Gene 554 (2015) 181–195

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Identification of conserved and novel microRNAs in *Catharanthus roseus* by deep sequencing and computational prediction of their potential targets

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ARTICLE INFO

Article history:
Received 27 August 2014
Received in revised form 8 October 2014
Accepted 25 October 2014
Available online 28 October 2014

Keywords:
Catharanthus roseus
Deep sequencing
MicroRNA
Real-time PCR

ABSTRACT

MicroRNAs are small endogenous non-coding RNAs of ~19–24 nucleotides and perform regulatory roles in many plant processes. To identify miRNAs involved in regulatory networks controlling diverse biological processes including secondary metabolism in *Catharanthus roseus*, an important medicinal plant, we employed deep sequencing of small RNA from leaf tissue. A total of 88 potential miRNAs comprising of 81 conserved miRNAs belonging to 35 families and seven novel miRNAs were identified. Precursors for 16 conserved and seven novel cro-miRNAs were identified, and their stem-loop hairpin structures were predicted. Selected cro-miRNAs were analyzed by stem-loop qRT-PCR and differential expression patterns were observed in different vegetative tissues of *C. roseus*. Targets were predicted for conserved and novel cro-miRNAs, which were found to be involved in diverse biological role(s) including secondary metabolism. Our study enriches available resources and information regarding miRNAs and their potential targets for better understanding of miRNA-mediated gene regulation in plants.

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1. Introduction

MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are regulatory nucleic acid molecules which have been widely studied during the past two decades. MiRNAs are small noncoding RNA (ncRNA) molecules of ~19–23 nucleotides in length and play important regulatory roles in most of the biological processes (Bartel, 2004). Plant miRNAs are transcribed from intergenic regions as primary microRNA (pri-miRNAs) and cleaved within nucleus by ribonuclease enzyme Dicer-like 1 (DCL1) to form a precursor-miRNA (pre-miRNA) hairpin which is further cleaved to form a miRNA:miRNA* duplex (Kurihara and Watanabe, 2004). The characteristic feature of plant miRNA processing is 2′-O-methylation of miRNA:miRNA* duplex by Hua-Enhancer1 (HEN1), a nuclear RNA methyl transferase protein (Yu et al., 2005). The miRNA:miRNA* duplex is transported to cytoplasm by HASTY, a plant ortholog to exportin 5 (Bollman et al., 2003) and subsequently incorporated into the RNA-induced silencing complexes (RISCs) where argonaute family proteins direct the mature miRNA to interact with its target gene for regulation (Baumberger and Baulcombe, 2005). The miRNA* strand is usually destined for degradation but it might also become functional guide strands and perform regulatory roles (Guo and Lu, 2010; Hsieh et al., 2009; Li et al., 2010). Within RISC the miRNAs are completely or near perfectly complementary to target mRNA(s), thus, cleave them and ultimately silence the gene concerned. Besides endonucleolytic cleavage plant miRNAs also repress the target gene through translational inhibition (Brodersen et al., 2008). The first miRNA-encoding gene reported was *lin-4* from *Caenorhabditis elegans* that is involved in the regulation of genes during early stages of larval development (Lee et al., 1993), since then several miRNAs have been identified in metazoans and found to be involved in diverse regulatory functions. In plants, miRNAs play crucial roles in regulating many events of developmental activities such as flower and root morphogenesis (Aukerman and Sakai, 2003; Wang et al., 2005), anther development (Millar and Gubler, 2005), and, leaf and vascular development (Kim et al., 2005; Palatnik et al., 2003; Song et al., 2012). The role(s) of plant miRNAs have also been implicated in environmental stresses like cold (Zhou et al., 2008), drought (Zhao et al., 2007; Zhou et al., 2010), salinity (Sunkar et al., 2008), pathogen infection (Navarro et al., 2006), UV-B radiation (Zhou et al., 2007) and mechanical stress (Lu et al., 2005). Cloning of small RNAs has been an indispensible method for discovery of conserved as well as novel microRNAs (Sunkar and Zhu, 2004; Sunkar et al., 2005). Apart from cloning, computational methods

Abbreviations: A, adenosine; AMFE, adjusted minimal folding free energy; BLAST, Basic Local Alignment Search Tool; bp, base pair; C, cytidine; cDNA, DNA complementary to RNA; cro-miRNA, *Catharanthus roseus* micro ribonucleic acid; DCL1, Dicer-like protein 1; DTT, dithiothreitol; dNTP, deoxyribonucleoside triphosphate; EST, expressed sequence tags; G, guanosine; GO, gene ontology; GSS, genomic survey sequences; miRNA, micro ribonucleic acid; μl, microliter; MFE, minimal folding free energy; MFEI, minimal folding free energy index; ng, nanogram; ncRNA, non-coding ribonucleic acid; NCBI, National Centre for Biotechnology Information; nt, nucleotide(s); pre-miRNA, precursor micro ribonucleic acid; qRT-PCR, quantitative real time polymerase chain reaction; RISC, RNA-induced silencing complex; siRNA, small interfering ribonucleic acid; T, thymidine; TIA, terpenoid indole alkaloid; TSA, transcriptome shotgun assembly; U, uridine.

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http://dx.doi.org/10.1016/j.gene.2014.10.046
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also provide a useful means for mining conserved microRNAs in different organisms which rely on the sequences available in the public databases. Expressed sequence tags (ESTs) and genomic survey sequences (GSSs) were analyzed for the identification and prediction of miRNAs in Barley (Colaiacovo et al., 2010), *Brachypodium* (Unver and Budak, 2009), sorghum (Katiyar et al., 2012), soybean (Zhang et al., 2008) and tomato (Yin et al., 2008) etc. Next-generation high-throughput sequencing technologies are robust tools for the identification of poorly expressed, species-specific, non-conserved and novel miRNAs. Deep sequencing of RNAs in combination with transcriptome data is proving helpful in identifying conserved and novel miRNAs in plants lacking whole genome sequence information. Several miRNAs have been reported from plants like *Arabidopsis* (Fahlgren et al., 2007), rice (Sunkar et al., 2008; Sunkar and Jagadeeswaran, 2008), *Vitis vinifera* (Pantaleo et al., 2010), *Populus trichocarpa* (Puzey et al., 2012), *Vitis amurensis* (C. Wang et al., 2012), *Hevea brasiliensis* (Gébelin et al., 2012), *Prunus persica* (Luo et al., 2013), *Brassica oleracea* (Lukasik et al., 2013), *Solanum tuberosum* (Lakhotia et al., 2014), *Triticum aestivum* (Su et al., 2014) etc. by using high-throughput sequencing and subsequent computational analysis.

The *Catharanthus roseus* belongs to the Apocynaceae family and synthesizes over 130 secondary metabolites of economic importance that include a variety of terpenoid indole alkaloids and phenolic compounds via Tepenoid Indole Alkaloid (TIA) and phenylpropanoids pathways, respectively (El-Sayed and Verpoorte, 2007; Mustafa and Verpoorte, 2007). Important alkaloids produced by *C. roseus* include anti-neoplastic vinblastine and vincristine, anti-hypertensives reserpine and ajmalicine, and anti-arrhythmic ajmaline. *C. roseus* leaf contains specialized cells and is considered as a major site for biosynthesis of high value secondary metabolites (Murata and De Luca, 2005; St-Pierre et al., 1999). MicroRNA-like molecules have recently been identified and reported from few secondary metabolite-rich plants having medicinal and pharmaceutical importance like *Taxus chinensis* (Qiu et al., 2009), *Papaver somniferum* (Unver et al., 2010) and *Artemisia annua* (Pérez-Quintero et al., 2012). Due to the lack of genome sequence and availability of limited number

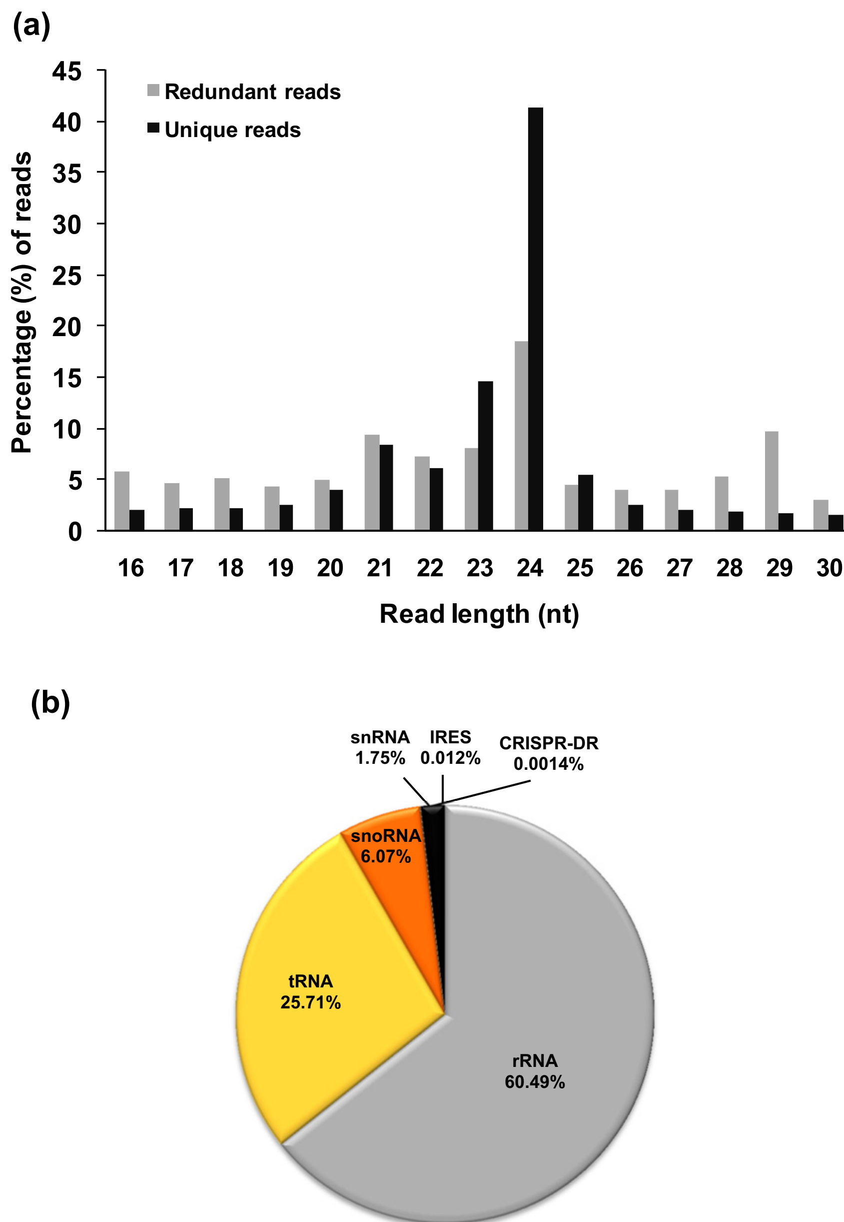

Fig. 1. Analyses of small RNA reads generated by deep sequencing of *C. roseus* leaf small RNA library. (a) Size distribution of small RNA sequences identified in the library. Majority of small RNA reads ranged between 21 and 24 nt in length among which 24, 23 and 21 nt long were the most abundant. (b) Percentage of major categories of non-coding RNAs other than miRNAs identified in the library.

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