Plant Cell Rep (2010) 29:757–769
DOI 10.1007/s00299-010-0862-4

ORIGINAL PAPER

Identification of conserved micro-RNAs and their target transcripts in opium poppy (*Papaver somniferum* L.)

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Received: 21 January 2010 / Revised: 6 April 2010 / Accepted: 15 April 2010 / Published online: 5 May 2010

Abstract Micro-RNAs (miRNA) are regulatory non-coding class of small RNAs functioning in many organisms. Using computational approaches we have identified 20 conserved opium poppy (*Papaver somniferum* L.) miRNAs belonging to 16 miRNA families in Expressed Sequence Tags (EST) database. The existence of ESTs suggested that the miRNAs were expressed in *P. somniferum*. Lengths of mature miRNAs varied from 20 to 23 nucleotides located at the different positions of precursor RNAs. Uracil was found to be a dominant nucleotide in both poppy pre-miRNA sequences (31.28 ± 7.06% of total nucleotide composition) and in the first position at the 5′ end of the mature poppy miRNAs. We have applied quantitative real-time PCR (qRT-PCR) assays to compare and validate expression levels of selected *P. somniferum* miRNAs and their target transcripts. As a result, some of the predicted miRNAs and their target genes were found to be differentially expressed in *P. somniferum* leaf and root tissues. A meaningful correlation between three of the four analyzed pairs of miRNAs and their target transcript expression levels was detected. Additionally, using these predicted miRNAs as queries, 41 potential target mRNAs were found in National Center for Biotechnology Information (NCBI) protein-coding nucleotide (mRNA) database of all plant species. Some of the target mRNAs were found to be transcription factors regulating plant development, morphology, and flowering time. Other target mRNAs of identified conserved miRNAs involve in metabolic processes, signal transduction, and stress responses. This study reports the first identification of opium poppy miRNAs.

Keywords *Papaver somniferum* L. · Micro-RNA · Stem-loop hairpin structure · Target mRNA · qRT-PCR

Communicated by H. Judelson.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-010-0862-4) contains supplementary material, which is available to authorized users.

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Introduction

Micro-RNAs (miRNAs) are small, non-protein coding regulatory RNAs, which posttranscriptionally regulate gene expression in many organisms by targeting mRNAs for cleavage or suppression of translation (Carrington and Ambros 2003; Bartel 2004; Zhang et al. 2006a). Micro-RNA genes are transcribed by RNA polymerase II into long primary precursor miRNA (pri-miRNA) transcripts (Chen 2005; Zhang et al. 2006a; Unver et al. 2009). Those pri-miRNAs are processed into hairpin (stem-loop) structure precursors (pre-miRNA) by ribonuclease enzyme included microprocessors. Then the precursor is cut into small, double-stranded, RNAs and the loop region of the pre-miRNA is removed by the Ribonuclease III like enzyme, DICER Like I (Kurihara and Watanabe 2004). Mature miRNAs are incorporated into RNA-induced silencing complex (RISC) to guide cleavage and

translational inhibition of their sequence-specific complementary target mRNAs by RNA interference mechanism (Lin et al. 2005; Brodersen et al. 2008). Therefore, plant miRNAs regulate expression of genes functioning in diverse developmental (Bao et al. 2004; Laufs et al. 2004; Mallory et al. 2004; Guo et al. 2007; Kim et al. 2005; Lauter et al. 2005; Mlotshwa et al. 2006; Jung and Park 2007; Schwarz et al. 2008), stress response to environment and pathogens (Kasschau et al. 2003; Chen et al. 2004; Jones-Rhoades and Bartel 2004; Sunkar and Zhu 2004), metabolism (Zhang et al. 2007a), signal transduction and protein degradation (Achard et al. 2004; Guo et al. 2007; Zhang et al. 2006a) processes. Since the first miRNA identification in plants (Park et al. 2002), a number of plant miRNAs have been detected via experimental and computational approaches. In the latest release of miRBase (release 14.0 September 2009, http://microrna.sanger.ac.uk/sequences/index.shtml), 2,043 plant miRNAs ranging from dicotyledons (such as 190 of *Arabidopsis thaliana* and 234 of *Populus trichocarpa*) to monocotyledons (such as 414 of *Oryza sativa*, 32 of *Triticum aestivum*, and 109 of *Zea mays*) are represented. Despite its significant medicinal and economical value, however, no miRNAs for opium poppy (*Papaver somniferum*) are yet found in the database.

With the availability of bioinformatics-based miRNA identification methods employing sequence-homology-based searches and structure-similarity-based searches (reviewed in Zhang et al. 2006c and Unver et al. 2009), prediction of conserved miRNAs of a particular organism is possible through utilizing available mature miRNA sequences (e.g. in Genomic Survey Sequences and Expressed Sequence Tags databases) conserved across species. Furthermore, identification of miRNAs by computer-based approaches is accurate, fast, and cheap thanks to the application of web-accessible free algorithms such as BLASTn algorithm (Altschul et al. 1997) for homology-complementary-based searches, and the MFOLD3.2 algorithm (Zuker 2003) for secondary structure prediction. Target mRNAs of predicted or known miRNAs, on the other hand, can be searched via BLASTn and/or BLASTx searches. Additionally, plant mRNA targets can be predicted using specialized algorithms such as miRU (Zhang 2005) and MIRCheck (Jones-Rhoades and Bartel 2004). Likewise, expressed sequence tags (ESTs) and genomic survey sequences (GSSs) were analyzed in terms of sequence homology and secondary structure-similarity-based search strategies for cotton (Zhang et al. 2007b), rice (Zhang et al. 2005), maize (Zhang et al. 2006c), potato (Guo et al. 2007), oil rape (Xie et al. 2007), tomato (Yin et al. 2008), and Brachypodium (Unver and Budak 2009).

Poppy is an agronomically and economically important plant as it is the primary source of opiate production (Allen et al. 2004). It produces a great number of benzylisoquinoline alkaloids. Dried latex of the poppy capsule is composed of ~12% morphine and relatively lesser amount of other alkaloids such as codeine, papaverine, tebaine, and noscapine (Page 2005). Morphine and codeine alkaloids are used as most important and effective analgesics in medicine worldwide. Moreover, intermediates in the morphine biosynthetic pathway have medicinal importance (Allen et al. 2004). Hence, it is important to elucidate regulation of gene expression involved in the pathway of morphine biosynthesis. As regulatory RNAs, miRNAs are possibly involved in the process of regulation of gene expression for morphine biosynthesis pathway. In this study, we aimed to identify *P. somniferum* miRNAs and their target genes using a combined computer-based approach, and then to validate their function experimentally. To achieve this goal, we have compared all known plant miRNA sequences with *P. somniferum* ESTs. We have then searched the NCBI EST database of *P. somniferum* and protein-coding nucleotide (mRNA) database of all plant species to predict the target genes of the identified miRNAs. Some of the selected putative *P. somniferum* miRNAs and miRNA target transcripts were validated through differences in expression levels measured by qRT-PCR.

Materials and methods

Reference miRNAs

The set of miRNAs that we used, downloaded from miRBase (version 13.0, March 2009, http://microrna.sanger.ac.uk/sequences/) consists of a total number of 1,328 known mature miRNA sequences from 9 species; *A. thaliana* (187), *Glycine max* (78), *Medicago truncatula* (38), *O. sativa* (377), *Physcomitrella patens* (230), *P. trichocarpa* (234), *Saccharum officinarum* (16), *Sorghum bicolor* (72) and *Zea mays* (96). Those miRNAs were compared with *P. somniferum* EST sequences to computationally identify *P. somniferum* miRNAs.

EST source for *P. somniferum* and computer-based identification of *P. somniferum* miRNAs

A total of 20,382 ESTs of *P. somniferum* were obtained from GenBank, at the NCBI (http://www.ncbi.nlm.nih.gov, May 2009) were used in this study. Identification of *P. somniferum* miRNAs have been achieved according to previously published computational methods (Zhang et al. 2005, 2007b; Yin et al. 2008; Unver and Budak 2009; Unver et al. 2009). We have compared all available *P. somniferum* ESTs with previously known plant mature miRNA sequences using the strategy developed by Zhang

et al. (2005). In miRNA search, two important parameters were considered: (a) sequence complementarities/homology/conservation of miRNAs and (b) properties of pre-miRNA secondary hairpin structures. Known plant mature miRNA sequences were uploaded on BLASTn algorithm, BLAST 2.2.21 (28 July 2009) for homology search in ESTs of *P. somniferum*. BLASTn settings were adjusted as follows: expect value cutoff of 1,000; the number of descriptions and alignments were 10 and automatically adjusted parameters for short input sequences. Then, the EST sequences with only 0–4 nucleotide mismatches compared with the miRNA sequence have been selected manually. To *in silico* predict the secondary structures of the chosen ESTs, the Zuker folding algorithm (Zuker 2003) was used. The web-based computational software MFOLD 3.2 (Zuker 2003) is publicly available at http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi. Outputs of MFOLD 3.2, including minimum free energy (ΔG kcal/mol), number of arms per structure, number of nucleotides (A, G, C and U), size and symmetry of internal loops within arms, predicted secondary structure in the usual dot-bracket notation, size of helices within arms, miRNA-like helicity, and mfold-style connect (ct) files were saved for further analysis. The minimal folding free energy index (MFEI) was then calculated for each pre-miRNA sequence as previously described (Yin et al. 2008) (Table 1). The parameters applied on mature and pre-miRNAs were (a) mature miRNAs' lengths are in the range of 19–24 nucleotides, (b) mismatches between candidate mature miRNAs and known mature miRNAs are 0–4, (c) minimum folding energy index (MFEI) are relatively higher than other types of RNAs, and negative minimum folding energy (MFE) of pre-miRNAs is relatively lesser according to Yin et al. (2008) and Zhang et al. (2006b). MFEI is one of the useful parameters to distinguish miRNAs from other types of RNA. It is assumed that MFEI of RNA with approximately >0.67 is more likely pre-miRNA (Yin et al. 2008), (d) a loop or a large break in the mature miRNA sequence should not be present, (e) miRNAs should be located in the arms of stemloop structure, and (f) in the pre-miRNA structure, no more than six nucleotide mismatches are allowed between miRNA and its opposite sequence (miRNA*) (Zhang et al. 2007b, 2008, 2009; Yin et al. 2008; Unver and Budak 2009). Applying the above criteria, we have detected a number of candidate mature miRNA (Table 1) and pre-miRNA sequences (not shown).

Total RNA isolation

Papaver somniferum seeds were planted in greenhouse and were grown for 2 months. Greenhouse conditions were maintained at approximate day/night temperatures of 25/20°C (±3). Total RNA was isolated from leaves and roots of 2-month-old seedlings using Trizol reagent (Invitrogen, CA, USA), according to manufacturer's protocol. The quality and quantity of isolated leaf and root RNA samples were measured using Nanodrop ND-100 (Nanodrop Technologies, Wilmington, DE, USA).

Stem-loop reverse-transcription

Stem-loop RT primers for Pso-MIR 397a, Pso-MIR 414, Pso-MIR 171b, Pso-MIR 169a, miR 167, and miR 408 were designed according to Varkonyi-Gasic et al. (2007) (Supplementary Table 1). The miRNA stem-loop reverse transcription experiments were performed using 2, 20, and 200 ng of total RNA samples of leaf and/or root samples (1 μL), 0.5 μL 10 mM dNTP mix, 1 μL stem-loop RT primer (1 μM) and 10.5 μL nuclease free water. Those components were mixed separately for the different dilutions of total RNA stem-loop RT primer cDNA syntheses and incubated for 5 min at 65°C, and then put on ice for 2 min. After that, 4 μL first-strand buffer (5×), 2 μL 1 M DTT, 0.1 μL RNAseOUT (40 units/μL), and 0.25 μL SuperScript III (200 units/μL) were added onto each tube. The RT reactions were performed as 30 min at 16°C followed by 60 cycles of 30°C for 30 s, 42°C for 30 s and 50°C for 1 s. As controls for stem-looped miRNA cDNA synthesis, we have also generated same reactions by adding all components except RT primer (no RT or −RT) and RNA template (no RNA or −RNA) in reaction tubes.

Experimental miRNA detection, SYBR Green I real-time assays

To experimentally confirm some of the predicted *P. somniferum* miRNAs and to measure and compare the expression levels of the miRNAs in leaf and root tissues, qRT-PCRs were performed by using SYBR Green Master mix of Stratagene (La Jolla, CA, USA) on a Stratagene Mx3000p real-time PCR Detection System (La Jolla, CA, USA). We used previously synthesized 2 μL RT Stem-looped cDNA products synthesized from 2, 20, and 200 ng total RNA dilutions as described by Varkonyi-Gasic et al. (2007) to calculate primer and qRT PCR efficiencies: 10 μL 2× master mix, 1 μL forward (10 pmol), 1 μL reverse (10 pmol) primers, and 8 μL nuclease-free water for qRT-PCR. Although forward primers were specifically designed for each individual miRNAs (Table 2), the reverse primer (5′-GTGCAGGGTCCGAGGT-3′) (Varkonyi-Gasic et al. 2007) was the same for all the reactions. Thermal cycler setup of the specified qRT-PCR was adjusted as 95°C for 15 min, followed by 40 cycles of 95°C for 5 s, 57°C for 10 s, and 72°C for 10 s. All of the

Table 1 List of computer-based identified miRNAs in *P. somniferum*

Conserved miRNA	miRNA family	*P. sominiferum* miRNAs	Accession	NM (nt)	Homologous miRNA	Location	ΔG (kcal/mol)	LP (nt)	LM (nt)	A + U (%)	MFEI
pso-MIR159	159	**U**UUGGCCUUUGAAGGGAG**C**UCUG	EST: FG601626	1	sbi-MIR159	5′	−27	138	23	65	0.56
pso-MIR166g	166	**U**CGGACCAGGCUUCAUUC**C**UU	EST: FG611490	1	osa-MIR166g	3′	−11.4	68	21	58.8	0.41
pso-MIR169a	169	**U**UGGCAAAUCAUCCUUGG**C**UG	EST: FG610705	2	ath-MIR169a	3′	−21.3	120	21	68.33	0.57
pso-MIR169b	169	CAGCCAAGGAUGAUUUGCCAA	EST: FG610705	3	ath-MIR169b	3′	−16.5	121	21	67	0.41
pso-MIR169d	169	GGAGCCAAGGAUGACUUA**C**UG	EST: FG603398	3	ath-MIR169d	5′	−26.8	100	21	58	0.64
pso-MIR169n	169	AAGCCAAGAAUGAAUUGC**C**UG	EST: FE967691	3	osa-MIR169n	5′	−24.4	103	21	53.4	0.51
pso-MIR171b	171	**U**UGACAGCCGUGCCAAUAUC	EST: FG607011	2	ath-MIR171b	3′	−22.4	94	21	47.8	0.47
pso-MIR172a	172	CUGCUCUUGAUGAUGCUG**C**AG	EST: FG613112	4	ath-MIR172d	3′	−16.1	80	21	58.7	0.5
pso-MIR172d	172	GGAUCUUGAUGAUGCUGGUA	EST: FG600448	3	ath-MIR172d	3′	−13.9	66	20	62.1	0.56
pso-MIR397a	397	**U**CAUUGAGCGCAGCGUUGAUU	EST: FG609471	2	ath-MIR397a	3′	−19.4	62	21	53.22	0.67
pso-MIR397b	397	**U**UAUUGAGUGCAGCAUUGAUG	EST: FE966243	2	osa-MIR397b	5′	−26.6	120	21	56.6	0.5
pso-MIR406	406	**U**UGAAUGCUAUUGUAAUUAUG	EST: EB388987	4	ath-MIR406	5′	−17	88	21	75	0.77
pso-MIR414	414	**U**AUCUUCAUCAUCCUCGUCA	EST: FG613095.1	1	ath-MIR414	5′	−19.2	101	21	58.4	0.45
pso-MIR446	446	GAUCAUAUGAAUAUGGGAAGUGG	EST: FG608817	2	osa-MIR446	5′	−28.8	120	23	57.5	0.56
pso-MIR476a	476	**U**ACCAAUCCUUCUUUGCAAAU	EST: FG611821	3	ptc-MIR476a	3′	−18.8	89	21	60.6	0.54
pso-MIR771	771	GUUUAUCUGUGGUAGCCCUCG	EST: FG607461	4	ath-MIR771	3′	−16.2	100	21	60	0.41
pso-MIR781	781	**U**UGGUGUUUUUCUGGAUA**C**UU	EST: FG608147	2	ath-MIR781	3′	−24.1	114	21	63.15	0.57
pso-MIR835	835	AACUUGCAUAUGUUCUUUAGC	EST: FG604403	3	ath-MIR835-5p	3′	−34.7	210	21	64	0.47
pso-MIR844	844	**U**UGUAAGAUUGCUUAUAAGAU	EST: FG610984	2	ath-MIR844	3′	−13.6	79	21	67	0.52
pso-MIR859	859	**U**CUCUCAGUUGUGAAGUCAAU	EST: FG607768	2	ath-MIR859	5′	−29.2	119	21	59.66	0.62

NM, number of mismatch; LM, length of mature miRNAs; LP, length of pre-miRNA; ΔG, folding free energies; MFEIs, minimal folding free energy indexes

Table 2 Major characteristics of identified *P. somniferum* pre-miRNAs

Characteristic	Minimal	Maximum	Median	Average	Standard deviation
Sequence length (nt)	59	210	101	96.85	27.72
G + C (%)	25	52.2	41.2	40.76	3.56
A + U (%)	47.8	75	58.8	59.24	3.56
MFE (−kcal/mol)	9.2	34.7	19.2	20.79	7.50
MFEI	0.4	0.77	0.51	0.49	0.07
A (%)	20.8	41.3	26.5	26.96	5.35
C (%)	9	28.9	20.75	18.53	3.78
G (%)	11.9	32.3	21.3	21.15	4.08
U (%)	20	44.9	33.4	31.28	7.06

reactions were repeated at least three times for statistical analysis.

Target mRNA identification of predicted miRNAs

The target transcripts of the predicted *P. somniferum* miRNAs were discovered using the BLASTn software with the benefit of high complementarity between plant miRNAs and their targets. Since the proteome of *P. somniferum* has not yet been fully annotated, the BLASTn searches were applied in both NCBI EST database of *P. somniferum* and protein-coding nucleotide databases of all other plant species. We applied same methods and parameters described by Zhang et al. (2006b) and Yin et al. (2008). Those criteria were (a) in total, more than four nucleotide mismatches are not allowed in complementary sites between miRNA and the target gene, (b) mismatches should not be located at the position of 10 or 11 in complementary sites between miRNA and their target mRNA, which are supposed to be cleavage sites in miRNA sequence, (c) no more than three continuous mismatches can be in the region of the miRNA–mRNA pair, but up to three mismatches between 12th and 23rd nucleotides are acceptable. Consequently, we predicted the target genes of identified miRNAs.

Target miRNA measurement by qRT-PCR

Target gene expression levels of miRNA target genes were measured with quantitative real-time PCR experiments. Some of the predicted opium poppy miRNA target transcripts were confirmed, and their relative expression level differences in root and leaf tissues were measured. Target genes of Pso-miR169a, Pso-miR171b, Pso-miR397a, and Pso-miR414, found by BLASTn searches and specific PCR primers were designed for quantification of above four miRNA target genes (Supplementary Table 2). qRT-PCR analysis was performed as previously outlined (Unver et al. 2008, 2009). Briefly, 2 μL of this cDNA was amplified with 1 μM of specific primers in a total of 20 μL volume using Brilliant SYBR Green qPCR Master mix (Cat no: 600548, Stratagene, La Jolla, CA, USA) with STRATAGENE Mx3000p Real-time PCR Detection Systems (Stratagene, La Jolla, CA, USA). According to Udvardi et al. (2008) we have tested multiple reference genes as Actin-1 (NM_179953.2) forward: 5′-CCGAGCGTGGTTACTCTTTC-3′/reverse: 5′-GCTGTCTCGAGTTCCTGCTC-3′, Tubulin Alpha-1 (AY091372.1) forward: 5′-CAACTGGATTCAAGTGCGGG-3′/reverse: 5′-TTCTCCACCAACTTCCTCATAATC-3′ and 18S rRNA (GenBank accession number: DQ912880.1, forward primer: 5′-TAGCGGGCCTCTTCTCTTTC-3′/reverse primer: 5′-CGCATTTCGCTACGTTCTTC-3′. Since the 18s RNA gene was found to be most consistent and applicable quantification and comparision experiments were performed using 18s RNA as a normalizer. Three independent real-time PCR results with acceptable efficiency (1.8–2.1) were averaged for each quantification.

Results

P. somniferum miRNA identification and miRNA characteristics

We applied a homology-based miRNA search on *P. somniferum.* BLASTn searches were performed using 1,328 known mature miRNA sequences from 9 plant species as queries and 20,382 *P. somniferum* ESTs as subjects. With the further structural MFOLD3.2 analysis, a total of 20 potential miRNAs were identified (Table 1) out of 0.11% of ESTs of *P. somniferum* that contained potential miRNAs. These miRNAs were either located in the 5′ arm or 3′ arm of the pre-miRNA sequences. Of the 20 identified *P. somniferum* miRNAs, 9 were located in the arm of 5′ of pre-miRNAs while the remaining 13 miRNAs were detected to be located in the 3′ of the stem-loop hairpin structures. The length of the mature miRNAs varied from

20 to 23 nucleotides (Table 1). In agreement with the previous results (Zhang et al. 2007b, 2008; Yin et al. 2008; Unver and Budak 2009), the majority of the miRNA sequences detected (12 of the 20) had uracil (U) as their first nucleotide. The identified 20 *P. somniferum* miRNAs were classified into 16 miRNA families. The family of miR-169 included four members, miR-172, miR-397, and miR-159 families contained two members and the rest of miRNA families were represented only by one member. Detected miRNAs had high conservation with previously identified plant miRNAs. One of them is Pso-MIR 414. We aligned the mature and pre-miRNA sequences of miRNA 414 to show the conservation of detected poppy miRNAs across other plant species at sequence level (Fig. 1). Pso-MIR414 had high sequence similarity with the known plant miRNA 414 sequences and mature miRNA sequence of Pso-MIR 414 was more conserved than its miRNA* site (Fig. 1). *P. somniferum* pre-miRNAs were diverse in both structure and size (Table 1, structures of six are shown in Fig. 2, Supplementary Fig. 1 and Supplementary Table 1). The size of the identified pre-miRNAs varied from 59 to 210 nucleotides with an average size of 96.85 ± 27.72 nucleotides. We also detected that most of the pre-miRNAs (14) were in the range of 70–160 nucleotides (Fig. 2) being in agreement with the previous results (Lin et al. 2005; Yin et al. 2008). The percentages of four nucleotides (A, C, G and U) in poppy pre-miRNAs were also analyzed (Table 2). Uracil was found to be dominant in poppy pre-miRNA sequences and comprised 31.28 ± 7.06% of total nucleotides, followed by adenine (26.96 ± 5.35), guanine (21.15 ± 4.08), and cytosine (18.53 ± 3.78%) with a great agreement of the results in previous study (Zhang et al. 2008). To distinguish miRNAs from other types of RNAs MFEI was used as a valuable criterion. The index included key characteristics for secondary structure and size of pre-miRNAs, which were MFE, sequence length, and G–C % in nucleotide contents. MFEI of RNAs has been studied previously and detected rate of miRNAs were reported higher than other types of RNA molecules such as tRNAs (0.64), rRNAs (0.59) and mRNAs (0.62–0.66) (Zhang et al. 2006c, 2008; Yin et al. 2008). In our study, we detected some of the identified miRNAs with relatively higher MFEIs (Table 1).

qRT-PCR confirmation and measurement of *P. somniferum* miRNA levels

In this study, qRT-PCR experiments were performed to validate computationally identified *P. somniferum* miRNAs and to measure expression level differences of some of them both in leaf and in root tissues. The miRNAs validated and quantified via SYBR Green I assay were Pso-MIR397a, Pso-MIR414, Pso-MIR171b, Pso-MIR169a, and Pso-MIR 167. The qRT-PCR analysis showed all the tested miRNA candidates were expressed in leaf and in root of *P. somniferum.* Comparisons of the detected opium poppy miRNA expression levels are relatively represented in Fig. 3a. For instance, expression level of Pso-MIR 414 in leaf tissue was significantly (4.65 ± 0.9 fold) higher than that of Pso-MIR397a. In root tissue, it was detected that expression level of Pso-MIR 414 was also significantly higher (4.10 ± 0.5 fold) than that of Pso-MIR397a. The expression profiles of miRNA 414 and miRNA 397a appeared to be related to different biological functions. The expression level of Pso-MIR414 potentially targets MYB transcription factor and was found to be significantly higher compared with other measured opium miRNAs (Fig. 3a). In *Arabidopsis*, MYB transcription factors have been detected to play roles in plant leaf development (Palatnik et al. 2003; Millar and Gubler 2005). On the

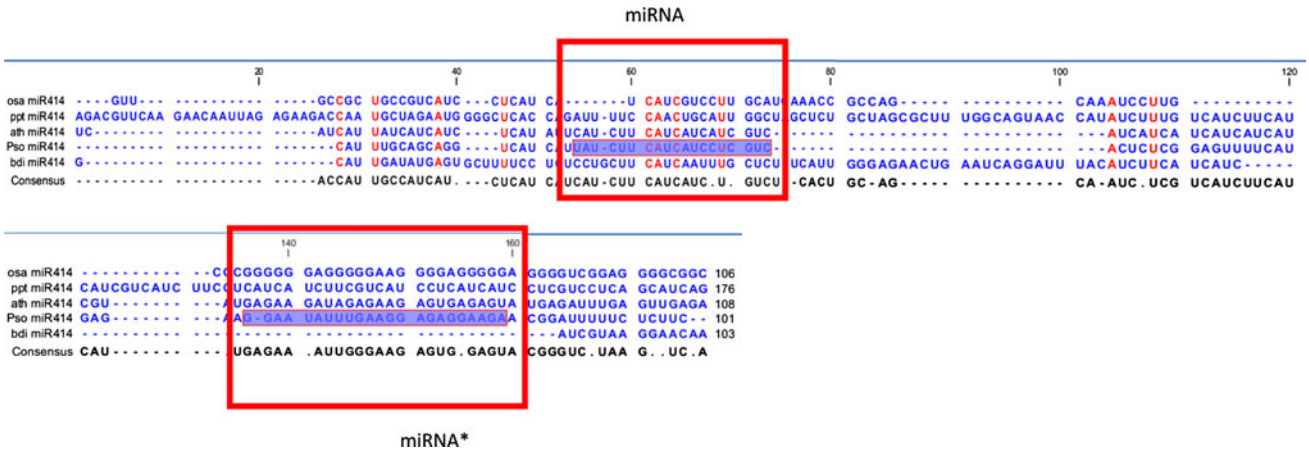

Fig. 1 Multiple sequence alignment of pre-miRNA 414 in different plant species. Comparison of the identified *P. somniferum* miRNA 414 and the miRNA 414s in other plant species deposited in miRBase (release 13.0) database. Mature miRNA sites in the precursors are more conserved than the miRNA* sites in the pre-miRNAs

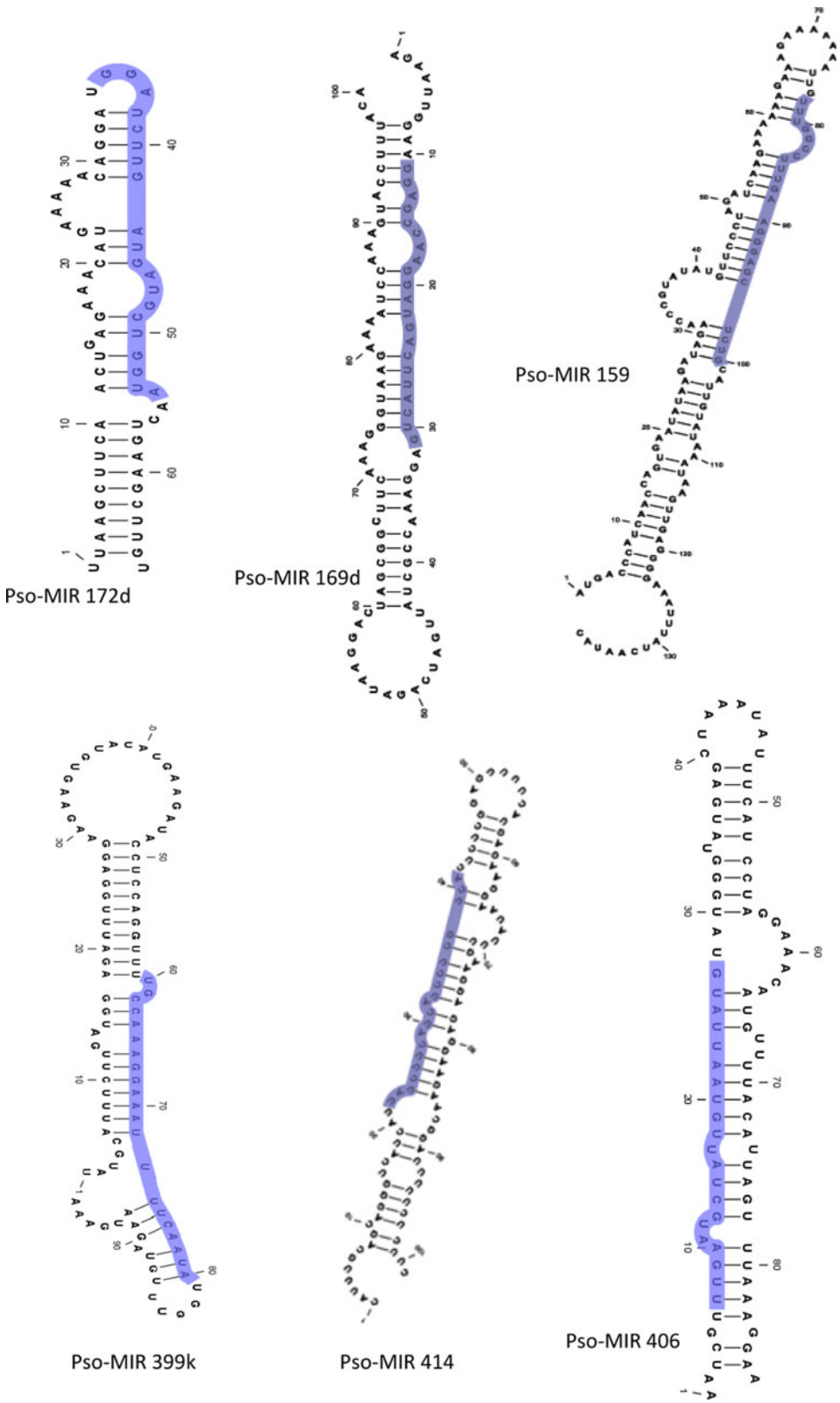

Fig. 2 Some of the secondary stem-loop structures of newly identified *P. somniferum* miRNAs. Mature miRNA sequences are colored (*blue*)

other hand, miR 397a potentially targets Laccase enzyme (Table 3) (Zhang et al. 2009). In rice, expression level of miR 397 is decreased during transition from undifferentiated to differentiated calli (Luo et al. 2006). Among five selected and measured opium poppy miRNAs, Pso-MIR414 were detected as relatively more abundant in

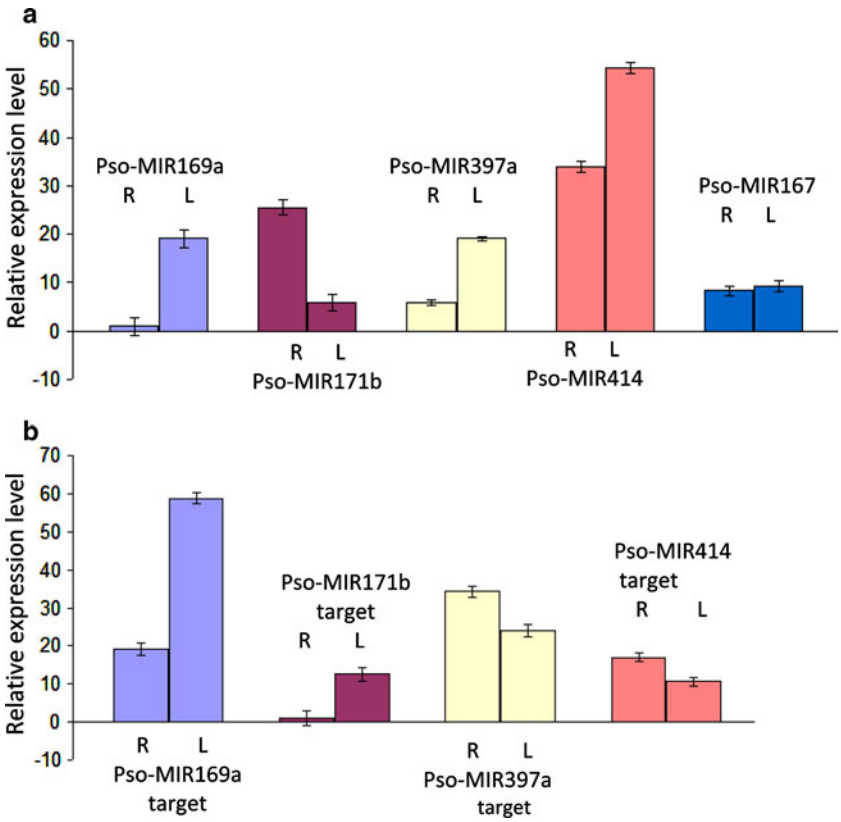

Fig. 3 Relative expression level comparisons in root (*R*) and leaf (*L*) tissues measured with qRT-PCR **a** miRNA expression levels **b** miRNA target transcript levels miRNA expression levels were calculated by dividing each sample's calibrated value by that of the lowest one. Relative expression levels of the target transcripts were also calculated the same way except they were first normalized with 18S rRNA expression levels

leaf and root tissues (Fig. 3a). Pso-miR169a potentially targeting nuclear transcription factor was quantified as the lowest in expression in both leaf and in root tissues compared with that of selected miRNAs (Fig. 3a).

P. somniferum miRNA targets

It has been proven that, due to high sequence complementarities between miRNA and mRNA target sites, miRNA-guided RISC matches with the target sites of mRNA to cleave the transcript or to inhibit the translation. Therefore, they regulate gene expression at posttranscriptional level (Rhoades et al. 2002; Bartel 2004; Jones-Rhoades and Bartel 2004). According to previous results, more than four mismatches between miRNA and target mRNA are not allowed (Bartel 2004; Schwab et al. 2005; Zhang et al. 2008). Because *P. somniferum* NCBI EST database is limited and proteins of *P. somniferum* have not yet been fully annotated, we could not find possible targets in EST database. Therefore, the identified miRNAs were subjected to BLASTn search in NCBI mRNA database of all plant species to predict the potential mRNA targets. We found 41 potential miRNA target mRNAs in all other plant species (only five of them are shown in Fig. 4 to save space). We have detected that the targets have distinct functions and they are diverse in sequence. The majority of target genes detected are transcription factors and functional proteins in plant metabolism and environmental stress response. In this respect, our results are consistent with previous studies (Supplementary Table 3) (Rhoades et al. 2002; Bonnet et al. 2004; Zhang et al. 2006c, 2008; Yin et al. 2008; Unver and Budak 2009). Additionally, the putative miRNA target transcripts were predicted using miRU plant target finder (http://bioinfo3.noble.org/miRNA/miRU.htm) (Supplementary Table 4) (Zhang 2005).

Target mRNA validation with qRT-PCR

We confirmed some of the computationally predicted conserved opium poppy miRNA targets with qRT-PCR. For normalization we used several control sequences as recommended in Rule 8 of Udvardi et al. (2008), 18s rRNA gene was selected as the most consistent reference. Expression levels of the target genes of Pso-miR169a (NM_001055667.1), Pso-miR171b (NM_116232.4), Pso-miR397a (NM_001154470.1), and Pso-miR414 (NM_001084524.1) were relatively measured in opium poppy root and leaf tissues (Fig. 3b). In leaf tissue, transcripts of Pso-MIR 414 target gene (NM_001084524.1) was found to be the lowest in expression compared with other genes, while Pso-MIR414 was detected as one of the most abundant miRNAs (Fig. 3a). Similarly, transcripts of Pso-MIR 171b target gene (NM_116232.4) were detected as

Table 3 List of computer-based predicted targets of *P. somniferum* miRNAs in GenBank mRNA database of all plant species

miRNA	Targeted protein	Target function	Targeted mRNAs or EST homologs of genes in all other plant species	Plant species
pso-MIR169a	Nuclear transcription factor	Transcription factor	ref|NM_001055667.1 |ref|NM_001153839.1 |ref|NM_001156465.1|	*Oryza sativa* *Zea mays* *Zea mays*
	Hypothetical protein		ref|NM_001139229.1|	*Zea mays*
	CCAAT-binding transcription factor			
pso-MIR397a	Ascorbate oxidase	Metabolism	ref|NM_001154470.1 |ref|NM_001051308.1|	*Zea mays* *Zea mays*
	Putative laccase LAC5-6	Signal transduction	ref|XM_002284437.1|	*Vitis vinifera*
			ref|XM_002280380.1|	*Vitis vinifera*
	Hypothetical protein		ref|XM_002278196.1|	*Vitis vinifera*
pso-MIR781	ATP dependent DNA ligase	DNA metabolism	ref|NM_105343.2|	*Arabidopsis thaliana*
pso-MIR835	Unknown protein		ref|NM_001084363.1|	*Arabidopsis thaliana*
pso-MIR844	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase	Signal transduction	ref|NM_001154322.1|	*Zea mays*
			ref|XR_077429.1|	*Vitis vinifera*
	Oligopeptide transporter	Stress response	ref|XM_002269631.1|	*Vitis vinifera*
		Transport	ref|XM_002270026.1|	*Vitis vinifera*
Pso-MIR172a	AP2 domain-containing transcription factor	Transcription factor	ref|XM_002310679.1|	*Populus trichocarpa*
pso-MIR169b	Aldehyde oxidase	Metabolism	ref|NM_001055667.1 |ref|NM_001111838.1 | ref|NM_001153839.1|	*Oryza sativa* *Zea mays* *Zea mays*
	Nuclear transcription factor	Transcription factor	ref|NM_001156465.1 |ref|NM_001139400.1 |ref|NM_001139229.1|	*Zea mays* *Zea mays* *Zea mays*
	CCAAT-binding transcription factor		ref|NM_001138257.1|	*Zea mays*
pso-MIR169d	Serine/threonine protein kinase	Signal transduction	ref|XM_002323991.1|	*Populus trichocarpa*
pso-MIR171b	Scarecrow-like transcription factor 6 (SCL6)	Transcription factor	ref|NM_116232.4|	*Arabidopsis thaliana*
pso-MIR397b	Laccase 90c	Signal transduction	ref|XM_002315095.1|	*Populus trichocarpa*
pso-MIR169n	Nuclear transcription factor	Transcription factor	ref|NM_001155626.1| ref|XM_002278813.1|	*Zea mays*
			ref| XM_002313271.1|	*Vitis vinifera*
				Populus trichocarpa
	CCAAT-binding transcription factor (CBF-B/NF-YA)		ref|XM_002299903.1|	*Populus trichocarpa*
			ref|NM_001057514.1|	*Oryza sativa*
			ref|NM_112983.4|	*Arabidopsis thaliana*
			ref|NM_001155603.1| ref|XM_002324680.1|	*Zea mays*
			ref|NM_001156465.1|	*Populus trichocarpa*
				Zea mays
pso-MIR414	AtM1/AtMYB101/MYB101 (myb domain protein 101)	Transcription factor	ref|NM_001084524.1|	*Arabidopsis thaliana*
pso-MIR166 g	Hypothetical protein, ATHB-15 (INCURVATA 4); DNA binding	Transcription factor	ref|XM_002310511.1| ref|NM_001084233.1|	*Populus trichocarpa* *Arabidopsis thaliana*
pso-MIR476a	Hypothetical protein, 40S ribosomal protein S3a	Metabolism	ref|XM_002283106.1| ref|XM_002314635.1|	*Vitis vinifera*
			ref|XM_002312509.1|	*Populus trichocarpa*
				Populus trichocarpa

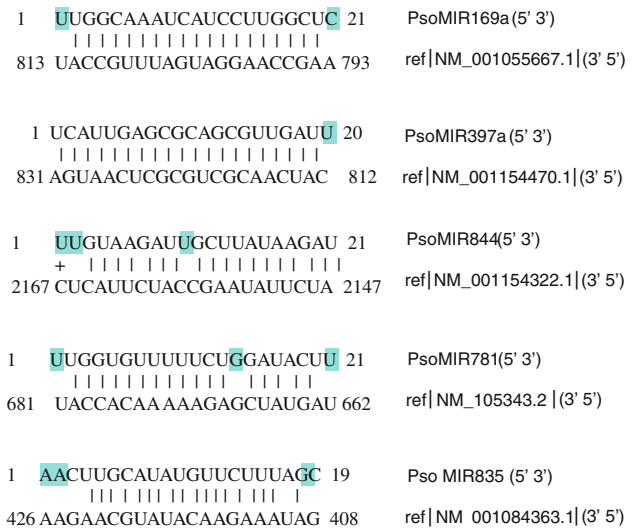

Fig. 4 Potential miRNA targets and their complementary sites within defined reference mRNAs in all plant species. *Dashed bases* show mismatches and G:U wobble pairing (*plus*). Watson–Crick pairing is seen (*vertical lines*)

having the lowest expression compared with other measured target genes in roots (Fig. 3b), while its miRNA (miRNA 171b) was measured as having the second highest expression in roots. Again, target gene (NM_001055667.1) expression of Pso-MIR169a was detected as the most abundant transcript in roots while its miRNA (Pso-MIR 169a) was detected as the lowest expression (Fig. 3a, b). Therefore, the target gene expression level measurements have been found to be correlated with miRNA quantification experiment outcomes, that is, a negative correlation was detected between three (Pso-MIR169a, Pso-MIR171b and Pso-MIR414) of the four pairs of miRNAs and their target mRNA transcripts in opium poppy root and leaf tissues. For Pso-MIR397a and its target transcript, there was a negative correlation between the root samples while no significant difference was observed for the leaf samples.

Discussion

Though several plant miRNAs have been identified via computational or experimental approaches, there is no available sequence or functional information about miRNAs of *P. somniferum* which is an important medicinal plant as a primary source of opiate production. In this study, we have identified 20 conserved miRNAs and their 41 potential targets in opium poppy. Of the limited number of EST sequences available in GenBank, 0.11% was found to contain potential miRNAs. Identified conserved miRNAs belong to 16 distinct miRNA families and many of their targets are transcription factors (such as CCAAT-binding transcription factor, scarecrow-like transcription factor 6/SCL6, INCURVATA4/ATHB15, MYB101, and nuclear transcription factor) playing a role in plant development, morphology and flowering time. Other targets are mainly involved in plant metabolism (ATP dependent DNA ligase, ascorbate oxidase, aldehyde oxidase), stress response (BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase), and signal transduction (serine/threonine protein kinase, laccase 90c) (Table 3). Although the target genes identified do not seem to be directly related to opiate biosynthesis, our miRNA identification results are consistent with previous data (Rhoades et al. 2002; Bonnet et al. 2004; Zhang et al. 2006c; Yin et al. 2008; Zhang et al. 2008; Unver and Budak 2009) with respect to functional categories of the target genes which could involve opiate metabolism directly or indirectly.

We have also detected that uracil nucleotide tends to be dominant at the first nucleotide position of the 5′ end of the mature miRNAs as previously reported (Zhang et al. 2006c, 2008). In several identified conserved poppy miRNAs, cytosine was found to be located at the 19th nucleotide position from 5′ end (bold in Table 1). In previous results such as Yin et al. (2008) and Zhang et al. (2007b, 2008) it has been shown that similarity between identified miRNAs and previously reported miRNAs are high. On the contrary, our findings present lesser similarity or conservation between identified conserved poppy miRNAs and previously known miRNAs. The possible reasons for this could be, (a) *P. somniferum* has limited or relatively low number of ESTs and no GSSs in GenBank, and therefore, we might not be able to capture the highly conserved miRNAs, (b) *P. somniferum* is phylogenetically less close to the aligned organisms used for miRNA search than tomato (Yin et al. 2008), soybean (Zhang et al. 2008), and cotton (Zhang et al. 2007b). Pso-MIR 169b and Pso-MIR 169n might specifically target CCAAT-binding transcription factor (CBF-B/NF-YA). Cai et al. (2007) showed that over-expression of *HAP3b* (a putative CCAAT-binding transcription factor that causes delayed flowering when mutated) in *Arabidopsis* causes induction of early flowering. Our finding suggests opium poppy miRNAs both target the transcription factor CBF-B/NF-YA and regulate flowering time in poppy. Pso-MIR 171b targets a plant-specific scarecrow-like transcription factor 6 (SCL6). It has been reported that the SCL6 regulates a variety of processes related to plant development (Llave et al. 2002; Reinhart et al. 2002). We have found that SCL6 is a potential target of Pso-MIR 171b, one of our identified miRNAs in poppy. Pso-MIR 172 family miRNAs potentially target AP2 (APETALA 2) transcription factor. It has been proven that the miR172 plays important roles in controlling the timing of flowering and floral morphology in plants. This miRNA was over-expressed in *Arabidopsis* by Aukerman and Sakai

(2003), and reported to cause early flowering and suppression in the floral organ specification. On the other hand, over-expression of an AP2-like protein, TARGET OF EAT1 (TOE1), causes late flowering (Aukerman and Sakai 2003). The translational repression of AP2 by miRNA 172 has also been reported (Chen 2004). Another role of miRNA 172 has been reported by Mlotshwa et al. (2006) in *Nicotiana benthamiana*. They presented that miRNA 172 acts in floral identity and flowering. Additionally, Lauter et al. (2005) studied miRNA 172 in maize and showed that miR172 down-regulates glossy15 (gl15), an AP2-like gene, and usually express in maize juvenile leaves to regulate transition of vegetative phase to the reproductive stage. Hence, Pso-MIR 414 is another identified miRNA which targets MYB101 transcription factor. MYB101 transcription factor has been reported to function in plant leaf development. Palatnik et al. (2003) produced a specific Arabidopsis transgenic line over-expressing a miRNA-resistant version of MYB33 and observed upward curled leaves. Similarly Millar and Gubler (2005) have transformed *Arabidopsis* with miRNA target site mutated MYB33 gene, and observed pleitrophic developmental defects along with abnormal curled leaves. We have presented here that Pso-MIR 414 potentially targets MYB transcription factor and regulates leaf development. Since miRNA 414 was found to be more expressed in the leaf and the miRNA 414 target (NM_001084524.1, MYB101) has been measured more suppressed in the root tissue (Fig. 3a, b), our findings support the involvement of miRNA 414 in leaf development. Pso-MIR 166g is another identified poppy miRNA playing important roles in plant development through specifically targeting INCURVATA4/ATHB15 transcription factor. It has been shown that INCURVATA4/ATHB15, a member of class III HD-ZIP transcription factor, participates in the control of leaf polarity, patterning of shoot and root apical meristem, and stem vascular differentiation (Prigge et al. 2005; Williams et al. 2005; Ochando et al. 2006). Identified *P. somniferum* miRNAs not only target the genes related with plant development but also target genes involved in several biological metabolisms including signal transduction and stress tolerance. In this study, we found that Pso-MIR 397b potentially targets Laccase gene. Zhang et al. (2009) have also detected miRNA 397 in potato and they predicted its target gene as Laccase. Luo et al. (2006) reported that expression level of miR 397 was decreased during transition from undifferentiated to differentiated calli. Pso-MIR 169d potentially targets serine/threonine kinase, Pso-MIR 844 targets BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase. These predicted miRNA targets are involved in signal transduction pathways. 40S ribosomal protein is potentially targeted by Pso-MIR 476a while ATP-dependent DNA ligase is targeted by Pso-MIR 781. These target genes are related to metabolism. Target discovery of some poppy miRNAs such as Pso-MIR 771, Pso-MIR 859, Pso-MIR 399k, Pso-MIR 406, Pso-MIR 159, Pso-MIR 446, and Pso-MIR 869 failed in the GenBank database. The possible reasons could be that (a) expression level of those mRNAs might be very low or their expression might be time or specific tissue/condition dependent, (b) GenBank database might not yet cover those transcript sequences, (c) identified miRNAs might be *P. somniferum*-specific unique miRNAs and therefore their targets might be poppy-specific, and (d) EST database of *P. somniferum* restricts the finding of other potential miRNAs and their targets. qRT-PCR analysis presented the expression level differences of the identified different poppy miRNAs and their target genes in different tissues, further confirming the functional activity of the detected miRNAs. Hence, the computer-based identified poppy miRNAs have been validated via experimental approach by SYBR Green I assay.

Conclusion

In this study we have discovered opium poppy, *P. somniferum*, miRNAs using computer based homology search approach in NCBI EST database. 20 conserved miRNAs with 1–4 mismatches have been identified based on conservation of previously identified plant miRNAs. The detected miRNAs' precursors are varied from 59 to 210 nucleotides in length with an average of 96.85 ± 27.72 (Supplementary Table 3) and the length of mature miRNAs have ranged from 20 to 23 nucleotides. We have observed that many of the mature miRNAs have uracil as the first nucleotide at the 5′-end, and also some of the mature miRNA sequences contain cytosine at the position 19 from the 5′ end. Additionally, uracil is found to be dominant nucleotide in pre-miRNA sequences of poppy. qRT-PCR analysis of some of the identified *P. somniferum* miRNAs and miRNA target genes showed that expression levels of miRNAs differ both in leaf and root tissues. This study presents the first report for identification of *P. somniferum* miRNAs and their potential targets validated experimentally through qRT-PCR.

Acknowledgments We would like to thank Prof. Neset Arslan of Ankara University for supplying poppy seeds and Prof. Hikmet Budak of Sabancı University for his kind assistance.

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