ORIGINAL ARTICLE

IDENTIFICATION OF MICRO-RNAS IN CALENDULA OFFICINALIS: NEW INSIGHTS INTO HERBAL MEDICINE

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ABSTRACT

MicroRNAs (miRNAs) are a class of single stranded, non-coding, small RNAs (18–22 nucleotides) that play important roles in plant and animal biological processes by acting as gene expression regulators at a post-transcriptional level. Nevertheless, no miRNA has been identified in marigold (Calendula officinalis), a very well known medicinal plant.In this study, total RNA from marigold was extracted, then, using Next-generation sequencing and homology approaches, potential miRNAs were predicted. A total of 4 conserved miRNAs, with 0 mismatches, were identified in marigold: ath-miR166a, osa-miR166h, ppt-miR894 and ath-miR817. The miR166 family is one of the most conserved family in the plant kingdom, as well as in many medicinal plants with a potential impact on mammals. The potentially conserved miRNAs from Calendula were identified only in the inflorescence, which is the part used for medicinal purposes. Our findings provide new information regarding plant miRNAs in marigold. This approach could open up the way for further researches regarding miRNAs and their role as possible novel therapeutic options in medicinal plants. The potential of miRNA therapeutics, could lead to a modern definition of pharmacognosy.

KeyWords: plant microRNA, Herbal medicine, Calendula officinalis, bioinformatics

INTRODUCTION

MicroRNAs (miRNAs) are small, noncoding, single-stranded RNAs that play a crucial role in gene expression regulation at the post-transcriptional level through mRNA translational repression [1].

Plant miRNAs were initially discovered in Arabidopsis thaliana in 2002 [2]. The most important difference between the two kingdoms involves target recognition [3]. Animal miRNAs almost always bind with mismatches to the target mRNAs they regulate, while plant miRNAs have highly complementary targets, therefor the gene expression regulation take place mostly by direct cleavage of the specific mRNAs [4]. Another difference worth mentioning is that plant miRNAs are methylated at their 3' ends by HEN1, which offers them stability and protection from exonucleases [5].

Recent research has shown that exogenous plant miRNAs can survive in mammals where they can act like one of their own. Zhang et al. reported that miRNA from plants (rice) can survive after oral ingestion in the mammalian gut and then enter the bloodstream where they can regulate mammalian gene expression [6].

Therefore, plant miRNAs play a role in root initiation, leaf morphology, flower development or stress response [3], but they could also have an impact on animals/humans. Because of their great value, more and more identification and detection techniques are becoming available to researchers.

Various computational tools have been used to identify plant miRNAs such as: a) genome survey sequence (GSS) [7], b) Expresses sequence tag (EST) that represent short, randomly selected complementary DNA sequences [8] c) high-throughput sequencing of small RNA [9]. Among these methods, high-throughput sequencing has contributed to small RNA discovery for those plant species in which information about the genome sequence is unavailable [10].

Calendula officinalis – marigold- a species form the Asteraceae family, is one of the best known medicinal plants [11]. The flowers have been used for anti-inflammatory [12], antibacterial [13] and antifungal activity [14]. It also has been shown to have antioxidant [15], wound healing [16] and immunomodulatory [17] benefits. Traditionally, marigold is used in the treatment of hemorrhoids, duodenal ulcers and dysmenorrhea [18]. Used topically, it's a remedy for inflammations of the oral and mucous membranes, ear infections, wounds and burns [19, 20].

In this article, we identified 4 potentials miRNAs in marigold but we also searched for better understanding of the molecular mechanism from the miRNA point of view associated to medicinal activities of Calendula officinalis.

MATERIAL AND METHODS

Plant materials and RNA extraction

The flowers from Calendula officinalis, grown under natural conditions, were collected from the University of Agricultural Sciences and Veterinary Medicine, Department of Agronomy, Timisoara. Immediately after collection, the fresh tissues were prepared for RNA isolation. Total RNA was extracted from the flowers and petals of marigold by phenol/chloroform using miRVana miRNA isolation kit from Ambion. To confirm the quality of plant RNA we used Agilent 2100 Bioanalyzer in order to determine that the RNA integrity number (RIN) is greater that 8.

Library construction and small RNA sequencing

cDNA libraries were obtained using the Ion Torrent RNA-seq kit v1 for small RNA libraries (ThermoFisher) following to manufacturer's protocol. Then, the resulting RNA library was sequenced using

a 316 chip on an Ion Torrent sequencing platform (ThermoFisher). The obtained raw data was trimmed on the 3p and 5p ends to remove low quality reads, sequences < 17 nucleotides, barcodes and the adapter sequences. Finally, the 18-30 nucleotides long unique sequences were stored until further analysis.

Reference set of miRNA

Previously known miRNAs were downloaded from miRBase v.21 [21] which contains 8,496 known plant miRNAs. To avoid the duplicate entries, we manually removed miRNA copies, so we could obtained the non-redundant, unique plant miRNAs.

Prediction of conserved miRNAs in Marigold

The small RNA raw sequences were converted from BAM/SAM format to FASTA, then they were aligned against the known plant miRNAs unique sequences from miRBase using homology approaches. Only sequences with 100% homology rate (0 mismatches) were considered conserved miRNAs from the flowers of Calendula.

RESULTS

Computational prediction of conserved miRNAs in Calendula

In order to identify the potential miRNAs in marigold, total RNA was extracted from the flowers and subjected to an Ion Torrent sequencing platform (ThermoFisher). We used Ion Torrent system, who is unique among Next-Generation sequencing technologies because the detection for sequencing is based upon measuring the pH change as the consequence of the release of a H+ ion and not upon fluorescent dyes [22]. To obtain high quality raw reads, we used a series of raw data cleaning processes. The remaining clean sequences were matched against known plant miRNAs from miRBase v.21. Fig. 1 shows a schematic representation of the marigold miRNA search procedure.

Due to the absence of the complete genome or transcriptome sequences and with no information regarding Calendula officinalis miRNAs, we used only the non-redundant mature plant miRNA sequences from miRBase against our massive dataset to predict miRNAs in Calendula using similarity searches. The small RNA sequences from the raw dataset were considered as miRNA candidates only if they correspond to the following criteria: at least 18 nt length and to have 0 mismatches in sequences with the known mature plant miRNAs.

A total of 4 miRNAs with 100% homology rate were identified in the flowers of marigold: ath-miR166a, osa-miR166h, ppt-miR894 and ath-miR817. The miR166 family is one of the most conserved family in the plant kingdom due to its important regulatory role.

As reported in Table 1, the sequence length distribution was between 21 and 20 nucleotides long. These are the first miRNAs ever identified in Calendula officinalis.

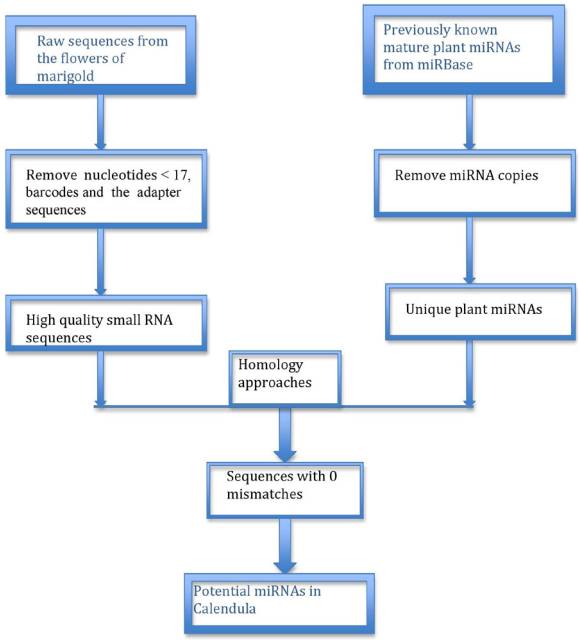

Figure 1.
Workflow for Calendula miRNA prediction

Name	Sequence	Length of mature miRNA (nt)	Reference plant	Plants with the same homolog
Ath-mir166a	UCGGACCAGGCUUCAUUCCCC	21	Arabidopsis thaliana	29
Lus-mir166e	UCGGACCAGGCUUCAUUCCUC	21	Oryza sativa	31
Ppt-mir894	CGUUUCACGUCGGGUUCACC	20	Physcomitrella patens	2
Ath-mir8175	GAUCCCCGGCAACGGCGCCA	20	Arabidopsis thaliana	1

Table 1.
Potential conserved miRNAs in marigold

Conservation between medicinal plants

As seen in Figure 2, the conservancy of miR166 family found in marigold shown high similarity with their homologs in other medicinal plants. We choose BLASTN to search for miR166a and miR166h homologs within miRBase. We selected only the miRNA candidates from plants used in herbal medicine.

Conserved miRNAs play important roles in regulatory biological processes [22]. To date, the miR166 family have been found to be associated with various developmental processes, including organ polarity, seed and root development and nutrition ion uptake [23,24].

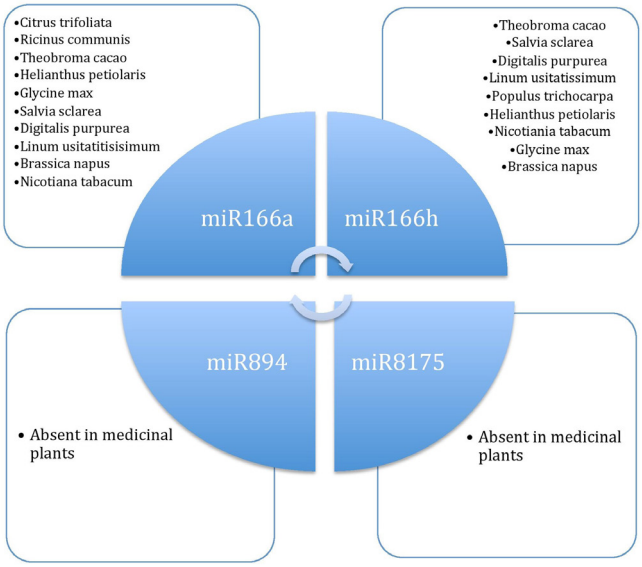

Figure 2.
Conservacy of miRNAs identified in maringold along different medicinal plants

DISCUSSION

In both animals and plants miRNAs are key regulators in gene expression at a post-transcriptional level by repressing or degrading translation [25]. In the last decade, a large number of experimental and computational studies have been made to identify miRNAs from plants. Today, a high amount of miRNAs is deposited in different databases, but still no miRNA from Calendula officinalis (marigold), a very well known medicinal plant, has been discovered.

Without the genome sequence, identification of miRNA in marigold is a great challenge. High-throughput sequencing technologies provide an efficient and inexpensive approach for identifying small RNAs in many plant species [26,27].

Phytotherapy has been used for treating many diseases for ages. Bioactive secondary metabolites such as tannins, flavonoids, polyphenols have been highly studied as an important tool for making less side effect drugs [28]. However, the mechanism of action regarding these plant-delivered active components has been permanently discussed. In particular, Calendula officinalis is a medicinal plant used for centuries due to its many therapeutic actions for treating hemorrhoids, duodenal ulcers, dysmenorrhea, inflammations, infections and wounds [11].

In the present study, using an Ion Torrent sequencing platform (ThermoFisher), we were able to identify 4 potential conserved miRNAs from the fresh flowers of marigold. We chose this specific part of the plant because only the inflorescence has significant value in herbal medicine [29]. We identified 2 members of the miR166 family, a very well conserved family in other plant species [30]. Interestingly, we found out that many medicinal plants have a large number of miRNAs from the miR166 family.

In an article regarding the cross-kingdom transfer of plant miRNA, several miRNA species from plants were identified in mammalian breast milk exosomes [31]. Moreover, the potential human targets of those plant miRNAs, coding several proteins, were predicted. One of the miRNA identified in marigold, ath-mir166a, had one of the highest abundance level in mammalian breast milk. In Table 2 we see that mir166a inhibits Interleukin1 Receptor-like 1 protein, showing that this specific plant miRNA has a role in inflammation and immune response [32,33]. The anti-inflammatory and immunomodulatory actions are among the main benefits of using marigold.

Here, we raise a question regarding a new aspect of plant biology that might have a powerful impact on understanding herbal medicine. The microRNA expressed in medicinal plants might act as a new bioactive compound capable to interact with the mammalian system [34].

Plant miRNA	Target Gene symbol	Protein name	Potential Biological Impact
Ath-mir166a	IL1RL1	Interleukin1 Receptor-like 1	• Reduction of inflamations • Inhibition of immune responses

Table 2.
Potential impact of mir166a on mammals

CONCLUSION

In this study, using high-throughput sequencing technologies, we have identified 4 potential miRNAs in marigold, one of the best known medicinal plants. This is, to our knowledge, the first report on Calendula miRNA identification. Further studies are yet necessary to identify more Calendula miRNAs, but also to validate their targets. This would be an important step forward regarding the role of miRNAs in medicinal plants and their possible influence on mammal metabolism.

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