AJAB

Asian J Agric & Biol. 2021(2).
DOI: 10.35495/ajab.2020.12.551

Original Article

Identification of conserved and novel mature miRNAs in selected crops as future targets for metabolic engineering

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Received:
December 04, 2020
Accepted:
January 07, 2021
Online First:
February 15, 2021
Published:
April 25, 2021

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Abstract

MicroRNAs (miRNAs) are small non-coding RNA molecules, involved in the post-transcriptional gene expression of countless metabolic pathways including plant biomass production. The current work was focused on identification of miRNAs involved in the growth metabolism of *Glycine max*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Brassica napus*, *Triticum aestivum*. In order to identify conserved miRNA clusters, the miRNA data were collected from miRBase database. Overall, 756, 738, 325, 241, 92, and 125 datasets of the mature miRNA sequences of *Glycine max*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Brassica napus*, *Triticum aestivum* were collected from miRbase. Using MEGA software, a total of 6, 6, 5, 6, and 3 conserved miRNA clusters were examined in aforementioned crops, respectively, with the aim of studying the conserved miRNA clusters belonging to same gene families. The conserved miRNA clusters were shown to belong to miR166, miR399, miR156, miR171, miR164, miR167, and miR394 families in the selected crops. This study may lead to elucidate the role of these miRNAs and their subsequent exploitation to enhance the biomass production via metabolic pathway engineering.

Keywords: Micro-RNA, Molecular phylogenetics, Biomass production, Crop improvement

How to cite this:

Noor F, Alshamrani R, Gull M, Mehmood MA and Aslam S, 2021. Identification of conserved and novel mature miRNAs in selected crops as future targets for metabolic engineering. Asian J. Agric. Biol. 2021(2):202012551. DOI: https://doi.org/10.35495/ajab.2020.12.551

Introduction

Energy has become the basic necessity in the social and economic development as well as in improving the standards of human being. Since 1970, researchers have paid a great deal of attention towards the development of technologies using renewable resource of energy in the context of high energy crises (Gokcol et al., 2009). Among various sources, biomass from agricultural practices (bagasse, wheat/rice straw), wastewater cultivated microalgae (Shahid et al., 2020) and biomass from non-arable lands has shown promising potential as a renewable and low-cost feedstock to produce energy either biological or thermochemical processes (Mehmood et al., 2017, Ahmad et al., 2017, Ye et al., 2018). Thermochemical

methods have shown dominance over biological methods in terms of robustness, efficiency, and cost-effectiveness (Mehmood et al., 2019). Besides, regardless of the method of choice, higher biomass productivity is one of the desired parameters to enhance the cost-effectiveness of the bioenergy production. Bioenergy crops are considered as an auspicious source of the renewable energy (Sims et al., 2006). Biogas, ethanol and biodiesel are leading bioenergy products with respect to modern bioenergy (Yuan et al., 2008). The utilization of bioenergy is particularly high in the countries good financial backing or tax incentives, as for example China, Sweden, and Brazil (Wright, 2006).

Various approaches have been adopted to enhance the biomass productivity of the biomass including agricultural management practices to metabolic engineering for the subsequent use of biomass to produce bioenergy. Among various targets of metabolic engineering, Micro-RNAs have come forward as targets of interest due to their diverse roles in plant physiology including biomass production (Joshi et al., 2017). MicroRNAs (miRNA) are small non-coding RNAs, comprising about 22 nucleotides (Zhang et al., 2006b) and perform diverse physiological roles in the development of plant ;(Nogueira et al., 2007, Chitwood et al., 2009, Rubio-Somoza et al., 2009), abiotic and biotic stress responses (Shukla et al., 2008, Ruiz-Ferrer and Voinnet, 2009), signal transduction, protein degradation (Guo et al., 2005, Zhang et al., 2006b), post-transcriptional gene expression, and cellular metabolism (Zhang et al., 2006b, Zhao et al., 2010). The miRNA sequences of monocotyledonous and dicotyledonous plants are available in the miRBase. The identification of novel miRNA is a preliminary step to figure out the evolution of miRNAs in plant species along with their role in plant physiology. Hence, may lead to cultivation of the selected crop plants under salt/drought stress, after modifying the stress-responsive metabolic pathways.

A large number of miRNA have been reported in *Glycine max* (756), *Oryza sativa* (738), *Zea mays* (325), *Sorghum bicolor* (241), *Brassica napus* (92), *Triticum aestivum* (125), in miRBase (http://www.mirbase.org/) (Griffiths-Jones et al., 2007). The present study was focused on the identification and phylogenetics-based molecular characterization of conserved mature miRNAs in the aforementioned crops. The miRNA families have been shown to regulate physiological processes in the studied crops under stress conditions. Hence, study was aimed to propose the targets, those can be used in future for the metabolic engineering as well as in biomass production to meet the need of energy crises in future

Material and Methods

Retrieval of miRNA data

Plant genome contains hundreds of miRNAs. A very limited data is available for miRNA. In the current study, miRNA data of bioenergy was retrieved from miRBase database for further analysis (Figure 1). The miRBase database is considered as one of the main storehouses to collect miRNA genes since its inception because it provides a user-friendly interface offering a detailed overview of miRNA of interest and includes mature miRNA sequence along with their genomic coordinates and gene family. The dataset of the mature miRNAs sequences of *Glycine max* (no=756 mature), *Oryza sativa* (no=738), *Zea mays* (no=325, mature), *Sorghum bicolor* (no=241), *Brassica napus* (no=92 mature), and *Triticum aestivum* (no=125 mature) were collected from miRBase.

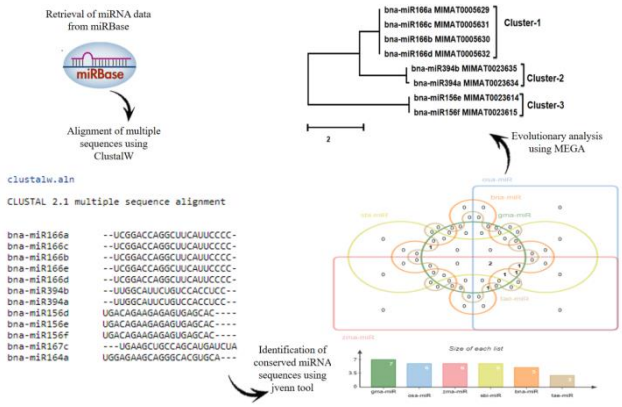

Figure-1. Schematic diagram representing the overall methodology used for the identification of conserved miRNA sequences in bioenergy crops

Multiple sequence alignment using ClustalW

The miRNA sequences of the bioenergy crops were then subjected to ClustalW for multiple sequence alignment (Chenna, 2003), which is a freely available and a frequently-used tool for the alignment of multiple sequences. It works on the basis of progressive alignment method. Therefore, it was used to reveal the conserved consensus by performing multiple alignments among mature miRNA sequence.

Phylogenetic analysis using MEGA

The phylogenetic analyses of aligned sequences were carried out by utilizing Molecular Evolutionary Genetics Analysis (MEGA). Due to its user-friendly interface and availability of multiple methods for phylogenetic tree building, MEGA is well known tool for evolutionary analysis. MEGA offers the comparative analysis of aligned sequences. In order to infer the evolutionary history among aligned sequences, the Neighbor-Joining method was used. The Maximum Composite Likelihood method was employed to calculate the evolutionary distances. The scale tree is drawn with branch length in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Identification of conserved miRNA

After performing alignment, a separate phylogenetic tree was constructed for each bioenergy crop. Because some conserved miRNA along with their corresponding gene family were found in more than one bioenergy crop, therefore Jvenn tool (http://jvenn.toulouse.inra.fr) was used to identify conserved miRNA (Bardou et al., 2014) among the selected bioenergy crops.

Results

Identification of intra-specific homologous mature miRNA sequences

On the basis of sequence identity and phylogenetic relationship, the homologous sequences in *Glycine max* were clustered (Figure 2). The present analysis was comprised of 13 nucleotide sequences. These sequences were further subjected to MEGA for phylogenetic analysis. From pairwise deletion option, all uncertain positions were deleted. The final dataset contained total of 26 positions and which were grouped into 6 clusters. All the miRNA sequences were shown belong to a cluster that showed 100% sequence identity and their associated genomic coordinates (Table 1). Likewise, a phylogenetic model was employed in *Oryza sativa* with the aim of identifying all identical miRNA sequence (Figure 3). The analysis was comprised of 20 different nucleotide sequences where final datasets were shown to contain 23 positions which grouped into 6 clusters. All miRNA sequences were shown to belong to a cluster that showed 100% sequence identity and their associated genomic coordinates (Table 2). Similarly, the same phylogenetic model was employed in *Zea mays* to identify identical miRNA sequence (Figure 4). The analysis comprised of 14 nucleotide sequences and the final dataset contained 23 positions and have been grouped into 5 clusters. In this case, all the miRNA sequences were shown to belong to a cluster that showed 100% sequence identity and their associated genomic coordinates (Table 3). Same phylogenetic model was employed in *Sorghum bicolor* where analysis comprised of 14 nucleotide sequences (Figure 5) and the final dataset had a total of 21 positions and were grouped into 6 clusters. The miRNA sequences which belonged to a cluster that showed 100% sequence identity and their associated genomic coordinates are shown in Table 4. The phylogenetic analyses of *Brassica napus* contained 8 nucleotide sequences where final dataset had total of 24 positions and were grouped into 3 clusters (Figure 6). The miRNA sequences which belonged to a cluster that showed 100% sequence identity and their associated genomic coordinates are shown in Table 5.

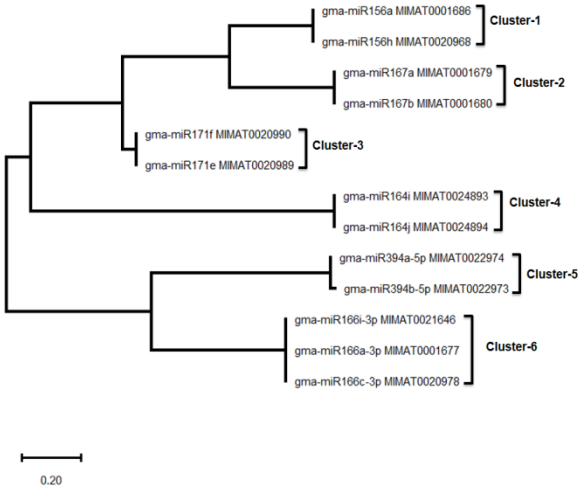

Figure-2. Evolutionary relationship in *Glycine max*. This analysis involved 13 nucleotide sequences.

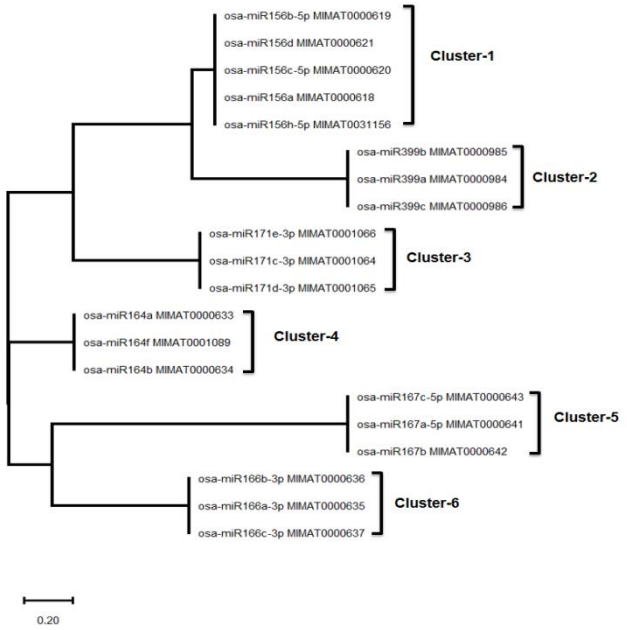

Figure-3. Representation of evolutionary relationship in *Oryza sativa* based on 20 nucleotide sequences.

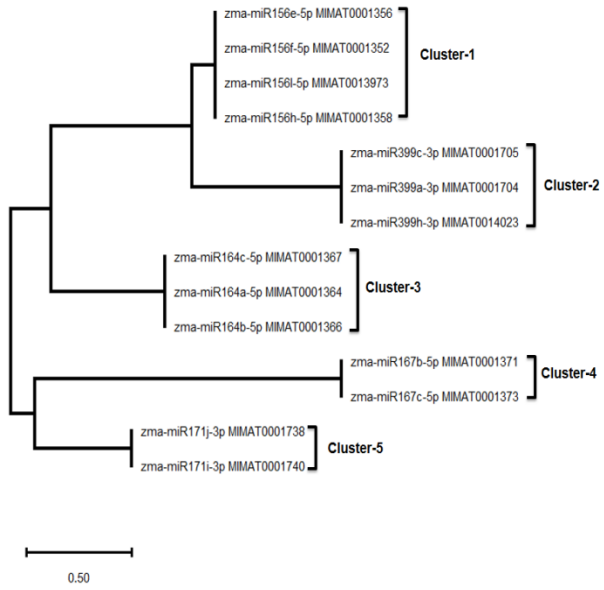

Figure-5. Representation of evolutionary relationship in *Sorghum bicolor* based on 14 nucleotide sequences.

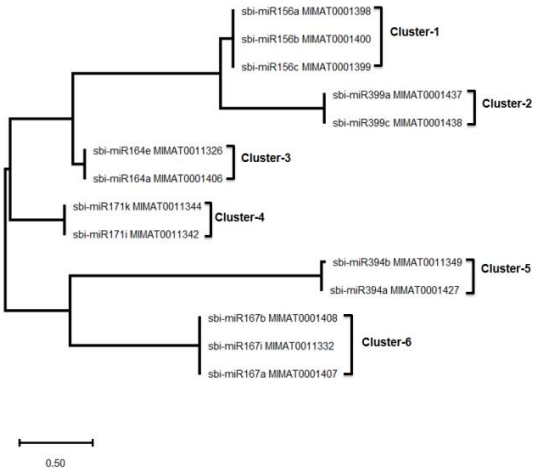

Figure-4. Representation of evolutionary relationship in *Zea mays* based on 14 nucleotide sequences.

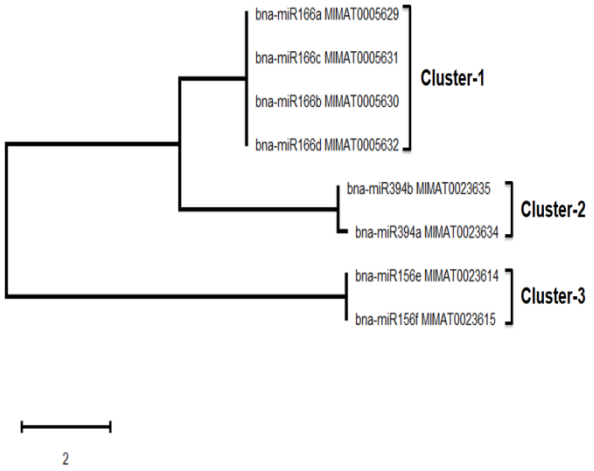

Figure-6. Evolutionary relationship in *Brassica napus* based on 8 nucleotide sequences.

Table-1. Summary of miRNAs grouped in clusters along with the members, genomic coordinates, gene families and the mature miRNA sequences in *Glycine max*

Sr. No.	Members	Gene family name	Genomic coordinates	Mature miRNA sequences
Cluster 1	gma-miR156a MIMAT0001686	MIPF0000008; MIR156	chr17: 5885507-5885628 [-]	ugacagaagagagugagcac
	gma-miR156h MIMAT0020968		chr4: 5047205-5047294 [-]	
Cluster 2	gma-miR167a MIMAT0001679	MIPF0000023; MIR167_1	chr19: 42073549-42073667 [+]	ugaagcugccagcaugaucua
	gma-miR167b MIMAT0001680		chr2: 14838068-14838188 [-]	
Cluster 3	gma-miR171f MIMAT0020990	MIPF0000030; MIR171_1	chr15: 8505075-8505164 [-]	ugauugagccgugccaauauc
	gma-miR171e MIMAT0020989		chr5: 38773113-38773202 [-]	
Cluster 4	gma-miR164i MIMAT0024893	MIPF0000045; MIR164	chr18: 49506475-49506631 [+]	uggagaagcagggcacgugca
	gma-miR164j MIMAT0024894		chr19: 48278744-48278831 [+]	
Cluster 5	gma-miR394a-5p MIMAT0022974	MIPF0000100; MIR394	chr17: 41517111-41517267 [+]	uuggcauucuguccaccucc
	gma-miR394b-5p MIMAT0022973		chr14: 48784355-48784458 [+]	
Cluster 6	gma-miR166i-3p MIMAT0021646	MIPF0000004; MIR166	chr2: 14544488-14544584 [+]	ucggaccaggcuucauucccc
	gma-miR166a-3p MIMAT0001677		chr16: 1934911-1935056 [-]	
	gma-miR166c-3p MIMAT0020978		chr7: 4502830-4502959 [-]	

Table-2. Summary of miRNAs grouped in clusters along with the members, genomic coordinates, gene families and the mature miRNA sequences in *Oryza sativa*

Sr. No.	Members	Gene family name	Genomic coordinates	Mature miRNA sequences
Cluster 1	osa-miR156b-5p MIMAT0000619	MIPF0000008; MIR156	Chr1: 4666341-4666516 [+]	ugacagaagagagugagcac
	osa-miR156d MIMAT0000621		Chr2: 4512884-4513012 [-]	
	osa-miR156c-5p MIMAT0000620		Chr1: 4665975-4666123 [+]	
	osa-miR156a MIMAT0000618		Chr1: 22524147-22524246 [-]	
	osa-miR156h-5p MIMAT0031156		Chr8: 21491232-21491417 [+]	
Cluster 2	osa-miR399b MIMAT0000985	MIPF0000015; MIR399	Chr2: 7664026-7664122 [-]	ugccaaaggagaauugcccug
	osa-miR399a MIMAT0000984		Chr1: 30478636-30478784 [+]	
	osa-miR399c MIMAT0000986		Chr5: 26305938-26306047 [-]	
Cluster 3	osa-miR171e-3p MIMAT0001066	MIPF0000030; MIR171_1	Chr3: 1970487-1970605 [+]	ugauugagccgugccaauauc

	osa-miR171c-3p MIMAT0001064		Chr4: 31713472-31713570 [-]	
	osa-miR171d-3p MIMAT0001065		Chr10: 21237313-21237447 [+]	
Cluster 4	osa-miR164a MIMAT0000633	MIPF0000045; MIR164	Chr7: 28523341-28523496 [-]	uggagaagcagggcacgugca
	osa-miR164f MIMAT0001089		Chr5: 23343908-23344117 [+]	
	osa-miR164b MIMAT0000634		Chr5: 15896163-15896271 [-]	
Cluster 5	osa-miR167c-5p MIMAT0000643	MIPF0000023; MIR167_1	Chr3: 33130619-33130781 [+]	ugaagcugccagcaugaucua
	osa-miR167a-5p MIMAT0000641		Chr12: 25476808-25476948 [+]	
	osa-miR167b MIMAT0000642		Chr3: 30546928-30547090 [-]	
Cluster 6	osa-miR166b-3p MIMAT0000636	MIPF0000004; MIR166	Chr6: 30327084-30327289 [-]	Ucggaccaggcuucauucccc
	osa-miR166a-3p MIMAT0000635		Chr10: 19987135-19987279 [+]	
	osa-miR166c-3p MIMAT0000637		Chr3: 3487788-3487912 [+]	

Table-3. Summary of miRNAs grouped in clusters along with the members, genomic coordinates, gene families and the mature miRNA sequences in *Zea mays*

Sr. No.	Members	Gene family name	Genomic coordinates	Mature miRNA sequences
Cluster 1	zma-miR399a-3p MIMAT0001704	MIPF0000015; MIR399	chr4: 233617206-233617328 [-]	ugccaaaggagaauugcccug
	zma-miR399h-3p MIMAT0014023		chr5: 150363095-150363188 [+]	
	zma-miR399c-3p MIMAT0001705		chr6: 163867932-163868138 [+]	
Cluster 2	zma-miR156h-5p MIMAT0001358	MIPF0000008; MIR156	chr10: 129642870-129642984 [-]	ugacagaagagagugagcac
	zma-miR156f-5p MIMAT0001352		chr2: 185613978-185614144 [-]	
	zma-miR156e-5p MIMAT0001356		chr2: 33005026-33005151 [+]	
	zma-miR156l-5p MIMAT0013973		chr5: 188595332-188595421 [-]	
Cluster 3	zma-miR171j-3p MIMAT0001738	MIPF0000030; MIR171_1	chr10: 59006552-59006680 [+]	ugauugagccgugccaauauc
	zma-miR171i-3p MIMAT0001740		chr1: 278093492-278093595 [+]	
Cluster 4	zma-miR167b-5p MIMAT0001371	MIPF0000023; MIR167_1	chr5: 7976369-7976495 [+]	Ugaagcugccagcaugaucua
	zma-miR167c-5p MIMAT0001373		chr5: 4414244-4414375 [+]	
Cluster 5	zma-miR164c-5p MIMAT0001367	MIPF0000045; MIR164	chr6: 157477837-157478106 [+]	Uggagaagcagggcacgugca
	zma-miR164a-5p MIMAT0001364		chr2: 223401157-223401308 [-]	

	zma-miR164b-5p MIMAT0001366		chr6: 145727415-145727542 [+]	

Table-4. Summary of miRNAs grouped in clusters along with the members, genomic coordinates, gene families and the mature miRNA sequences in *Brassica napus*

Sr. No.	Members	Gene family name	Genomic coordinates	Mature miRNA sequences
Cluster 1	sbi-miR156a MIMAT0001398	MIPF0000008; MIR156	chr4: 5326257-5326340 [-]	ugacagaagagagugagcac
	sbi-miR156b MIMAT0001400		chr3: 3415906-3415989 [-]	
	sbi-miR156c MIMAT0001399		chr3: 3416227-3416321 [-]	
Cluster 2	sbi-miR399a MIMAT0001437	MIPF0000015; MIR399	chr3: 61878757-61878892 [+]	ugccaaaggagaauugcccug
	sbi-miR399c MIMAT0001438		chr9: 55505804-55505933 [-]	
Cluster 3	sbi-miR164e MIMAT0011326	MIPF0000045; MIR164	chr9: 44994962-44995166 [+]	uggagaagcagggcacgugca
	sbi-miR164a MIMAT0001406		chr9: 38911782-38911907 [-]	
Cluster 4	sbi-miR171k MIMAT0011344	MIPF0000030; MIR171_1	chr6: 56762480-56762566 [-]	ugauugagccgugccaauauc
	sbi-miR171i MIMAT0011342		chr1: 59668493-59668582 [-]	
Cluster 5	sbi-miR394b MIMAT0011349	MIPF0000100; MIR394	chr4: 62923468-62923544 [-]	uuggcauucuguccaccucc
	sbi-miR394a MIMAT0001427		chr2: 66830969-66831078 [+]	
Cluster 6	sbi-miR167b MIMAT0001408	MIPF0000023; MIR167_1	chr1: 7229987-7230184 [+]	ugaagcugccagcaugaucua
	sbi-miR167i MIMAT0011332		chr8: 59223214-59223345 [+]	
	sbi-miR167a MIMAT0001407		chr1: 4354010-4354105 [+]	

Table-5. Summary of miRNAs grouped in clusters along with the members, genomic coordinates, gene families and the mature miRNA sequences in *Triticum aestivum*

Sr. No.	Members	Gene family name	Genomic coordinates	Mature miRNA sequences
Cluster 1	bna-miR166a MIMAT0005629	MIPF0000004;MIR166	EM:DX911364: 697-831 [-]	ucggaccaggcuucauucccc
	bna-miR166c MIMAT0005631		EM:AC189591: 82389-82516 [+]	
	bna-miR166b MIMAT0005630		EM:BH423978: 306-423 [+]EM:BH680789: 165-282 [-]	
	bna-miR166d MIMAT0005632		EM:AC189313: 1397-1524 [+]	
Cluster 2	bna-miR394b MIMAT0023635	MIPF0000100;MIR394	EM:AC189295: 85927-86038 [+]	Uuggcauucuguccaccucc
	bna-miR394a MIMAT0023634		EM:AC189318: 31776-31891 [+]	
Cluster 3	bna-miR156e MIMAT0023614	MIPF0000008;MIR156	EM:AC189375: 124714-124832 [+] EM:DU831758: 290-408 [-]	Ugacagaagagagugagcac
	bna-miR156f MIMAT0023615		EM:CV432746: 228-337 [+]	

Identification of Inter-specific homologous mature miRNAs

The multiple aligned sequences were subjected phylogenetic analyses (Figure 8). The analysis comprised of 73 nucleotide sequences, and the final dataset was shown to contain 26 positions which were grouped into 7 clusters. It was interesting to see that one miRNA- UGACAGAAGAGAGUGAGCAC was conserved in *Glycine max*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, and *Brassica napus*. Another miRNA-UGCCAAAGGAGAAUUGCCCUG was shown to be conserved in *Glycine max*, *Oryza sativa*, *Zea mays*, and *Sorghum Bicolor*. While another miRNA-UGAUUGAGCCGUGCCAAUAUC was shown to be conserved in *Glycine max*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, and *Triticum aestivum*. The miRNA- UGAAGCUGCCAGCAUGAUCUA was found to be conserved in *Glycine max*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Brassica napus*, and *Triticum aestivum*. The miRNA-UGGAGAAGCAGGGCACGUGCA was found to be conserved in *Glycine max*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Brassica napus*, and *Triticum aestivum*. The miRNA-UUGGCAUUCUGUCCACCUCC was found to be conserved in *Glycine max*, *Sorghum bicolor*, and *Brassica napus*. While, the miRNA-UCGGACCAGGCUUCAUUCCCC was shown to be conserved in *Glycine max*, *Zea mays*, *Sorghum bicolor*, and *Brassica napus*.

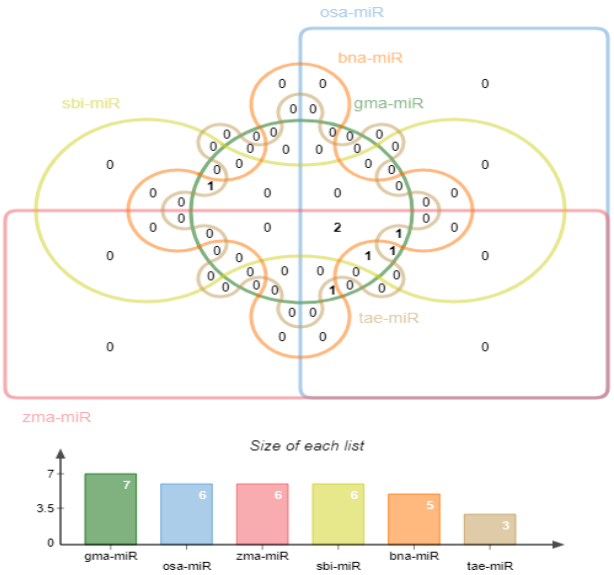

Figure-7. Representation of overlapped miRNA in *Glycine max, Oryza sativa, Zea mays, Sorghum bicolor, Brassica napus,* and *Triticum aestivum*

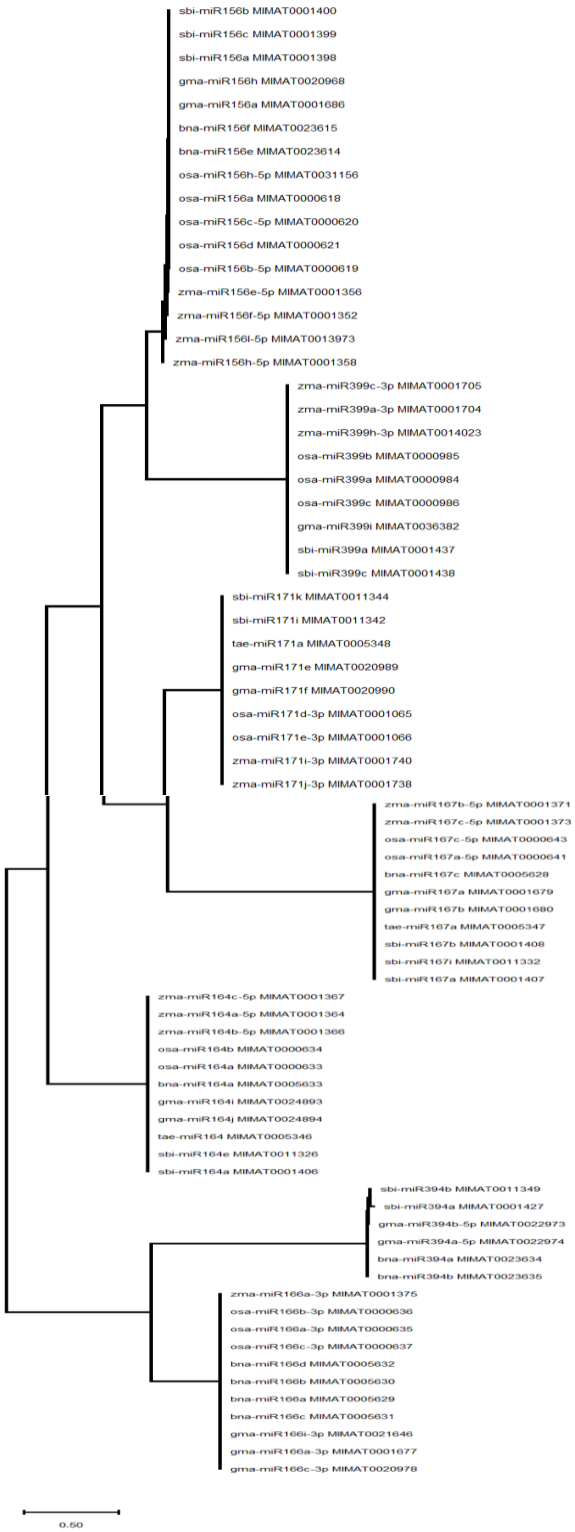

Figure-8. Representation of evolutionary relationship among *Glycine max, Oryza sativa, Zea mays, Sorghum bicolor, Brassica napus,* and *Triticum aestivum* based on 73 sequences.

Discussion

It is shown that miRNAs are involved the post-transcriptional gene expression (Zhang et al., 2006a). In spite of the fact that miRNA is common in animals, some miRNA clusters are found to be conserved in plants (Sunkar and Zhu, 2004, Guddeti et al., 2005, Zhang et al., 2007, Talmor- Neiman et al., 2006). However, insertion, deletion and duplication events in miRNA sequences suggested that evolutionary conserved clusters are present in plants. However, it has been estimated that gene duplication events occur more frequently in eukaryotic genomes (Lynch and Conery, 2000) and particularly in flowering plants (Blanc and Wolfe, 2004, Cui et al., 2006). It has been investigated that plant comprises more non-conserved clusters when compared to the conserved clusters (Fahlgren et al., 2007, Rajagopalan et al., 2006, Sunkar et al., 2008).

Several *in-silico* and *in-vitro* studies have identified conserved miRNA in various bioenergy crops, but none deciphered the conserved identical miRNA sequences in the group of bioenergy crops. The present study was focused to identify the intra-specific and inter-specific conserved miRNAs in six bioenergy crops. Through computational and experimental identification, miRBase is considered as one of the main storehouses to collect miRNA genes. The present study elucidated 7 miRNA families that found to be conserved in six bioenergy crops. The conserved miRNA clusters were shown to belong miR166, miR399, miR156, miR171, miR164, miR167, and miR394 families indicated that due to genomic duplication event the ancestral clusters might have been originated.

The miR156 represents an evolutionary conserved miRNA which indicated that it is common in plant species. Interestingly, the bioenergy crops overexpressing miR156 were shown to increase the plant biomass and altered lignin content and composition (Fu et al., 2012, Rubinelli et al., 2013, Schwab et al., 2005). The miR156 has also found associated with the phase transition, in the plant development, formation of floral meristem, and morphology of immature leaves and cell wall. Besides, miR156 has shown to be involved in abiotic stress responses including drought and low-nitrogen in bioenergy crops (Ferreira et al., 2012, Khraiwesh et al., 2012). A higher expression level of miR156 led to a shortened length of internode and hence the overall plant biomass decreased (Fu et al., 2012). Both of the

miR166 and miR167 are involved in metabolism, morphology, and development of *Zea mays* and *Sorghum bicolor* (Wei et al., 2009). Moreover, miR166 and miR167 are also involved in the early development of plant, hence could have potential application in abiotic stress, biofuel yield, bio confinement, and recalcitrance (Trumbo et al., 2015). The miR164 is another evolutionary conserved miRNA which is found associated with the metabolic processes, drought response, early development (Wei et al., 2009), regulation of lateral rooting. Hence, it could be another target of metabolic engineering to counteract stress and to enhance plant biomass production (Wei et al., 2009). In *Sorghum bicolor*, miR399 may have potential application in abiotic stress. During water deprivation, miR399 was upregulated and showed a positive stress response (Calviño et al., 2011, Katiyar et al., 2012, Paterson et al., 2009). In *Glycine Max*, miR399 could be an engineering target to control the phosphate regulation (Sun, 2012). In *Zea mays*, miR399 has shown to involved regulating the morphogenesis and embryonic development in the grain (Li et al., 2016). In *Oryza sativa*, miR399 has shown to be involved in phosphate signaling (Fang et al., 2009). In *Sorghum bicolor*, miR171 is associated with the stress responses and in the process of morphological development (Ram and Sharma, 2013). While the upregulation of miR171 have shown to be involved in abiotic stress and in floral development in *Oryza Sativa* (Zhou et al., 2010). Further *in vitro* studied are required on miR399, miR171, and miR394 for their exploitation as future targets of metabolic engineering to counteract the abiotic stress and to enhance biomass production of the bioenergy crops.

Conclusion

The study was focused on identification of conserved miRNAs in selected bioenergy crops as future targets of metabolic engineering to improve the biomass productivity. Based on phylogenetic analyses, conserved miRNA clusters were shown to belong to miR166, miR399, miR156, miR171, miR164, miR167, and miR394 families, while these families can be used as genetic engineering targets. Such studies can further be extended to other crops of agricultural and environmental importance to identify conserved miRNAs to understand their physiological roles and evolutionary relationships.

Disclaimer: None.
Conflict of Interest: None.
Source of Funding: None.

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Noor F: Performed all analysis of current study
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