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Computational identification of miRNAs and their targets from Niger (*Guizotia abyssinica*)

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ABSTRACT

MicroRNAs play a pivotal role in regulating a broad range of biological processes, acting by cleaving mRNAs or by translational repression. A group of plant microRNAs are evolutionarily conserved; however, others are expressed in a species-specific manner. In this study we used homology-based analysis with available expressed sequence tag (EST) of Niger (*Guizotia abyssinica*) to predict conserved miRNAs. Two potent miRNAs targeting 49 genes were identified. The newly identified miRNAs belongs to miR2592 and miR396 family. Targets recognized were F-box proteins, leucine zipper, DEAD box RNA helicase, disease resistant proteins. Gene annotations revealed miRNAs were involved in growth and development and Encyclopaedia of Genes and Genomes (KEGG) pathway analyses showed miRNAs were involved in metabolic pathways.

1. INTRODUCTION

miRNAs are small endogenous 20-24nt non-coding RNAs derived from single-stranded RNA precursors that can form stem-loop structures and have been proved to play a vital role in wide range of biological process. In plants, after transcription by Pol II or Pol III enzyme into primary miRNA (pri-miRNA), the miRNA gene is processed by Dicer-like (DCL) into a stem-loop that contain miRNA/ miRNA* duplex, called an miRNA precursor (pre-miR-NA). Subsequently, the duplex is cleaved from the pre-miRNA and transported from the nucleus into the cytoplasm which combines with Argonaute (AGO) forming the RNA-induced silencing complex (RISC). miRNAs, regulate the gene expression at post-transcriptional level by directing the mRNA cleavage or by repressing translation [1,2]. In plants, miRNAs regulate their target mRNAs by nearly perfect complementary base pairing, which leads to the degradation of target genes [3]. Several biological experiments indicate that miRNAs play key roles during development and in response to environmental stresses [4, 5]. There are different methods in identifying miRNAs; viz. direct cloning, high throughput sequencing and computational analysis which uses ESTs and GSS sequences. Although high throughput sequencing technology has made miRNA identification rapid and significant, computational analysis is also a promising way in identification of conserved and novel miRNAs. A majority of miRNAs are evolutionary conserved, which can be identified by sequence homology analyses [6]. Nevertheless, a proportion of miRNAs are species-specific and usually expressed at lower levels in comparison with other conserved miRNAs [7, 8]. Till date, miRNAs have been identified in variety of species using computational approach such as silkworm, where 16 novel miRNAs were identified using homology search of Genomic survey sequence (GSS) [9], 8 potential novel miRNAs were identified in *Festuca arundinacea* using ESTs and GSS [10], 6 miRNAs that regulate twenty potential targets were predicted in mulberry [11], in Soybean 521 novel miRNA genes belonging to 58 families were identified [12]. Three conserved miRNAs belonging to miR166 and miR1310 were identified in Finger millet using ESTs [13]. Niger (*Guizotia abyssinica* (L. f.) Cass, *Compositae*) is a dicotyledonous herb and oilseed crop cultivated in Ethiopia and India.

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Niger is usually grown on light poor soils with coarse texture. Unlike other plants Niger also faces abiotic and biotic stress in changing environmental conditions; hence studies on miRNAs in this plant, an untouched area till date will help in improving the crop yield and quality.

In the present study, for first time we have identified two conserved miRNAs belonging to miR396 and miR2592 family using available ESTs and characterized their target genes. Gene ontology, protein interactions and KEGG pathway analysis for target genes were also performed with available computational tools.

2. METHODOLOGY

2.1 Identification of conserved miRNA

EST sequences of Niger (*Guizotia abyssinica*) were downloaded from NCBI (http://www.ncbi.nlm.nih.gov) and plant miRNAs from miRBase v21 (www.mirbase.org) were used as reference for predicting the conserved miRNAs. The sequences were assembled using CAP3 program [14]. tRNA and rRNA sequences were filtered with successive BLAST searches over the ribosomal RNAs database from Rfam 12 (http://www.sanger.ac.uk/Software/Rfam/). The coding regions were removed using BLASTX. mirnaDetect (http://datamining.xmu.edu.cn/main/~leyiwei/mirnaDetect.html) a tool for detecting potential pre-miRNAs from the genome-scale data was also used to predict the possible miRNAs in Niger [15]. The secondary structures of putative pre-miRNAs were predicted by Mfold (http://mfold.rna.albany.edu) [16].

2.2 Target Prediction and GO analysis

Target predictions for the miRNAs are based on the principle of nearly perfect complementation between the miRNA and target mRNAs. The *Brassica rapa* transcripts, downloaded from phytozome v10 (www.phytozome.net) was used to determine the potential target mRNA candidates for miRNAs using psRNATarget with default parameters (http://www.plantgrn.org/psRNATarget/) [17]. Sequences with a score of less than 4 were regarded as miRNA target genes. Functional annotations of predicted targets were analysed using BGI WEGO platform (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) [18].

2.3 KEGG pathway analysis and predicted protein interactions

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database was used to identify the significantly enriched pathways of miRNA target genes. STRING (http://string-db.org/) computational tool was used to study protein-protein interactions for miRNA targets. These interactions are derived from genomic context, high throughput experiments and Co-expressions.

3. RESULTS AND DISCUSSION

3.1 Identification of conserved miRNAs in Niger

Till date there are 25711 Niger ESTs available in NCBI, which were downloaded for the analysis. microRNAs in Niger were identified using mirnaDetect and CAP3 tools. Initially all the ESTs sequences were assembled using CAP3 programme and Blast search was initiated with default parameters. Initially we found more than 32 probable miRNA candidates which were screened for their precursors, which were supposed to be the parent molecules of functional miRNAs. The obtained sequences were filtered for the tRNA, ribosomal RNA contamination by successive Blastn searches on Sanger's Rfam database. The presence of coding sequences among the predicted originator sequences were analysed through Blastx strategy against non-redundant protein database. Successive filtering resulted in 13 microRNAs, which were screened for stable secondary stem-loop structures, as the main feature of miRNA is to fold back with precursor sequences. Stem-loops were obtained from Zuker folding algorithm adopted form MFOLD 3.2. Out of 13 putative pre-miRNAs only 02 sequences satisfied the criteria described by Ambros *et al.* [19], which are 21nt in length, which is consistent with other reports [20-21]. The identified conserved miRNAs in Niger were designated as Gab-miR1 and Gab-miR2 which belonged to the family miR2592 and miR396 (Table 1). Precursor sequence of identified mature Gab-miR1 and Gab-miR2 was 165 and 120nt in length with the MEF of -41.1 and -26.5 kcal/mol respectively. Precursor sequences for identified miRNAs are given in Supl. 1 and their secondary structures are depicted in Figure 1.

Table 1 Conserved miRNAs identified in Niger by EST analysis.

miRNA	Gene ID	Sequence	Length	Precursor length	GC%	MFE*
Gab-miR1	gb|GE573440.1	AAAUGCUUGAGUCCUGUUGUU	21	165	40	-41.1
Gab-miR2	gb|GE552120.1	UUCCACAGCUUUCUUGAACUU	21	120	34	-26.5

Suppl. 1: Precursor sequences of conserved miRNAs identified in Niger.

miRNA	Precursor Sequence
Gab-miR1	GGAAGAGUAUUAGGGCUUGCUAGAGAUAAGGAUCUUGGAGUGGAGAACGUGAGGCAUUUUAUUCUCGAUGAGUGUGACA AAAUGCUUGAGUCACUUGAUAUGAGAAGAGAUGUUCAGGAGAUUUUUAAGAUGACACCUCAUGACAAGCAAGUAAUGAU GUUCUCU
Gab-miR2	AUUGAUAUCAGAUGAGAGCUUCAUCUUCUUCUUCAUCUUCUGAUGAUGAAUUUCUUCAAGAAACUGAGAAUUGAACAACA CCCAGAUCAAGAAACAAGAUCAGAAGCUCAAAUCUUCAAU

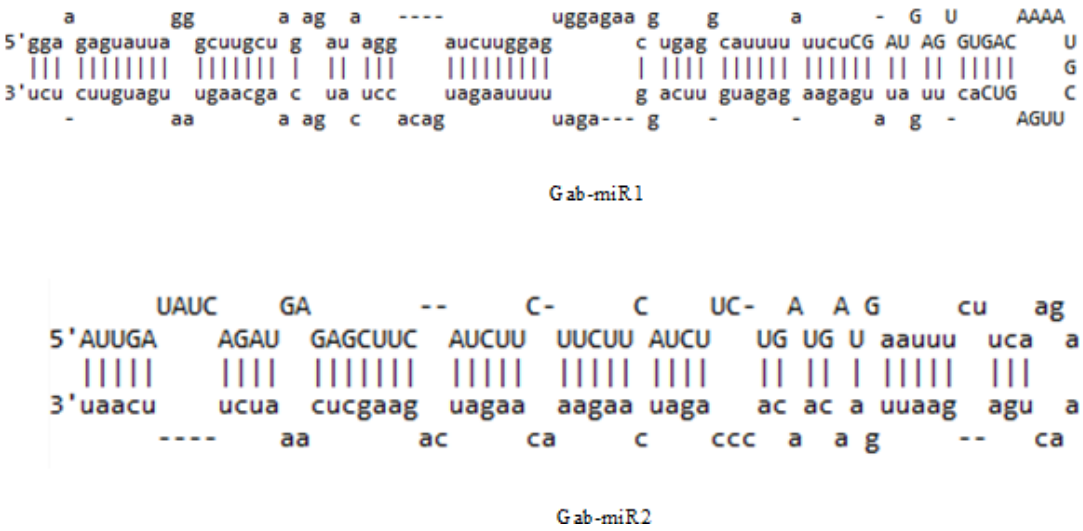

Fig. 1: Secondary structure of predicted conserved miRNAs in Niger.

3.2 **Target prediction and GO analysis**

Functional importance of the predicted miRNAs can be understood by studying their targets, targets identified using insilco approaches have been important till date. The systemic search for mRNA complementary to miRNAs led to the observation that many miRNAs targeted genes through perfect/nearly perfect matches in anti-sense manner leading to degradation of the target gene [22]. psRNATarget scan was used to identify targets in Niger with Brassica rapa transcripts. A total of 49 targets were identified for two conserved miRNAs, and the main mechanism of gene regulation by miRNA was through target degradation as evidenced by 92% of target cleavage. Both Gab-miR1 and Gab-miR2 showed multiple targets, Gab-miR1 targeted HMG box DNA binding protein, F-box protein and cytochrome p450. Gab-miR2 targeted DEAD box RNA helicase, carboxyltransferace, disease resistant protein, growth regulating factors, heat shock proteins etc. (Suppl. 2). In Arabidopsis HMG domain containing SSRP1 was found to be required in DNA demthylation and for activation and repression of many parentally imprinted genes in the central cell [23]. Recently in Arabidopsis it was shown that AT-rich interaction domain and the HMG-box domain of ARID-HMG proteins promote DNA mini-circle formation but are also capable of inducing negative supercoils into relaxed plasmid DNA suggesting the involvement of this protein in several nuclear events [24]. F-box proteins regulate diverse cellular processes, including cell cycle transition, transcriptional regulation and signal transduction, by playing roles in Skp1p-cullin-F-box protein (SCF) complexes or non-SCF complexes. Stefanowicz et al., showed that in Arabidopsis F-box-Nictaba gene is a stress-inducible gene responsive to salicylic acid, bacterial infection and heat stress, and is involved in salicylic acid related plant defense responses [25]. In rice (*Oryza sativa*) F-box gene MEIOTIC F-BOX (MOF), which is essential for male meiotic progression was studied, mof meiocytes display disrupted telomere bouquet formation, impaired pairing and synapsis of homologous chromosomes, and arrested meiocytes at late prophase I, followed by apoptosis [26]. DEAD-box proteins are ubiquitous in RNA-mediated processes and function by coupling cycles of ATP binding and hydrolysis to changes in affinity for single-stranded RNA. AtRH7, one of the *Arabidopsis thaliana* DEAD-box RNA helicases is an interactor of Arabidopsis COLD SHOCK DOMAIN PROTEIN 3 (AtCSP3), which is an RNA chaperone involved in cold adaptation [27]. Many disease resistance (R) proteins in plants detect the presence of disease-causing bacteria, viruses, or fungi by recognizing specific pathogen effector molecules that are produced during the infection process [28]. NBS-LRR disease resistant gene are isolated and characterized from Pea (*Pisum sativum*) [29], Mango [30] which shows that NBS genes recognize many different pathogenic virulence factors and play a very important role in disease defence.

Gene annotations of miRNA targets showed they belong to all three GO categories; molecular function, cellular component and biological process. Molecular functions were highlighted with metabolism, transcription, signal transduction, development and response to stress. Cellular component was enriched with nucleus and biological process was enriched with binding and enzyme activity as depicted in Figure 2 and the GO terms for identified targets are given in Suppl. 3.

Suppl. 2: Targets genes identified for conserved miRNAs in Niger.

miRNA_Acc.	Target_Acc.	Target Description
Gab-miR1	Brara.B00171.1	HMG-box (high mobility group) DNA-binding family protein
	Brara.I04066.1	F-box/RNI-like superfamily protein
	Brara.C00853.1	F-box/RNI-like superfamily protein
	Brara.D00129.1	cytochrome P450, family 76, subfamily C, polypeptide 4
Gab-miR2	Brara.A02187.1	Pentatricopeptide repeat (PPR) superfamily protein
	Brara.G02611.1	P-loop containing nucleoside triphosphate hydrolases superfamily protein
	Brara.C03329.1	3-phosphoinositide-dependent protein kinase-1, putative
	Brara.D02484.1	Rhodanese/Cell cycle control phosphatase superfamily protein
	Brara.F02877.1	Frigida-like protein
	Brara.I01711.1	NAD-dependent epimerase/dehydratase family protein
	Brara.J02674.1	Chalcone-flavanone isomerase family protein
	Brara.E02713.1	Tetratricopeptide repeat (TPR)-like superfamily protein
	Brara.E00714.6	acetyl Co-enzyme a carboxylase carboxyltransferase alpha subunit
	Brara.E00714.5	acetyl Co-enzyme a carboxylase carboxyltransferase alpha subunit
	Brara.E00714.4	acetyl Co-enzyme a carboxylase carboxyltransferase alpha subunit
	Brara.E00714.3	acetyl Co-enzyme a carboxylase carboxyltransferase alpha subunit
	Brara.C04527.1	alpha/beta-Hydrolases superfamily protein
	Brara.E00714.2	acetyl Co-enzyme a carboxylase carboxyltransferase alpha subunit
	Brara.E00714.1	acetyl Co-enzyme a carboxylase carboxyltransferase alpha subunit
	Brara.B03601.1	DEAD box RNA helicase (RH3)
	Brara.F02796.1	DEAD box RNA helicase (RH3)
	Brara.A00236.1	Disease resistance protein (TIR-NBS-LRR class) family
	Brara.H01668.1	Disease resistance protein (TIR-NBS-LRR class) family
	Brara.D00663.1	Pentatricopeptide repeat (PPR) superfamily protein
	Brara.H02781.1	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
	Brara.E01444.1	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein
	Brara.J01076.1	Protein kinase superfamily protein
	Brara.G03519.2	Protein of unknown function (DUF630 and DUF632)
	Brara.G03519.1	Protein of unknown function (DUF630 and DUF632)
	Brara.A01418.1	growth-regulating factor 8
	Brara.D02744.1	growth-regulating factor 9
	Brara.I01630.1	cytochrome P450, family 96, subfamily A, polypeptide 15
	Brara.D00563.1	growth-regulating factor 4
	Brara.B01438.1	growth-regulating factor 7
	Brara.G01562.1	growth-regulating factor 4
	Brara.C01828.1	growth-regulating factor 4
	Brara.I03590.1	growth-regulating factor 4
	Brara.D02218.1	growth-regulating factor 3
	Brara.E00841.1	growth-regulating factor 3
	Brara.J00997.1	Leucine-rich repeat (LRR) family protein
	Brara.K00742.1	growth-regulating factor 8
	Brara.K01237.1	growth-regulating factor 2
	Brara.A00122.1	growth-regulating factor 2
	Brara.C02492.1	growth-regulating factor 1
	Brara.D01740.1	GRAS family transcription factor
	Brara.A00536.1	2Fe-2S ferredoxin-like superfamily protein
	Brara.C01276.1	HEAT SHOCK PROTEIN 81.4
	Brara.J02094.1	Aldolase-type TIM barrel family protein
	Brara.B00492.1	Aldolase-type TIM barrel family protein

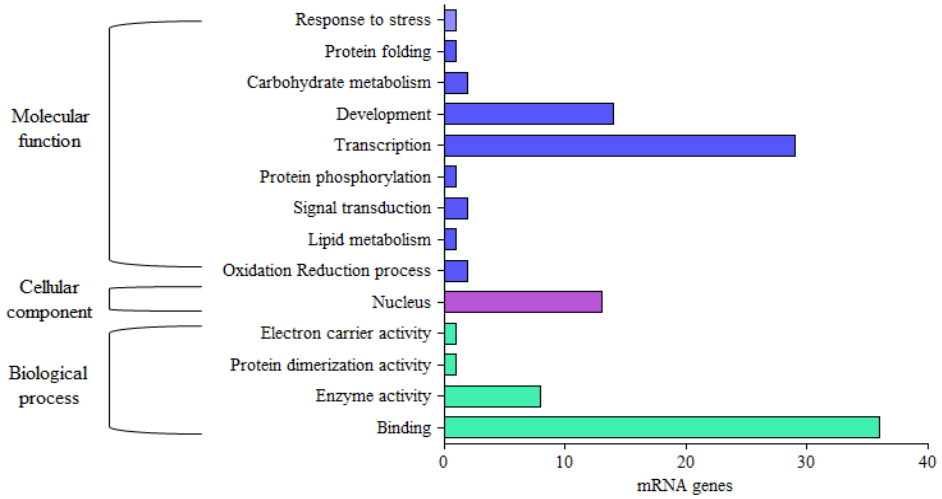

Fig. 2: Gene ontology classification of predicted targets genes for miRNAs identified in Niger.

Suppl. 3: Gene ontology annotations of miRNAs in Niger.

miRNA_Acc.	Target_Acc.	GO term
Gab-miR1	Brara.B00171.1	GO:0005515
	Brara.I04066.1	GO:0005515
	Brara.C00853.1	GO:0005506, GO:0016705, GO:0020037, GO:0055114
Gab-miR2	Brara.J02674.1	GO:0016872
	Brara.C04527.1	GO:0006629
	Brara.B03601.1	GO:0003723, GO:0004386, GO:0005634, GO:0003677, GO:0005524, GO:0016787, GO:0003676, GO:0008270
	Brara.F02796.1	GO:0003723, GO:0004386, GO:0005634, GO:0003677, GO:0005524, GO:0016787, GO:0003676, GO:0008270
	Brara.A00236.1	GO:0043531, GO:0005515, GO:0007165
	Brara.H01668.1	GO:0043531, GO:0005515, GO:0007165
	Brara.H02781.1	GO:0046983
	Brara.J01076.1	GO:0004672, GO:0006468
	Brara.G03519.2	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.G03519.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.A01418.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.D02744.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.I01630.1	GO:0005506, GO:0016705, GO:0020037, GO:0055114
	Brara.D00563.1	GO:0005634, GO:0006351, GO:0032502
	Brara.B01438.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.G01562.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.C01828.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.I03590.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.D02218.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.E00841.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.J00997.1	GO:0005515
	Brara.K00742.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.K01237.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.A00122.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.C02492.1	GO:0006351, GO:0032502, GO:0005524, GO:0005634, GO:0006355
	Brara.A00536.1	GO:0009055, GO:0051536
	Brara.C01276.1	GO:0005524, GO:0006457, GO:0006950, GO:0051082
	Brara.J02094.1	GO:0005975
	Brara.B00492.1	GO:0005975

3.3 **KEGG pathway analysis and Protein interactions**

KEGG pathway analysis showed 07 significantly enriched pathways in Niger such as pentose phosphate pathway, polycyclic aromatic hydrocarbon degradation, Fatty acid biosynthesis and metabolic pathways of glycerolipid, glycero-phospholipid, pyruvate and propaanote (Table 2).

Table 2 KEGG analysis for miRNA target genes in Niger.

KEGG ec	Target_ID	Pathways
2.2.1.2	bra00030	Pentose phosphate pathway
3.1.1.26	bra00561	Glycerolipid metabolism
3.1.1.3	bra00561	Glycerolipid metabolism
	bra00564	Glycerophospholipid metabolism
	bra00030	Pentose phosphate pathway
	bra00624	Polycyclic aromatic hydrocarbon degradation
3.1.1.32	bra00564	Glycerophospholipid metabolism
6.4.1.2	bra00061	Fatty acid biosynthesis
	bra00620	Pyruvate metabolism
	bra00640	Propanoate metabolism

Web based protein-protein interactions of the identified miRNAs showed that putative predicted proteins such as Bra022667, Bra039334, Bra0333281, Bra011781, Bra000575, Bra006956, and Bra005268 which are growth regulating factors work in network and may play a role in regulation of gene expression (Figure 3).

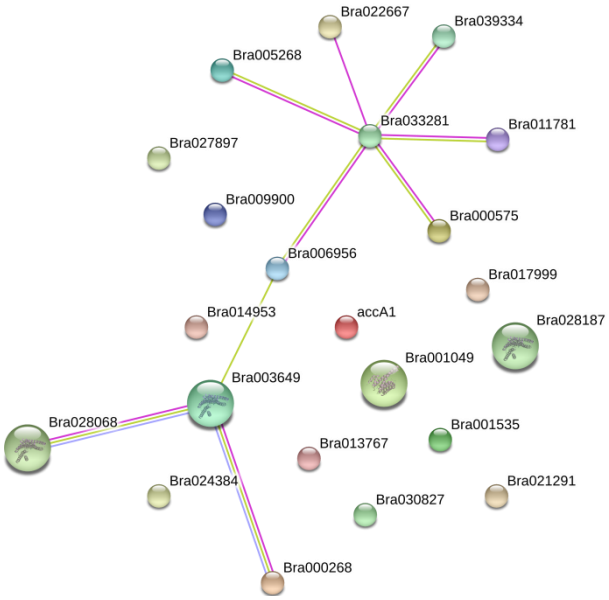

Fig. 3: Protein-protein interaction networks of predicted targets.

4. CONCLUSION

The present study for the first time identifies miRNAs in Niger. The evolutionary conservation of miRNA across the taxa renders powerful approach in their identification using EST analysis. We were able to identify two microRNAs targeting 49 genes. To further analyse the role of miRNAs target interactions, GO analysis and protein interactions of target genes were studied which showed miRNAs may play an important role in growth and development. These finding will contribute for future investigations of miRNAs in Niger under abiotic and biotic stress.

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