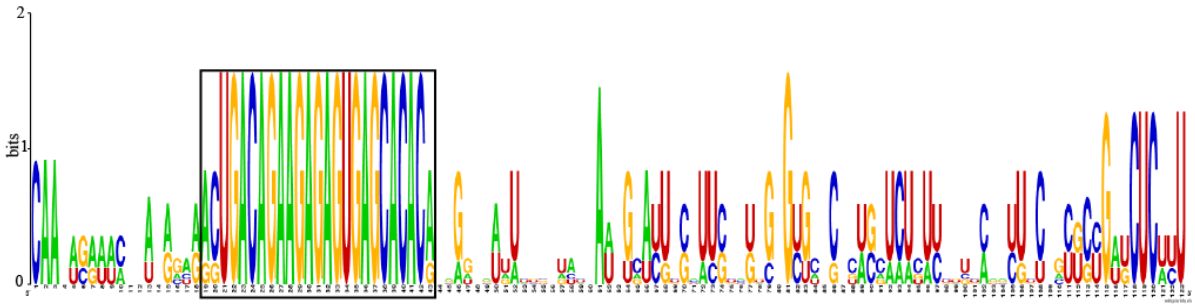

Fig. 2. The Radish miRNA conservation studies.
Alignment of the radish pre-miRNAs (156) with *Arabidopsis thaliana* (ath), *Oryza sativa (osa)*, *Zea mays (zma)* and *Populus trichocarpa* (ptc) miRNAs, using Weblogo: a sequence logo generator, showing miRNA sequences conservation. The conserved mature sequence is highlighted in a box.

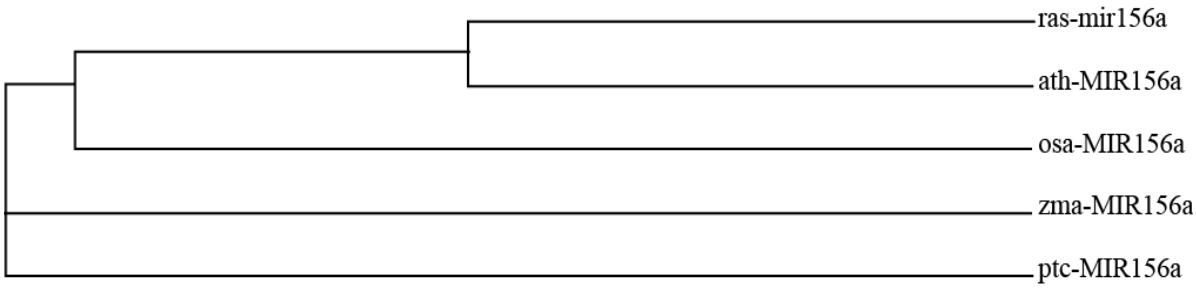

Fig. 3. The Radish miRNA phylogenetic analysis.
The Phylogenetic analysis of the radish (ras) pre-miRNA (156) with *Arabidopsis thaliana* (ath), *Oryza sativa (osa)*, *Zea mays (zma)* and *Populus trichocarpa* (ptc) miRNAs, was done with the help of ClustalW and cladogram tree was generated using neighbor joining clustering method. The Phylogenetic tree showed that on the basis of pre-miRNA sequences, the Radish (ras) is more closed to *Arabidopsis thaliana* (ath) than the *Oryza sativa (osa)*, *Zea mays (zma)* and *Populus trichocarpa* (ptc).

The radish miRNAs potential targets: The prediction of the targets is a crucial step for validation of computationally identified miRNAs. Total 42 targets were predicted for the new identified radish miRNAs, as shown in Table 2. Almost all of these targets are already reported as miRNA targets in other plants (Barozai *et al*., 2008; Feng *et al*., 2009; Barozai *et al*., 2011a, 2011b).

Transcription factors are the central targeted proteins of plant miRNAs (Kozak, 2007; Feng *et al*., 2009). Same targets family is predicted for radish miRNAs. The newly identified radish miRNAs 156, 160, 319 and 414 target Squamosa promoter-binding-like proteins, Auxin response factor 17, Splicing factor 4-like protein and General transcription factor respectively.

The metabolic processes proteins are also reported as a class targeted by miRNAs (Feng *et al*., 2009; Barozai *et al*., 2011a, 2011b). The metabolomics proteins like; Phosphonopyruvate decarboxylase, Histone deacetylase complex subunit SAP18, Long chain acyl-CoA synthetase 5, 26S protease regulatory subunit 6A, Aldehyde dehydrogenase 22A1 precursor and Ubiquinol-cytochrome C reductase complex protein, also predicted as targets for the radish miRNAs.

Other radish miRNAs targets are hypothetical proteins, Histone H2A variant 1, GrpE like protein, Serine/threonine protein phosphatase 2A and NAM (No apical meristem)-like protein. Similar findings were reported for various plants (Barozai *et al*., 2008; Feng *et al*., 2010).

Table 2. Putative Radish miRNA targets.

Radish miRNA Family	Putative targets
156	Squamosa promoter-binding-like protein 9, Squamosa promoter-binding-like protein 2 Squamosa promoter-binding-like protein 15, Squamosa promoter-binding-like protein 3 Hypothetical protein, Phosphonopyruvate decarboxylase-like protein
160	Auxin response factor 17
162	Flavoprotein oxidoreductase
163	Protein phosphatase, Uncharacterized protein AAD25612.1, Villin-3
164	NAM (No apical meristem)-like protein, Histone deacetylase complex subunit SAP18
167	Protein phosphatase 2C family protein, Nucleoside transmembrane transporter
170	Long chain acyl-CoA synthetase 5, 26S protease regulatory subunit 6A, Protein kinase-like protein
319	Aldehyde dehydrogenase 22A1 precursor, Splicing factor 4-like protein, Predicted protein
414	Hypothetical protein, General transcription factor IIIC, Mitochondrial glycoprotein family protein, Expressed protein, Putative RNA binding protein, Uncharacterized protein At1g55365.1, Riboflavin biosynthesis related protein, Transducin/WD40 repeat-like superfamily protein, Expressed protein, Histidine-containing phosphotransfer protein 3, Serine/threonine protein phosphatase 2A, GrpE like protein, Uncharacterized protein AT5g24690
413	Hypothetical protein, Ubiquitin-fold modifier 1, Ubiquinol-cytochrome C reductase complex
841	Histone H2A variant 1
5030	Hypothetical protein
5661	Hypothetical protein similar to Os01g0953100, Hypothetical protein, Peroxidase 20

List of the potential targets of the newly identified miRNAs in Radish

Conclusion

This study produced 25 new miRNAs and their 42 targets for the first time in the radish plant. These novel miRNAs will be useful in the future for the improvement of this most important vegetable plant and these will also be good functional genomic resources to understand the gene regulatory mechanism in radish.

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