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Bioinformatic Prediction and Experimental Validation of miRNAs in Two Species of *Taraxacum*

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MicroRNAs (miRNAs) are short, non-coding, regulatory RNAs that are produced naturally by cells. Consisting of 18-26 nucleotides (nt), they perform significant functions when it comes to the post-transcriptional phase of gene regulation. These remain found to be evolutionarily conserved in eukaryotic organisms. The conserved nature of these small RNAs not only implies their conserved function but also makes them an important tool for hunting conserved miRNAs among various species through homologous analysis by means of different computational methods. Family Asteraceae is the largest angiospermic dicot family comprising of 1600 genera and 24000 species. *Taraxacum* is an important genus of family Asteraceae comprising of 60-500 species. This work focuses two species of this important genus viz *Taraxacum officinale (T. officinale*) and *Taraxacum kok-saghyz* (*T. kok-saghyz*). A comparative transcriptomic strategy was applied to identify new conserved miRNAs in both species which led to the prediction of 34 newly conserved miRNAs (precursor miRNAs) pertaining to 33 different families of miRNAs. Out of the 34 conserved miRNAs 26 come from *T. officinale* and 8 from *T. kok-saghyz*. Among these two of the *T. kok-saghyz* miRNAs (tkn-MIR165 and tkn-MIR166) were found as cluster. Another *T. officinale* miRNA (tof-MIR5032) was also found to be transcribed from the same genomic location in the opposite direction. Annotation and prediction of overall 35 targets for these newly profiled miRNAs was also done. The targets are found to be involved in major processes and 35 enriched gene ontology (GO) terms. Significant targets include copper-dependent protein binding, nucleic acid binding, RNA binding, regulation of DNA damage checkpoint and RNA-dependent DNA replication. The recently discovered pre-miRNAs were annotated for their length of precursor, mature sequence arm, minimum free energy, mature sequence length, sense/ antisense orientation and organ of expression. Additionally, ten (10) miRNAs were chosen at random for RT-PCR expressional validation.

Keywords: Bioinformatics, Gene regulation, miRNAs, *Taraxacum.*

INTRODUCTION

Specifically, the tiny miRNAs are endogenous and vary in length from 18 to 26 nucleotides. They play significant roles in regulating genes during posttranscriptional events (Ambros *et al.*, 2003). The RNA polymerase II commonly regulates the transcription of miRNAs. The Primary miRNAs (Pri-miRNAs); product of this transcription, mostly vary in length up to hundreds of nucleotides, containing several of the pre-miRNAs with loops on the stems of hairpins. Like messenger RNAs (mRNAs) the Pri-miRNAs also undergo 5' end capping and 3' end polyadenylation (Lee *et al.*, 2004). By self-hybridization these pri-miRNAs get converted into pre-miRNAs and transported into cytoplasm where other special proteins, the RNAs III and DICER like (DCL1) enzymes make an unstable duplex structure of miRNAs (Meyers *et al.*, 2008) .The duplex structure gets converted into mature miRNA (Liu *et al.*, 2012; Song *et al.*, 2010; Voinnet, 2009). The mature miRNAs perform vital role in plants during growth and development (Barozai *et al.*, 2013). These functions of miRNAs include regulation of biotic and abiotic stresses, tissue specific development, developmental timing, transgene inactivation, signaling processes, stem cell differentiation and maintenance etc.

After a decade of their discovery, the non-coding miRNAs became a hottest topic for research due to their versatile regulatory mechanisms both in plants and animals. Many researchers have identified and reported thousands of plant miRNAs via several computational tools (Bennasser *et al.*, 2004) and experimental approaches (Achakzai *et al.*, 2018;

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Baloch *et al.*, 2018; Barozai *et al.*, 2012; Benes *et al.*, 2010; Han *et al.*, 2010; Jike *et al.*, 2018; Mica *et al.*, 2009)

Asteraceae, the second-biggest family of Angiosperms in terms of species after Orchidaceae but the largest in terms of genera, contains over 1600 genera and 24000 species and is found practically everywhere except Antarctica (PANERO *et al.*, 2005). The plants of this family can be identified easily because of their capitulate florets. Economically the incalculable importance of Asteraceae family is based on its type of vegetation in tropics, subtropics, dry and temperate regions. *Taraxacum* is an important genus of family Asteraceae comprising of 60-500 species throughout the world. This work focuses two species of this important genus i.e. *T.officianale* and *T. kok-saghyz*. *T. kok-saghyz*. The common Dandelion (*T.officianale*) is an important medicinal herb of family Asteraceae distributed in geographically warm and temperate areas throughout the world (Quer, 1962). This perennial weed has been used as a traditional medicine from several years (Schütz *et al.*, 2006). In 1930s, The Russian dandelion (*T. kok-saghyz*), was discovered as a substitute for natural rubber (Lipshitz, 1934), and it is suitable for growing home rubber in temperate climates (Buranov *et al.*, 2010; Cornish *et al.*, 2016).

The identification of conserved miRNAs in non-model plants is a commonplace to comprehend the gene regulation mechanism in plants. To date thousands of miRNAs have been reported in hundreds of plants but *Taraxacum* has remained neglected. This research is an attempt to explore *Taraxacum* miRNAs and their diverse molecular functions.

MATERIALS AND METHODS

The computational tools are widely used methods for profiling conserved miRNA orthologs by comparative approaches. The methodology of (Achakzai *et al*, 2018; Baloch *et al*, 2018) was used for miRNA prediction in *Taraxacum*. Many computational methods which includes miRbase, Mfold, Clustal W, BLASTn, RNA hybrid, BLASTx, WebLogo and QuickGo were used to determine and annotate newly pertained miRNAs in *Taraxacum*.

The plant miRNA database, miRbase and other available literature about miRNAs in *Taraxacum* were surveyed and from overall survey it was suggested that until now there is no any miRNA reported for *Taraxacum*. Therefore, this research is an effort for profiling conserved miRNAs in *Taraxacum*.

***Fetching of reference miRNAs*:** All the available (6,868) angiospermic plant pre-miRNAs and (8,957) mature miRNAs belonging to (20) dicot families (Amaranthaceae, Araliaceae, Asteraceae, Brassicaceae, Caricaceae, Cucurbitaceae, Euphorbiaceae, Fabaceae, Lamiales, Linaceae, Malvaceae, Myrtaceae, Paeoniaceae, Ranunculaceae, Rhizophoraceae, Rosaceae, Rutaceae, Salicaceae, Solanaceae and Vitaceae) and (4) monocot families (Arecaceae, Asparagaceae, Bromeliaceae and Poaceae) were fetched from latest release of miRBase 22.1 (Kozomara *et al.*, 2019) and saved in FASTA format.

***Prediction of potential sequences of miRNAs in Tarxacum*:** The (6,868) reference pre miRNAs from other plants were subjected to the BLASTn against *Taraxacum* ESTs. Potential candidate *Taraxacum* miRNAs in FASTA format with no more than four mismatches with the mature sequences of the reference miRNAs were chosen, saved for downstream analyses.

***Taraxacum miRNA secondary structure prediction*:** A key requirement for identifying and characterizing novel conserved miRNAs in *Taraxacum* is the annotation of secondary structures stem-loops of initially potential candidate sequences. The potential *Taraxacum* miRNA sequences were initially used in order to create structures with stem-loops using, secondary structure prediction program MFOLD version 3.6. (Zuker, 2003). The original candidate sequences that were unable to form steady miRNA structures all eliminated. Only potential miRNA sequences residing in stem portion, stable stem-loop structures with at least 12 bases involved in Watson-Crick or G/U base pairing were saved and put under physical inspection.

***Physical inspection*:** A physical inspection is necessary to eliminate the erroneous positive miRNAs. Therefore, all of the *Taraxacum* EST-derived candidate miRNAs that met the criteria for potential candidate miRNAs having no more than four mismatches with the reference miRNAs, being non-coding, forming a stable stem-loop secondary structure, and being single-tone in nature were physically examined to weed out sequences with noticeable bulges, mature sequences that weren't in the stem-region, and sequences with higher MFEs. Each newly profiled *Taraxacum* miRNA's organ of expression is also noted from its EST.

***RT-PCR validation*:** Ten miRNAs were randomly chosen from the recently profiled *Taraxacum* miRNAs and were subjected to RT-PCR (Reverse Transcription) expressional investigation along with a control sample. The ESTs of 10 randomly chosen miRNAs were utilized to build stem-loop primers using the Primer-3 method (http://bioinfo.ut.ee/primer3-0.4.0) (for detail, S.1. Table). *Taraxacum* leaves were used to extract total RNA using a Qiagen plant RNA kit. The RevertAid™ First Strand cDNA synthesis Kit (Fermentas) was then used to create cDNA in accordance with the manufacturer's instructions. The following PCR procedure was performed with a 60µg cDNA template: initial denaturation at 95 °C for 3 min, for 35 cycles; denaturation at 94 °C for 35 sec; annealing temperature was set 56°C - 62 °C for 35 sec; extension at 72 °C for 30 sec; and final elongation step at 72 °C for 10 min. With a 100 base pair DNA leader, a 1.7% (w/v) agarose gel was used to separate the PCR products.

***Phylogenetic and Conservation study of recently found identified miRNAs*:** Since miRNAs are found to be evolutionary conserved among plants (Zhang *et al.*, 2006) and

the nature of conservation among plant miRNAs provide a sound approach for newly conserved miRNAs in plants (Baloch *et al.*, 2015; Barozai *et al.*, 2008). Thus, tof-MiR398 selected for identifying the conservation among miRNAs of various plant species. A software WebLogo (https://weblogo.berkeley.edu/logo.cgi) (Crooks *et al.*, 2004) has been used for conservation analysis of MiRNAs. Another *Taraxacum* miRNA (tof-MiR393) was subjected to CLUSTALW for phylogenetic analysis. The neighbor-joining clustering method was used to create cladograms and the results were saved

***Identification of miRNA targeted genes*:** It is a crucial stage to find targets of newly conserved micro RNAs to confirm the predicted miRNAs. Therefore, all sequences of mature miRNA targets have being determined by using psRNATarget (Dai *et al.*, 2011) for target prediction of newly identified miRNAs. The QuickGO was used to perform functional and enrichment analyses on the predicted putative *Taraxacum* miRNA targets (Binns *et al.*, 2009).

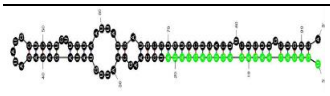
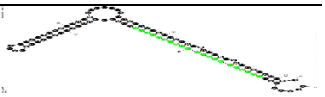
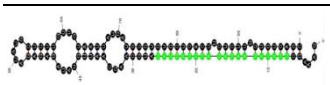
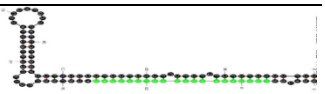
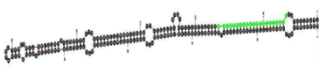
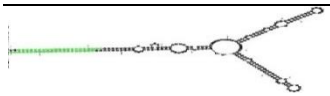
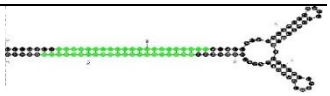
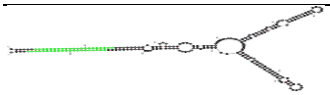
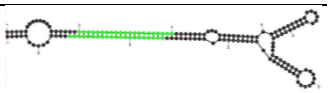
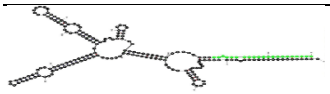
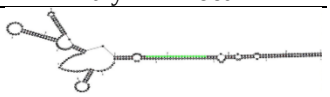
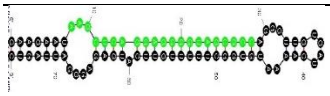
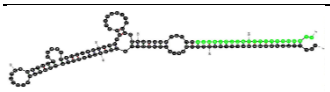
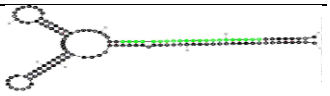
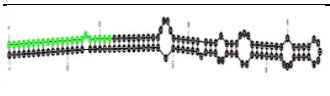
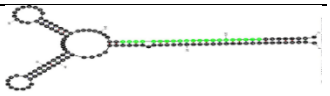
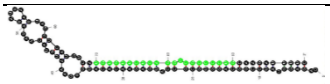
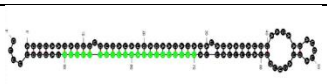
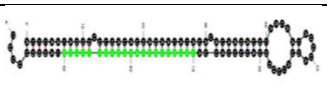
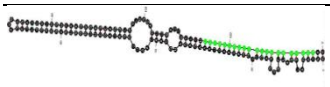
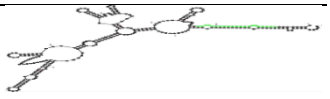
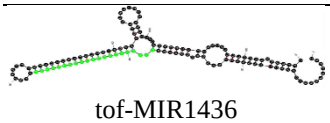
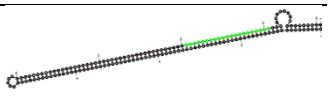
RESULTS AND DISCUSSION

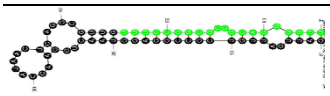
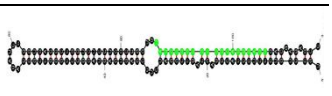
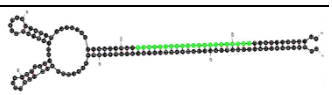
Research based on genome comaprison is a prominent strategy for discovering novel, intriguing discoveries in a variety of organisms (Wahid *et al.*, 2016), (Jahan *et al.*, 2017). The computational identification of miRNAs resulted 26 conserved miRNAs in *T. officinale* and 8 in *T. kok-saghyz.* These 34 miRNAs belonged to 32 miRNA families, i.e. miR 156, 159, 165, 166, 169, 390, 393, 398, 858, 1436, 1846, 1852, 2873, 4224, 5032, 5141, 5161, 5630, 5634, 5640, 5641, 5653, 6167, 6174, 6482, 8155, 8182, 10223 and 11604. Based on recent identifications, these 34 miRNAs are documented first time in *Taraxacum*. The 34 newly identified miRNAs are derived from previously identified miRNAs of *A. thaliana* (32.3%), *O. sativa* (20.5%), *H. brasiliensis* (11.7), *A. lyrata* (8.8%), *C. cardunculus* (8.8%), *G. max* (5.8%), *C. papaya* (2.9%), *E. uniflora* (2.9%), *P lactiflora* (2.9%) and *R. glutinosa* (2.9%). The empirical formula based on B, C and criterion proposed by (Ambros *et al*, 2003) was used to confirm recently identified miRNAs in *Taraxacum* as possible candidates. (Meyers *et al*, 2008) established this criterion for plants and it was suggested that the D criterion is enough for orthologous sequences for validation of newly identified conserved micro RNAs in various species of plants plant (Ambros *et al*, 2003).

***Taraxacum miRNAs characterization*:** Mature miRNA sequences with mismatches, Pre-miRNA length, MFE of pre-miRNAs, number of mismatches, orientation of strand, length of mature sequence, GC percentage, organ of expression, and mature sequences arm were used to characterize and annotate the newly profiled *Taraxacum* miRNAs [for detail, S.1. Table]. The stem portions of the stem-loop structures include the entire mature sequence of the novel conserved *Taraxacum* miRNAs (shown in Fig 1). The stem-loop structures of predicted miRNAs revealed a minimum of 8-16 nucleotides involved in G/U base pairings/Watson-Crick among mature miRNA and precursor miRNAs within stem region, and the hairpin precursors lack significant internal loops. Similar outcomes for miRNAs in other species of plants and animals have been documented by other studies.

A reduced minimum folding energy (MFE) indicates a greater thermodynamics stability in the miRNA secondary structures. Hence, the MFE of the recently identified *Taraxacum* pre-miRNAs stands as a pivotal feature in the characterization of these miRNAs. As prescribed by Mfold (Zuker, 2003) the average MFE of the newly identified *Taraxacum* miRNAs spanned from-85.60 Kcal mol–1 to -10.60 Kcal mol–1 with an average of -31 Kcal mol–1. Pre-miRNAs with MFE values between -30 and -49 Kcal mol-1 (10) made up 29% of the total pre-miRNAs based on class boundaries, whereas those in the range of -50 to -69 (1) made up 3% and -70 to -89 (2) 6%. Approximately 21% of pre-miRNAs had MFE values between -10 and -29 (21) with 62%, which is the bulk of pre-miRNAs. These results were also supported by other researchers who previously had similar findings for MFE (Din, Barozai, & Baloch, 2016; B. Zhang, Pan, & Stellwag, 2008). It is known that precursor miRNAs yield mature miRNAs (Bartel, 2004). Pre-miRNAs from plants have a wide variety of structures and sizes, in contrast to those from mammals (B. Zhang *et al*., 2006). The lengths of the preserved *Taraxacum* pre-miRNAs ranged from 57 to 227 nucleotides, with an average length of 109 nucleotides. According to classification based on class boundaries the precursor miRNAs ranged from 57-84 nucleotides (11 out of 34) 32%, 85-112 nucleotides (9 out of 34) 27%, 113-140 nucleotides (10 out of 34) 29%, 141-168 nucleotide (1 out of 34) 3%, 169-196 nucleotides (0 out of 34) 0%, 197-224 nucleotides (1 out of 34) 3% and 225-252 nucleotides (2 out of 34) 6% (Gul, Barozai, & Din, 2017; B. Zhang *et al*., 2006; B. Zhang *et al*., 2008).

The lengths of the newly identified conserved mature *Taraxacum* miRNAs been found range from 19-24 nucleotides with an average of 21. Furthermore class boundaries were falling as 19-20 nucleotides (5 out of 34) 15%, 21-22 nucleotides (25 out of 34) 73%, and 23-24 nucleotides (4 out of 34) 12% of aggregate. The range of mature sequence lengths seen in *Taraxacum* is consistent with that of other plant species' miRNAs (Gasparis, Yanushevska, & Nadolska-Orczyk, 2017; Qiu, Hai, Guo, Li, & Zhang, 2016). The current study found that 23 of the 34 newly analyzed miRNAs were found on the sense strand, accounting for 68% of all miRNAs found. 11 of the 34 miRNAs, or 32% of the total miRNA population, are found in the anti-sense strand orientation. 14 mature sequences, or 41% of the total, are found on the stem-loop secondary structure's 5' arm, while the remaining 20 mature sequences, or 59% of the total are found on the 3' arm.

***Taraxacum* miRNA structures**	**Reference miRNA structures**
 tof-miR156	 cca-miR156b
 tof-MIR156a	 osa-MIR156a
 tof-MIR159	 osa-MIR159c
 tkn-MIR165	 ath-MIR165a
 tkn-MIR166	 aly-MIR166a
 tof-MIR169	 aly-MIR169c
 tkn-MIR390	 ath-MIR390a
 tof-MIR393	 ath-MIR393a
 tkn-MIR393	 ath-MIR393a
 tof-MIR398	 cca-MIR398
 tkn-MIR398	 cca-MIR398
 tof-MIR858	 ath-MIR858b
 tof-MIR1436	 osa-MIR1436

***Taraxacum* miRNA structures**	**Reference miRNA structures**
 tof-MIR1846	 osa-MIR1846d
 tof-MIR1852	 osa-MIR1852
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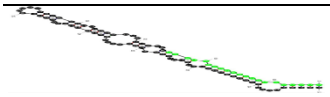
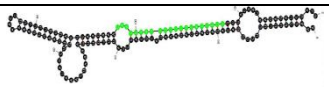
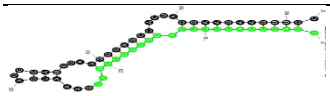
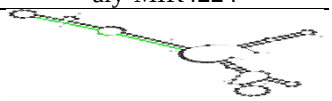
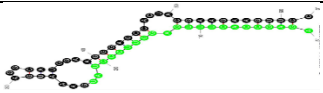
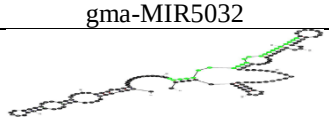
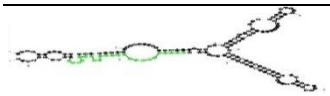
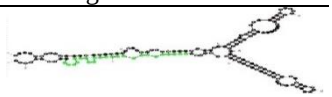
 tof-MIR4224	 aly-MIR4224
 tof-MIR5032	 gma-MIR5032
 tof-MIR5032	 gma-MIR5032
 tkn-MIR5141	 rgl-MIR5141
tof-MIR5161	osa-MIR5161
tof-MIR5630	ath-MIR5630b
tof-miR5634	ath-miR5634
tof-MIR5640	ath-MIR5640
tof-MIR5641	ath-MIR5641
tof-MIR5653	ath-MIR5653

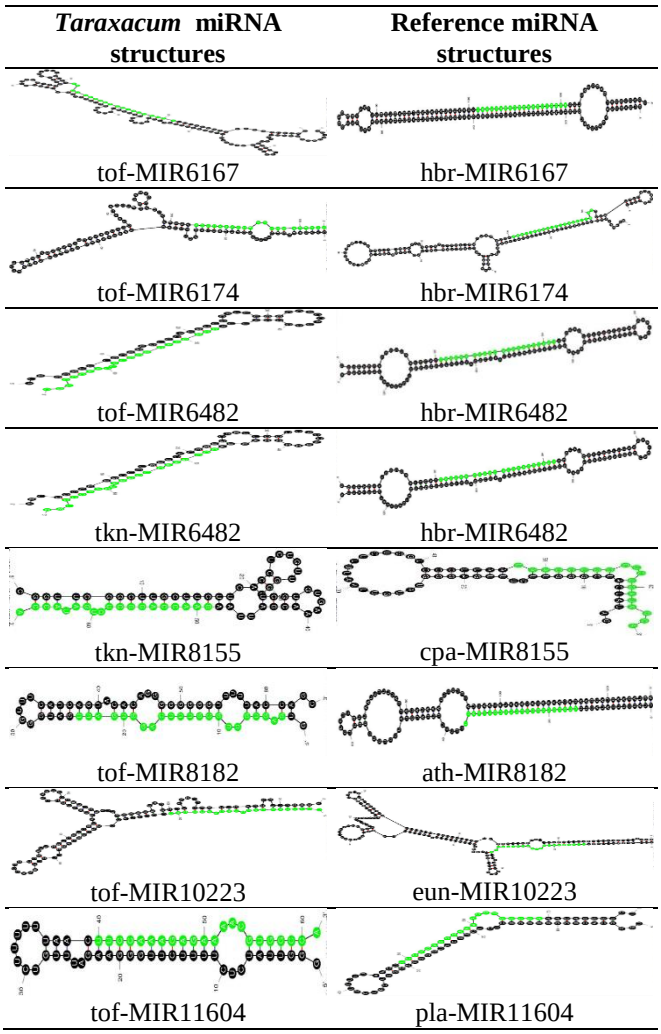


Figure 1. *Taraxacum* miRNA secondary structures. The newly identified *Taraxacum* miRNAs' secondary structures with their reference miRNA structures were developed through the Mfold algorithm. These structures clearly show similarity with their reference miRNA structures.

***Cluster and Sense/antisense miRNAs in Taraxacum*:** MiRNA clusters are collections of two or more miRNA-encoding genes that are co-transcribed to form polycistronic miRNAs (Cui *et al.*, 2009). It is crucial to understand that how these miRNAs behave, and how they serve different biological purposes mainly because a mature miRNA have the ability to target numerous gene sequences and the cluster miRNA comprises multiple miRNA sequences that may target several genes. Similar to protein-coding genes, the miRNA clusters are controlled by genetic and epigenetic processes. These clusters have the capacity to control each phase of cellular activity, including, but not limited to, metabolism, growth, biogenesis, proliferation, DNA repair, differentiation, development, infection, cell death and messenger signaling (Cui *et al*, 2009; Kabekkodu *et al.*, 2018). In current study two precursor miRNAs (tkn-MIR165 and tkn-MIR166) were found as cluster each with two mature sequences.

It is widely believed that only a small subset of promoters may initiate bidirectional transcription to produce antisense RNA (Swami, 2009). Natural antisense transcripts (NATs) and microRNAs (miRNAs) regulate a variety of biological functions and have been widely used to modify the expression of genes in eukaryotic cells (Li *et al.*, 2020). In this study a sense/antisense miRNA (tof-MIR5032) was found to transcribe from a same genomic loci.

***Amplification and validation of Taraxacum miRNAs*:** The newly profiled *Taraxacum* miRNAs need to be experimentally validated, which requires the RT-PCR analysis. The ten *T. officinale* miRNAs with one control sample were selected for RT-PCR expressional analysis, as illustrated in Fig 2. The ten *T. officinale* miRNAs were identified as follows: 1 control (sample prepared with PCR mix and a set of reverse and forward primers, excluding cDNA template), 2 (tof-MIR156), 3 (tof-MIR159), 4 (tof-MIR393), 5 (tof-MIR398), 6 (tof-MIR858), 7 (tof-MIR2873), 8 (tof-MIR5641), 9 (tof-MIR8182), 10 (tof-MIR5634) and 11 (tof-MIR11604). All of the chosen miRNAs supported their experimental validation.

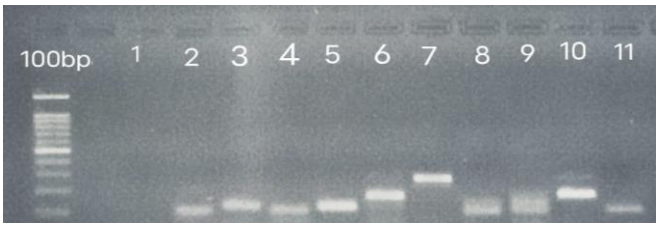


Figure 2. *Taraxacum* miRNAs RT-PCR Validation. One control sample ant 10 *Taraxacum* miRNAs 1 control (sample prepared with PCR mix and a set of reverse and forward primers, excluding cDNA template), 2 (tof-MIR156), 3 (tof-MIR159), 4 (tof-MIR393), 5 (tof-MIR398), 6 (tof-MIR858), 7 (tof-MIR2873), 8 (tof-MIR5641), 9 (tof-MIR8182), 10 (tof-MIR5634) and 11 (tof-MIR11604) were chosen and analyzed for RT-PCR expression. On an agarose gel with a 1.7% (w/v) concentration and a 100 base pair DNA leader, the product of each sample was separated.

***Conservation and phylogeny analysis of Taraxacum miRNAs*:** A newly conserved miRNA (tof-MIR398) was selected for conservation analysis via WebLogo which found to conserve among other plant species i.e *Cynara cardunculus*, *Arabidopsis thaliana*, *Vitis vinifera*, *Oryza sativa* (Figure. S.1.).The phylogeny analysis of one of the predicted miRNA (tof-MIR393) was done by using Clustal W, with other plant species *i.e Arabidopsis thaliana, Cynara*

cardunculus, Cucumis melo, Oryza sativa and *Vitis vinifera* by using Clustal W method. As shown in (S.2. Figure).

***Prediction of Taraxacum miRNAs potential targets*:** Prediction of miRNA targets is a crucial part of characterizing the profiled *Taraxacum* miRNAs. By using a very strict methodology, as previously stated, an aggregate of 35 targeted genes have been predicted for 34 newly conserved *Taraxacum* miRNAs. These targets are comprised of 35 GO-terms, (as illustrated in Table 1) are involved in important processes such as transporters, metabolism, transcription factors, cell signaling, growth and development, structural proteins, and stress-related processes.

Table.1. *Taraxacum* targets enrichment analysis. Analysis of putative *Taraxacum* targets enriched in GO keywords. Molecular function, biological process, and cellular component are denoted as MF, BP, and CC, respectively.

GO Terms	Ontology	Description
GO:0008157	MF	protein phosphatase 1 binding
GO:0036002	MF	pre-mRNA binding
GO:0004834	MF	tryptophan synthase activity
GO:0044508	MF	glucagon-like peptide 1 receptor activity
GO:0102694	MF	kinetin UDP glycosyltransferase activity
GO:0032767	MF	copper-dependent protein binding
GO:0008134	MF	transcription factor binding
GO:0030741	MF	inositol 1-methyltransferase activity
GO:0043425	MF	bHLH transcription factor binding
GO:0097162	MF	MADS box domain binding
GO:0003789	MF	actin filament severing activity
GO:0032767	MF	copper-dependent protein binding
GO:0015250	MF	water channel activity
GO:0030042	BP	actin filament depolymerization
GO:2000001	BP	regulation of DNA damage checkpoint
GO:0032612	BP	interleukin-1 production
GO:0043629	BP	ncRNA polyadenylation
GO:0032610	BP	interleukin-1 alpha production
GO:0034130	BP	toll-like receptor 1 signaling pathway
GO:0097359	BP	UDP-glucosylation
GO:0055070	BP	copper ion homeostasis
GO:0019086	BP	late viral transcription
GO:0006009	BP	glucose 1-phosphate phosphorylation
GO:0009299	BP	mRNA transcription
GO:0034660	BP	ncRNA metabolic process
GO:0070422	CC	G-protein beta/gamma-Raf-1 complex
GO:0043674	CC	Columella
GO:0005723	CC	alpha-heterochromatin
GO:0008305	CC	integrin complex
GO:0031533	CC	mRNA cap methyltransferase complex
GO:1903113	CC	copper ion transmembrane transporter complex
GO:0035976	CC	transcription factor AP-1 complex
GO:0030121	CC	AP-1 adaptor complex
GO:0070985	CC	transcription factor TFIIK complex
GO:0005849	CC	mRNA cleavage factor complex

According to GO-molecular functions, as shown in Fig. 3, the potential targets of recently reported *Taraxacum* miRNAs heavily engaged with processes related to regulation of response to biotic stimulus (GO: 0002831), response to stress (GO: 0006950), cell surface receptor signaling (GO: 0007166), immune response regulating cell (GO: 0002768), positive regulation of response (GO: 0031347) and toll-like receptor 1 signaling (GO: 0034130). In contrast to animals, plants lack an adaptive immune system and specialized immune cells. However, plants utilize distinct integrated defense mechanism to protect themselves from pathogens attack and manage advantageous symbiotic and commensal associations with bacteria [30].

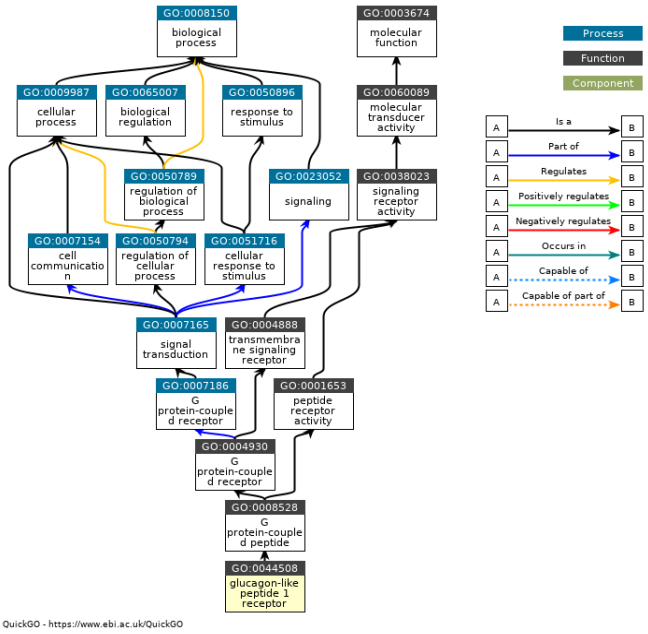


Figure 3. *Taraxacum* GO Molecular Function. *Taraxacum* miRNAs are involved in different molecular functions, therefor more complex enriched molecular functional processes were developed using QuickGo.

Higher plants have a huge number of immune receptors that are dispersed both intracellularly and on the surface and that can recognize a wide range of immunological.

Signals related to pathogen infections. The majority of surface-localized plant immunological receptors are proteins like receptor and receptor kinases that are comparable in principle to animal receptors called toll-like receptor molecules. Pattern recognition receptors (PRRs), also known as toll-like receptors, are able to recognize particular Cutin and apoplastic peptide fragments, as well as microbe-associated molecular patterns (MAMPs) like bacterial flagellin and lipopolysaccharides, are examples of host-derived, damage-associated molecular patterns (DAMPs) (also known as DAMPs). A major and significant initial defense mechanism in plants is pattern-triggered immunity

(PTI), is activated by PRRs once they have been activated. PTI is activated by several different signaling pathways, including as mitogen-activated protein kinase (MAPK), Ca2+, along with signaling mediated by reactive oxygen species (ROS) (Couto *et al.*, 2016).

According to GO-Biological Process as illustrated in Fig. 4, the probable targets of the recently profiled *Taraxacum* miRNAs have a substantial role in mRNA transcription (GO:0009299), ncRNA polyadenylation (GO:0043629), regulation of DNA damage checkpoint (GO:2000001), mRNA transcription (GO:0009299), coper ion homeostasis (GO:0055070), ncRNA metabolic processes (GO:0034660).

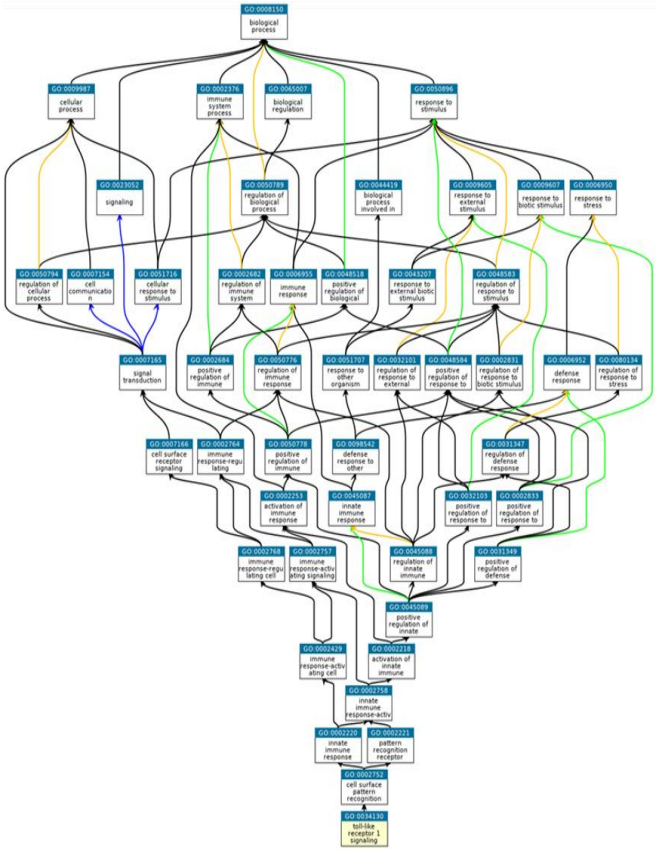


Figure 4. *Taraxacum* GO Biological Process. *Taraxacum* miRNAs are involved in a variety of complex enriched biological processes were developed using QuickGo.

Environmental circumstances are continually changing, which exposes plants to biotic and abiotic stressors. The disruption in reactive oxygen species (ROS) homeostasis has been observed in plants impacted by different growing circumstances (Mittler *et al.*, 2004). Various antioxidant mechanisms with enzymatic and non-enzymatic activities of glutathione reductases, catalases, and peroxidases, effectively scavenge ROS under steady-state circumstances. However, when stress is present, the rate of ROS synthesis might outpace the scavenging systems, leading to an increase in ROS that is particular to a cell or tissue. The oxygen derived compounds have potent oxidizing prospective and break single- and double-stranded DNA as well as a varied range with other genetic substances, which with electron-rich bases (Amor *et al.*, 1998; Roldán-Arjona *et al.*, 2009). Water is considered as significant ROS component that can go through cellular tissues and in many compartments. Such property gives water the capacity to harm a range of cellular structures as well as operate as a signaling molecule, enabling the activation of pathways that regulate metabolic, defense, and developmental pathways (Mittler *et al.*, 2011). Water signaling effects cell division cause activation of cellular checkpoint (Tsukagoshi, 2012).

Cell cycle checkpoints regulate cellular proliferation by preventing the primary cellular regulators, consisting on regulatory cyclins and cyclin dependent kinases (CDKs) to process heterodimeric complexes (Norbury *et al.*, 1992; Tsukagoshi, 2012). The exceptionally conserved ATM AND RAD3-RELATED (ATR) kinases and ATAXIA TELANGIECTASIA MUTATED (ATM), which become recruited dependent on the kind of DNA damage, turn on these checkpoints. (Abraham, 2001; Kurz *et al.*, 2004; Zhou *et al.*, 2000).

The activation of ATM occurs due to breakage of double strands however activation of ATR is due to stalled replication forks, which inhibit DNA replication. Both the ATR and ATM stimulation in mammals causes Chk1 and ChK2 phosphorylation. These kinases then cause phosphorylation of transcription factors such as p53 which are responsible for DNA damage. (Rozan *et al.*, 2007; Shieh *et al.*, 2000). Plant orthologs for p53, Chk2 and Chk1 do not act to exist, though another transcription factor, SUPPRESSOR OF GAMMA RESPONSE1 (SOG1), which maintains genomic stability and participates in reaction to DNA damage (Yoshiyama *et al.*, 2013).

Plants have different characteristic to inhibit CDKs (cyclin-dependent kinases) as DNA stress responses. Plants have CDK inhibitors, which can bind to CDK/cyclin complexes and reduce their function. As checkpoints, these inhibitors make sure that the cell cycle does not continue until the DNA damage has been repaired. SIAMESE (SIM) and WEE1 kinase are two examples of CDK inhibitors found in plants. (Francis, 2011). Whereas in budding yeast and in mammals, inactivation of CDC25 phosphatase leads to cell cycle arrest and activates checkpoint of DNA replication (De Schutter *et al.*, 2007). Plants lacking WEE1 become more vulnerable to replication limiting drugs like hydroxyurea (HU) that depletion of deoxynucleotide triphosphates (dNTPs) by limiting the activity of ribonucleotide reductase (RNR) protein. On the other hand plants with WEE1 deficiency, respond to various DNA damages in the same way that control plants do (De Schutter *et al*, 2007).

GO cellular process [shown in Fig. 5] targeted genes are identified to involved in transcription factor AP-1 complex (GO:0035976), AP-1 adaptor complex (GO:0030121), mRNA cleavage factor complex (GO:0005849), transcription factor TFIIK complex (GO:0070985), G-protein beta/gamma-Raf-1 complex (GO:0070422), alpha-heterochromatin (GO:0005723), integrin complex (GO:0008305), mRNA cap methyltransferase complex (GO:0031533) and copper ion transmembrane transporter complex (GO:1903113). These are significantly identified targeted genes of tof-MIR159, tof-MIR169, tof-MIR393, tof-MIR398 and tof-MIR858. Copper (Cu) is an essential cofactor for many proteins in plants. Whereas the unknown number of cuproproteins play critical roles within plant cells.

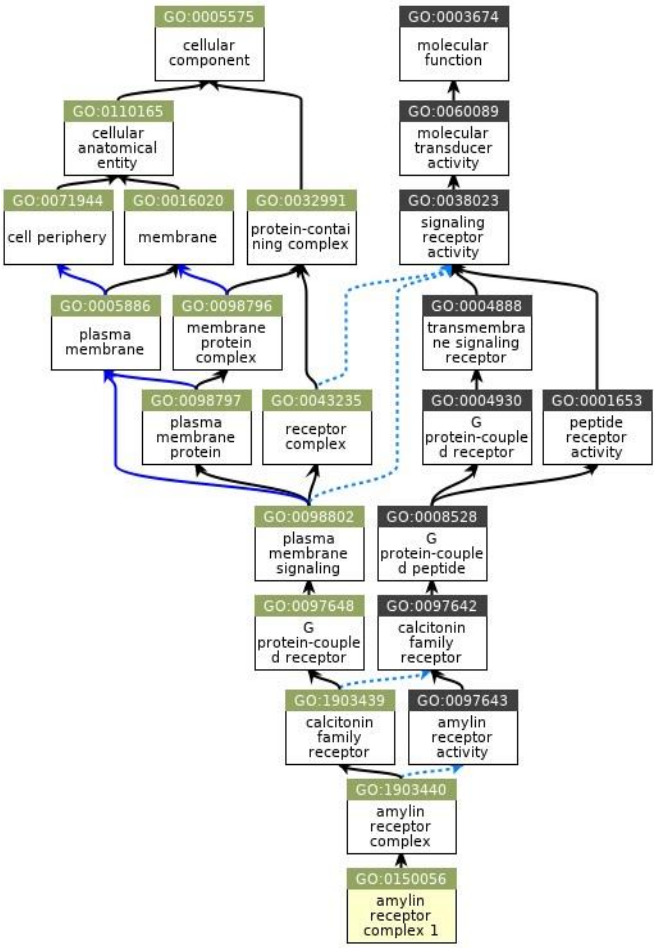


Figure 5. *Taraxacum* GO Cellular Process. *Taraxacum* miRNAs involved various in cellular elements, which were established based on QuickGo.

The micronutrient Cu, is only required in trace amounts to maintain cellular functions. Cu excess, on the other hand, may have a negative impact on plant primary production and even survival. As a result, plants having a tightly maintained the Cu homeostasis, which influenced by tissue development (Printz *et al.*, 2016). According to (Zhou *et al.*, 2012) the MiRNAs playing a crucial role in plant species' responses to metal stress. They discovered six miRNAs in *Medicago truncatula* that are sensitive to Mercury (Hg), Aluminium (Al) and cadmium (Cd). Current research has similarly found that Cd exposure alters the expression of some miRNAs in *Brassica napus* (Huang *et al.*, 2010) and rice (Huang *et al.*, 2009). (Cuypers *et al.*, 2011; Sunkar *et al.*, 2006) discovered that various metals have various sound effects the expression of miRNA. As, copper (Cu) and iron (Fe) inhibit miR398 expression, resulting in increased copper/zinc superoxide dismutase (CSD) expression. Beside this, too much cadmium (Cd) causes response to miR398, which also evident when there is little Cu available. SQUAMOSA Promoter Binding Protein-Like7 (SPL7), an active transcription factor thought to be a key regulator in Cu homeostasis, controls the expression of miR398 under Cu deprivation (Abdel-Ghany *et al.*, 2008; Yamasaki *et al.*, 2009).

According to (Salih *et al.*, 2016) the miRNAs involved in regulation of TFs are mir159, mir169, mir172, mir319 and mir164 which are typically found in multigene families. TFs are typically modular proteins with DNA-binding domains which interact with ciselements of many of their targeted genes (Boeva, 2016; Orenstein *et al.*, 2017). Additionally, it contains a protein-protein interaction domain that aids in the oligomerization of other regulators with TFs (Boeva, 2016; Padi *et al.*, 2015). Most of the miRNA targets are found as TFs that control plant development and growth. Flowering is one of the key stages in plant development, and it is controlled by intricate gene networks that take into account a variety of extrinsic and intracellular stimuli to ensure that flowering occurs when it should (Shriram *et al.*, 2016; Shu *et al.*, 2016; Spanudakis *et al.*, 2014). The activation of three major TFs, LEAFY (LFY), FRUITFULL (FUL), and APETALA1, ultimately govern this pathway (AP1). Subsequently the absence of LFY function results in pronounced interruption in the flowering among these three genes, it has been assumed that LFY functions as a key regulator. However, TF SQUAMOSA PROMOTER BINDING PROTEINLIKE 3 regulates these three genes (SPL3). The SQUAMOSA PROMOTER-BINDING (SBP) domain, which is made up of a distinctive zinc finger having two zinc ion binding sites, is the distinguishing feature of SQUAMOSA PROMOTER BINDING PROTEINLIKE (SPL) genes.

***Conclusion*:** Utilizing comparative genomics techniques, the 34 novel conserved miRNAs from *Taraxacum* EST sequences that are members of 33 families were discovered. The discovery of each of these miRNAs in *Taraxacum* is novel. Additionally, 35 targets are predicted for these 34 *Taraxacum* miRNAs, and they play parts in 35 GO-enriched pathways. All targets are found to be involved in a variety of developments, including cell signaling, metabolism, stress-

related processes, transporter, growth and development, transcription factor, and structural protein. Additionally, RT-PCR is used to confirm a few randomly chosen *Taraxacum* miRNAs. All newly identified miRNAs and their targeted proteins also were thoroughly characterized and annotated. These results will aid in understanding the *Taraxacum* miRNA-mediated life processes.

Supporting information

S.1. *Taraxacum* Characterization Table. The *Taraxacum* predicted miRNAs are described regarding source miRNAs, length of Precursor (PL), Mature sequence (MS), Mature sequence length (MSL), Minimum free energy Kcal/mol (MFE), Mature sequence arm (MSA), Number of mismatches (NM) and Data Base (DB).

S2 Table. *Taraxacum* primer sequences for RT-PCR Validation. The *Taraxacum* Primer sequences for pre-miRNAs experimental validation using RT-PCR. ten randomly chosen *Taraxacum* miRNAs that were submitted to RT-PCR expression investigation, along with primers, source EST, melting temperature (Tm) and product size (bp).

S.1. Figure tof-miR398 WebLogo. Alignment of the *Taraxacum* pre-miRNAs with their ortholog pre-miRNAs for sequence conservation analyses using WebLogo: a sequence logo generator, showing a) mature miRNA conservation with *Cynara cardunculus, Arabidopsis thaliana, Vitis vinifera* and *Oryza sativa* highlighted in box.

S.2. *Taraxacum* Phylogeny Figure. A cladogram tree was created using the neighbor-joining clustering approach to analyse miRNAs. *Taraxacum officinale with Arabidopsis thaliana, Cynara cardunculus, Cucumis melo, Oryza sativa* and *Vitis vinifera* were selected for phylogeny analysis.

Author's contribution

***Conceptualization*:** Shehnaz Shair, Iftekhar Ahmed Baloch and Muhammad Naeem Shahwani

***Formal analysis*:** Shahjahan Shabir Ahmed Rana and Asma Abro

***Investigation*:** Shehnaz Shair

***Methodology*:** Iftekhar Ahmed Baloch

***Supervision*:** Muhammad Naeem Shahwani and Iftekhar Ahmed Baloch

***Writing – original draft/ Review and editing*:** Shehnaz Shair

Conflict of interest: All authors declared no conflict of interest related to this study.

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