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Original Article

Identification of some *Echinophora platyloba* miRNAs using computational methods and the effect of these miRNAs in the expression of *TLN2* and *ZNF521* genes in different human body organs

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ABSTRACT

MicroRNAs (miRNAs) are small (~22 nucleotides) non-coding endogenous RNA molecules that negatively regulate gene expression at the post-transcriptional level by degrading the target protein-coding mRNA genes or suppressing translation in plants, which consequently participate in a variety of biological and metabolic processes in both animals and plants. Detection of miRNAs is chiefly carried out by microarray, real-time-PCR, northern blot, and bioinformatics approaches. Bioinformatics or in silico-based approaches are the easiest and cheapest ways to identify desired miRNAs. In this study, several miRNAs in *Echinophora platyloba* were identified, and their potential roles were reported. *E. platyloba*, which belongs to the Umbelliferae family, is an endemic plant in Iran found in the Kermanshah, Hamedan, and Lorestan provinces; it has important medicinal uses such as cytotoxic activity in breast cancer, treatment of dysmenorrhea, central and peripheral analgesic effects, and hepatoprotective effects on acute acetaminophen-induced liver injuries. To this end, the RNA was extracted from *E. platyloba* leaf and sent to the Beijing genome institute for RNA sequencing. After quality control, low-quality data was filtered, and *de novo* assembly was performed. Detection of miRNAs was then performed by miRDeep (v37) and miRBase tools. Accordingly, we identified seven miRNAs from the leaf dataset, and their secondary structures were evaluated. Target genes of the detected miRNAs were identified through the psRNA target website.

1. Introduction

Lots of research has been done in the last 20 years on the various functions of non-coding RNAs, such as microRNAs. These are small RNAs that are known to act as regulators of post-transcriptional processes [1-3]. Mature miRNAs are processed from much longer primary transcripts, called pri-miRNAs, via stem-loop-structured intermediates called pre-miRNAs.in plant, Mature miRNAs are generated from Pre-miRNAs by Dicer-like RNA endonucleases through intermediate steps of pre-miRNA synthesis and finally, the RNA-induced silencing complex (RISC) controlled by the ARGONAUTE 1 (AGO1) protein target miRNAs to their complementary targets. point to mRNA sequence[4]. They are mainly incorporated into the RNA-induced silencing

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complex, interacting with their target transcripts [1, 5, 6]. miRNAs play a crucial role in various biological processes [7].

Echinophora genus has 10 species *such as: E. tournefotiijoub*, *E. tenuifolia*, *E. vadiaus boiss*, *E. anatolica boiss*, *E. platyloba*, *E. cinera*, *E. sibthorpiana*, *E. orientalis*, *E. spinose*, *E. trichophyllasmm* [8, 9]. There are four species in Iran, including the endemic species platyloba, orientalis, and cinerea sibtrubiana, known as Karawi, Fiale, and Khosharidze [10-13]. This plant has antiseptic, antibacterial, and antifungal properties. And the aerial parts of this plant are almost everywhere, used in wet and dry forms to flavor dairy products and extend shelf life [14].

The *E. platyloba* plant is one of the four endemic species of this genus in Iran, belongs to the family Umbelliferae, and has many traditional and biological characteristics [15-17], But until today, no molecular study has been done on this plant and its metabolic pathways; also the genes involved in the synthesis of these metabolites remain unknown. This plant produces valuable metabolites such as trans-β-ocimene, 2-furanone, myrcene, linalool, and thymol[18, 19]; The amount of these metabolites varies for each ecotype (depending on the altitude and environment in which it grows)[20]. A previous study showed that a crude methanolic extract of *E. platyloba* inhibited proliferation, induced the apoptotic mechanism and caused cell cycle arrest at the S phase in human breast cancer MDA-MB-231 Cells[21]. Also, its use in treating dysmenorrhoea has been identified during the luteal phase and the first three days of the menstrual cycle [22, 23]. Other studies have used *E.*

Platyloba hydroalcoholic extract in Suppression of chronic and acute pain. Even though the analgesic mechanism of this herb is still unknown, the extract could be both Peripheral and central pain relief and lead to increased resistance to pain and decreased response to chronic and acute pain [24-27].

RNA sequencing (RNA-Seq) is a technology based on new-generation sequencing, which can determine the presence and amount of RNA in a biological sample at a specific time without the need for previous information about its genome [28, 29]. Modern RNA sequencing uses deep sequencing techniques. Briefly, the steps of transcriptome analysis include RNA extraction and cDNA library construction, sequencing by new-generation sequencing technologies, initial manipulation of raw data, integration of short sequences, extraction of single genes, and finally includes, functional interpretation of single genes using databases[30, 31].

Calculation methods are one of the most useful methods for identifying protected miRNAs in different organisms[32]. Today, using bioinformatics tools, it is possible to determine the putative roles of miRNAs in cellular responses. The performance and function of a miRNA depend on its structure, which is determined by bioinformatics tools based on specific patterns of secondary structures, as miRNA genes in secondary structures are highly conserved compared to the primary sequence[33, 34].

Research shows that the first factor for wound healing is the patient's genetics. Genes determine the wound microbiome and its recovery. Diversity in genetics determines different phenotypes and different levels of cell adhesion, which are important drivers of infection. Specific genes in patients are associated with the number of bacteria and frequency of common pathogens in wounds. In general, microbiomes can determine how a wound heals and how long it takes. Also, the more diversity there is in a wound microbiome, the shorter the healing time. Many studies have been conducted on Echinophora platyloba in wound healing in rats, the results of which show that this plant is effective in wound healing and causes it to heal [35]. Many studies have been conducted on the role of *TLN2* and *ZNF521* genes in wound healing, which shows that these genes play a significant role in healing infection, therefore, in this study; we will investigate the bioinformatics of these genes [36-38]. Considering that Echinophora platyloba is effective in wound healing, it is possible to discuss the standard connections between this plant and these genes. Finally, use the gene transfer technique for therapeutic effects as much as possible to heal wounds. For this

purpose, in this study, we examine the expression of these genes in the human body and their other characteristics.

2. Material and methods

2.1. RNA extraction

In the first step, RNA was extracted from the leaf tissue of *the E.platyloba* plant via DENAzist Column RNA Isolation Kit (DENAzist Asia Co., Mashhad, Iran). The quantity and quality of extracted RNA were then checked by spectrophotometry and agarose gel electrophoresis, respectively. After, the extracted RNA was sent to Beijing Genomes Institute for RNA sequencing.

2.2. RNA-seq data

Sequencing data were checked by Fastp software (v0.20.1) in terms of quality and quantity, and the reads that did not have the required quality were removed. In the next step, de novo assembly of the reads was done by Trinity software (v2.6) in a Linux environment.

2.3. miRNA identification

miRNAs were identified by miRDeep2 (v37) software. In order to identify potential miRNAs, 38461 genes were checked by miRDEEP. For the in silico prediction of the second structure of the selected transcriptome sequences, the MFOLD(http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) web computing database based on Zoker's algorithm [39] was used. The second predicted structure has the highest MFEI (Minimal Folding Free Energy Index) energy and the lowest MFE (Minimal Free Energy) energy. Both of these parameters are important for distinguishing miRNAs from other small RNAs. The MEF energy represents the negative conformational free energy (ΔG) of the predicted secondary structures, and the MFEI energy value is calculated according to the following equation.

MFEI = [(MFE/sequence length) x 100]/(G+C%)

Finally, all miRNAs were checked by the miRNA-dis tool (http://bliulab.net/miRNA-dis/server) in order to distinguish true from false miRNAs. In addition to identifying miRNAs, one of the most important issues is identifying the genes which were regulated by miRNAs. Identification of miRNA targets was done by checking their homology in the psRNA target database (https://plantgrn.noble.org/psRNATarget/analysis). cDNA library of the *Arabidopsis thaliana* plant was used as putative targets of desired miRNAs. Next, the expression of each gene that contained the putative miRNAs were calculated by the Salmon tool, following the function of those genes analyzed using the NCBI-BLASTx tool.

3. Results

Bioinformatics and laboratory methods have identified several miRNA sequences in various plants; Mature miRNAs in plant species are evolutionarily conserved, while precursor miRNAs are not conserved and vary between species in the plant kingdom[40]. To date, no *E. platyloba* precursor miRNAs have been reported in miRNA databases such as the Plant miRNA Database (PMRD) and microRNA Database (miRBase). A total of 38,589 known plant miRNAs were downloaded from the miRBASE database (http://www.mirbase.org/) and used as a reference miRNA set for identifying conserved miRNAs in *E. platyloba*. The output of the miRDeep2 software was seven miRNAs (DN3405, DN3519, DN3796, DN6691, DN11845, DN26260, DN32511), whose secondary structures and target proteins were shown in Table 1.

Table 1. miRNAs features

microRNA	The sequence of mature miRNA	GC%	MFE	MEFI	miRNA precursor length
DN3405_c1_g1_i2_117	ucgauaaaccucugcauccag	42.7%	-35	-0.74	110
DN3519_c0_g1_i1_22	ugccaaaggagaauugcccug	38.7%	-38.9	-0.90	111
DN3796_c0_g1_i1_36	ugccaaaggagaauugcccug	45%	-47.4	-0.94	111
DN6691_c0_g1_i1_198	uuccacggcuuucuugaacug	38.5%	-39.5	-0.94	109
DN11845_c0_g1_i1_187	augcacugccucuucccuggc	54.9%	-47.8	-0.78	111
DN26260_c0_g1_i1_176	ugccuggcucccugcaugcca	51.4%	-52.5	-0.92	111
DN32511_c0_g1_i1_200	uugacagaagagagagagcac	41.4%	-30.7	-0.66	111

Based on the obtained results, the average MFE energy is -41.6, with a range of -52.5 kcal/mol to -30.4 kcal/mol. The minimum and maximum lengths of miRNA precursors are 109 and 111 nucleotides, with an average length of 110 nt (Table 1), respectively. Precursors of miRNAs should have the highest MFE and MFEI parameters to create a secondary structure compared to non-coding and coding RNAs. In our results, MEFI energy ranged from -0.66 to -0.94, and the average was -0.84 (Table 1). Moreover, the precursor miRNAs had the G + C content of 38.5% to 54.9%, with an average of 44.65%. The miRNAs were checked with miRNA-dis, and only one of the seven candidates was a false miRNA (DN3405). A total of 80 potential target proteins of the characterised *E. platyloba* miRNAs were also identified, including cytochrome P450, ubiquitin-conjugating enzymes, phosphate 2, Disease resistance protein, DEAD-box ATP-dependent RNA helicase 3 and auxin response factor (Table 2). Seven genes contain these miRNAs, three of which are only miRNA precursors, while the remaining genes have other functions, including producing squamosa promoter, endoglucanase 12-like, and 3-ketoacyl-CoA synthase 11., their TPM ranged from 223 to 1755with the average of 622.2 (Table 3).

Table 2. miRNAs targets and their features

miRNA	Target protein	Target protein function	Accession number
DN3405_c1_g1_i2_117	Pentatricopeptide repeat-containing protein At3g62470, mitochondrial	Facilitate processing, splicing, editing, stability, and translation of RNAs.	AT3G62470.1
DN3405_c1_g1_i2_117	Cytochrome P450, family 96, subfamily A, polypeptide 1	Involve in NADPH- and O2-dependent hydroxylation reactions, and detoxification of xenobiotics.	AT2G23180.1
DN3405_c1_g1_i2_117	Transferase	catalyse the transfer of a group of atoms.	AT5G17540.1
DN3519_c0_g1_i1_22	Probable ubiquitin-conjugating enzyme E2 24	Perform the second step in the ubiquitination, reaction that targets a protein for degradation via the proteasome.	KAH9748702.1
DN3519_c0_g1_i1_22	phosphate 2	Essential to maintain Pi homeostasis and hence plant growth.	AT2G33770.1
DN3519_c0_g1_i1_22	Disease resistance protein (TIR-NBS-LRR class)	Involve in recognition of specialised pathogen effectors.	AT1G31540.1
DN3796_c0_g1_i1_36	probable ubiquitin-conjugating enzyme E2 24	Perform the second step in the ubiquitination, reaction that targets a protein for degradation via the proteasome.	XP_006467799.1
DN3796_c0_g1_i1_36	Ubiquitin-conjugating enzyme/RWD-like	catalyze the covalent attachment of ubiquitin to target proteins	KAG7638386.1
DN6691_c0_g1_i1_198	DEAD-box ATP-dependent RNA helicase 3	Binds specific group II introns in chloroplasts and facilitates their splicing. Required for normal development of chloroplasts.	KAH9733739.1
DN6691_c0_g1_i1_198	RNAse E/G-like protein	Required for the biogenesis and accumulation of native cytochrome b6 in the thylakoid membrane.	NP_001325371.1
DN6691_c0_g1_i1_198	ALC-interacting protein 1	May interact with ACI1 and its homologs to control cell separation during fruit dehiscence in Arabidopsis.	NP_195757.1
DN11845_c0_g1_i1_187	Basic blue protein-like	Forms a concentration gradient along the pollen tube growth path, with a lower level in the stigma papilla cell wall and a higher level in the transmitting tract extracellular matrix of the style.	XP_006479305.2
DN11845_c0_g1_i1_187	Plantacyanin	unknown function	NP_178388.1
DN11845_c0_g1_i1_187	Proteasome component (PCI) domain protein	A scaffold for the other complex subunits and other binding partners.	NP_001319448.1
DN26260_c0_g1_i1_176	Auxin response factor 17	Transcriptional factors that bind specifically to the DNA sequence 5'-TGTCTC-3' are found in the auxin-responsive promoter elements (AuxREs).	KAH9756788.1
DN26260_c0_g1_i1_176	Endonuclease/exonuclease/phosphatase superfamily	involved in intracellular signaling	KAG7578016.1
DN32511_c0_g1_i1_200	Squamosa promoter-binding protein-like (SBP domain) transcription factor family protein	Constitute a diverse family of transcription factors that play fundamental roles in plant growth and development.	NP_001332253.1

3.1. *TLN2* gene

TLN2 gene is located on chromosome number 15. This gene encodes talin 1 protein and has 59 exons (Table 4). This protein is a cytoskeleton protein that plays an important role in the assembly of actin filaments. This gene also plays an effective role in the expansion and migration of different types of cells, including fibroblasts and osteoclasts. This protein is related to transmembrane receptors and creates effective links between extracellular matrices and the actin cytoskeleton. Table 4 shows other bioinformatics characteristics related to the *TLN2* gene [41].

3.2. *ZNF521* gene

The *ZNF521* gene is located on chromosome number 18. This gene has 8 exons and plays a role in regulating transcription through RNA polymerase II and is involved in the function of neurons (Table 4). This gene is located in the nucleus and regulates bone growth through pathways related to RUNX2 gene expression and transcription. Among the diseases related to this gene, we can mention lung cancer and bladder transitional cell cancer. The important paralog of this gene is ZNF423. Table 4 shows other bioinformatics characteristics related to the *ZNF521* gene [42].

3.3. Analysis of *ZNF521* gene expression in different body organs

As can be seen in Figures 1 and 2, the expression level of the *TLN2* gene in different organs of the body is relatively higher than that of the *ZNF521* gene. The results of the gene expression study showed that the expression of the *TLN2* gene in the brain and kidney is higher than in other organs of the body, and the lowest level of expression is related to the pancreas and lymph nodes. In the *ZNF521* gene, as mentioned, the level of expression is lower than that of the *TLN2* gene, and the highest level of expression is related to the ovary, spleen, and endometrium. The lowest level of expression was observed in the pancreas and liver.

Table 3. Genes that include a miRNA and their feature

Gene	TPM	Function	Protein ID
DN32511_c0_g1_i1_200	1755	squamosa promoter-binding-like protein 13A	XP_017238206.1
DN3796_c0_g1_i1_36	302.207	Precursor of miRNA	Precursor of miRNA
DN6691_c0_g1_i1_198	322.207	endoglucanase 12-like	XP_017252703.1
DN3519_c0_g1_i1_22	223	Precursor of miRNA	Precursor of miRNA
DN11845_c0_g1_i1_187	437	Precursor of miRNA	Precursor of miRNA
DN26260_c0_g1_i1_176	572	hypothetical protein DCAR_014980	KZM97658.1
DN3405_c1_g1_i2_117	744.207	3-ketoacyl-CoA synthase 11	XP_017235501.1

Table 4. Genes sequence results of *TLN2*, and *ZNF521*

Name	*TLN2*	*ZNF521*
ORGANISM	Homo sapiens (Human)	Homo sapiens (Human)
Accession number nucleotide	NM_015059.3	NM_015461.3
Accession number protein	NP_055874.2	NP_056276.1
Gene ID	83660	25925
Chromosome	15	18
Inheritance	Autosomal dominant	Autosomal dominant
Cytogenetic location	15q22.2	18q11.2
Chromosome location bp	62390550-62844631	25061924-25352166
nucleotide length	12023bp	4887bp
protein length	2542aa	1311aa
Molecular weight (Da)	271613.07	147866.10
Isoelectric point	5.40	6.65
Total Exon	59	8

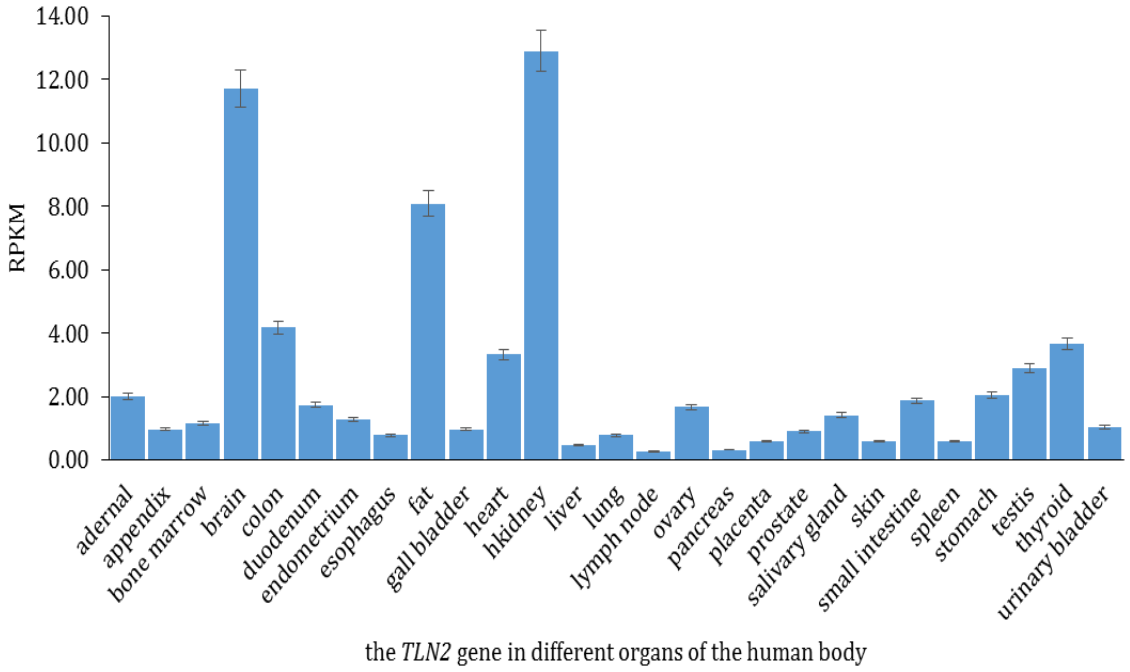

Fig. 1. Examining the expression of the *TLN2* gene in different organs of the human body

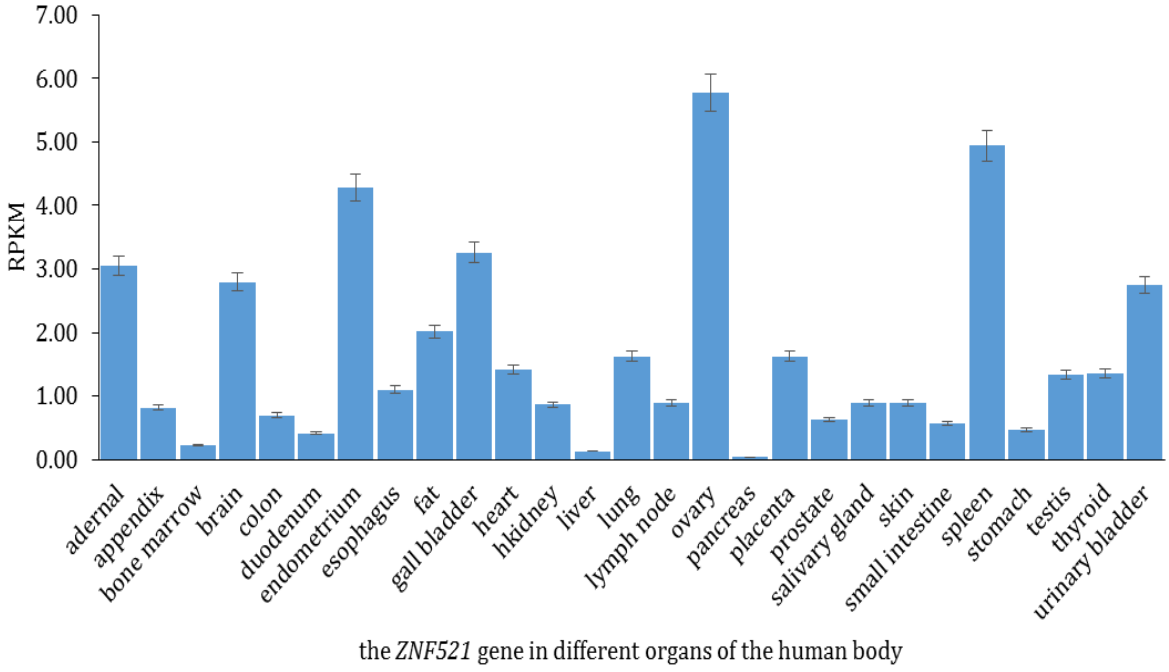

Fig. 2. Examining the expression of the *ZNF521* gene in different organs of the human body

3.4. Analysis of domains and motifs of *TLN2* and *ZNF521* genes

Examining the domains and motifs of the *TLN2* and *ZNF521* genes showed that the *TLN2* gene has a Band 4.1 homologs (B41) domain and a Coiled-Coil (CC) motif (Figure 3 A). The B41 domain is associated with protein domains such as myosins, ezrin, radixin, moesin, and protein tyrosine phosphatases and has a structural and regulatory role in the assembly and stabilization of plasma membrane domains. A coiled-coil motif is a protein domain that forms a bundle of two or three alpha helices. This motif is generally involved in protein interactions and plays an effective role in structural and movement proteins. The *ZNF521* gene has a 30-domain

protein called ZnF_C2H2 zinc finger and does not have a motif (Figure 3B). In general, the ZnF_C2H2 zinc finger domain is associated with small motifs and is often found in clusters. ZnF_C2H2 zinc finger domain is effective in gene transcription, translation, cytoskeleton organization, epithelial development, cell adhesion, protein folding, and chromatin remodeling.

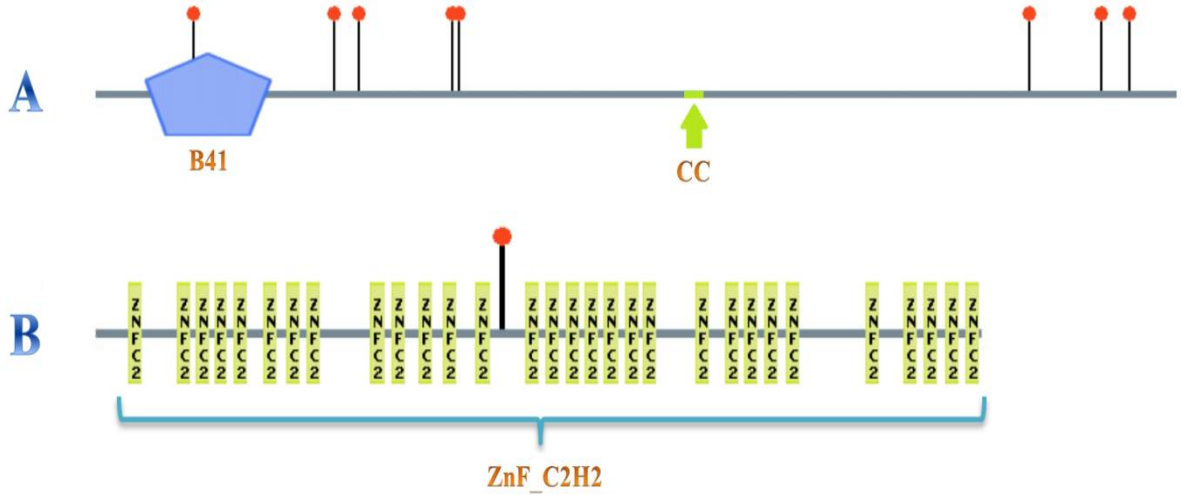

Fig. 3. Analysis of domains and motifs of *TLN2* and *ZNF521* genes. A) Domains and motifs of *TLN2* gene. B) Domains of the *ZNF521* gene.

4. Discussion

MicroRNAs can be used as new breeding tools, and one of their applications is the genetic improvement of plants, the development of miRNA identification and expression analysis, including direct cloning, EST analysis, and deep sequencing techniques, has provided opportunities for studying miRNAs [40]. So far, no miRNA has been reported in *E. platyloba* before this research. In addition, a large number of secondary metabolites (such as spathulenol and beta-ocimene) and other important nutrition components in *E. platyloba* make it a valuable medicinal plant.In this research, seven miRNAs have been found, and the analysis revealed their structure and target protein; also, it was found that 6 of them are true miRNAs(only DN3405 was a fake miRNA). In this study, different target genes were obtained for seven miRNAs associated with several gene families with different biological functions.

The TPMs of genes containing miRNAs varied from 223 to 1755, with the highest and lowest TPMs belonging to DN32511 and DN3519, respectively. These miRNAs directly influence growth and development, morphology, flowering time, and metabolism and contribute to responsive stress.

5. Conclusion

These results open new avenues for researchers to explore the role of these novel miRNAs in *E. platyloba* and use them to regulate the production of secondary metabolites. In silico prediction of miRNAs is only the first step of miRNA study and should be followed by other investigations such as gene ontology and functional annotation for a comprehensively understand of its biological roles.

Conflict of Interest

The authors hereby declare that they have no conflict of interest.

Author's contributions

All authors equally participated in designing experiment analysis and interpretation of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

No human or animals were used in the present research.

Consent for publications

All authors have read and approved the final manuscript for publication.

Availability of data and material

The authors have embedded all data in the manuscript.

Informed Consent

The authors declare not used any patients in this research.

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