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Review

Overview of the potential of microRNAs and their target gene detection for cassava (*Manihot esculenta)* improvement

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Production and utilization of cassava (*Manihot esculenta*) is significantly constrained by pests, diseases, poor yields and low nutritional content. Various approaches are currently being applied to mitigate these constraints. However, an aspect of plant developmental genetics little known in cassava is the role that microRNAs (miRNAs) play in gene regulation. miRNAs are 20 - 24 nucleotide long non-protein-coding RNAs that play important roles in post-transcriptional gene silencing in many organisms. Thorough understanding of the mechanisms involved in miRNAs mediated post-transcriptional gene regulation will have implications for crop production improvement. The potential of miRNAs for cassava improvement and also some data obtained on cassava miRNAs using comparative genomics analysis have been briefly discussed. 17 miRNA families and target genes in cassava that are also conserved in other plant species have been revealed. However, the ESTs representing seven of these miRNA families produced foldbacks that show more than 3 nucleotides not involved in canonical base pairing within a loop or bulge in the mature miRNA: RNA* dimer, thus were not considered miRNA secondary structures. Consistent with previous reports, majority of the target genes identified are transcription factors. Other targets appear to play roles in diverse physiological processes. Furthermore, a detailed description and insight into some of the current bioinformatic resources and approaches applicable to cassava have been discussed. Such information will further enhance the rapid discovery and analysis of more novel miRNAs in cassava towards its improvement.

Key words: Cassava, microRNAs, target genes, improvement, characterization.

INTRODUCTION

Cassava (*Manihot esculenta*) is one of the most important root crops in Africa. It has high adaptability to low-fertility soils and is able to withstand erratic rainfall and long drought conditions (Mathews et al., 1993). For this reason, cassava is a major food security crop and a source of cash for resource-poor farmers. Today, over half of the world's cassava is produced in Africa. Nonetheless, it is faced with production constraints of pest, diseases and poor yields everywhere it is grown on the continent. The widespread use of cassava for food and industry places a high demand for increased production.

To achieve significant production will require the development of cultivars with higher yields, improved resistance to biotic and abiotic stresses and high nutritional content. Some significant progress is being made with various strategies in this regard. However, an aspect of plant development regulation that has not been exploited in our attempt to develop cassava varieties is the potential application of the knowledge of microRNAs (miRNAs) in gene regulation during plant growth and development. Thorough understanding of the mechanisms involved in miRNAs mediated post-transcriptional gene regulation will have implications on the strategies currently being developed to enhance the crop. Newly discovered miRNAs may play important role in cassava development

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and the response to biotic and abiotic stresses. Although miRNAs have been studied for years, to date no study has been performed on cassava. Accurate detection and expression profiling of these miRNAs will enable a better understanding of their role in plant growth and development (Millar and Gubler, 2005; Rhoades and Bartel, 2004; Jagadeeswaran et al., 2009). Breakthrough in this area is likely to reveal developmental regulation and disease mechanisms related to miRNAs.

In an attempt to stimulate more research interest in cassava miRNAs, an overview of miRNAs and some current bioinformatic resources and approaches available for prediction and analysis of plant miRNAs have been discussed. Furthermore, relevant bioinformatic resources and approaches applicable to cassava as evident from data presented here have been described. Comparative genomics across vastly divergent taxa has shown that many miRNAs are highly conserved from species to species in the plant and animal kingdoms (Reinhart et al., 2002; Sunkar and Jagadeeswaran, 2008). The extensive evolutionary conservation of miRNAs provides an effective approach to their identification using comparative genomics. In plants, miRNAs have perfect to near perfect homology to their target genes and generally have only single site within their target (Subramanian et al., 2009). With the development of new algorithms based on these characteristics, computational approaches to plant miRNA prediction and characterization are on the increase. Computational methods have been shown to be efficient for the identification of putative miRNAs in a wide variety of plant species including *Arabidopsis thaliana* (Rhoades and Bartel, 2004; Sunkar and Zhu, 2004), Rice (Sunil and Nagaraju, 2007), Maize (Mica et al., 2006), Tomato (Pilcher et al., 2007), Irish potato (Qiang et al., 2007) and Soybean (Zhang et al., 2008).

The recent breakthrough in deciphering the cassava genome sequence (Cassava Genome Project 2009, http:://www.phytozome.net/cassava) coupled with a number of available up-to-date bioinformatic approaches has now made it possible to identify and characterize miRNAs in cassava towards enhanced crop improvement.

MICRO-RNAs: BIOGENESIS AND MECHANISM OF GENE REGULATION

A brief description

miRNAs are 20 - 24 nucleotide small endogenous non-protein-coding regulatory RNA sequences that are produced by genes distinct from the genes that they regulate. Biogenesis of miRNAs involves nucleolytic processing of a precursor transcript with extensive foldback structure (Han et al., 2004; Vazquez et al., 2004; Yang et al., 2006). Evidence provided by Allen et al. (2004) and Felippes et al. (2008) show that some miRNAs evolved by inverted duplications of target gene sequences whereas others originated from random sequences that either have self-complementarity by chance or sequences that represent highly eroded inverted duplications. Since their discovery, miRNAs have become the interest of intensive research in various model organisms. Several miRNAs have been computationally and/or experimentally identified and characterized in many species. A number of studies have shown that miRNAs play key roles in regulatory functions of gene expression for most eukaryotes (Sunkar and Zhu, 2007; Jagadeeswaran et al., 2009), mainly at the posttranscriptional levels (Chapman et. al., 2004; Mallory et. al., 2004).

miRNA sequences have been shown to be highly conserved among plant species (Sunkar and Jagadeeswaran, 2008) and have perfect or near perfect complementarity with their specific messenger RNA (mRNA) targets. As a result, a plant miRNA guides the cleavage, degradation or translational inhibition of its target mRNA with a resultant repression of gene expression. Apart from the miRNA section, other parts of the corresponding precursor pre-miRNA sequences are less conserved (Lau et al., 2001). Initially, it was thought that the sole purpose for the miRNA gene regulatory mechanism was to defend plants against RNA viruses and transposons. However, several recent findings have implicated miRNAs in a number of biological mechanisms including leaf growth (Palatnik et al., 2003), stem growth (Mallory et al., 2004), root growth (Subramanian et al., 2009), floral organ identity, control of female gamete formation and reproductive development (Millar and Gubler, 2005; Olmedo-Monfil et al., 2010), auxin signaling (Rhoades and Bartel, 2004), biotic and abiotic stress response (Jagadeeswaran et al., 2009).

Biogenesis pathways

A simplified scheme of the plant miRNA biogenesis pathway is shown in Figure 1. miRNA genes are first transcribed in the nucleus by RNA polymerase II into primary transcripts referred to as pri-miRNAs, which are subsequently capped at the 5' end and polyadenylated at the 3' end (Lee et al., 2004). The pri-miRNA is further processed by an enzyme microprocessor complex which includes Dicer like-1 nucleases (members of an RNase III endonuclease family) and Arabidopsis hyponastic leaves (HYL1), a dsRNA-binding domain containing protein, bound to the pri-miRNA complex (Jones-Rhoades et al., 2006). The activity of the microprocessor complex culminates in pre-miRNA stem loop structures from which miRNAs are eventually processed.

In *A. thaliana*, all of the miRNA biogenesis steps are processed by one of four Dicer-like RNase III endo-nucleases (Filipowicz et al., 2008). Dicer-like-1 processes primary miRNA transcripts into an miRNA-miRNA* duplex with 2 nucleotide overhangs at the 3' end (Reinhart et al., 2002), while the other Dicer-like enzymes are involved with another type of small RNA biogenesis and accu-mulation known as short interfering RNAs (siRNAs)

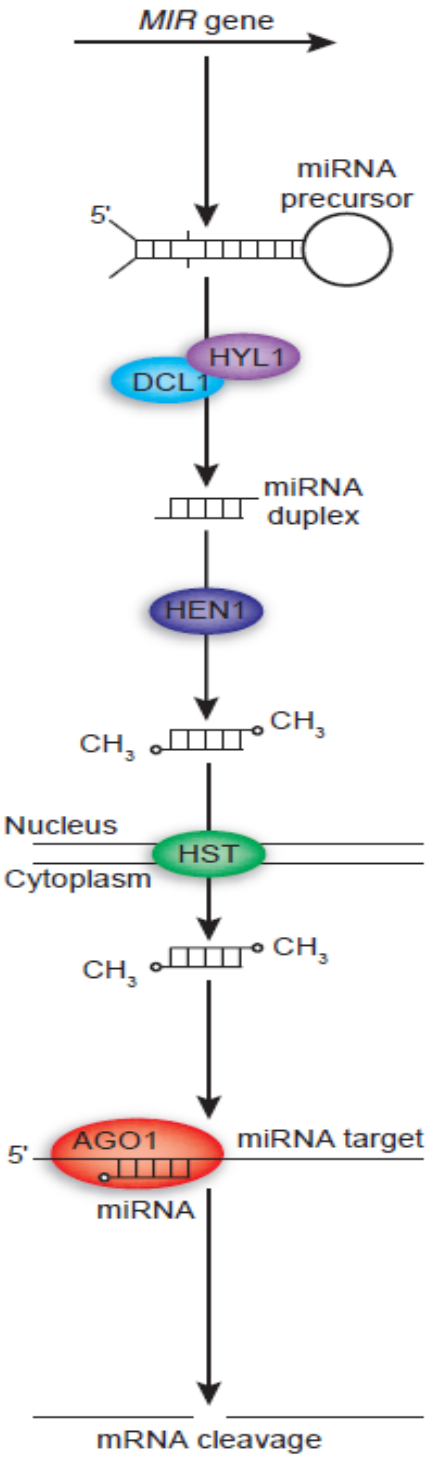


Figure 1. Simplified scheme of the plant miRNA biogenesis pathway (Mallory and Vaucheret et al., 2006).

(Filipowicz et al., 2008). HYL1 and SERRATE, a C_2H_2 zinc finger protein, assist DCL1 in releasing the miRNA duplex (Han et al., 2004; Vazquez et al., 2004; Yang et al., 2006). Next, HUA ENHANCER1 (HEN1) enzyme, a methyl transferase, adds methyl groups to the 3' ends of the miRNA duplex and stabilizes it (Yu et al., 2005). The miRNA duplex is then exported into the cytoplasm with the help of HASTY (HST), a plant ortholog of a miRNA transporter enzyme, Exportin-5, which in animal systems transports pre-miRNAs to the cytoplasm (Yi et al., 2003). Based on the relative internal thermodynamic stability of the two ends of the duplex, one strand becomes the mature miRNA whereas the other complementary strand is degraded by an unknown nuclease (Khvorova et al., 2003; Schwarz et al., 2003).

Mechanism of miRNA-mediated gene repression

The mature strand of the miRNA is loaded into a complex of ribonucleotide proteins to form the miRNA-induced silencing complex (miRISC) to cleave its specific target mRNA or to inhibit the translation of its target transcript (Vaucheret et al., 2004; Baumberger and Baulcombe, 2005). The miRISC complex comprises mainly Arganoute (AGO1) and an RNaseH-like P-element induced wimpy testis (PIWI) domain containing protein. The AGO protein family is the most important and key component of the miRNA-RISC complex (Schwarz et al., 2003). The primary proteins in this complex are members of the Argonaute (AGO) family, each of which possesses repressive capabilities. The single stranded mature miRNA together with the PIWI domain are used as guide in the miRISC to recognize the target mRNA to which it may be perfectly or near perfectly complementary in plants unlike in animals.

In all known cases, most plant miRNAs bind to the protein-coding region of their target mRNAs induce target mRNA degradation via an RNAi-like mechanism where the AGO protein cleaves the miRNA-mRNA duplex (Figure 1), thereby repressing expression of that particular mRNA (Rhoades et al., 2002). Gene repression can also be effected by translational inhibition through deadenylation of the 3' poly (A) tail and decapping of the 5' end in mRNAs, which leads to progressive mRNA decay and degradation (Chen, 2004; Brodersen et al., 2008). Generally, miRNA biogenesis is similar to a large extent between plants and animals, however, fundamental differences exist. A comparison of the main differences have been summarised in Table 1. These and other differences suggest that the two systems may have evolved independently, possibly as a prerequisite to suite efficiently the regulatory mechanisms of the respective developmental complexities.

MITIGATING CASSAVA PRODUCTION AND UTILIZATION CONSTRAINTS

Many initiatives both local and international recognize the importance of cassava in Africa. Most of these initiatives aid increased productivity through biotechnological

Table 1. Comparison of miRNA biogenesis in plant and animal systems.

Parameter	Plant system	Animal system
Pre-miRNAs processing	Processed from pri-miRNA by Dicer-like 1 in the nucleus[a]	Processed from pri-miRNA by Drosha protein in the nucleus[b]
miRNA processing	Processed from pre-miRNA by Dicer-like 1 in the nucleus[c]	Processed from pre-miRNA by Dicer protein in the cytoplasm[d]
miRNA methylation	miRNA duplexes are methylated at 3' ends by HEN1 enzyme[e]	Methylation not known[f]
Molecular transporters	miRNA is moved from nucleus to cytoplasm by the HASTY protein[g]	pre-miRNA is moved from the nucleus to cytoplasm by Exportin-5[h]
Stem-loop structure	Stem-loops are longer and highly variable[i]	Stem-loops are shorter and more uniform[j]
Source of miRNAs	Mainly from non-coding transcriptional units[k]	Arise from introns and protein coding sequences[l]
SmallRNAs in transcriptome	miRNAs constitute a small fraction of smallRNA populations[m]	smallRNA populations are predominantly miRNAs[n]
Presence of miRNA clusters	Clusters are rare[o]	Clusters occur frequently[p]
miRNA target binding	Have perfect or near perfect complementarity with target site[q]	miRNA could bind imperfectly[r]
miRNA binding sites	Predominantly bind coding regions of target genes[s]	miRNAs bind the 3' UTRs of target genes[t]
Number of miRNA binding sites	Generally single binding sites in target genes[u]	Generally targets have multiple binding sites[v]
Repression of gene expression	Generally by direct mRNA cleavage[w]	In most cases by translational attenuation[x]

[a,c]Filipowicz et al., (2008); [b]Lee et al., (2003); [d]Bernstein et al., (2001); [e,f]Yu et al., (2005); [g,h]Yi et al., (2003); [i,j]Bartel (2004); [m]Lu et al., (2005); [n]Mineno et al., (2006); [k,l,o,p]Millar and Waterhouse (2005); [q,r,u,v]Subramanian et al., (2009); [s]Sunkar and Zhu (2004); [t]Rajewsky (2006); [w,x]Baumberger and Baulcombe (2005).

research for development of improved varieties, provision of improved germplasm and also offer training in new methods of cassava molecular breeding. One of such initiatives is the International Centre for Tropical Agriculture (CIAT) project "Genetic Enhancement of Cassava" (http://www.ciat.cgiar.org/africa/cassava.htm). CIAT has developed Cassava Mosaic Disease (CMD) markers and tested their suitability in marker-assisted selection for CMD resistance on several progenies. The centre has also conducted marker assisted breeding of lines to combine CMD resistance, high carotene content and good dry matter yield. These lines will further be recombined with high protein lines currently being developed.

In order to make available important biotechnology tools to complement conventional breeding programs in cassava, the International Institute of Tropical Agriculture (IITA), Nigeria has generated and characterized an 18,166 expressed sequence tag (ESTs) dataset enriched for drought responsive genes. The institute has also developed a 60-mer oligo DNA microarray comprising 35,785 ESTs as well as genetic transformation protocols for the genetic modification of model cassava genotypes.

Furthermore, the International Laboratory for Tropical Agricultural Biotechnology (ILTAB), USA with support from USAID, the Bill and Melinda Gates Foundation and in collaboration with scientists from East Africa is working to enhance virus resistance in cassava. They are employing two main strategies; ssDNA binding protein based strategy and gene silencing strategy to produce cassava disease resistant varieties. They are also examining the processing of short interfering RNAs (siRNAs) in relation to cassava geminiviruses. It is hoped that their results will have implications on the strategies being developed to control cassava geminiviruses in Africa. Another interesting aspect of cassava being studied by ILTAB and an international team of cassava researchers is the development of cassava varieties with enhanced nutritional content. In this regard, the laboratory has developed transgenic cassava plants that are currently under greenhouse investigations. ILTAB has also indicated a successful development of an efficient tool to express at significantly high levels, a variety of proteins in cassava roots (http://content.yudu.com/Library/A1d00p/DonaldDanforthPlantS/resources/11.htm).

In Ghana, the Biotechnology and Nuclear Agriculture Research Institute (BNARI) in collaboration with the International Atomic Energy Agency (IAEA), Austria, is applying gamma irradiation mutagenesis technology for

cassava improvement. Application of induced mutation techniques in Ghana has led to the production of an improved mutant cassava. Irradiation of stem cuttings using gamma irradiation resulted in the production of "Tek bankye," a mutant variety with high dry matter content (40%) and good poundability. BNARI is also currently using irradiation techniques in combination with molecular methods to develop disease resistant and high starch yielding cassava varieties to enhance the production and utilization of the crop.

APPROACHES FOR CASSAVA MICRORNAS AND TARGET IDENTIFICATION

Screening for previously known miRNA families

miRBase (http://microrna.sanger.ac.uk/) is the main database of miRNAs from most plant and animal species as well as viruses and fungi. The latest version of miRBase released on September 14, 2009, (http:// microrna. sanger.ac.uk/sequences/), consists of 10,581 miRNA and 10,883 precursor hairpin structures entries from 115 species. These miRNAs have been identified via computational and/or experimental methods. The database is constituted into three sections: the *miRBase:: Registry*, where novel miRNA data is uploaded prior to publication; information on miRNA sequences, nomenclature and target mRNAs can be obtained from the *miRBase:: Sequences* section; and, the third section *miRBase:: Targets* provides the prediction of the mRNA target from all published miRNAs (Griffiths-Jones et. al., 2008). A total of 212 previously reported *A. thaliana* miRNAs and their precursor sequences were screened from *miRBase:: Sequences*. These miRNAs served as a reference set of miRNA sequences for the identification of putative miRNA families in cassava (Table 2). The sequences were used as query sequences for BLASTn search against the publicly available cassava ESTs in the cassava genome database (http://www.phytozome.net/ cassava.php). ESTs that aligned 19 - 22 nucleotides with known *A. thaliana* miRNAs were selected for prediction of putative secondary stem-loop structures. The maximum number of mismatches allowed was 0 - 3 with no gaps.

Work undertaken by Smalheiser (2003) and Zhang et al. (2005) show successful identification of miRNAs by mining the repository of available ESTs. This is feasible because miRNAs are generated from long precursor hairpin structures that can be found by searching ESTs. The strategy is to look for the sequences containing conserved mature miRNAs and then check if these miRNA bearing ESTs can form stable secondary stem-loop structures (Figure 2; Table 2). Another useful miRNA database is the Plant miRNA Database (PMRD) released October 1, 2009, (http://bioinformatics.cau. edu. cn/PMRD). This first edition of the database contains 8433 miRNAs from 121 plant species, their secondary structures, target genes, expression profiles and a genome browser.

High-throughput 454 pyrosequencing of short RNAs and transcriptomes

Bioinformatic methods have been invaluable in the identification of many plant miRNAs, however, these approaches identify mainly conserved or evolutionally "old" miRNAs. Besides, some non-conserved miRNAs mostly considered recently evolved "young" miRNAs have been discovered and appear to be species-specific (Allen et al., 2004; Felippes et al., 2008). Until recently, most sequence information such as ESTs or genome survey sequences (GSS) used for computational prediction of miRNAs were generated by the traditional Sanger sequencing method. Compared to conserved miRNAs, non-conserved miRNAs are often expressed at lower levels which make their detection more daunting using small-scale sequencing. The development of high-throughput 454 pyrosequencing technology has, therefore, allowed the discovery of several non-conserved or lowly expressed miRNAs through cloning and deep sequencing of small RNA and transcriptome libraries in *A. thaliana* (Rajagopalan et al., 2006; Fahlgren et al., 2007), wheat (Yao et al., 2007), tomato (Moxon et al., 2008).

Prediction of miRNA secondary stem-loop structures

Irrespective of the method used to generate sequence information, the prediction of putative pre-miRNA structures is achieved mainly using bioinformatic softwares some of which are publicly available. To predict putative pre-miRNA secondary hairpin structures for selected cassava ESTs, the programme Mfold (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/ rna-form1.cgi), a webserver was used. This programme is widely used for predicting the minimum fold energy (MFE) secondary structure using the algorithm originally proposed by Zuker and Stiegler, (1981). Equilibrium base-pairing probabilities of MFE structures are calculated via McCaskill's partition function (PF) algorithm (McCaskill, 1990). Besides, RNAmFold (http:// rna.tbi.univie.ac.at/ cgi-bin/RNAfold.cgi) can also be used to find potential miRNA hairpin structures from EST sequences (Hofacker, 2003). These and many other useful programmes, their applications and methodologies for miRNA identification, characterization and analysis have been recently reviewed by Unver et al. (2009).

The secondary structure output of the candidates of pre-miRNAs sequences obtained in cassava were analysed and scored for their potential to form miRNA precursors with the following widely accepted characteristics (Xie et al., 2007); (1) The RNA sequences should be able to fold into a hairpin structure, such that one arm of the hairpin contains the 20 - 22 nucleotide mature miRNA sequence. (2) The predicted secondary structure of the miRNA precursor should have lower minimal fold energy (MFE) and minimal fold energy index (MFEI) than other types of RNA (example, tRNA, rRNA). (3) The

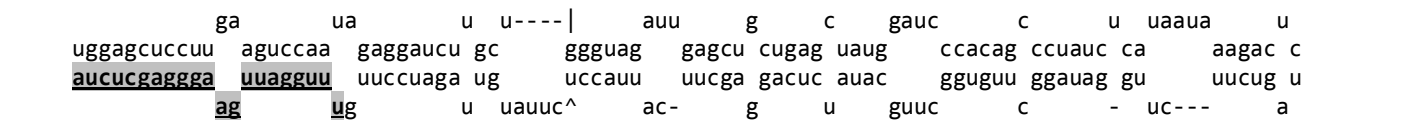
Table 2. Characteristics of identified putative miRNAs and stem-loop structures from ESTs of cassava.

miRNAs	Sequences	NM	LP	ARM	A+U%	AMFE	MFEI	EST ID
miR156a-f	UGACAGAAGAGAGAGAGCAC	0	-	-	-	-	-	FF535946[a]
miR156g	***U***GACAGAAGAGAGAGAGCAC	1	-	-	-	-	-	FF535946[a]
miR157a-c	***A***UGACAGAAGAGAGAGAGCAC	1	-	-	-	-	-	FF535480[a]
miR157d	UGACAGAAGA***G***AGAGAGCAC	1	-	-	-	-	-	FF535480[a]
miR159a	UUUGGAUUGAAGGGAGCUCUA	0	181	3'	55.25	47.07	1.05	DV457265
miR159b	UUUGGAUUGAAGGGAGCUCU***A***	1	89	3'	59.55	32.47	0.80	DB921728
miR160a-c	UGCCUG***A***CUCCCUGUAUGC***AG***	3	98	3'	44.90	35.41	0.64	DB935104
miR162a,b	UCGAUAAACCUCUGCAUCCAG	0	-	-	-	-	-	DR087131[a]
miR166a-g	UCGGACCAGGCUUCAUUCCCC	0	179	3'	58.10	36.92	0.76	DV449619
miR166a-g	UCGGACCAGGCUUCAUUCCC***G***	1	149	3'	62.42	43.75	1.16	DV457519
miR166a-g	U***U***GGACCAGGCUUCAUUCCCC	1	122	3'	62.42	32.21	0.86	DV457519
miR166a-g	UCGGACCAGGCUUCAUUCCC***G***	1	171	3'	60.82	41.57	1.04	DB922056
miR166a-g	U***U***GGACCAGGCUUCAUUCCCC	1	122	3'	60.82	33.77	0.86	DB922056
miR167a,b	UGAAGCUGCCAGCAUGAUCU***G***	1	391	5'	62.92	33.58	0.91	DV447797
miR167d	UGAAGCUGCCAGCAUGAUCUGG	0	391	5'	62.92	33.58	0.91	DV447797
miR168a,b	UCGCUUGGUGCAGGUCGGGAA	0	173	5'	52.60	41.96	0.89	DB925940
miR171a	UGAUUGAGCCGCG***U***CAAUAUC	1	132	3'	59.85	45.15	1.12	DV454131
miR172a,b	AGAAUCUUGAUGAUGCUGCAU	0	177	3'	53.67	36.89	0.80	CK645041
miR172a,b	AGAAUCUUGAUGAUGCUGCAU	0	73	5'	64.38	13.56	0.38	DV449432
miR172c,d	AGAAUCUUGAUGAUGCUGCA***U***	1	177	3'	53.67	36.89	0.80	CK645041
miR172c,d	AGAAUCUUGAUGAUGCUGCA***U***	1	73	5'	64.38	13.56	0.38	DV449432
miR172e	***A***GAAUCUUGAUGAUGCUGCAU	1	177	3'	53.67	36.89	0.80	CK645041
miR172e	***A***GAAUCUUGAUGAUGCUGCAU	1	73	5'	64.38	13.56	0.38	DV449432
miR390a,b	AAGCUCAGG***A***GAGAUA***U***CGCC	2	-	-	-	-	-	DV447689[a]
miR394a,b	UUGGCAUUCUGUCCACCUCC	0	151	5'	54.30	45.96	1.01	CK652213
miR397a,b	***UU***AUUGAGUGCAGCAUUGAUG	2	-	-	-	-	-	FF379852[a]
miR398a	UGUGUUCUCAGGUC***G***CCCCU***G***	2	-	-	-	-	-	DV449231[a]
miR398b,c	UGUGUUCUCAGGUC***G***CCCCUG	1	-	-	-	-	-	DV449231
miR399a-c	UGCCAAAGGAGA***A***UUGCCCUG	1	121	3'	56.20	45.12	1.03	DB931132
miR399a-c	UGCCAAAGGAGA***A***UUGCCCUG	1	112	3'	58.04	44.38	1.06	DB945966
miR399a-c	UGCCAAAGGAGA***A***UUGCCCUG	1	127	3'	55.12	44.33	0.99	DB947384
miR399f	UGCCAAAGGAGAUUUGCCCGG	0	111	3'	52.25	40.81	0.85	DB932374
miR408	AUGCACUGCCUCUUCCCUGGC	0	134	3'	50.00	49.03	0.98	DV440994
miR408	AUGCACUGCCUCUUCCCUGGC	0	217	3'	52.07	43.13	0.90	DV444153
miR414	UCAUCUUCAUCAUCAUCGU***U***A	1	-	-	-	-	-	DV444343[a]

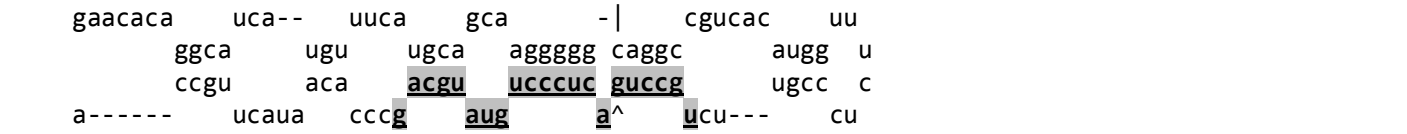
NM, Nucleotide substitutions between known *A. thaliana* query miRNAs and the corresponding new miRNA in cassava; LP, length of pre-miRNA; ARM, miRNA location in hairpin structure; AMFE, adjusted minimum fold energy; MFEI, minimum fold energy index; EST ID, identifier of the EST from which miRNA was derived. Italicized, underlined and bold letters show nucleotide substitutions in new cassava miRNAs. [a]EST could not form secondary stem-loop structures.

predicted mature miRNA should have an A+U content of 30-70%. (4) The mature miRNA sequences within the hairpin loop segment must have less than six mismatches to the opposite miRNA* sequence in the other arm. (5) Any loop or bulge in the mature miRNA:RNA* dimer should contain a maximum of 3 nucleotides not involved in canonical base pairing. Using these criteria a number of pre-miRNA sequences corresponding to identified miRNAs in cassava were obtained that are similar to hairpins previously reported for other plant species (Figure 2). However, the ESTs FF535946, FF535480, DR087131, DV447689, FF379852, DV449231 and DV444343 representing seven miRNA families produced foldback structures that show more than 3 nucleotides not involved in canonical base pairing within a loop or bulge in the mature miRNA:RNA* dimer, thus were not considered miRNA secondary structures (Table 2). This may be because the miRNA regions are located at the 5' end of the sequences. Interestingly, Song et al. (2009) could show the expression in specific citrus tissues, some miRNAs whose precursor sequences could not fold back into stable hairpins.

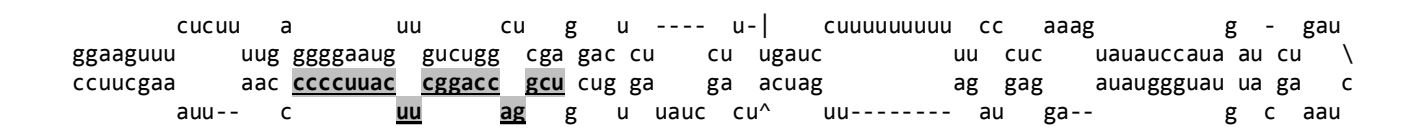
miR159a



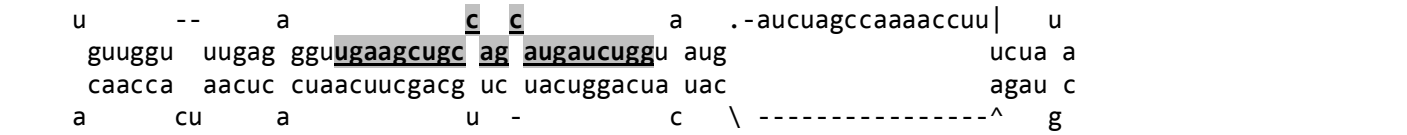
miR160a-c



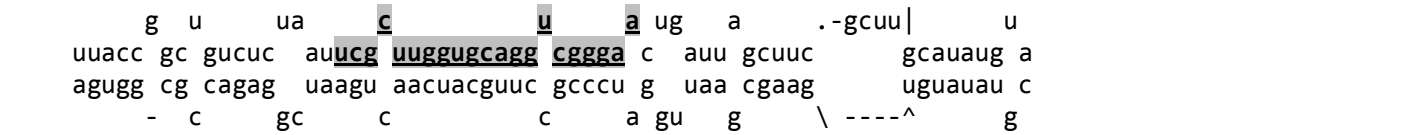
miR166a-g



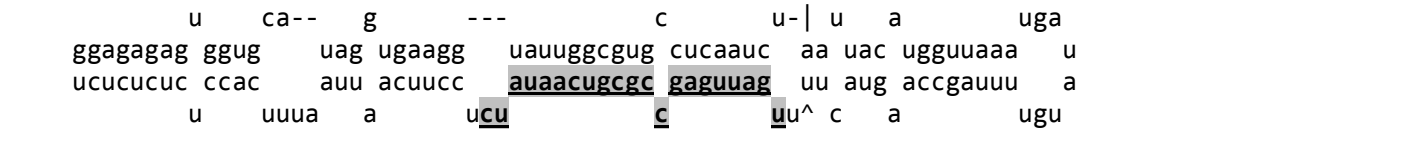
miR167a,d



miR168a,b



miR171



miR172a,b

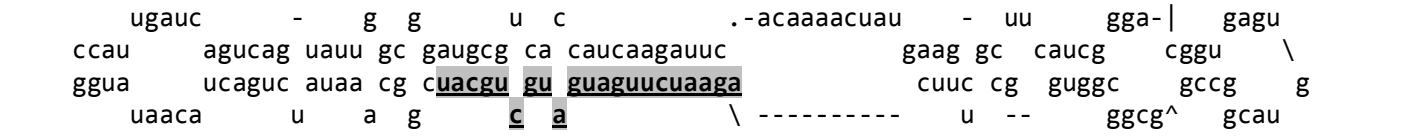


Figure 2. Representative stem-loop structures of identified cassava miRNA families. Mature miRNAs are underlined, bold and shaded. miRNA precursors could be slightly longer than the sequences shown in this figure.

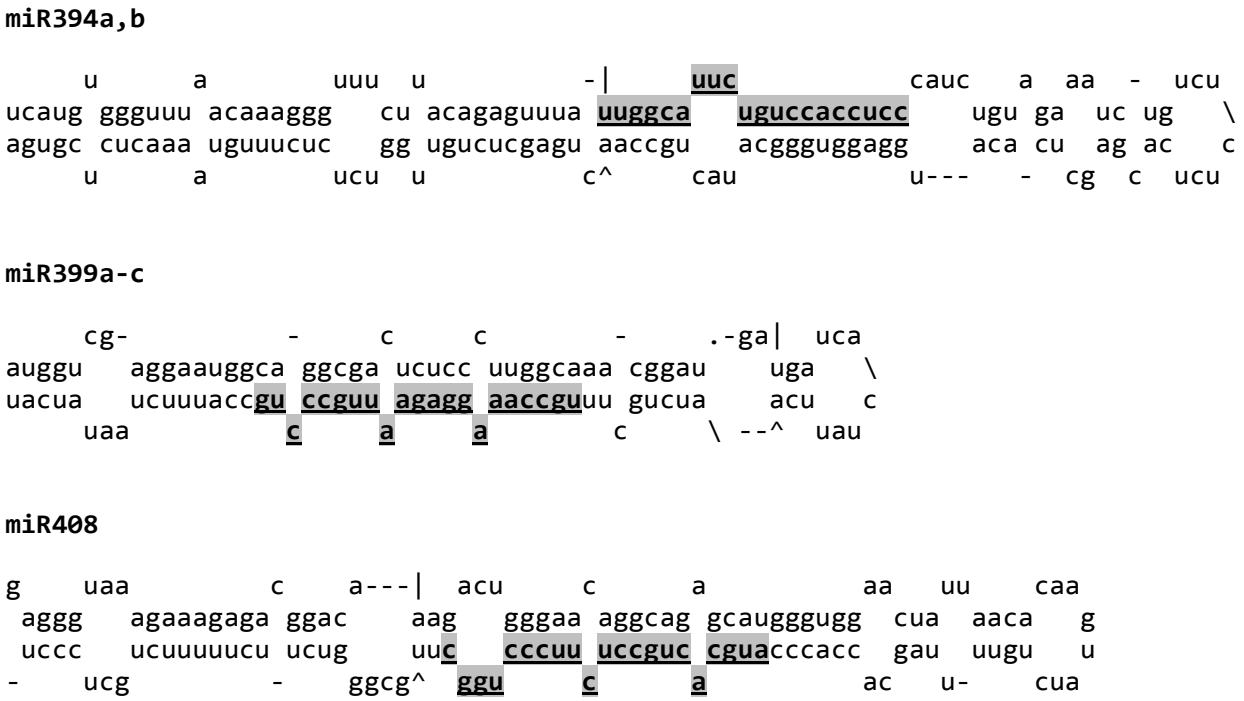


Figure 2. Continued.

Computer-based procedure for prediction of miRNA targets

Several algorithms are currently available for prediction of putative miRNA-mRNA targets in plants (Hofacker, 2003; Zuker, 2003). One of the most widely employed miRNA target finding softwares is the miRU (http://bioinfo3.noble.org/miRNA/miRU.htm) (Zhang, 2005). The miRU system searches for target sites from selected databases for potential complementary target sites in miRNA-target recognition with acceptable mismatches. It is required to input a mature miRNA sequence followed by selection of the dataset for prediction of mRNA target in the organism of interest. The search could be done using default parameters otherwise, the allowable complementary mismatches between the target mRNA and the uploaded miRNA sequence can be adjusted. The output provides for each predicted miRNA target, the gene identifier, target site position, mismatch score, number of mismatches and target complementary sequence. This software was used extensively for the prediction of target genes in the identified cassava miRNA families (Table 3). In addition, targets were cross checked with BLASTn search of the Arabidopsis Information Resource (TAIR) database. Default parameters were used in these analyses.

Experimental validation of predicted miRNAs and miRNA target genes

Computationally predicted miRNAs and their targets are normally validated experimentally in order to avoid false positives (Meyers et al., 2008). This has been achieved in many cases using conventional technologies such as cloning, northern hybridization and microarray analysis. However, in plants, miRNAs are highly under-represented in the small RNA fraction rendering conventional technologies not sensitive enough to detect less abundant miRNAs (Varkonyi-Gassic et al., 2007). To circumvent these limitations, reverse transcription-polymerase chain reaction (RT-PCR) detection methods have been developed and are at present the most powerful techniques due to their comparatively higher effectiveness, specificity and sensitivity. However, because of their small size, detection of miRNAs by PCR is technically demanding. A quantitative real-time PCR (qRT-PCR) method based upon reverse transcription (RT) reaction with a miRNA specific stem-loop primer has been described (Chen et al., 2005; Tang et al., 2006, Varkonyi-Gassic et al., 2007). Following stem-loop RT, either TaqMan or SYBR Green qRT-PCR and analysis are performed using miRNA-specific forward primer and a universal reverse primer designed to bind the stem-loop RT primer sequence. Chen et al. (2005) explained that stem-loop RT primers provide better specificity and sensitivity than linear primers because of base stacking and spatial constraint of the stem-loop structure. Detection sensitivity is further enhanced by a pulsed RT reaction (Tang et al., 2006).

On the other hand, identified miRNA gene targets are commonly confirmed by 5' RACE (Rapid Amplification of cDNA Ends) procedure. In this technique, cleaved mRNA

Table 3. Potential targets of identified miRNA families in cassava.

miRNA family	Target protein	Function of target	Target gene (MS, NM)	E-value
miR156	Squamosa promoter-binding-protein-like 2 (SPL2)	Transcription factor	AT5G43270 (0,0)	3.0e-04
miR157	Squamosa promoter-binding-protein-like 2 (SPL2)	Transcription factor	AT5G43270 (0,1)	9.0e-05
miR159	Myb domain protein 101 (MYB101); DNA binding	Transcription factor	AT2G32460 (2,2)	9.0e-05
miR160	Auxin Response Factor 17 (ARF17); transcription factor	Transcription factor	AT1G77850 (2,0)	33e-02
miR162	Dicer-like1 (DCL1); ATP-dependent helicase/ ribonuclease III	miRNA biogenesis	AT1G01040 (2,0)	9.0e-05
miR166	DNA binding / transcription factor	Transcription factor	AT1G52150 (1.5,2)	9.0e-05
miR167	Auxin response factor 8 (ARF8)	Transcription factor	AT5G37020 (2,3)	3.0e-05
miR168	Argonaute 1; AGO1	miRNA biogenesis	AT1G48410 (3,3)	9.0e-05
miR171	Scarecrow-like transcription factor 6 (SCL6)	Transcription factor	AT4G00150 (0.5,1)	21e-03
miR172	TOE2; DNA binding / transcription factor	Transcription factor	AT5G60120 (0.5,1)	21e-03
miR390	Protein kinase family protein	Metabolism	AT5G03640 (2.5,3)	13e-01
miR394	F-box family protein	Signal transduction	AT1G27340 (1,1)	73e-03
miR397	LAC2 (laccase 2); copper ion binding / oxidoreductase	Metabolism	AT2G29130 (0.5,1)	50e-04
miR398	Heterogeneous nuclear ribonucleoprotein, putative (hnRNP)	Metabolism	AT1G08830 (3,3)	13e-01
miR399	PHO2/UBC24 (Phosphate 2); ubiquitin-protein ligase	Metabolism	AT2G33770 (2,3)	33e-02
miR408	Peptide chain release factor, putative	Metabolism	AT2G47020 (0,0)	9.0e-05
miR414	Lon protease homolog 1, mitochondrial (LON)	Signal transduction	AT1G80960 (0,0)	9.0e-05

MS, miRU Score; NM, number of mismatches.

products of miRNA guided activity are amplified by ligation of an oligo-nucleotide adapter to the 5' phosphate of the cleaved mRNAs followed by reverse transcription and PCR amplification with a gene specific primer (Sunkar et al., 2005). The resulting RACE products are subsequently gel purified, cloned, sequenced and analysed to ascertain the gene target of a particular miRNA.

CASSAVA miRNA FAMILIES AND THEIR TARGET GENES

Consistent with reported studies in other plant species, the majority of target proteins of identified cassava miRNAs are transcriptional factors, whereas others are associated with metabolism and response to environmental stress. Eight miRNA families representing about half the total number of miRNA families identified appear to target transcriptional factor encoding mRNAs. Seven other miRNA families target genes coding a range of different proteins which may play important roles in the aspects of metabolisms, development, signal transduction and stress response. Two target genes are components of the miRNA biogenesis process. Generally, plant miRNAs have single binding sites in their target genes but it was found that each of the ESTs (DV457519 and DB922056) has two binding sites for the miRNA family miR166 (Table 2). The transcription factor regulatory miRNAs identified among others include the miR156 and miR157 families. Several studies indicate that miRNA156/157 targets squamosa promoter binding protein (SBP)-like (SPL) genes (Gandikota et al., 2007). The function of these miR156/157 miRNA families is well conserved across plant species as evident from several previous reports. Other transcription factor regulatory miRNA families identified in cassava and consistent with other plant species include miR159, miR160, miR166, miR167, miR171 and miR172. Hagen and Guilfoyle (2002) showed that auxin response factors (ARF), a plant-specific family of DNA binding proteins and the target of miRNA family miR167, are involved in hormone signal transduction (Tables 2 and 3). Wu et al. (2009) also recently found that in *A. thaliana* miR172 acts downstream of miR156 to promote adult epidermal identity.

Another group of interesting miRNA families identified include the miR162 and miR168 which respectively target the miRNA biogenesis proteins Dicer-like1 and Argonaute 1 (Table 3). This indicates that the mechanism of miRNA biogenesis is self regulating. The other miRNAs could potentially play important role in plant responses to different biotic and abiotic environmental stresses. Studies into the roles of these transcription factors and other identified genes will further deepen our understanding of plant gene regulation during growth and development.

It was observed also that the identified cassava miRNAs exhibit a wide variation in the length of pre-miRNA sequences (Table 2). The pre-miRNA length varies from 73 to 391 with most between 100 and 200 nucleotides long. It has been suggested that the differences in size among the different miRNA families is indicative they have unique functions in the regulation of miRNA

biogenesis or gene expression. The location of the new mature miRNAs in the precursor pre-miRNAs also vary among the miRNA families. 18 miRNAs were found to be located in 3' arm of the stem-loop hairpin structures while 7 are in 5' arm (Table 2). These characteristics of cassava miRNAs are similar to those reported for other plant species.

Evaluation of the new miRNAs in cassava was also based on A+U content. The new miRNA precursors have A+U content ranging from 44.90 to 64.38% (Table 2). Consistent with general notion, majority of the identified cassava pre-miRNA precursors contain more A+U nucleotides than G+C (Zhang et al., 2006). It is also important to note that formation of hairpin structure is not a unique feature of miRNAs since other RNAs such as mRNA, rRNA and tRNA can also form similar structures. For this reason, uniform systems for annotating new miRNAs comprising negative minimal fold energy (MFE), adjusted minimal fold energy (AMFE) and the minimal fold energy index (MFEI) have been developed (Seffens and Digby, 1999; Bonnet et al., 2004; Zhang et al., 2006) and have become generally accepted. Zhang et al. (2006) explained that since plant miRNA precursors are highly variable in length, a comparison based only on their MFEs which are inversely proportional to length of pre-miRNA sequence (Seffens and Digby, 1999), is not appropriate hence the need to adjust the MFE values to AMFEs. AMFE is defined as the MFE of a 100-nucleotide length of sequence and is calculated as AMFE = (MFE/Sequence length) *x* 100 (Zhang et al., 2006). Besides, it is noteworthy that the folding energy in miRNAs is not always higher than that of other RNAs (Bonnet et al., 2004). The predictions are therefore normally based on MFEI which is considered the best criterion for prediction of miRNAs, a commonly used index for distinguishing miRNAs from other non-coding and coding RNAs. Zhang et al. (2006) indicated that most miRNA precursors identified have an MFEI greater than 0.85 which is much higher than in tRNA (0.64), rRNA (0.59), or mRNA (0.65). However, it is not out of place to find pre-miRNAs with lower MFEIs provided the number of nucleotide substitutions in the particular miRNA compared with other species does not exceed three (Table 2). The MFEI is calculated by the equation: MFEI = AMFE/(G+C)% (Zhang et al., 2006).

CONCLUSION

Plant miRNA studies are increasingly revealing a lot of insight into a number of biological events. A better understanding of the role of miRNAs in post-transcriptional gene silencing in response to biotic and abiotic stresses will be vital in our attempt to develop superior cassava varieties with higher disease and pest resistance. As a pre-requisite, a complete catalogue of cassava miRNAs is essential to enable systematic and fundamental comparison of characteristics of miRNA species in cassava as well as in other plants. In this regard, the achievement in the identification and characterization of some conserved miRNAs in cassava may initiate further study of cassava miRNAs regulation mechanism. This first effort will also serve as a major step forward for us to learn more about the important regulatory roles of miRNAs in cassava growth and development. Besides, the fairly detailed description of currently available procedures and resources presented here for the identification and characterization of miRNAs in cassava will enable the rapid discovery of more novel miRNAs towards the improvement of the crop.

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Abbreviations

ESTs, Expressed sequence tag; **HYL1,** hyponastic leaves; **siRNAs**, short interfering RNAs; **miRNA,** microRNA; **miRISC,** miRNA-induced silencing complex; **AGO,** argonaute; **CIAT,** International Centre for Tropical Agriculture; **CMD,** cassava mosaic disease; **ILTAB,** International Laboratory for Tropical Agricultural Biotechnology; **BNARI,** Biotechnology and Nuclear Agriculture Research Institute; **IAEA,** International Atomic Energy Agency; **PMRD,** plant miRNA database; **GSS,** genome survey sequences; **MFE,** minimum fold energy; **MFEI,** minimal fold energy index; **AMFE,** adjusted minimal fold energy; **TAIR,** arabidopsis information resource; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcription-PCR; **qRT-PCR,** quantitative real-time PCR; **SBP,** squamosa promoter binding protein.

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