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Identification of miRNAs from French bean (*Phaseolus vulgaris)* under low nitrate stress

[Fransız fasülyesinde (*Phaseolus vulgaris*) düşük nitrat stresi altında miRNA'ların tanımlanması]*

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ABSTRACT

Objective: In this study, we report the role of miRNAs involved under nitrogen starvation from widely grown vegetable crop, French bean. In recent years, a great deal of attention has been paid to the elucidation of miRNAs involved in low nitrate stress.

Methods: To identify miRNAs expressed under stress, cDNA libraries were analyzed.

Results: We reported the nine potential miRNAs with 67 targets involved in nutrient transporters and other stress specific genes. Among the miRNA sequences obtained 6 sequences belong to miR172 family, one with miR169. RT-PCR analysis of expression of miR172 family was induced upon low nitrate stress while miR169 family was repressed. In addition, Pvu-SN7b and Pvu-miR16 may be new members of miRNA172 and miR169 families, respectively.

Conclusion: The targets of Pvu-SN7b were major protein kinases, one among which is the Protein Kinase CK2. CK2 Kinase is found to involve in transcription-directed signaling, gene control and cell-cycle regulation**.** Other targets of Pvu-SN7b were involved in DNA-dependent transcription regulation, photo-periodism, calcium-mediated signaling. Pvu-miR16 targets Thymidine kinase, the key enzyme of deoxy-nucleotide synthesis. The cleavage of these targets affects cell proliferation there by affecting nodule formation. Pvu-miR8 inhibits translation of its target protein Pre-protein translocase, a membrane-bound protein transporter involved in trans-membrane protein transportation. Together these results denote the response and role of miRNAs to nitrate-limiting conditions in French bean.

Key Words: French bean; low nitrate; protein kinase; photo-periodism

Conflict of Interest: There were no potential conflicts of interest disclosed by the authors

ÖZET

Amaç: Çalışmada yaygın olarak yetiştirilen bitki tohumu olan Fransız fasulyesinde düşük nitrat koşulları altında miRNA'ların rolü araştırılmıştır. Son yıllarda düşük nitrat stresi ile ilişkili miRNA'ların aydınlatılması gittikçe önem kazanmaktadır.

Yöntem: Stres altında ifadelenen miRNA'ları tanımlamak amacıyla cDNA kitaplıkları analiz edilmiştir.

Bulgular: Dokuz potansiyel miRNA ve bunların besin taşıyıcıları ve strese özgül genlerle ilişkili 67 hedefi saptanmıştır. miRNA dizilerinin 6 tanesi miR172, bir tanesi ise miR169 ailesine ait olarak bulunmuştur. RT-PCR analizlerinde, miR172 ailesinin ifadelenmesi düşük nitrat stresi koşullarında artarken, miR169 ailesi baskılanmaktadır. Ayrıca Pvu-SN7b ve Pvu-miR16'nın sırasıyla miRNA172 ve miR169 ailelerinin yeni üyeleri olabileceği düşünülmektedir.

Sonuç: Pvu-SN7b'nin hedefleri protein kinaz CK2'nin de içinde bulunduğu protein kinazlardır. CK2 kinaz transkripsiyon yönlendirmeli sinyal yolu, gen kontrolü ve hücre döngüsünün düzenlenmesi ile bağlantılıdır. Pvu-SN7b'nin diğer hedefleri DNA-bağımlı transkripsiyonun düzenlenmesi, photoperiodism, kalsiyum aracılı sinyal yollarında görev almaktadır. Pvu-miR16 deoksinükleotid sentezinde anahtar enzim olan timidin kinazı hedef almaktadır. Bu hedeflerin kesilmesi hücre büyümesini dolayısıyla nodül oluşumunu etkilemektedir. Pvu-miR8 hedef proteini olan Pre-protein translokazın translasyonunu inhibe etmektedir. Pre-protein transkolaz membran-bağlı protein taşıcısı olup, transmembran protein taşınmasından sorumludur. Bütün bu bulgular Fransız fasülyesinde sınırlı nitrat bulunması durumunda miRNA'ların yanıt ve rollerini göstermektedir.

Anahtar Kelimeler: Fransiz fasulyesi, düşük nitrat, protein kinaz, foto-periodism

Çıkar Çatışması: Yazarlar çıkar çatışması olmadığını beyan etmiştir.

Introduction

MicroRNAs (miRNAs) are small RNA molecules, 20-24 nucleotides in length, recognized as important regulators of gene expression in animals and plants [1,2]. In plants, each miRNA precursor (pre-miRNA) is processed by the Dicer-like enzyme DCL-1 through two consecutive cleavage reactions to generate a single small duplexed-RNA containing the miRNA and its partially complementary strand or miRNA*, leaving a 5'-phosphate and a two-nucleotide overhang at the 3'-end [3]. The miRNA precursor may be transported out of the nucleus by HASTY or retained in the nucleus and further processed by DCL-1 to release the stem portion of the hairpin as an miRNA: miRNA* duplex [1,4]. The duplex, which comprises a mature miRNA of 21 nucleotides and a similarly sized miRNA* fragment on the opposite arm of the miRNA precursor, is then presumably unwound by a helicase, releasing the single stranded mature miRNA. The miRNA then enters the RNA induced silencing complex and guides the complex to identify target messages for post-transcriptional gene silencing through direct target cleavage.So far, a large number of miRNAs have been found in various plant species [3, 5-14]. In plants, miRs were shown to regulate diverse aspects of development like leaf polarity [15], leaf shape [7], the transition from the juvenile to the mature growth phase [16], flowering time [17], stomatal development and nodule development. miRNAs also regulate the adaptation of plants to abiotic stresses, including macro-nutrient limitations [11,18,19]. Little information about stress or nutrient-responsive plant miRs is available. This is due to their often low expression levels and the absence of miR or MIRNA gene probes on widely used transcriptome platforms such as Affymatrix Gene-Chips. Custom-made microarrays can be designed to include probes for miRNAs and MIRNA genes for a broader response analysis, but these are not very sensitive [20,21]. Reverse transcription followed by quantitative-PCR analysis (qRT-PCR) with non-specific double-stranded DNA-binding fluoro-phores, such as SYBR Green, is a powerful alternative for highly sensitive, rapid, multi-parallel, and cost-effective expression analysis [22-24] reported two qRT-PCR-based methods to measure the levels of mature miRs. The first approach relies on *in-vitro* polyadenylation of mature miRs followed by reverse transcription with an oligo dT adapter primer and amplification using SYBR Green with a miR-specific forward primer and a compatible reverse primer. In the second approach, each specific miR is reverse transcribed from total RNA using a specific stem-loop primer, followed by TaqMan PCR amplification. Although it is desirable to quantify the biologically active mature miR species, a limitation of both qRT-PCR methods is that they are unable to differentiate the expression strengths of MIRNA genes that yield (nearly) identical mature miR molecules. Although pri-miRs are not the biologically active molecules, several previous studies have shown that the response of a pri-miR can reflect that of the encoded mature miR [10,19,25] and thus can serve as a valid indicator. Therefore, qRT-PCR profiling of pri-miR scan serve as a useful tool to discover responses to particular stimuli, which can then be confirmed by analysis of the mature species.

French bean is one of the most important vegetable crops grown worldwide. Furthermore, the crop typically gives high yields due in major measure to the use of large amounts of nitrogen fertilizer, which also contributes to a large release of active nitrogen to the environment. Studies on French bean have also contributed significantly to our understanding of plant development and evolution as a genetic model system. More recently, this knowledge has been employed to elucidate the regulatory functions of miRNA genes. A genome-wide computational prediction of miRNA genes and their characterization with respect to expression, putative targets, whole genome duplication, and allelic diversity has been reported. However, information about the way by which miRNA are regulated by abiotic stresses in general and by low nitrate in particular is unavailable. In the present work, we used the traditional cloning method to detect the regulation of miRNAs in French bean leaves and roots under transient low nitrate availability. The corresponding mature miRNAs along with some predicted target genes have also been analyzed for their expression pattern by real time qRT-PCR. Finally, the analysis and prediction of the miRNAs interaction with target genes was performed.

Materials and Methods

Plant material and growth conditions

French bean (*Phaseolus vulgaris Selection - 9*) seeds were surface-sterilized and germinated in sand in a plastic containers 27 ^{o}C with a 16 h light/8 h dark photoperiod cycle for 8 days with half-strength modified Hoagland nutrient solution containing two nitrate concentrations : 4 mM $Ca(NO_3)_2.4H_2O$, optimal nitrate condition (+N) and 0.04 mM $Ca(NO_3)_2.4H_2O$, low-nitrate (-N) and then the seedlings were collected at 0, 48 h and 96 h after the onset of stress.

Isolation and cloning of miRNAs

Total RNA was isolated from both control and stressed seedlings by TRizol (Invitrogen), and then treated with RNase-free DNase I according to the manufacturer's instructions. A small pooled RNA library was constructed using mirVana^{TM} miRNA Isolation Kit (Ambion). Cloning of miRNAs was performed using miRNA cloning kit (Takara). Small RNAs (200 nt) were separated on a denaturing 15% polyacrylamide gel. 18 to 26 nt bands were excised and recovered using 30 µl RNase-free water. The recovered small RNAs were ligated sequentially with a 3' and 5' adapters and were purified by 10% denaturing polyacrylamide gel

electrophoresis; the small RNAs ranging in size from 62 to 70 nt were eluted from the gel. Reverse transcription was performed using the adapter primers, and the recovered DNA amplification product was cloned into pGEMT-Easy (Promega) and transformed into DH5α Competent cells for plasmid cultivation. Plasmids were isolated from individual colonies for amplified cultivation, sequenced and processed for BLAST analysis against the NCBI genomic data sets for French bean.

Sequence analysis and prediction of fold-back structures

All sequences were used to search the Rfam (www.sanger.ac.uk/Software/Rfam) database with BLASTN, to identify sequence tags originated from coding exons, repeats, rRNA, tRNA, snRNA, and snoRNA, which were removed from the small RNA sequences, and the remaining sequences were compared against rice and Arabidopsis ncRNAs deposited in the NCBI GenBank database and Rfam 8.0 database. Only the miRNAs that perfectly mapped onto the genome were considered in the current study. Candidate miRNA sequences with perfect matches against these sets were used for fold-back secondary structure prediction, which was conducted on web-based program Mfold 3.1 [26], according to the criteria described by [27].

Target gene prediction

In order to indentify the accuracy of the target genes, we adopted a set of rules proposed in earlier reports for predicting miRNA targets [28,29]. These criteria are as follows: allowing one mismatch in the region complementary to nucleotide positions 2–12 of the miRNA, but not at position 10/11, and three additional mismatches between positions 12 and 22 but with no more than two continuous mismatches. Therefore, candidate miRNA target genes were determined using publicly available prediction algorithms, including psRNATarget program (http://plantgrn.noble.org/psRNATarget) with default parameters. Newly identified miRNA sequences were used as custom miRNA sequences; *Phaseolus vulgaris* (French bean) DFCI Gene Index (PVGI) Release 3.1 was used as custom plant database.

Expression analysis of miRNAs by stem -loop RT-PCR

Five µg sample of total RNA was used for cDNA synthesis using the Invitrogen Reverse Transcription reagents kit (Invitrogen, USA). This was completed by reverse transcription using the Applied Biosystems TaqMan microRNA Reverse Transcription Kit and miRNA-specific stem-looped RT primers. Many studies show that miR159, miR167, miR169, miR172, miR393, miR395, miR396, miR398, and miR399 are important for plant growth as well for response to environmental stress [10,19,30-33]. Thus, we selected these nine miRNAs. Mitochondrial 5S RNA was used as an internal control to normalize all data. The resulting products with all primer combinations were initially visualized on 2% agarose gel to confirm the generation of a single product of the correct size.

Validation of miRNA expression by Northern blot analysis

For miRNAs quantification, northern blot hybridization was conducted using high sensitive miRNA Northern blot assay kit (Signosis, USA). 30 µg total RNA of each sample was electrophoresed on 15% polyacrylamide gel and transferred to membrane. Antisense RNA biotin labeled in the 5' end (Invitrogen) was used for hybridization probes. The SYBR Green® II stained (Biotech) 5S RNA used as loading control.

Results

Characterization of stress associated miRNAs from French bean

To identify the conserved and novel miRNAs involved in response to low nitrate stress; we constructed a pooled small RNA library (16 to 30 nt). More than 50 clones were selected from this library and 24 clones ranging in size from 19 to 24 nt were obtained for further sequencing and fold-back structure prediction. For validation of candidate miRNAs, cDNA libraries were constructed by poly (A)-tailed RT-PCR. The libraries are suitable for further validation of newly isolated and known miRNAs and for expression pattern analysis by semi-quantitative RT-PCR and Northern blot analysis. Thirty three sequences were obtained ranging in size from 18 to 24 nt. BLASTN searches revealed that 15 (46%) of these sequences were known rRNAs, nine (27%) sequences were well matched against French bean ESTs. However, the remaining nine sequences were not matched, either from French bean or related species genomic and EST data sets, these sequences were excluded from further analysis. The size distribution information is listed in Figure 1. Nine sequences were selected for miRNA prediction found by sequence similarity search against miRBase (http://www.mirbase.org/search.html). Five of the newly found miRNAs belong to one size family of 20 nt, two of them are of 22 nt in size. Four of the nine newly identified miRNAs begin with a 5' uridine, which is a characteristic feature of miRNAs; Pvu-miR13, Pvu-miR17a begins with C, Pvu-miR8 with G, Pvu-SN7b, Pvu-miR16 with A (Table-1). Pvu-miR17a and Pvu-miR17b have identical sequences while differing from Pvu-miR17d. The precursors of these 9 predicted miRNAs varied in their length between 70 to 180 nt and all were capable of forming stable stem-loop structure (Supplementary File S1). The homology search of precursor sequences and the mature sequences of the predicted miRNAs was done by BLAST with existing sequences downloaded from miRBase (http://www.mirbase.org/search.html). Out of the nine sequences obtained, eight sequences showed homology with

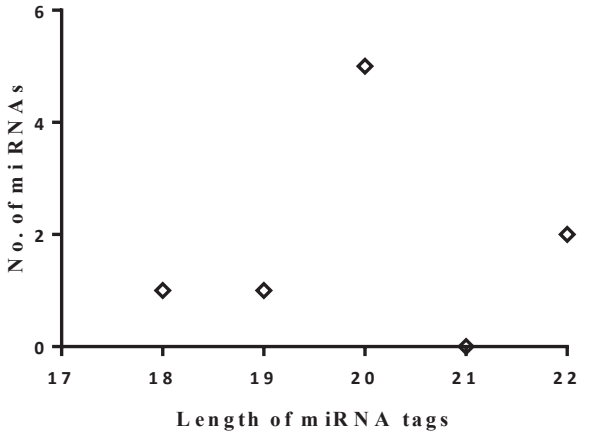

Figure 1. Length of miRNA expressed in French bean under low nitrate stress

miR172 members and one (Pvu-miR16) with miR169. So far, 124 members of miRNA172 family and 116 members of 169 were deposited in miRBase (http://www.mirbase.org).

Target gene prediction

Micro RNAs regulate gene expression by either translation inhibition or target mRNA cleavage thus altering the cellular protein composition in response to stress. Regulation of gene expression is one of the ways by which the plants adapt and become capable of surviving under adverse environmental conditions. Sixty seven putative target genes were identified for nine newly predicted miRNA sequences. The number of genes targeted by each miRNA varies significantly with Pvu-SN7b having highest number of targets (52) and Pvu-miR16 having single target gene. Two among the nine (Pvu-SN17a and Pvu-miR17b) had targeted none of the genes (Table-2). The targets were involved in transcription regulators, protein transporters, ubiquitin modification and metabolic enzymes involved in nucleotide synthesis and polymerization. Pvu-SN7b targets found to be involved in various cellular processes including membrane bound protein kinases, circadian system, Ca^{2+} cell signaling, auxin regulation, ATP binding proteins, DNA dependent transcription regulation (APETALA) genes. The targets of Pvu-miR13 and Pvu-miR15 showed ROS removal and tRNA processing O-sialo glycoprotein, Ethylene over production (Table 2).

Validation of new cloned miRNAs by semi-quantitative RT-PCR and Northern blot analysis

To confirm the existence of the newly cloned miRNAs and validate their temporal expression trends obtained from French bean seedlings under low nitrate conditions. Semi-quantitative RT-PCR technique was used. It was shown that all the identified miRNAs were consistent with the previous computational prediction. Nine miRNAs showed several different expression patterns and would be categorized into three types; Pvu-Sn7b, Pvu-miR13, and Pvu-miR15 showed substantial increase in their expression while Pvu-miR16 showed repression under stress. miR159, mir167, and miR399 remain unaltered showed similar expression to that of 5S RNA as control. (Figure 2)

Discussion

MiRNA cloning is extensively used for miRNA detection in eukaryotes as a classic method [11,29,34]. It can detect the known miRNAs, and more importantly, it can discover the novel, species-specific or stress-regulated specific miRNAs. Thousands of plant miRNAs have been discovered and deposited in miRBase. However, many stress-specific miRNAs were yet to be characterized. The studies of miRNAs mainly concentrated on model plants such as rice and *Arabidopsis* and recently, more efforts have been made to detect miRNAs in other plants including French bean [35,36]. In this study, 9 miRNAs

Table 1. miRNAs obtained from French bean seedlings under low nitrate stress

miRNA	Sequence	Length	Pre-miRNA length	miRNA Family
Pvu-SN7	UAUUAAUCUUGAUGAUGCUGCA	22	210	miR172
Pvu-SN7b	AGAAUCUUGAUGAUGCUGCA	20	168	miR172
Pvu-miR8	GGAAUAUUGAUGAUGCUGAU	20	210	miR172
Pvu-miR13	CAAUUUGGGUGCCCCUGCUG	20	144	Unknown
Pvu-miR15	UUGGGUGCCCCUGCUGUUUCUU	22	144	Unknown
Pvu-miR16	AAGACAUCGCCAAGGAGACU	20	141	miR169
Pvu-miR17a	CUAUUAAUCUUGAUGAUC	18	166	miR172
Pvu-miR17b	UUAAUCUUGAUGAUGCUGC	19	144	miR172
Pvu-miR17d	UGGUGCACUUGAUGAUGCUG	20	151	miR172

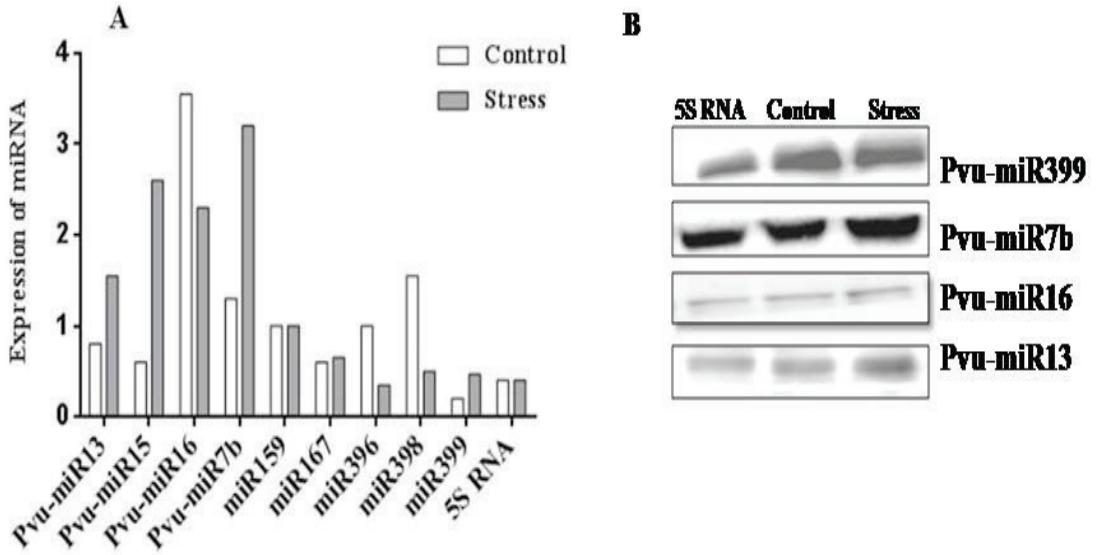

Figure 2. (A) Differential expression of stress specific miRNAs under low nitrate stress analyzed by sqRT-PCR **(B)** Gel-blot analysis of miRNAs expressed under low nitrate stress. SYBR Green® II stained (Biotech) 5S RNA were used as control.

were characterized from cDNA library constructed utilizing miRs cloned from French bean seedling under low nitrate stress. Two miRNAs (Pvu-SN7b and Pvu-miR16) were not conserved among the cloned ones. It seems to be consistent with the reports that most miRNAs are conserved in plants [1]. However, reports on species, tissue and stress-specific miRNAs have shown that some of those miRNAs involved in tissue development and stress response might be unique. In addition, the sequence of Pvu-SN7b and Pvu-miR16 were almost identical with *Arabidopsis* miRNA172 and miR169 but different from the others. So it is possible that our newly cloned French bean miRNAs are tissue or stress specific and these may be new members of the families.

The knowledge of target gene's function is helpful if we are going to decide whether the further study of candidate miRNAs is necessary. Earlier research in *Arabidopsis* has demonstrated that target genes of candidate miRNAs were predominantly transcription factors [11] and most miRNAs were involved in many diverse biological processes [37].The full list of the predicted target genes of identified miRNAs had been listed in this study (Table 2). In the long course of evolution, plants have developed highly specialized and complicated molecular networks to counter low nitrate stress; the activation of specific stress response-genes seems to be a universal adaptation strategy. Among those target genes, transporters are particularly important, because they have been extensively studied in model plants such as rice and Arabidopsis [38-40]. According to the function of the target genes (Table 2), we can divide the identified miRNAs into three categories. The first includes miR169 and miR172, which target transcription factors involved in further regulation of gene expression and signal transduction. The targets of miR169s have several HAP2 transcription factors associated with nutrient deficiency and drought stress [29]. Our experiments showed that the expression of miR169 species had been repressed under low nitrogen and was consistent with the response of the pri-miR169 under low nitrogen treatment in *Arabidopsis*. It mainly targeted with Thymidine kinase, the key enzyme of thymine nucleotide biosynthesis which is also the regulatory enzyme balancing cellular ribonucleotide to deoxy-ribonucleotide concentration. The inhibition of this enzyme has its impact on cell proliferation during nodule formation. Thus ultimately affecting nitrate uptake and plant growth. Repression of miR169s by nitrate limitation, as detected in our experiments, points toward a potential mechanistic link between low nitrate status and nodule development in legumes. High abundance of miR169 in phloem sap during nitrate replete growth and the sharp decrease during N and P limitation also flags miR169 as a potential long-distance signal that is able to report shoot N and P status to the roots, similar to the role of miR399 [25]. The targets of miR169s are several HAP2 transcription factors (i.e. nuclear factor YA subunits (NF-YA) [41-43]. In *Arabidopsis,* miR169 was reported to influence drought resistance via inhibition of the A5 subunit of NF-Y, a ubiquitous transcription factor that is highly expressed in guard cells and crucial for the expression of a number of drought stress-responsive genes [43]. In addition to the effects of the nitrate transporter CHL1 [44] or nitrate reductase mediated nitric oxide generation [45] on stomatal opening, low expression of

miR169 during nitrate limitation could thus contribute to drought tolerance of N-limited plants [46,47]. In legume species, nodule development is dependent on the presence of previously established nodules and N/nitrate availability, creating a root-to-shoot signal that activates the CLAVATA1-like receptor kinase SUNN in *M. truncatula* or HAR1 in *Lotus japonicus*. A recent report suggests that a nitrate-induced CLAVATA3/ESR-related (CLE) peptide is this root-to-shoot signal [48]. MiR169 over expression or knockdown of HAP2-1 leads to a developmental block of nodule formation [41].

MiR172 has eight potential target genes including APETALA-2(AP2) like transcription factors. In French bean, miR172 (Pvu-SN7b), also known as tasselseed4 (ts4), was shown to be involved in the regulation of French bean floral organ identity and meristem acquisition through the target gene which is the APETALA-2 (AP2) transcription factor 1 [49]. Pvu-SN7b targeted as many as 52 genes most of which are Protein kinases such as, casein kinase (CK2). Studies concerning functions of plant CK2 have shown that this enzyme is involved in many processes, including light responses and growth control, cell division and cell cycle regulation, seed development, and salicylic acid (SA), and abcisic acid (ABA) signaling pathways. CK2 interacts and phosphorylates many transcription factors, affecting their DNA binding activity. Among these transcription factors, CCA1 (circadian clock-associated 1) and LHY (late elongated hypocotyl) are essential for the regulation of the endogenous circadian clock in *Arabidopsis*. CK2's association with and phosphorylation of diverse chromatin components and modifiers, our results strongly suggest a global role for protein kinase CK2 in nucleosomal remodeling processes that are particularly important at transition points such as cell cycle (re-)entry [50]. Regarding the circadian function, CK2 has emerged as a conserved molecular component modulating the subcellular localization and stability of key clock proteins. CK2 phosphorylates the Arabidopsis central clock components CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY). Furthermore, the CCA1 phosphorylation was proposed to be important for CCA1 clock function. Over-expression of CKB3 or CKB4 leads to period shortening and altered day-length-dependent regulation of developmental output. The circadian clock is responsible for the integration of temporal and photic information that regulates hypocotyl elongation [51].

Conclusions

The aim of this study was to identify the new candidate miRNAs involved in low nitrate stress response in French bean seedling. Nine miRNAs were discovered, including newly cloned and two conserved (miRNA172 and miRNA169). In addition, Pvu-SN7b and pvu-miR16 may be new members of miRNA172 and miR169 families respectively and French bean specific miRNAs in our miRNA library. Target prediction of candidate miRNAs and functional analysis showed that some of

Table 2. Description of predicted targets of miRNA expressed under low nitrate stress

miRNA	Target Description	Target involved
Pvu-SN7	Chromosome chr17 scaffold_101, Nodulin- like protein	Copper ion binding, electron transport, trans-membrane channeling
Pvu-SN7b	Protein kinase CK2 alpha chain S-receptor kinase-like protein 2 Floral homeotic protein APETALA 2 PHAP2B protein	Regulating casein kinases Cell signaling, development, stress response DNA dependent transcription regulation and meristem development and maintenance DNA dependent transcription regulation
Pvu-miR8	Pre-protein translocase	Trans-membrane, signal directed protein transportation
Pvu-miR13	L-ascorbate peroxidase 1, cytosolic	Stress induced ROS scavenging
Pvu-miR15	O-sialoglycoprotein endopeptidase	tRNA processing; Required for the formation of a threonylcarbamoyl group on adenosine at position 37 (t_6A37) in tRNAs that read codons beginning with adenine
Pvu-miR16	Thymidine kinase	Pyrimidine synthesis
Pvu-miR16	Chromosome chr17 scaffold	unknown
Pvu-miR17d	RING-H2 finger protein A cellular membrane protein involved in RNA binding.	Protein involved in ubiquitin-like modifier processing, activation, conjugation or deconjugation such as Ubl-activating enzymes (E1s), Ubl-conjugating enzymes (E2s), Ubl-protein ligases (E3s)

them are directly or indirectly involved in stress response. The expression patterns of miRNAs were analyzed by stem- loop RT-PCR and real-time PCR. It provided an indication that miRNA172 was involved in French bean seedlings response to low nitrate stress and showed an Arabidopsis or rice like regulation mechanism. Other species or tissue specific miRNAs might also be directly or indirectly involved in this process.

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Conflict of Interest: There were no potential conflicts of interest disclosed by the authors

References

[1] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281–297.

[2] Voinnet O. Origin, biogenesis, and activity of plant microRNAs. Cell 2009; 136:669–687.

[3] Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. MicroRNAs in plants. Genes Dev 2002; 16: 1616–1626.

[4] Kidner CA and Martienssen RA. The developmental role of microRNA in plants. Curr Opin Plant Biol 2005; 8: 38–44.

[5] Llave C, Kasschau KD, Rector MA, Carrington JC Endogenous and silencing-associated small RNAs in plants. Plant Cell 2002; 14: 1605–1619.

[6] Park W, Li J, Song R, Messing J, Chen X. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. Curr Biol 2002; 12: 1484–1495.

[7] Palatnik JF, Allen E, Wu X, Schommer C, Schwab R,Carrington JC, Weigel D. Control of leaf morphogenesis by microRNAs. Nature 2003; 425: 257–263.

[8] Bonnet E, Wuyts J, Rouze P, Van de Peer Y. Detection of 91 potential conserved plant microRNAs in *Arabidopsis thaliana* and *Oryza sativa* identifies important target genes. Proc Natl Acad Sci USA 2004; 101: 11511–11516.

[9] Floyd SK and Bowman JL. Gene regulation: Ancient microRNA target sequences in plants. Nature428, 485–486 from moss. Plant J 2004; 43: 837–848.

[10] Jones-Rhoades MW and Bartel DP. Computational identification of plant microRNAs and their targets, including a stress induced miRNA. Mol Cell 2004; 14: 787–799.

[11] Sunkar R and Zhu J-K. Novel and stress-regulated micro-RNAs and other small RNAs from Arabidopsis. Plant Cell 2004; 16: 2001–2019.

[12] Wang J-F, Zhou H, Chen Y-Q, Luo Q-J, Qu L-H. Identification of 20 microRNAs from *Oryza sativa*. Nucleic Acids Res 2004; 32: 1688–1695.

[13] Adai A, Johnson C, Mlotshwa S, Archer-Evans S, Manocha V, Vance V, Sundaresan V. Computational prediction ofvmiRNAs in *Arabidopsis thaliana*. Genome Res 2005; 15: 78–91.

[14] Bedell JA. et al. Sorghum genome sequencing by methylation filtration. PLoS Biol 2005; 3: e13.

[15] Emery JF, Floyd SK, Alvarez J, Eshed Y, Hawker NP, Izhaki A, Baum SF, Bowman JL. Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. Curr Biol 2003; 13: 1768–1774.

[16] Wu G, Poethig RS. Temporal regulation of shoot development in Arabidopsis thaliana by miR156 and its target SPL3. Development 2006; 133: 3539–3547.

[17] Aukerman MJ and Sakai H. Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. Plant Cell 2003; 15: 2730–2741.

[18] Fujii H, Chiou TJ, Lin SI, Aung K, Zhu JK. A miRNA involved in phosphate-starvation response in Arabidopsis. Curr Biol 2005; 15: 2038–2042.

[19] Bari R, Datt Pant B, Stitt M, Scheible WR. PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol 2006; 141: 988–999.

[20] Axtell MJ and Bartel DP. Antiquity of microRNAs and their targets in land plants. Plant Cell 2005; 17: 1658–1673.

[21] Liu HH, Tian X, Li YJ, Wu CA, Zheng CC. Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. RNA 2008; 14: 836–843.

[22] Udvardi MK, Czechowski T, Scheible WR. Eleven golden rules of quantitative RT-PCR. Plant Cell 2008; 20: 1736–1737.

[23] Shi R, Chiang VL. Facile means for quantifying microRNA expression by real-time PCR. Biotechniques 2005; 39: 519–525.

[24] Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33: e179.

[25] Pant BD, Buhtz A, Kehr J, Scheible WR. MicroRNA399 is a long distance signal for the regulation of plant phosphate homeostasis. Plant J 2008; 53: 731–738.

[26] Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003; 31: 3406–3415.

[27] Ambros V et al. A uniform system for microRNA annotation. RNA 2003; 9: 277–279.

[28] Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D. Specific effects of microRNAs on the plant transcriptome. Dev Cell 2005; 8: 517–527.

[29] Sunkar R, Girke T, Jain PK, Zhu JK. Cloning and characterization of microRNAs from rice. Plant Cell 2005; 17: 1397–1411.

[30] Raghothama K. Phosphate acquisition. Annu Rev Plant Biol 1999; 50: 665–693.

[31] Tesfaye M, Liu J, Allan DL, Vance CP. Genomic and genetic control of phosphate stress in legumes. Plant Physiol 2007; 144: 594- 603.

[132 Lopez-Bucio J, Herna´ndez-Abreu E, Sa´nchez-Caldero´n L, Pe´rez- Torres A, Rampey RA, Bartel B, Herrera-Estrella L. An auxin transport independent pathway is involved in phosphate stress-induced root architectural alterations in Arabidopsis. Identification of BIG as a mediator of auxin in pericycle cell activation. Plant Physiol 2005; 137: 681–691.

[33] Jiang C, Gao X, Liao L, Harberd NP, Fu X. Phosphate starvation root architecture and anthocyanin accumulation responses are modulated by the gibberellin-DELLA signaling pathway in Arabidopsis. Plant Physiol 2007; 145: 1460–1470.

[34] Arazi T, Talmor-Neiman M, Stav R, Riese M, Huijser P, Baulcombe DC. Cloning and characterization of micro-RNAs from moss. Plant J 2005; 43(6): 837-48.

[35] Lauter N, Kampani A, Carlson S, Goebel M, Moose SP. MicroRNA172 down-regulates glossy15 to promote vegetative phase change in maize. Proc Nat Acad Sci USA 2005; 102: 9412–9417.

[36] Mica E, Gianfranceschi L, Pe` ME. Characterization of five microRNA families in maize. J Exp Bot 2006; 57: 2601–2612.

[37] Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JDG. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 2006; 312: 436–439.

[38] Muchhal US, Pardo JM, Raghothama K. Phosphate transporters from the higher plant *Arabidopsis thaliana*. Proc Nat Acad Sci 1996; 93: 10519–10523.

[39] Karthikeyan AS, Varadarajan DK, Mukatira UT, D'Urzo MP, Damsz B, Raghothama KG. Regulated expression of Arabidopsis phosphate transporters. Plant Physiol 2002; 130: 221–233.

[40] Jung JH, Seo PJ, Park CM. MicroRNA: biogenesis and function in higher plants. Plant Biotechnol Rep 2009; 3: 111–126.

[41] Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, et al. MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. Genes Dev 2006; 20: 3084–3088.

[42] Fahlgren N, Howell MD, Kasschau KD, Chapman EJ, Sullivan CM, et al. High throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes. PLoS ONE 2007; 2: e219.

[43] Li WX, Oono Y, Zhu J, He XJ, Wu JM, Iida K, Lu XY, Cui X, Jin H, Zhu JK. The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. Plant Cell 2008; 20: 2238–2251.

[44] Guo FQ, Young J, Crawford NM. The nitrate transporter AtNRT1.1(CHL1) functions in stomatal opening and contributes to drought susceptibility in Arabidopsis. Plant Cell 2003; 15: 107–117.

[45] Desikan R, Griffiths R, Hancock J, Neill S. A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in Arabidopsis thaliana. Proc Natl Acad Sci USA 2002; 99: 16314–16318.

[46] Lodeiro AR, Gonza´lez P, Herna´ndez A, Balague´ LJ, Favelukes G. Comparison of drought tolerance in nitrogen-fixing and inorganic nitrogen-grown common beans. Plant Sci 2000; 154: 31–41.

[47] Castaings L, Camargo A, Pocholle D, Gaudon V, Texier Y, et al. The nodule inception-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. Plant J 2008; 57: 426–435.

[48] Okamoto S, Ohnishi E, Sato S, Takahashi H, Nakazono M, Tabata S, Kawaguchi M. Nod factor/nitrate-induced CLE genes that drive HAR1-mediated systemic regulation of nodulation. Plant Cell Physiol 2009; 50: 67–77.

[49] Chuck G, Meeley R, Irish E, Sakai H, Hake S. The maize tasselseed4 microRNA controls sex determination and meristem cell fate by targeting Tasselseed6/indeterminate spikelet1. Nat Genet 2007; 39: 1517–1521.

[50] Barz T, Ackermann K, Dubois G, Eils R, Pyerin W. Genome-wide expression screens indicate a global role for protein kinase CK2 in chromatin remodeling. Journal of Cell Science 2003; 116 (8): 5163-5177.

[51] Portoles S and Mas P.The functional interplay between protein kinase CK2 and CCA1 transcriptional activity is essential for clock temperature compensation in Arabidopsis. PLoS Genet 2010; 6(11): e1001201.

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Supplementary File 1 [Ek Dosya 1]

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Identification of miRNAs from French bean (*Phaseolus vulgaris)* under low nitrate stress

[Fransız fasülyesinde (*Phaseolus vulgaris*) düşük nitrat stresi altında miRNA'ların tanımlanması]*

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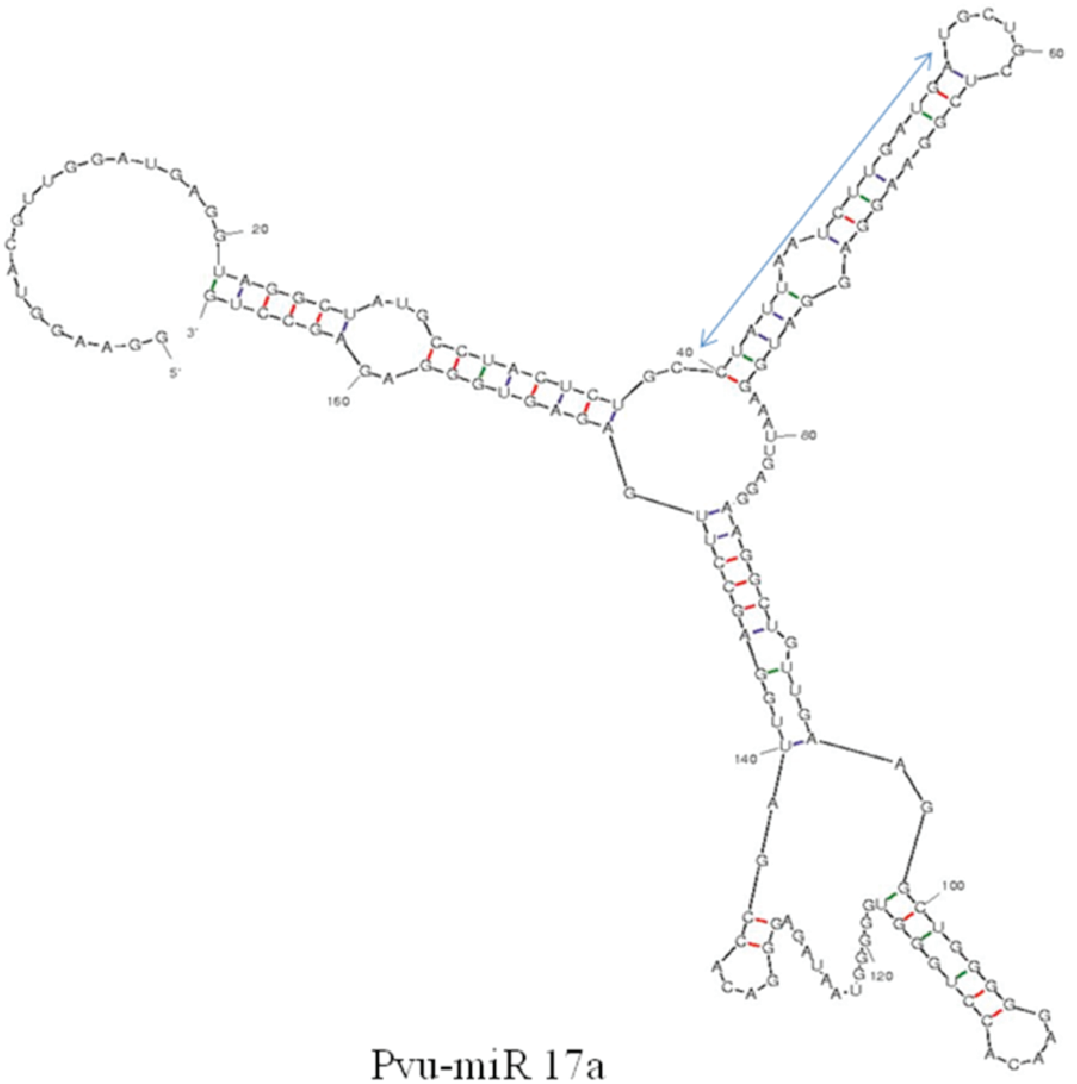
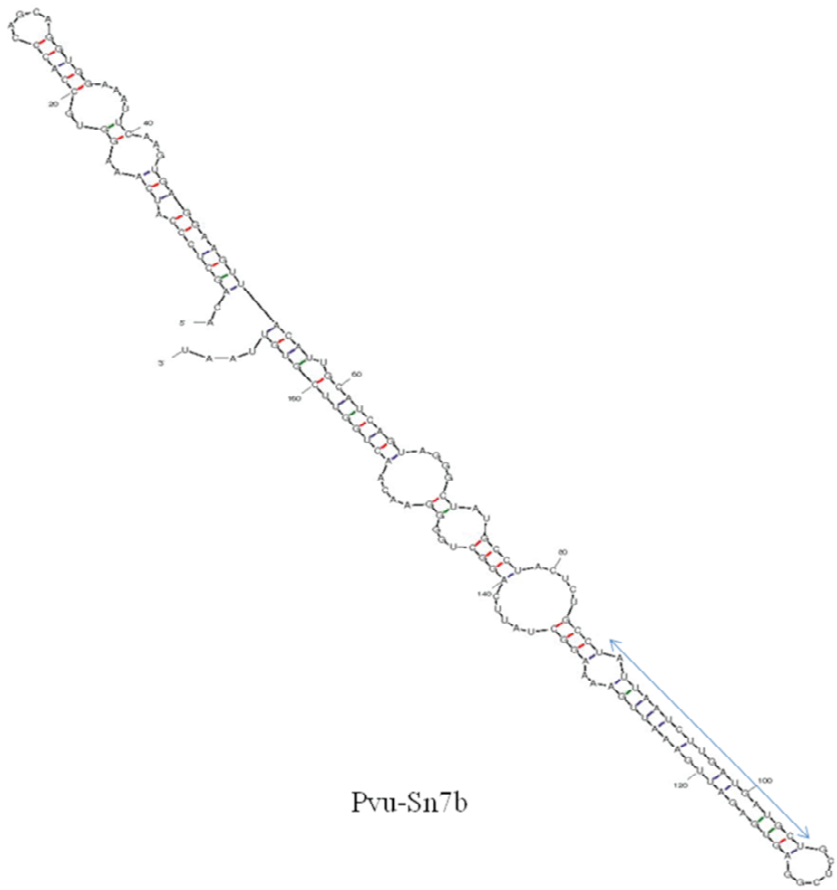
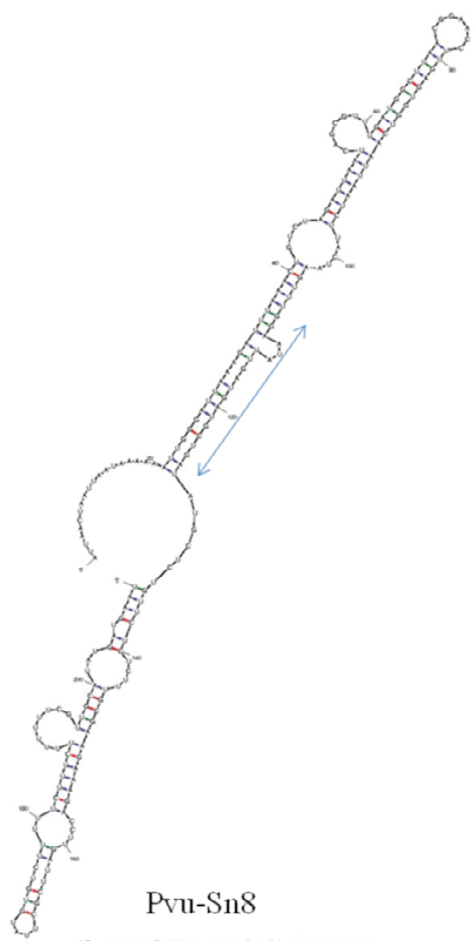
Supplementary File S1: Sequences selected for miRNA prediction



Pvu-miR 17a



Pvu-Sn7b



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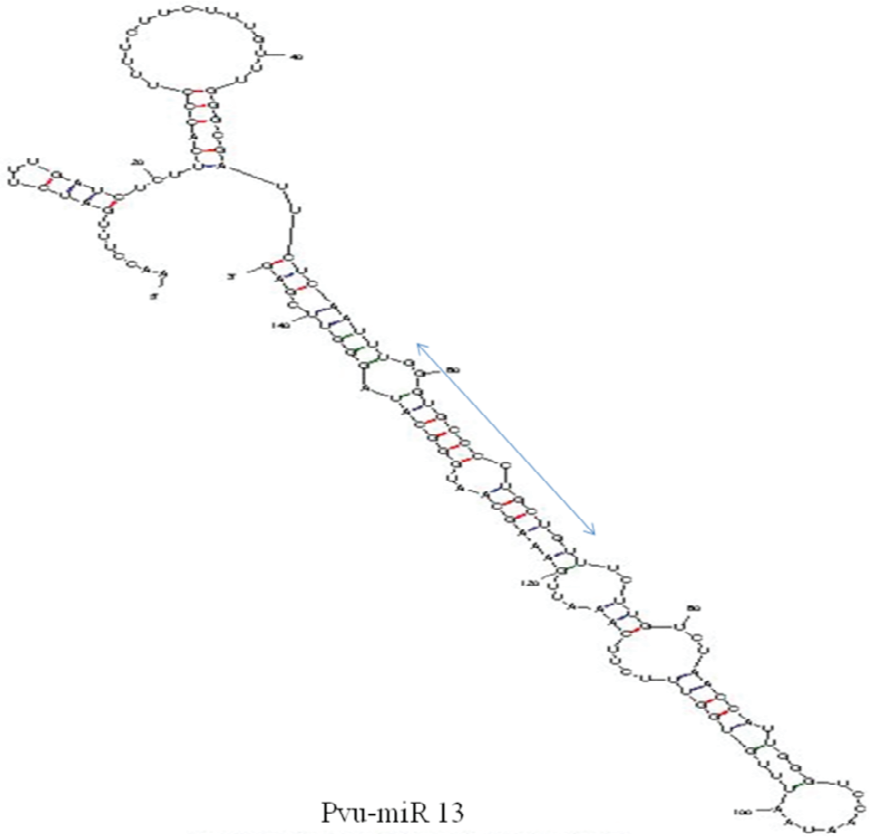

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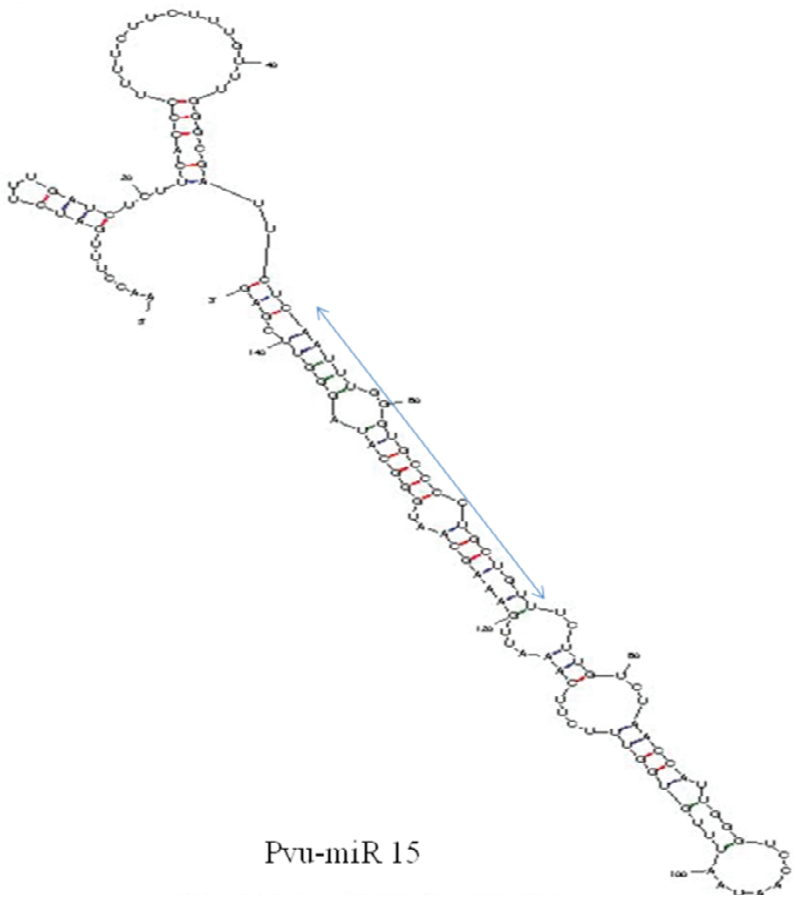

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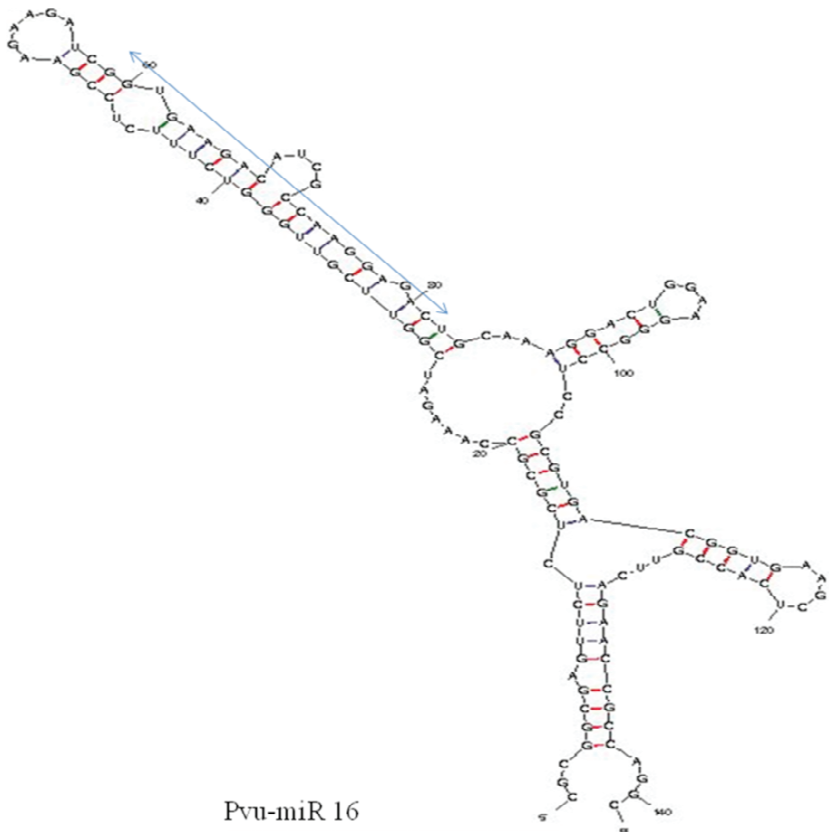

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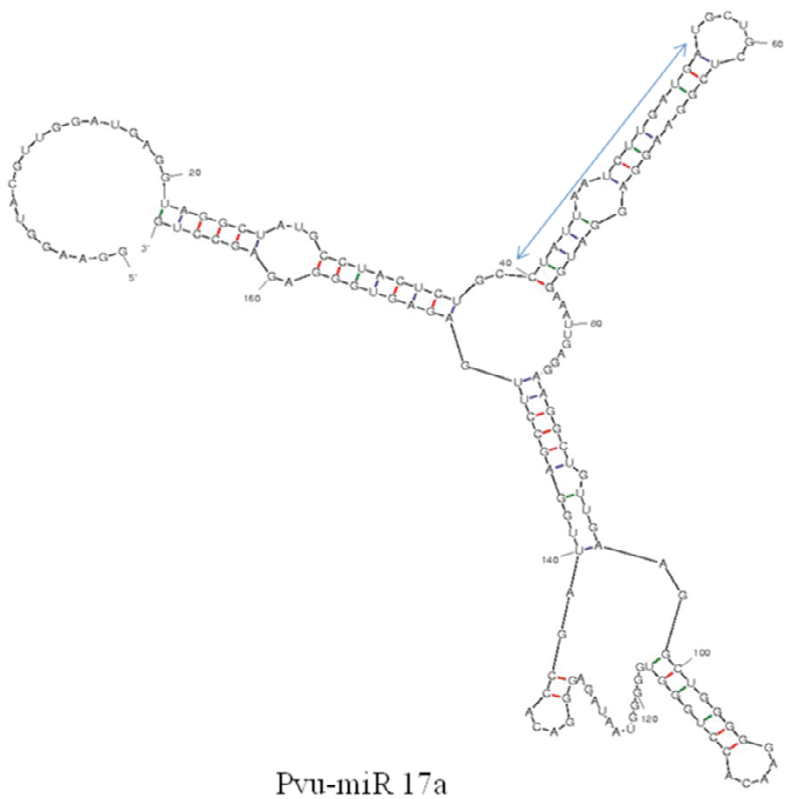
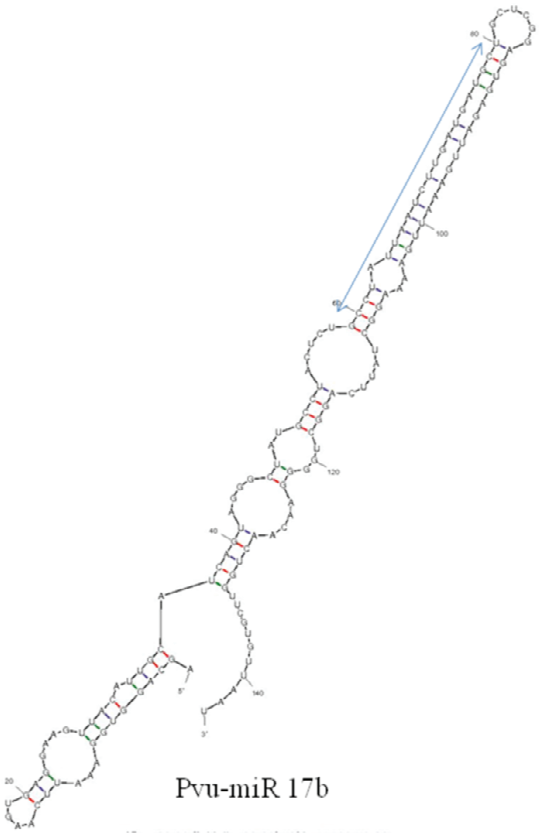


Pvu-miR 17a



Pvu-miR 17b

Pvu-miR 17d