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In silico Identification and Characterization of Conserved miRNAs and their Targets in Pigeon pea (*Cajanus cajan* L.) Expressed Sequence Tags

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A B S T R A C T

MicroRNAs (miRNAs) are highly conserved class of short endogenous non-coding small RNA molecules of about 18–22 nucleotide in length. MicroRNAs negatively regulate the gene expression by degrading target mRNA. In the present study, comparative genomic based approach was used to identify and characterize new conserved microRNAs in "orphan legume crop" pigeon pea (*Cajanus cajan* L.) using expressed sequence tags (ESTs) by *in silico* method. A total of 4,621 unique previously reported microRNAs were used for homology search in 25,576 ESTs of pigeon pea for identifying conserved miRNAs. The results upon stringent selection found five conserved miRNAs namely cca-miR6483, cca-miR5219, cca-miR393a, cca-miR395a, cca-miR169b belonging to five different families. The target analysis through psRNATarget server found 27 mRNA targets which code for eukaryotic translation initiation factor 6 (EIF-6)-like protein, apyrase-like protein, ferric reductase, ATP sulfurylase, CCAAT-box transcription factor complex WHAP12, Lipoxygenase-9, Transport inhibitor response 1, and MYB transcription factor MYB102 which play an important role in response to both biotic and abiotic stresses. Our results have laid the foundation for further research on miRNAs, which will lead to understand the gene silencing mechanisms at post transcriptional level for various stresses.

Introduction

Pigeon pea (*Cajanus cajan* L.) is an often cross pollinated legume crop (2n = 2x = 22) with genome size 808 Mb conferring 48,680 genes. In India, pigeon pea is cultivated in an area of 3.88 mha contributing to total production of 3.29 mt with average productivity of 849 kg/ha (Agricultural statistics at a glance, 2014). Pigeon pea serve has a multipurpose crop with unique benefits like firewood, fence, thatch and making baskets from its byproducts apart from economic yield. The decomposition of fallen leaves will enrich soil with nutrients and also symbiotic nitrogen fixation increases fertility (Varshney *et al.*, 2010). The nutritional benefits include 45 % dietary fibers, 23 % protein, 7 % calcium, trace amounts of thiamin, riboflavin, and niacin. Despite of many advantages, pigeon pea has remained "orphan legume crop" with less genetic improvement (Varshney *et al.*, 2009). Recognizing its importance, substantial amount of genomic resources have been generated, largely owing to the efforts of

Indo-US Agricultural Knowledge Initiative (AKI), NSF and GCP funded projects and its genome sequence has been drafted (Varshney *et al.,* 2010; Dutta *et al.,* 2011*;* Bohra *et al.,* 2011). To exploit advantages from pigeon pea, the knowledge about the genetic basis of yield, quality and stress tolerance is important for genetic improvement. Under such circumstances microRNAs (miRNA) are one of the major players in controlling biotic and abiotic stress responses in plants (Schwab *et al.,* 2005). The miRNAs are approximately 21-nucleotide (nt) noncoding small RNAs that are encoded by MIR genes located in the intergenic (between protein-coding genes) or intragenic regions (within protein-coding genes) on the chromosomes which play critical roles in gene regulation at the post-transcriptional level (Schwab *et al.,* 2005*;* Unver *et al.,* 2009*;* Yang *et al.,* 2012).

The molecular mechanism of miRNA-mediated gene expression involves perfect or near perfect complement with targeted mRNA sequences, and then degrade targeted mRNAs or repress mRNA translation (Bartel *et al.,* 2004). The miRNAs are therefore negatively regulate the gene expression (Voinnet *et al.,* 2009). A wide range of miRNAs have been discovered in model crops like arabidopsis, rice and maize which code for several developmental programs, such as root initiation and development, vascular development, leaf morphogenesis and polarity as well as floral differentiation (Marin *et al.,* 2010*;* Donner *et al.,* 2009*;* Mallory *et al.,* 2004*;* Chuck *et al.,* 2008).

Reviewing the important of miRNAs in plant development, many methods were developed to identify the miRNAs among them computational method was effective, quick and less time consuming. The biogenesis of miRNAs suggests that it is possible to find miRNAs by searching expressed sequence tags (ESTs) with known miRNAs. This method provided avenue for identification of conserved miRNAs based on comparative genomics in many species like *Allium sativum, Camellia sinensis, Zea mays, Glycine maxand Gossypium herbacium* (Panda *et al.,* 2014*;* Das and Mondal, 2010*;* Zhang *et al.,* 2006a*;* Liu *et al.,* 2011; Boopathi and Pathmanaban, 2012).

As mentioned above, pigeon pea an "orphan legume crop" despite of many advantages with less information was registered about miRNAs. This article mainly provides genetic basis for different responses in pigeon pea to stress and enhance the genetics of the crop. The identification of new miRNAs was done through *in silico* mining in expressed sequence tags (ESTs). Our efforts resulted in identification of five novel microRNAs in pigeon pea.

Materials and Methods

Collection of miRNA sequences and ESTs of pigeon pea

To search potential new miRNA in pigeon pea expressed sequence tags (ESTs), the sequences of previously known plant mature miRNA sequences from viridaeplantae kingdom were downloaded from miRBase release 21 (http://wwwmirbaseorg/ftpshtml). The total count of plant mature miRNA sequences was around 8486 which includes lots of similarities and duplicates.

The redundancy in miRNA sequences were removed by cd-hit online sever with Sequence identity cut-off of 1 which means 100% similarity between the sequences. This resulted in 4621 unique miRNA sequences. These miRNA sequences were BLASTed to assembled EST sequences of pigeon pea (*Cajanus cajan* L.). The EST sequences (25,576 as on 2014) were downloaded from NCBI's dbEST database (http://www.ncbi. nlm.nih.gov/).

Processing of pigeon pea ESTs in to contigs and singletons

All downloaded ESTs are partial sequences of a gene which consist of wide range of contamination like, polyA tails, repetitive regions, vector sequences and more over many of the ESTs are duplicates. All redundant and poor quality sequences were subjected to EGassembler online server, which processes the data in series of sequence cleaning, repeat masking, vector masking, organelle masking and finally sequence assembly by using CAP3 to give contigs (1,378) and singletons (14,814) with all parameter kept default (Masoudi-Nejad *et al.*, 2006). These contigs and singletons represent the non redundant part of downloaded ESTs.

Precursor miRNA prediction

The processed unique miRNA reference set was used for homology search in pigeon pea contigs and singletons using BLASTn option in BioEdit software version 7.2.5 (Hall *et al.*, 1999). The blast parameters like e-value was kept 1, word match size 11 and match-mismatch score (1,-4) and filtered low complexicity regions. All those pigeon pea EST sequences which are having query coverage of 95 to 100% and with mismatch less than 2 with miRNA sequences were selected and forwarded for further analysis to remove protein coding sequences among the identified ESTs. Since all the miRNA genes are non protein coding (Lee *et al.*, 1993), BLASTx online server was used to remove the protein coding sequences through blasting of precursor's to non redundant (NR) protein data base of pigeon pea. The non coding sequences were selected based on criteria, E value less than e^{-5} along with the identity percent less than 25 (Dehury *et al.*, 2013). The workflow for identification and characterization of new miRNAs were presented in figure 1.

The leftover non coding sequences were used for prediction of precursor miRNA using Zuker folding algorithm in MFOLD software version 3.2 (http://mfoldrnaalbanyedu /?q=mfold/rna-folding-form) with all default parameters (Zuker, 2003). The precursor sequences were searched at 50 nucleotides upstream or downstream from the location of mature miRNAs with an increament of 10 nucleotides. A stringent selection criteria was followed to select novel miRNA (Zhang *et al.*, 2005). The selection criteria in order to identify the appropriate hair pin structure includes 1) The minimum length of the pre-miRNAs to be 60 nt, 2) The pre-miRNA should fold into an appropriate stem loop hairpin secondary structure, 3) The miRNA/miRNA* duplex *i.e.*, the mature miRNA sequence and its opposite miRNA strand (miRNA*) should not have more than 7 nt mismatches (Das and Mondal, 2010) 4) The mature miRNA sequence should be placed in one arm of the hairpin structure, 5) The A+U content should be within 30–70% and 6) Predicted secondary structure should have higher minimal folding free energy index (MFEI)

MEFI= [(MEF/length of the RNA sequence) ×100]/ (G+C) %

Target prediction

The newly identified miRNA sequences were submitted to psRNA Target tool for target prediction (http://plantgrnnobleorg/psRNA Target/) by specifying search on *Glycine max* (soybean), unigene, DFCI Gene Index (GMGI), version 16.

The earlier reports have found that pigeon pea genome has high synteny relation with soybean genome (Varshney *et al.*, 2011). All the parameters were kept constant except expected value 3, to have good number of targets.

Functional annotation of target proteins

The genome annotation of the identified targets was done by using QickGo (http://wwwebiacuk/ QuickGO) tool. Furthermore, three important components such as biological process, cellular component and molecular function associated with each GO term were retrieved. The predicted miRNAs were named in accordance with miRBase (Griffiths-Jones *et al.,* 2006).The mature sequences are designated 'miR', and the precursor hairpins are labeled as 'mir' with the prefix. In case of *Cajanus cajan* it will be cca-miR395a for homologue of sly-miR395a.

Results and Discussion

In silico identification of novel miRNA

A total of 25,576 ESTs of pigeon pea (*Cajanus cajan*) were assembled to form contigs (1,378) and singletons (14,814) which were used for identification of new miRNAs based on homology relationship with previously reported non redundant mature miRNAs. The BLASTn programme with specific parameters (see materials and method) led to detection of 7 ESTs (4 singletons and 3 contigs) showing conserved miRNAs sequence with mismatch less than 2 nucleotide (nt) with previous miRNAs. The BLASTx operation these 7 ESTs resulted in retrieving 5 ESTs sequences which do not code for any protein. These 5 EST sequences were analyzed for presence of characteristic secondary hairpin structure. All the five ESTs fold into appropriate secondary structure and proved to be new miRNAs in pigeon pea (Fig. 1). The size of the newly identified miRNAs was in range of 20 nt to 22 nt which are considered as ideal length for typical miRNA (Zhang *et al.,* 2006a). The A+U content ranged from 49 % to 69 % which is in agreement with Zhang *et al.,* (2005) with an average of 58 % and similarly the G+C content ranged from 30 % to 50 % with an average of 41 % (Table 1). The length of the precursor miRNAs ranged from 93 to 194 nt with an average of 133 nt. The newly identified precursor miRNAs have minimum folding free energies (MFE) ranging from -74 to -162 kcal/mol which are not in consistence with folding energies of tRNA –275 kcal /mol and rRNA –33 kcal/ mol (Barozai *et al.,* 2008). The Minimal folding free energy index (MFEI) and MFE have been considered as significant features that distinguish miRNAs from other non coding RNAs (rRNA, tRNA, mRNA). The MFEI of newly identified precursor miRNAs ranged from 0.97 to 5.2 which is significantly higher than that of tRNAs (0.64), rRNAs (0.59) and mRNAs (0.62–0.66) with an average of 2.4 proving that these newly identified miRNAs in pigeon pea are likely to be actual miRNAs than any other kind of non-coding RNA (Zhang *et al.,* 2006b). All of above findings and analysis indicated that these five small RNAs were probably new miRNAs. The hairpin structure of newly identified miRNAs was presented in figure 2.

Computation based identification of putative target of new miRNAs

Result from psRNATarget server showed that the newly identified miRNAs have 27 target mRNAs, which code for different proteins involved in metabolism, response to stress, transcriptional regulation, signal transduction, growth, development, sulfate assimilation, protein lipoylation, coenzyme and, oxidoreductase activity. Our results found that the cca-miR6483 has highest number of targets ie., 10 genes followed by cca-miR395a with 7 targets genes, cca-miR5219 (2 target genes), cca-miR169b (4 target genes) and cca-miR393a (4 target genes). In the present study, it was observed that, one miRNA has more than one target mRNA, which were in

consistence with the reports of Boopathi and Pathmanaban (2012). The majority of targets genes includes enzymes 42.1 % having important role in assimilation, resistance mechanisms and biological functions followed by genes for proteins 26.3 %, transcription factors 21.1%, and transporters 10.5 % (Fig. 3). Our results were in consistent with the studies of Nodine and Bartel (2010) and Das and Mondal (2010) who predicted that miRNAs regulates the functional genes in plants that were involved in various physiological processes, leaf morphogenesis, stress responses and signal transduction. Many previous reports revealed that miRNAs of plants also regulates transcription factors (Lu and Yang, 2010)**.** The newly identified miRNAs have following targets proteins, Eukaryotic translation initiation factor 6 (EIF-6)-like protein, Apyrase-like protein, Protein kinase GhCLK1, Extensin, Ferric reductase, ATP sulfurylase, Dihydroflavonol-4-reductase, CCAAT-box transcription factor complex WHAP12, Lipoxygenase-9, Transport inhibitor response 1, 50S ribosomal protein L20 and MYB transcription factor MYB102. The functional annotation of target mRNAs is presented in table 2.

Table.1 Details of newly identified miRNAs in pigeon pea

New miRNA	Source	Mature miRNA	Homologue miRNA	L	PL (nt)	LM (nt)	NM (nt)	E-value	(A+U) %	(G+C) %	MFE (ΔG,kcal/mol)	MFEI
cca-miR393a	Contig1015	CATCCAAAGGGATCGCATTG	ppe-miR393a	3’	116	20	0	6.00E-06	49.1	50.9	-90	2.2
cca-miR169b	Contig1261	TGAGCCAAGGATGGATTGCC	vvi-miR169b	5'	165	20	1	0.006	59.4	40.6	-161	2.4
cca-miR395a	gi|251739378	TGAAGTGTTTGGAGGAACTCC	sly-miR395a	3’	93	21	1	0.012	63.4	36.6	-74.1	2.1
cca-miR5219	gi|251739202	TCATGGAATTTCAGCTGCTGCA	mtr-miR5219	3’	194	22	1	0.003	53	46.9	-88.5	0.97
cca-miR6483	gi|284456793	TATTGTAGAAATTTTCAGGATC	hbr-miR6483	5’	101	22	0	3.00E-06	69.3	36.6	-162	5.2

The novel identified miRNAs were characterized in terms of L = Location of miRNA; PL = precursor miRNA length; LM = mature sequence length; NM = number of mismatches; MFE = minimal folding free energies; MFEI = minimal folding free energy index.

Table.2 Prediction of miRNA target genes and their functional annotation

miRNA ID	Target Acc	Target description	Target function	Biolobical process
cca-miR6483	TC436145 TC465796	Eukaryotic translation initiation factor 6 (EIF-6)-like protein	Translation initiation factor Ribosomal large subunit binding	Mature ribosome assembly Translation initiation
	BE440256	Apyrase-like protein	Hydrolase activity	Metabolic process
	TC452083	Protein kinase GhCLK1	Protein serine/threonine kinase activity Transferase activity Nucleotide binding	Protein phosphorylation
	TC452253	Extensin	Structural constituent of cell wall	Plant-type cell wall organization
	TC473325	Ferric reductase	Oxidoreductase activity	Oxidation-reduction process
cca-miR5219	TC421771	60S ribosomal protein L9	Structural constituent of ribosome	Translational elongation
	TC423861	Peptidyl-prolyl cis-trans isomerase	Isomerase activity	Protein peptidyl-prolyl isomerization
cca-miR395a	BG789910 TC432008	ATP sulfurylase	Sulfate adenylyltransferase (ATP) activity	Sulfate assimilation
	TC421817	Plastidial lipoyltransferase 2	Transferase activity	Protein lipoylation
	GD787823	Dihydroflavonol-4-reductase	Coenzyme binding	Metabolic process
	BI426387	Cytochrome P450 82A2	Oxidoreductase activity	Oxidation-reduction process
cca-miR169b	TC474864	CCAAT-box transcription factor complex WHAP12	Sequence-specific DNA binding transcription factor activity	Regulation of transcription
	TC485068	Lipoxygenase-9	Linoleate 13S-lipoxygenase activity, Oxidoreductase activity	Oxidation-reduction process
cca-miR393a	TC431817	Auxin-responsive factor TIR1 protein	Inositol hexakisphosphate binding	Auxin-activated signaling
	TC429100	50S ribosomal protein L20	Structural constituent of ribosome Rrna binding	Translation
	TC432236	MYB102	Sequence-specific DNA binding transcription factor	Biotic and abiotic stress

Fig.1 Workflow for identification and characterization of new miRNAs and target genes in pigeon pea using ESTs

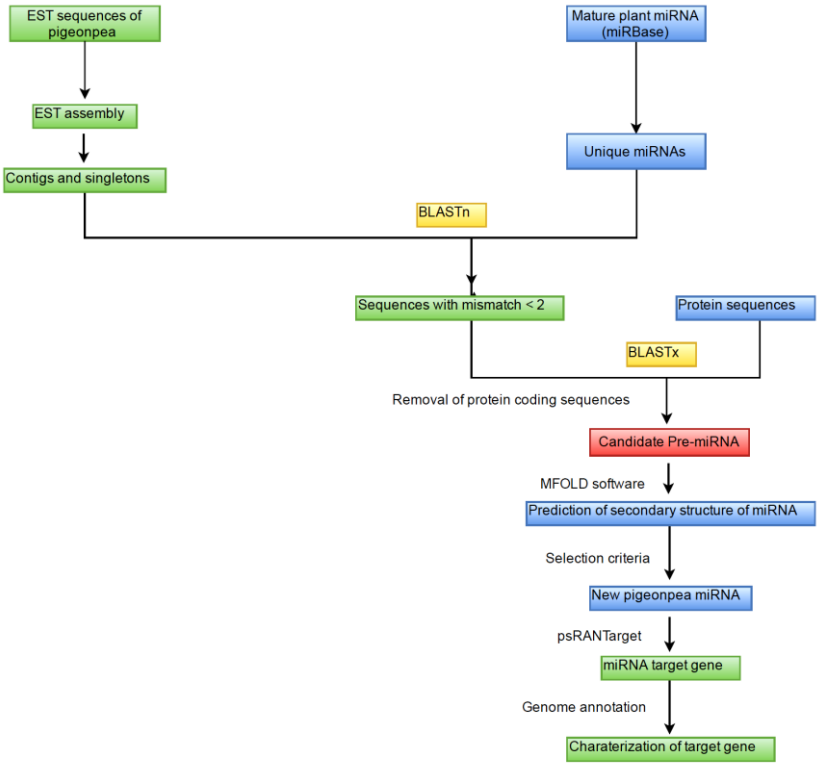
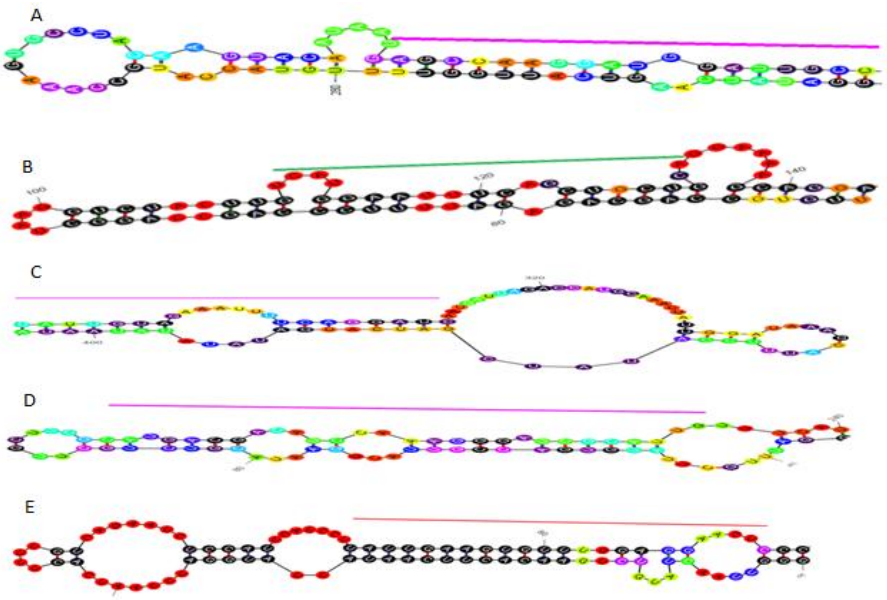
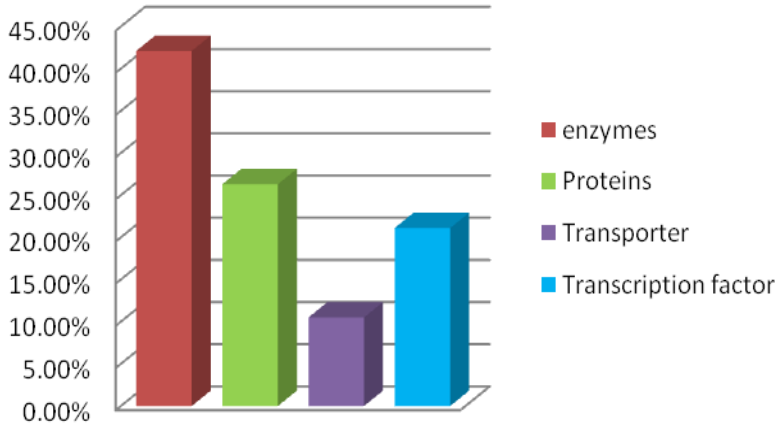


Fig.2 Secondary hair pin structure of newly identified miRNA in pigeon pea



A) cca-miR169b, B) cca-miR5219, C) cca-miR6384, D) cca-miR393a, E) cca-miR395a. The pink color bar on the stem loop structure of miRNA indicates the mature miRNA sequences

Fig.3 Major groups of miRNA targets



The cca-miR6483 targets multiple genes, it includes Eukaryotic translation initiation factor 6 (EIF-6)-like protein which has translation initiation factor activity, apyrase like protein, extensin and ferric reductase. In Arabidopsis and Rice EIF-6 has important role in embryogenesis (Kato *et al.,* 2010). The enzyme apyrase play important role in plant nutrition and nodulation. In soybean, cell wall protein extensin composed of hydroxyproline-rich glycoproteins helps in cell wall metabolism and another enzyme ferric reductase regulated iron homeostasis in plants, thus helps in preventing Fe deficiency (Hong *et al.,* 1994*;* O'Rourke *et al.,* 2007).

The miRNA cca-miR395a targets ATP sulfurylase that involves in sulfur assimilation and improves nutrient content of the plant, Dihydroflavonol-4-reductase enzyme that controls seed coat and flower colour in soybean (Herrmann *et al.,* 2014*;* Yan *et al.,* 2014). The miRNA cca-miR5219 suppresses the expression of 60S ribosomal protein L9 and peptidyl-prolyl cis-trans isomerase which help in translation elongation and protein peptidyl-prolyl isomerization respectively. The cca-miR169b negatively regulates transcription factors like CCAAT-box transcription factor complex WHAP12, nuclear transcription factor Y subunit A-3. The suppression of cca-miR169b leads to expression of Lipoxygenase-9, an important enzyme that confers resistance against the plant parasitic nematode, *Heterodera glycines* in soybean (Klink *et al.,* 2009). cca-miR393a regulates transport inhibitor response 1(TIR1), involved in auxin-activated signaling pathway. The tir1mutants leads to abnormal hypocotyl elongation and lateral root formation (Ruegger *et al.,* 1998). The miRNA cca-miR393a also regulates MYB102, which express under wide range of biotic and abiotic responses (Vos *et al.,* 2006).

In conclusion we identified five new miRNAs in EST of pigeon pea having 27 mRNA targets, most of the them involved in stress responses, development, physiological process, protein phosphorylations and other metabolic processes This finding from our study leads to further investigation of miRNAs functions and regulatory mechanism under wide range of biotic and abiotic stress which leads to crop improvement of Pigeon pea "an orphan legume crop"

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