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# *In silico* Prediction of MicroRNAs in Plant Mitochondria

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***Abstract:*** *MicroRNAs are endogenous, short (~21 base), non-coding, post transcriptional, regulatory RNA molecules. These microRNAs (miRNAs) are complementary to their target messenger RNAs, and bind principally to its 3' UTR. The conserved nature of miRNAs, and their high sequence complementarities of miRNA and its targets in plants, provides the basis for the easy identification of miRNA and its targets. Presence of miRNA in plant mitochondria is scantily studied. Identification of miRNA targets in plant mitochondria might indicate the involvement of miRNA in mitochondrial gene regulation and nuclear mitochondrial interactions. In this study, we used a computational approach to predict miRNA targets in plant mitochondria. The mitochondrial gene targets identified for miRNAs are located both in mitochondrial and nuclear compartments. This observation points to a fairly early origin of miRNAs. Besides, most of the targets identified can have copies in two compartments and suggest the possibility of miRNA mediated regulation. This study unfurls the possibility of regulating the plant mitochondrial genes by amending the miRNA genes in the nuclear compartment.*

***Keywords:*** *miRNA, Mitochondria, Chloroplast, Arabidopsis, Sorghum, Grapes.*

## Introduction

Small RNAs are regulatory RNA molecules that include miRNAs (miRNAs), Piwi associated RNAs (pi-RNAs) and small interfering RNAs (siRNAs). These are grouped together owing to their short sequence (21-40 nt).

The siRNAs are exogenous molecules generated from the perfect stem loop precursors, which mediate the cleavage of the target RNA when incorporated into RNA Induced Silencing Complex (RISC). On the other hand, the miRNAs are a class of endogenous, short, non protein coding RNA molecules. Genes coding for the miRNAs are mostly observed in the intronic regions [3], the transcription of which generates the miRNA precursor, primary miRNA (pri-miRNA). Drosha enzyme mediated cleavage of the long fold-back hairpin like precursor, pri-miRNA, generates 80-100 nucleotide stem-loop precursor, premature miRNA (pre- miRNA). These pre-miRNAs are exported to the cytoplasm by Exportin5 proteins. In the cytoplasm, the pre-miRNA is acted upon by the ribonuclease III Dicer-like (DCL) enzyme in plants, to release the miRNA/miRNA* duplex. The miRNA released by the helicase activity, gets loaded into the RNA Inducing Silencing Complex (RISC), and guides the assembly to the target mRNA. The miRNA duplex is degraded [6]. The miRNA is called the guide strand, while miRNA* is called the passenger strand.

MiRNA sequences, being complementary to their target mRNAs, causes sequence specific regulation, which includes translational repression or the post-transcriptional gene silencing by mediating mRNA cleavage/degradation [2, 10, 12, 13, 18]. The nature of the target determines the significance of miRNA regulation in plant development [6]. In plants, the miRNAs have perfect or near perfect complementarity to their respective target mRNAs, while in animals, the miRNA binds to its targets with numerous bulges and loops. This makes the identification of miRNA targets in plants easier when compared to those in animals [17]. The high degree of miRNA:mRNA sequence complementarity facilitates target cleavage. In accordance to the above fact, mRNA cleavage is observed in plants, unlike in animals. MiRNA mediated cleavage of the target mRNA usually occurs at the middle ($10^{th}$ or $11^{th}$ nucleotide) of the binding region [10], which is called the seed region [9].

MiRNAs were identified to target the transcription factors that regulate the development of vascular tissues, floral meristem, flowering time in thale cress, and sex determination in maize [4], and in regulating the plant immune responses to pathogen challenges [15]. MiRNAs are expressed in various plant tissues like leaf, flower, fruit and roots [10]. MiRNA do not always regulate the mRNAs native to its tissue, instead these were reported to function autonomously in the parts of plants where it is not expressed. The miR172, which is highly expressed in leaves, is validated to regulate the APETALA2 transcription factor, which negatively regulates fruit ripening in tomato [10].

Besides the above regulations, surprisingly, miRNAs have been reported to show cross kingdom regulation. miR168a, which is abundant in rice, was primarily found to be acquired orally through food intake. The sera of the Chinese subjects revealed the presence of miR168a, which was found to reduce the low-density lipoprotein receptor adapter protein 1 (LDLRAP1) expression in liver, consequently decreasing the LDL removal. This was validated both *in vitro* and *in vivo* [22].

The *inverted duplication hypothesis* drives in, when dealing with the evolutionary origin of miRNAs. According to this, miRNA genes arose from inverted duplications of target genes, or fragments of target genes, the transcription of which produced hairpin precursors of miRNA [3]. The evolution of miRNAs is still conflicted. Several plant miRNAs are conserved among many land plants. However, the evidence for any miRNAs conserved between animals and plants is slim. This investigation on the presence of miRNA targets in mitochondrial genome will shed light on the origin of miRNAs and inter compartmental interactions in plant cell.

## Materials and methods

### *Collection of miRNA*

The available miRNA sequences of all 10 monocots and 11 dicots were collected from Sanger's miRBase (http://www.mirbase.org/). The mature miRNA sequences were downloaded in the unaligned FASTA format. A multi FASTA format of the miRNA sequences were used and a local database was constructed. This was used as the query.

### *Retrieval of mitochondrial Coding sequences (CDS)*

Mitochondrial sequences of 47 plants were available in NCBI's Organelle Genome Database (http://www.ncbi.nlm.nih.gov/genomes/GenomesHome.cgi?taxid=2759&hopt=html) as on April 2012. 10 of 47 plants had their miRNAs deposited in the Sanger's miRBase. The Coding sequences (CDS) of such plants were collected and a local database was constructed. This database was used as the reference against the miRNA query database.

### *Complementarity search*

The publicly available Standalone BLAST version 2.2.25+ was downloaded from http://www.ncbi.nlm.nih.gov/books/NBK1762. This was used for the complementarity search to identify all possible miRNA targets, depending upon the sequence complementarity. The program was run in the command prompt. E value was set to 1000, which is recommended for short RNAs. The output format 6 was chosen, and the blast results were generated in notepad.

### *Identification of potential candidates*

AWK programming language version 95 is publicly available. AWK95 was downloaded from http://cm.bell-labs.com/cm/cs/awkbook/index.html, and was used to identify the candidates having perfect base pairing of >7 nucleotides from the 5' end of the miRNA. The result of Standalone BLAST was used as the input for screening using AWK program.

### *Prediction of secondary structure*

The RNAhybrid tool offered by the Bibiserv is a freely accessible simple online tool to calculate the minimum free energy (mfe), and to deduce the secondary structure of miRNA:mRNA hybrid. The RNAhybrid tool can also be downloaded from http://bibiserv.techfak.unibielefeld.de/download/tools/rnahybrid.html and worked offline. These results were screened for parameters such as (1) 100% complementarity, or perfect base pairing of > 7 nucleotides from the 5' end of the miRNA (2) minimum free energy < -28 kcal/mol. (3) Bulges with the maximum of three nucleotides, and (4) loops with one mismatch on either side. Such candidates were chosen as the potential targets. The mature miRNA sequences were used as the query to identify its plausible targets in the mitochondria of various crop plants. The analysis revealed four targets in *Arabidopsis thaliana*, one in *Sorghum bicolor* and two in *Vitis vinifera.*

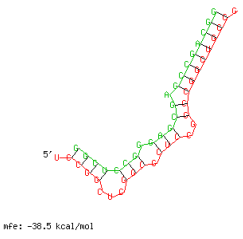
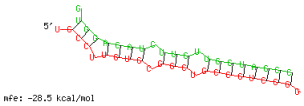
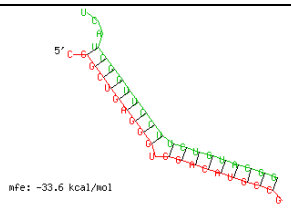
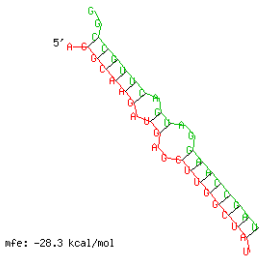
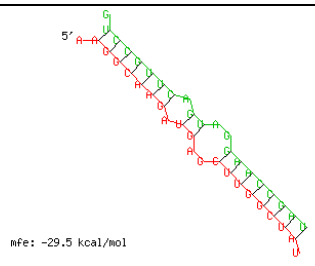
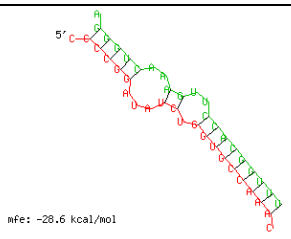
## Results and discussion

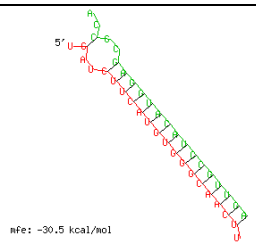
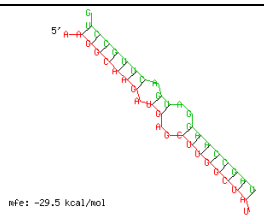
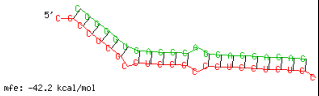
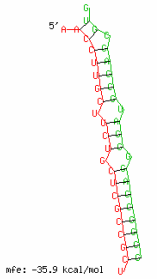
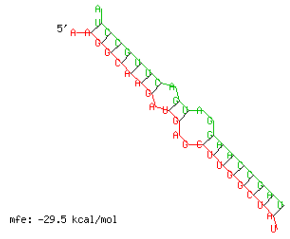
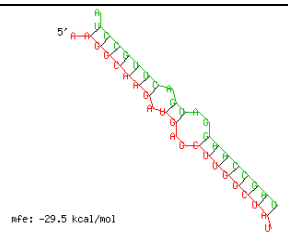
A computational analysis to identify the putative miRNA targets in wheat mitochondria was performed. All the miRNAs identified in wheat were used in the search. The screening parameters used were 100% complementarity, or perfect base pairing of > 7 nucleotides from the 5' end of the miRNA , minimum free energy < -28 kcal/mol, bulges with the maximum of three nucleotides, and (4) loops with one mismatch on either side. Due to this stringent parameters, no target candidates were detected both in wheat mitochondrial and nuclear compartments.

Hence a search using all the miRNAs listed in monocot plants were used against wheat genes. This yielded several wheat mitochondrial genes as targets (Table 1). However all these targets are located in the nuclear compartment and target their proteins to mitochondria.

To investigate whether mitochondrial genes located in mitochondrial genome are regulated by miRNA, coding sequences of all completely sequenced mitochondrial genomes were searched against all miRNAs available in the miRBase. This resulted in the identification of seven potential miRNA targets, four in *A. thaliana*, one in *S. bicolor* and two in *V. vinifera* (Table 2 and Table 3). The plants *Brassica napus, Brassica oleraceae, Carica papaya, Lotus japonicus* and *Ricinus communis* did not have any potential targets fitting the screening parameters used in this study.

Table 1. Monocot miRNAs targeting both the nuclear encoded mitochondrial mRNAs and mitochondrial encoded mitochondrial mRNAs in *Triticum aestivum*

| **Name of the Crop** | **miRNA** (miRBase acc. no.) | **Target mRNA** (GenBank acc. no.) | **Product encoded by the target mRNA** | **Secondary structure of the miRNA:mRNA hybrid** | **Stability of the hybrid mfe* (kcal/mol)** |
|---|---|---|---|---|---|
| **miRNAs targeting nuclear encoded mitochondrial mRNAs** | | | | | |
| *Oryza sativa* | osa-miR2907a | >gi\|4138868\|gb\|AF104107.1\| | Small heat shock protein Hsp 23.5 |  | -38.5 |
| | osa-miR5515 | >gi\|68449776\|gb\|DQ057344.1 | mitochondrial L2 ribosomal protein |  | -28.5 |
| *Zea mays* | zma-miR169f-3p | >gi\|4138868\|gb\|AF104107.1\| | small heat shock protein Hsp23.5 |  | -33.6 |
| **miRNAs targeting mitochondrial encoded mitochondrial mRNAs** | | | | | |
| *Brachypodium distachyon* | bdi-miR169b | >gi\|81176508:102097-103176 | ORF 359 |  | -28.3 |
| | bdi-miR169e | >gi\|81176508:102097-103176 | ORF 359 |  | -29.5 |
| *Hordeum vulgare* | hvu-miR5051 | >gi\|81176508:314992-315807 | mttB |  | -28.6 |

| | | | | | |
|---|---|---|---|---|---|
| *Oryza sativa* | osa-miR3982-3p | >gi\|341107\|gb\|M24084.1\|WHTMTATP6A | F1F0-ATPase precursor (atp6) gene |  | -30.5 |
| | osa-miR169m | >gi\|81176508:102097-103176 | ORF 359 |  | -29.5 |
| | osa-miR1858a | >gi\|6958203\|gb\|AF091838.1\| | single-subunit RNA polymerase G |  | -42.2 |
| | osa-miR2104 | >gi\|2326761\|emb\|Y14435.1\| | nad2 |  | -35.9 |
| *Sorghum bicolor* | sbi-miR169n | >gi\|81176508:102097-103176 | ORF 359 |  | -29.5 |
| | zma-miR169g | >gi\|81176508:102097-103176 | ORF 359 |  | -29.5 |

*mfe – minimum free energy; miRNA – green strand; mRNA – red strand

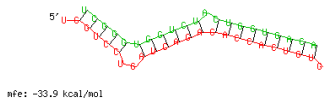
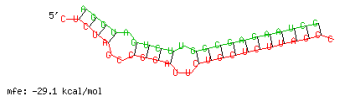
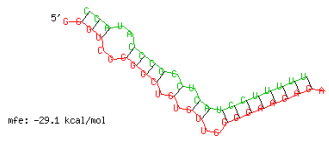
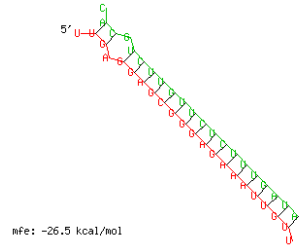
In order to validate the authenticity of the screening parameters, a comparative search on all the Arabidopsis miRNA against mRNA targets was carried out. A subset of the identified targets which were found to be experimentally validated either by quantitative real time PCR (qT-PCR) or by Northern blotting and Microarray analysis are listed in Table 4. The validated targets not only show higher (> -28) minimum free energy values, they also have bigger loops and bulges (Table 4). Alves-Junior et al. [2] used the RNAhybrid tool to identify the miRNA targets. The specificity and the signal-to-noise ratio were then assessed using the SHUFFLE program from the HMMER package [2]. This study uses standalone BLAST to generate all possible miRNA:mRNA combinations. The AWK program was used to screen the BLAST

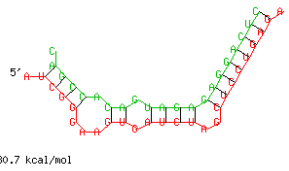
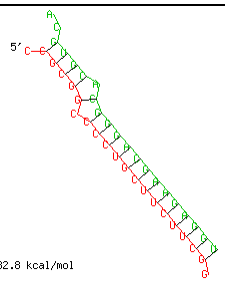
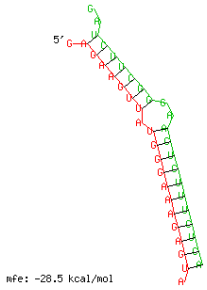
result with the specified parameters, which is close to the parameters used by Alves-Junior et al. [2]. Finally, the RNA hybrid tool was used to obtain the secondary structure of the hybrid and to test the stability (mfe) of the miRNA:mRNA hybrid.

Table 2. Identification of miRNA targets in the mitochondria of dicot plants

| Name of the crop | No. of premature miRNAs | No. of mature miRNAs | No. of mitochondrial mRNAs | No. of identified targets |
|---|---|---|---|---|
| *Arabidopsis thaliana* | 201 | 375 | 117 | 4 |
| *Brassica napus* | 46 | 48 | 78 | 0 |
| *Brassica oleraceae* | 6 | 7 | 79 | 0 |
| *Carica papaya* | 1 | 1 | 39 | 0 |
| *Lotus japonicus* | 3 | 4 | 34 | 0 |
| *Ricinus communis* | 63 | 63 | 37 | 0 |
| *Sorghum bicolor* | 171 | 172 | 32 | 1 |
| *Vitis vinifera* | 163 | 186 | 74 | 2 |

Table 3. miRNAs and their targets in the mitochondria of *A. thaliana, S. bicolor* and *V. vinifera*

| Name of the Crop | miRNA (miRBase acc. no.) | Target mRNA (GenBank acc. no.) | Product encoded by the target mRNA | Secondary structure of miRNA:mRNA hybrid | Stability of the hybrid mfe* (kcal/mol) |
|---|---|---|---|---|---|
| *Arabidopsis thaliana* | ath-miR5638b | >gi\|26556996:256865-257476 | cytochrome c biogenesis orf203 |  | -33.9 |
| | ath-miR5630a | >gi\|26556996:201729-202097 | hypothetical protein ArthMp061 |  | -29.1 |
| | ath-miR472 | >gi\|26556996:318588-319463 | hypothetical protein ArthMp104 |  | -29.1 |
| | ath-miR413 | gi\|26556996:132071-132213, 133177-133245, 134309-134775, 135829-136072, 137892-138153 | nad7 |  | -26.5 |

| | | | | | |
|---|---|---|---|---|---|
| *Sorghum bicolor* | sbi-miR1432 | >gil115278525:138746-139096 | rps13 |   | -30.7 |
| *Vitis vinifera* | vvi-miR164a, c,d | >gil224365609:43291-44718 | rbcL |   | -32.8 |
| | vvi-miR447b | gil224365609:755164-755624, 757059-757573, 761995-762417, 764462-764550 | nad4 |   | -28.5 |

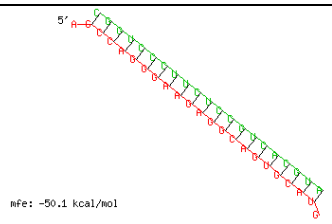
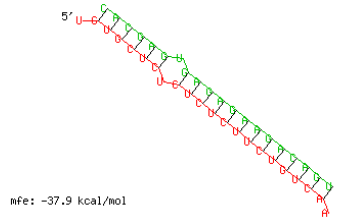
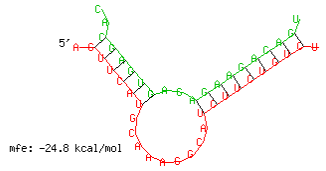
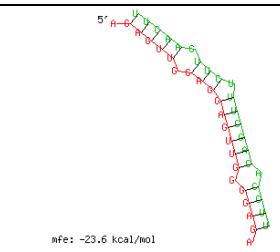
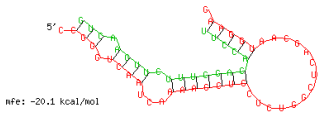
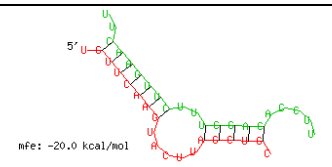
*mfe – minimum free energy

The screening strategies used in this study also led the identification of mitochondrial and chloroplast target genes located in the nuclear genome of *Sorghum bicolor* (Table 5). Although the Sbi-miR5381 targeting the ALDH2b does not fit into the screening parameter, having two mismatches at the 5' end of the miRNA, the secondary structure and the minimum free energy are convincing. Hence, this is included as a potential target.

MiRNAs add another level of gene regulation to the already existing plethora of factors in nuclear genes. MiRNAs are highly conserved among land plants. For example, miR156/157, miR172, miR170/171, miR165/166, miR159/319, miR396, miR168, miR160 and miR390 are highly conserved, while, miR394, miR164, miR169, miR167, miR162, miR398, miR414, miR393, miR397 and miR163 are moderately conserved among various families of plants [21]. In this study, the conserved nature of miR169 in *Oryza sativa, Sorghum bicolor, Zea mays, Brachipodium distachyon* and *Festuca arundinaceae* is observed. This hints the possibility of early origin of miRNAs, and their conserved nature among families.

A computational analysis was performed to find if similar miRNA mediated gene regulation is present in plant mitochondria. MiRNA target prediction is an indirect method for the identification of miRNA mediated regulation in plant mitochondria. Our computational prediction has lead to the identification of miRNA targets in plant mitochondria (Table 2 and Table 3). We have also demonstrated that our computational prediction strategy is robust in identifying miRNA targets, as many of them have been already validated experimentally (Table 4).

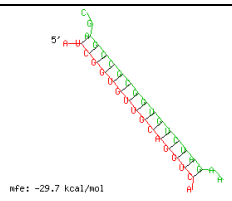
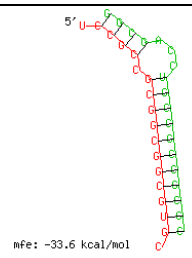
Table 4. Computationally identified miRNA targets in *Arabidopsis thaliana* that are validated experimentally

| **miRNA** (miRBase acc. no) | **Target mRNA** (GenBank acc. no) | **Product encoded by the target mRNA** | **Secondary structure of miRNA:mRNA hybrid** | **Stability of the hybrid mfe* (kcal/mol)** |
|---|---|---|---|---|
| ath-miR408 | >gi\|145362208\|ref\|NM_201977.2\| | Peptide chain release factor 1 |  | -50.1 |
| ath-miR156f | >gi\|30688928\|ref\|NM_129782.2\| | Squamosa promoter binding like Protein 9 (SPL9) |  | -37.9 |
| ath-miR156f | >gi\|42571846\|ref\|NM_202285.1\| | Squamosa promoter binding like Protein 4 (SPL4) |  | -24.8 |
| ath-miR396b | >gi\|30682906\|ref\|NM_112250.2\| | Growth regulating factor 5 (GRF 5) |  | -23.6 |
| ath-miR396a | >gi\|18396272\|ref\|NM_126630.1\| | Growth regulating factor 6 (GRF 6) |  | -20.1 |
| ath-miR396b | >gi\|18396272\|ref\|NM_126630.1\| | Growth regulating factor 6 (GRF 6) |  | -20.0 |

*mfe – minimum free energy

Hitherto, miRNAs have been identified to target the genes of virus [16], bacteria [7], and nuclear genes of algae [8], plants [2, 10], animals [3, 11] and humans [5]. Though miRNAs were not identified in eubacteria and archae bacteria, the presence of small RNA processors like argonaute protein have been identified in them [7]. This suggests an early origin for small RNA like molecules. Hence presence of miRNA mediated regulation of mitochondrial genes is highly likely. Our results also strongly suggest that miRNA mediated regulation is present in mitochondria. Lung et al. [14] also reported the presence of small RNAs in mammalian mitochondria and plant chloroplasts.

Table 5. Identification of miRNAs targeting the nuclear encoded organellar mRNAs in *Sorghum bicolor*

| **miRNA** (miRBase acc. no.) | **Target mRNA** (GenBank acc. no.) | **Product encoded by the target mRNA** | **Secondary structure of miRNA:mRNA hybrid** | **Stability of the hybrid mfe* (kcal/mol)** | **Remark** |
|---|---|---|---|---|---|
| Sbi-miR5381 | >gi\|20530130\|dbj\|AB084898.1 | ALDH2b Aldehyde dehydrogenase gene |   | -29.7 | Nuclear encoded mitochondrial protein |
| Sbi-miR5384 | >gi\|1255713\|gb\|U23945.1\|SBU23945 | Granule-bound starch synthase precursor |   | -33.6 | Nuclear encoded chloroplast protein |

*mfe – minimum free energy

According to the endosymbiotic theory, α-proteobacteria is the present mitochondria, in which most of the genes were transferred to nucleus, few were lost, and the remaining is still present in the mitochondria. The nuclear encoded mitochondrial genes could once have been in mitochondria prior to its evolution. The regulation of such genes might require miRNA mediated regulation. In this analysis, 12 potential candidates targeting the nuclear genes, three potential candidates targeting nuclear encoded mitochondrial genes, one potential candidate targeting the nuclear encoded chloroplast gene and 7 potential candidates targeting the mitochondrial genes have been identified, again supporting the fact that primitive prokaryotes probably had been regulated by miRNAs. The ratio of nuclear genes to the mitochondrial genes, targeted by the miRNAs, if analyzed in a broad spectrum of crop plants in the near future, might provide better insights in accordance with the above hypothesis.

In this study, ribosomal proteins, rpL2 and rps13 and mitochondrial protein cytochrome c (Table 1 and Table 3) were found to be the potential miRNA targets. Among the ribosomal protein targets, L2 was found to be located in either nucleus or mitochondria or in both the compartments of an organism [20]. Osa-miR5515 was found to target the ribosomal protein L2. This insinuates the possibility of miRNAs being used as the key regulatory molecule across various cellular compartments owing to their short size. Nuclear mitochondrial interaction might also be mediated by miRNA in certain cases. Besides, some of the NADH dehydrogenase genes and ATP synthase genes (Table 3) are present in both chloroplast and mitochondrial compartments in plants [1, 19]. This might also warrant the requirement of additional regulatory factors such as miRNA to coordinate the processes across various compartments.

## Conclusion

The computational approach used in the analysis is very stringent to identify the potential miRNA targets. Experimentally validated miRNA targets reveal that, their structures are greatly relaxed from the scrutiny parameters set in this study. Hence, the miRNA candidates identified in this study have a higher possibility of having targets, if validated by experiments *in vivo*. This study enlightens the researchers for the possibility of regulating the mitochondrial genes by manipulating the appropriate miRNA genes in the nuclear compartment.

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