Pak. J. Bot., 47(4): 1281-1285, 2015.

COMPUTATIONAL IDENTIFICATION OF 18 MICRORNAs AND THEIR TARGETS IN THREE SPECIES OF ROSE

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Abstract

MicroRNAs (miRNAs) are non-protein coding, small endogenous RNAs. Their length ranges from 18-26 nucleotides (nt). The miRNAs convergence property becomes a rational approach for the hunt of novel miRNAs in other organisms by homology search. As presently very little miRNAs are reported for rose species, so this study deals with the identification of miRNAs in different species of rose. Consequently 18 miRNA belonging to 17 miRNA families were identified in 3 species of rose (*Rosa hybrid*, *Rosa chinensis* and *Rosa virginiana*). All of the identified miRNA families (miR156, 160, 164, 166, 398, 482, 831, 837, 838, 841, 847, 3436, 3627, 6135, 6285, 6287 and 6288) are being reported for the first time in rose. Precursors of the identified miRNAs form stable minimum free energy (MFE) stem-loop structures and the mature miRNAs are found in the stem portions of their corresponding precursors. 11 putative targets of the miRNAs have also been identified. The identified targets are various proteins including transcription factors. Identification of 18 miRNAs will be supportive to explore the gene regulation phenomenon in various species of roses and it will be a good contribution for understanding the post transcriptional gene regulation in various stages of the life cycles of roses.

Key words: MicroRNAs; Post transcriptional gene regulation; Rosa

Introduction

MicroRNAs; once called small temporal RNAs (Hutvagner *et al.,* 2001) are an important class of non-coding regulatory RNAs. They are endogenous in nature and their length ranges from 18-26 nucleotides (nt) (Tang *et al*., 2003). They play important job in gene regulation after transcription (Ambros *et al.*, 2003). The genes of miRNAs are usually transcribed by RNA polymerase II (Lee *et al.*, 2004). The product is called a primary miRNA (Pri-miRNA) which may be thousands of nt in length and contains one or more miRNA stem loops (Lee & Ambros, 2001). This transcript is capped with a specially modified nt at the 5' end, polyadenylated with multiple adenosines at 3' end (Lee *et al.*, 2004) and spliced. This first transcript, known as Primary miRNAs (pri-miRNAs) self-hybridized into a stable stem-loop structure creating precursor miRNAs (pre-miRNAs). A special protein complex, consisting of an RNase III Drosha, with a double-stranded RNA (dsRNA) binding protein, i.e., Pasha/DGCR8 act on pri-miRNAs in the cell nucleus and this is called pri-miRNA processing (Denli *et al*., 2004). After being processed the pri-miRNA is changed into pre-miRNA. The resulting pre-miRNAs are approximately 90-240 nt and are sent to the cell cytoplasm where a special RNase III protein Dicer generate an unstable, 19-25 nt miRNA duplex structures (Sontheimer, 2005) and in case of plants it is processed by a Dicer like enzyme (Vaucheret *et al.*, 2012). This unstable duplex structure of double RNA strands is assimilated into a multiple-protein nuclease complex, the RNA induced silencing complex (RISC), which is also known as microRNA ribonucleoprotein complex: (miRNP) (Schwarz & Zamore, 2002). The RISC with miRNA act as negative gene regulator of gene expression either by hindering translation process or by causing messenger RNA (mRNA) demolition depending on the level of complementarity of miRNA to its target (Tang *et al.*, 2003). In contrast to their name and size, the miRNAs perform mega functions in eukaryotic organisms. They perform important functions in plant and animals during growing (Chen, 2003), organ-development, (Chen, 2003; Barozai *et al*., 2012a), transgene inactivation (Allen *et al*., 2005), signaling processes, (Yoshikawa *et al.*, 2005; Barozai, 2012a; Barozai *et al.*, 2013a), environmental stresses, (Sunkar & Zhu, 2004; Barozai *et al.*, 2013b), disease development and defense against the attacking viruses (Johnson *et al.*, 2004).

Because of their versatile regulatory functions in eukaryotic organisms, they were named as the mega regulators of eukaryotic genomes (Baloch *et al.*, 2013). Most of the miRNAs are conserved in the animals and plants (Bai *et al*., 2012; Barozai, 2012a, b; Baloch & Din, 2014; Din & Barozai, 2014a, b). The conserved nature of the miRNA becomes a sensible logic for the finding of new homologs by comparative genomics. Rosa is an important genus of family Rosaceae. This genus is famous for its ornamental plants. Surveying the latest releases of microRNA Registry Database (Version Rfam 20 released June 2013) (Griffiths-Jones, 2004) and Plant MicroRNA database (PMRD) (Zhang *et al.*, 2010), reveals that no miRNAs have been reported in the genus Rosa. This produced an idea to work and explore miRNAs for Rosa. Finally 18 miRNAs were identified from the three species of genus Rosa.

Materials and Methods

Prediction of candidate pre- miRNA sequences: Nearly the same method as adopted earlier (Barozai, 2012c), was used. The plant pre-miRNAs from the microRNA Registry Database (Version Rfam 20 released June 2013) (Griffiths-Jones, 2004), and PMRD (Zhang *et al.*, 2010) were downloaded and submitted to BLAST against publicly accessible 16379 *Rosa ESTs* from the database, i.e., dbEST release 130101 at http://blast.ncbi.nlm.nih.gov/Blast.cgi

using blastn (Altschul *et al.,* 1990). To find the candidate homologue sequences, the homology based search with the available miRNA sequences was done in three species of Rosa. The algorithm BLAST parameters were set as given: expect standards were adjusted at 1000; low complexity was selected as the sequence filter, database (others: ESTs), organism (Rosa, taxid: 3764), program selection (somewhat similar sequence) and all remaining parameters were set as default. The candidate EST sequences having maximum 4 mismatches with the mature reference sequences were saved in FASTA formats. A single represented EST was generated for each miRNA by eliminating the repetitive ESTs of the same gene.

Removal of protein coding sequences: To confirm the above selected candidate *Rosa* miRNAs as non-coding RNA, their saved sequences were submitted for protein similarity examination in the protein database at NCBI applying Blastx with default parameter (Stephen *et al.,* 1997). The outcomes were saved and the protein coding sequences were excluded.

Prediction of stem-loop structures: Zuker folding bioinformatics tool, MFOLD (version 3.2) (Zuker, 2003) openly accessible at http://www.bioinfo.rpi.edu/ applicaions/ mfold/rna/form1.cgi, was applied to predict the stem-loop structure of the initial miRNA sequences. The parameters were set similar as adopted earlier (Barozai, 2012d). The stem sections of the stem-loop were inspected for the mature miRNA sequences with minimum of 10 base pairs engaged in Watson-Crick or G/U base pairing between the mature miRNA with its opposite strand (miRNA*).

Conservation and phylogenetic analysis of Rosa miRNAs: For the conservation and phylogenetic analysis the Rosa miRNA family MIR-164 was studied with *Brassica napus* (bna), *Arabidopsis thaliana* (ath) and *Populus trichocarpa* (ptc) miRNAs orthologues with the help of openly accessible weblogo: a sequence logo generator tool (Crooks *et al.,* 2004) and ClustalW to make cladogram tree by neighbor joining clustering approach (Larkin *et al.,* 2007) respectively. The outcomes of these were saved.

Prediction of Rosa miRNA targets: A similar method (Barozai, 2013) was adopted to predict the Rosa miRNA targets using the NCBI Blastn program. The mRNA sequences having 75% query coverage were retrieved and submitted to RNA-hybrid, a miRNA target prediction tool for the verification of the targets (Kruger *et al.,* 2006). The targets with stringent seed site situated at either sites 2-7 or/and 8-13 after the 5' side of the miRNA along with the supplementary position and having hybridization MFE of -15 kcal/mol were chosen. The results were saved.

Results and Discussion

The Novel *Rosa* miRNAs: Total eighteen new *Rosa* pre-miRNAs were identified in three species of rose after filtration and finalizing of the procedure. The eighteen potential *Rosa* miRNAs belong to 17 families (i.e. miR 156, 160, 164, 166, 398, 482, 831, 837, 838, 841, 847, 3436, 3627, 6135, 6285, 6287 and 6288) and 3 species of rose. Sixteen miRNAs are from *Rosa hybrida* and one from each *Rosa chinensis and Rosa virginiana*. All the eighteen novel *Rosa* miRNAs are considered as valid candidates when satisfying the empirical formula of biogenesis and expression for the miRNAs, as suggested by Ambros *et al.* (2003). All the identified novel *Rosa* pre-miRNAs satisfied the criteria B, C with D. According to Ambros *et al.* (2003) just the criterion D is sufficient for homologous sequences to authenticate as new miRNAs in diverse species. Later, Meyers *et al.* (2008) more confirmed it in case of plants miRNA annotation. The present study is also in agreement with the homology based bioinformatics studies (Barozai *et al.,* 2012b; Barozai *et al.,* 2014; Noor-us Sabah *et al.,* 2015)

***Rosa* miRNAs characterization:** As predicted by MFOLD (Zuker, 2003), the MFE) for the newly explored *Rosa* pre-miRNAs have a range from -13.50 to -66.24 with an average -30.98 Kcal·mol^{-1}. The pre-miRNAs size observed from 51 to 242 nt with an average of 126 nt. The mature miRNA sequences length ranges from 20 to 23 nt. The most (61% i.e., 11 out of 18) of the *Rosa* miRNAs have 21 nt in size, followed by 20 nt (22%), 22 nt (11%) and 23 nt (6%). The maximum (44% i.e., 8 out of 18) *Rosa* miRNAs are observed to have 4 mismatches to their homologs, followed by 3 (39%) and 2 (11%) mismatches. Majority (61% i.e., 11 out of 18) of *Rosa* miRNAs are resided on the 5' and rest (39%) are on the opposite 3' arms of the stem-loop secondary structures as illustrated in Figure 1. The identified miRNA stem-loop structures were observed with minimum of 10 nucleotides engaged in Watson-crick or G/U base pairings of the mature miRNA with the opposite arms (miRNAs*) in the stem site and the stem-loop precursors structures do not show big internal loops or bulges. The *Rosa* miRNAs description such as Reference miRNAs (Ref miRNAs), pre-miRNAs length (PL), minimum free folding energies (MFE), mature miRNA sequences (MS) mature sequence arm (MSA), mature sequence length (ML), number of mismatches (NM) and source ESTs (SE) are presented in Table 1. These results are comparable to the published reports of various researchers working on miRNAs. (Barozai *et al*., 2012a; Bai *et al.,* 2012; Din & Barozai, 2014a, b).To confirm the newly profiled miRNAs as solid candidates of miRNAs the association between them and well-known proteins is very important. The *Rosa* miRNAs were submitted for similarity search in protein database at National Center for Biotechnology Information (NCBI) using Blastx and resulted in non-significant homology with reported proteins. This further validated our profiled miRNAs as strong candidates in *Rosa*.

Conservation and phylogenetic studies of *Rosa* miRNAs: The newly discovered *Rosa* miRNA (mir-164) is subjected for conservation and phylogeny. The *Rosa* miRNA (miR- 164) has shown conservation with *Brassica napus* (bna), *Arabidopsis thaliana* (ath) and *Populus trichocarpa* (ptc) miRNAs as illustrated in Figure 2. The Phylogenetic analysis for the same miRNA (mir-164) sequences proposed that the *Rosa* is more closer to *Populus trichocarpa* compared to *Brassica napus* and *Arabidopsis thaliana* as illustrated in Figure 3. These findingss are in agreement with the published research (Ghani *et al.,* 2013 and Barozai *et al.,* 2012a).

Table 1. The newly identified Rosa conserved miRNAs characterization.

Rosa miRNAs	Ref miRNAs	PL	MFE	MS with their positions in Precursor	MSA	ML	NM	SE
rhy-miR156	cme-miR156d	66	-18.70	**43**-GAAGAGAGGAGCAA**AT**CAGAG-**62**	3’	20	2	BQ104550
rvi-miR156	cme-miR156e	105	-26.39	**15**-GAAGAAGAGGGC**CA**AGAA**A**GAG-**35**	5’	21	3	JZ196919
rhy-miR160	cme-miR160a	193	-55.56	**172**-**T**CC**T**CGAGCCAGAGAG**G**CA**G**A-**192**	3’	21	4	BQ105663
rhy-miR164	aly-MiR164a	140	-40.30	**53**-**C**GGAGAAGCAGG**A**CAC**A**TGC**G**-**73**	5’	21	4	JK622622
rhy-miR166	osa-miR166c	232	-66.24	**202**-AAGGA**G**GCCGAGACCAAC**GA**-**221**	3’	20	3	JK621878
rch-miR398	cpa-miR398	157	-32.96	**1**-**AA**GACGACAGAGACACA**A**GA**T**-**21**	5’	21	4	BI978328
rhy-miR482	ppe-MiR482a	108	-21.10	**6**-GG**T**TG**GT**GAGGTTGCCGGAAAGA-**27**	5’	22	3	JK617370
rhy-miR831	aly-MiR831	81	-17.90	**58**-AGAAGAGG**CTGG**AGGAGATGAGA-**80**	3’	23	4	JK620877
rhy-miR837	aly-MiR837	154	-45.18	**118**-AAA**GA**AACAAGAAACT**G**TTGA-**138**	3’	21	3	BQ105854
rhy-miR838	aly-MiR838	83	-22.70	**2**-**C**TTTCTTCTTCTTCTT**C**CA-AAG-**22**	5’	21	3	EC587374
rhy-miR841	aly-MiR841	96	-34.70	**16**-TACGACCCAC**A**GGAAACT**G**AA-**36**	5’	21	2	EC587070
rhy-miR847	aly-MiR847	140	-30.95	**18**-TC**T**CTCCTCTTCTTC**TT**AATG-**38**	5’	21	3	JK617337
rhy-miR3436	aly-MIR3436	89	-14.30	**2**-**T**GAGCAAAAATCAT**G**GTTG**CA**-**22**	5’	21	4	JK621026
rhy-miR3627	mdm-MiR3627a	51	-13.50	**26**-TCGCAGGAGAGATGGCAC**CG**-**45**	3’	20	2	BQ104621
rhy-miR6135	pgi-miR6135g	105	-18.00	**3**-CAA**U**GCAAAAGAAAGA**G**AA**G**-**22**	5’	20	3	EC586684
rhy-miR6285	ppe-MiR6285	88	-16.10	**61**-TAGTGAAGTTTGA**GC**TAGGG**TC**-**82**	3’	22	4	BQ105849
rhy-miR6287	ppe-MiR6287	135	-31.33	**5**-CAA**A**AA**T**T**CC**AAGTTTTGGGC-**25**	5’	21	4	BQ106349
rhy-miR6288	ppe-MiR6288	242	-51.73	**219**-GAAAATGAC**G**A**T**T**T**GCT**T**GTT-**239**	5’	21	4	JK617333

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

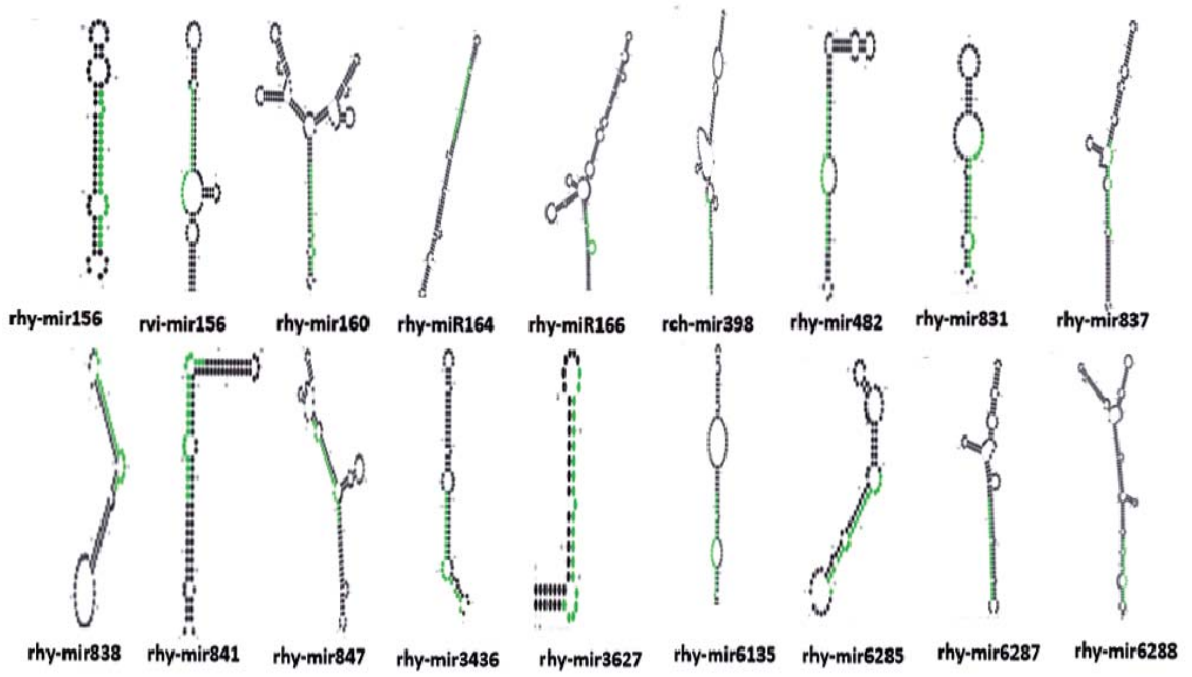

Fig. 1. The new conserved Rosa miRNA secondary structures.
The Rosa pre-miRNAs secondary structures were predicted using Mfold algorithm. These structures are clearly showing the mature miRNAs in stem region of the stem-loop structures, highlighted in green.

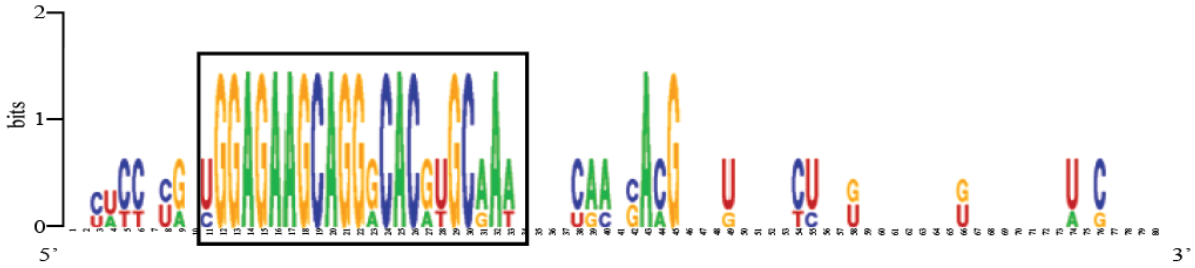

Fig. 2. The Rosa miRNA conservation studies.
Alignment of the Rosa pre-miRNA (164) with *Brassica napus* (bna), *Arabidopsis thaliana* (ath) and *Populus trichocarpa* (ptc) miRNAs was done by using Weblogo: a sequence logo generator, showing miRNA sequences conservation. The conserved mature sequence is highlighted in a box

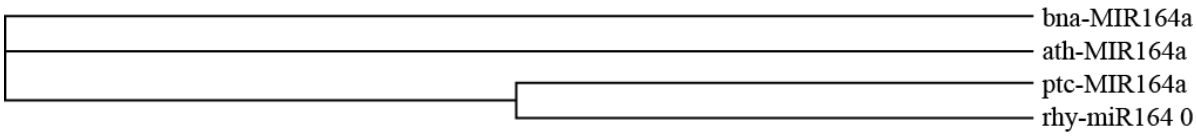

Fig. 3. The Rosa miRNA phylogenetic analysis.
The Phylogenetic analysis of the Rosa pre-miRNAs (164) with *Brassica napus* (bna), *Arabidopsis thaliana* (ath) and *Populus trichocarpa* (ptc) miRNAs, was done with the help of ClustalW and cladogram tree was generated using neighbor joining clustering method. The phylogenetic tree shows that on the basis of pre-miRNA sequences, the Rosa is closer to *Populus trichocarpa* as compared to *Brassica napus* and *Arabidopsis thaliana.*

Table 2. Putative *Rosa* miRNA targets. The *Rosa* miRNA families and their putative targeted proteins function and Genbank Acc. No. are provided here.

Rosa miRNA	Target Acc. No.	Target description
156	FR828553	MYB transcription factor
482	AB426520	mRNA for beta-glucosidase
831	JF806633	SOC1-like protein (SOC1)
837	JN903506	EXP4 protein (EXP4)
838	KF530803	BRC1 protein
841	AF394913	zinc-finger protein COP1
847	FR828553	MYB transcription factor
3627	JF806633	SOC1-like protein
6135	AM411468	NBS-LRR resistance protein
6287	JN857363	NAC2 protein
6288	JF806633	SOC1-like protein (SOC1)

***Rosa* miRNA targets:** The findings of new conserved *Rosa* miRNAs targets is another important phase for confirmation of miRNAs identified on homology basis. Total 11 targets (Table 2) were annotated for the new conserved explored *Rosa* miRNAs. Nearly all of the discovered targets are described as miRNA targeted genes in several organisms (Xie *et al.*, 2010; Barozai, 2012c).

The well-known targeted proteins class of miRNAs is transcription factors, reported in nearly all plants and animals (Xie *et al*., 2010; Din & Barozai, 2014a, b). The newly identified *Rosa* miRNAs also regulate this class of proteins. The identified putative *Rosa* targets of miRNAs; 156, 482, 831, 837, 838, 841, 847, 3627, 6135, 6287 and 6288 are MYB transcription factor, mRNA for beta-glucosidase, SOC1-like protein (SOC1), EXP4 protein (EXP4), BRC1 protein, zinc-finger protein COP1, MYB transcription factor, SOC1-like protein, NBS-LRR resistance protein, NAC2 protein and SOC1-like protein (SOC1) respectively. Our outcomes are in agreement to the prior research works reported by several researchers group in the same field (Frazier *et al*., 2010; Xie *et al*., 2010; Din & Barozai, 2014a, b).

Conclusions

We have identified eighteen (18) new conserved miRNAs belonging to seventeen (17) families in three species of *Rosa* from ESTs. All seventeen (17) families and their eighteen members are being reported for the first time. These findings will be valuable in understanding the gene regulation process in the various species of genus *Rosa*.

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(Received for publication 15 March, 2014)