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Short Communication

Identification of mirtrons in rice using MirtronPred: A tool for predicting plant mirtrons

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ABSTRACT

Studies from flies and insects have reported the existence of a special class of miRNA, called mirtrons that are produced from spliced-out introns in a DROSHA-independent manner. The spliced-out lariat is debranched and refolded into a stem–loop structure resembling the pre-miRNA, which can then be processed by DICER into mature ~21 nt species. The mirtrons have not been reported from plants. In this study, we present MirtronPred, a web based server to predict mirtrons from intronic sequences. We have used the server to predict 70 mirtrons in rice introns that were put through a stringent selection filter to shortlist 16 best sequences. The prediction accuracy was subsequently validated by northern analysis and RT-PCR of a predicted Os-mirtron-109. The target sequences for this mirtron were also found in the rice degradome database. The possible role of the mirtron in rice regulon is discussed. The MirtronPred web server is available at http://bioinfo.icgeb.res.in/mirtronPred.

1. Introduction

The microRNAs (miRNAs) have emerged as an important class of non-coding small RNAs that play crucial roles in plant regulatory networks by modulating gene expression [1,2]. The miRNAs are 21–22 nucleotide long RNA molecules that are mainly involved in regulating the gene expression at the post-transcriptional levels [3–6]. A miRNA gene is mostly transcribed by a DNA-dependent RNA polymerase (RNA Pol II), as a large primary transcript called the pri-miRNA. It is then processed within the nucleus into a highly folded precursor called the pre-miRNA. In plants the precursor transcript is processed by a RNase III type enzyme DICER-LIKE 1 (DCL1), a double strand binding protein HYPERNASTIC LEAVES 1 (HYL1) and the zinc-finger protein SERRATE (SE) to form a miRNA duplex within the nucleus [7–10]. The mature miRNA duplex invariably contains a 2 nucleotide overhang at the 3′ end which is methylated in plants by HUA ENHANCER1 (HEN1), for protection against endonucleases [11,12]. The duplex is transported from the nucleus to the cytoplasm by HASTY [13]. The guiding strand (miRNA) of the duplex gets incorporated into the RNA-induced silencing complex (RISC) with ARGONAUTE 1 (AGO1) as its main component and is targeted to the mRNA transcript leading to its degradation by cleavage or translational repression [14–16]. A large repertoire of miRNA exists in plants and animals [17].

Recently an alternative pathway for miRNA biogenesis has been reported from invertebrates [18,19]. Through this pathway molecules similar to the pre-miRNA are produced by splicing of the introns rather than by the DROSHA-defined processing. These were termed as 'mirtrons' [19,20]. The mirtrons have conserved canonical splice sites, with 'AG' splice acceptor of mirtronic introns typically adopting a 2 nucleotide 3′ overhang to these hairpins [19]. The slight diversion in structural features of mirtrons has made it possible to differentiate them from other intronic sequences.

The plant miRNAs are different from their animal counterparts both in biogenesis and function [21–25]. Though a large number of mirtrons have been characterized from non-plant systems, plant mirtrons need to be screened and compared with the animal mirtrons. Here we attempted to screen the rice genome to look for the mirtrons. The number of introns in the eukaryotic genomes is very large, apart from the fact that these vary in length as well as in structure [26]. It is difficult to filter mirtronic introns having hairpin structure from a huge repertoire of introns. In this work, we demonstrate the efficacy of a computational algorithm developed to predict mirtrons from a pool of plant introns. This was used to predict 70 mirtrons from the rice transcriptome. These sequences were further put through a stringent selection filter considering the distinct sequence, structure and genomic information of established miRNAs and mirtrons to shortlist 16 best sequences. The prediction accuracy was subsequently validated by northern analysis and RT-PCR. The possible role of the mirtron

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in rice regulon is discussed. The algorithm developed by us is available publicly as the MirtronPred web server (http://bioinfo.icgeb.res.in/mirtronPred). The server accepts FASTA formatted sequences and runs the algorithm to screen mirtronic features in the RNAfold secondary structures and outputs the probable miRNA/miRNA* sequences along with their secondary structures.

2. Results

2.1. The structural features of mirtrons

The computational algorithm for prediction of mirtrons is based on the analysis of the secondary structure features of introns that can be directly folded to acquire stem–loop structures similar to the pre-miRNA, instead of having a lower stem found in typical pri-miRNA transcripts which mediates the recognition and cleavage by PASHA/DROSHA complex [18,19,27]. Thus mirtronic introns exhibit extensive pairing between 5′ to 3′ splice sequences. Once the spliced mirtron is debranched it can adopt a pre-miRNA like hairpin structure which can be cleaved by DICER [18,19,28,29] to generate the mature miRNA.

The spliced-out introns that are ~100 nt in length, can be classified as mirtrons if the hairpin ends of the folded intron corresponds precisely to splice sites. The 5′ splice junction of mirtron is constrained by primary nucleotide motifs, i.e., 5′ GURAGU splice donors, 3′ polypyrimidine tracts and CAG or much less frequently UAG splice acceptor sites. The 'AG' splice acceptor of mirtrons typically adopts a 2nt-3′ overhang to these hairpins, thereby mimicking a DROSHA product [18–20]. This was considered as the most important feature while selecting for the correctly folded structures.

The G:U wobble pair also plays an important role but may have a negative effect. Often, such a pair is known as a disturbing factor. More than five G:U wobble pairs in the 5′ seed region are believed to impair mirtron structure, which is consistent with the features observed for plant pre-miRNAs. Therefore, G:U related features were ranked high in the prediction criteria. Moreover, the terminal loop in pre-miRNAs is frequently large and is believed to play a critical role in the specific cleavage of primary miRNA transcripts by DROSHA to liberate the pre-miRNA stem–loop [23]. However, in plants the size of the terminal loop is not that important once the pre-miRNA is formed. Recently an extensive *in vivo* structure–function analysis of *Arabidopsis thaliana* pri-miRNA 172a suggested that a defined distal structure with a 4 nucleotide terminal loop is sufficient for efficient miRNA processing [30]. In view of the small size of the spliced intron as compared to that of the large pri-miRNA molecules, the terminal loop length of parameter was fixed at ≤10 nt during mirtron predictions. Like plant miRNAs, most mirtronic RNAs were presumed to have 'A/U' residue at the beginning (5′) and at the tenth position, so this was considered as a desirable yet optional criterion.

2.2. Development of MirtronPred algorithm for computational prediction of mirtrons

The MirtronPred algorithm accepts FASTA formatted sequences with an option of selecting specific size ranges. It can analyze intronic sequences of sizes between 10 and 1000 nucleotides (see Supplementary Fig. 1). The PERL implementation of the algorithm initially runs RNAfold, Vienna RNA package [31], on input sequences to exclude improper hairpin structures. The RNAfold program is a widely used tool that predicts Minimum Free Energy (MFE) secondary structures in single linear RNA sequences, using dynamic programming algorithm [31]. In the next step the selected sequences are searched for the presence of one or more CAG motifs and a GA overhang at the 3′ end. To increase the selection stringency and thus the accuracy, secondary structures of retained sequences having energy higher than −10 kcal/mol are excluded in the subsequent step. There is an option of further increasing the stringency by decreasing the minimum energy requirements to a minimum of −25 kcal/mol in steps of 5 kcal/mol. Subsequently sequences with more than five wobble pairs in the 5′ seed region or terminal loop size greater than 10 base pairs in their secondary structures are discarded (see Supplementary Fig. 1). As a final optional step the filtered sequences are checked for the presence for A/U nucleotides at the first and tenth position from the 5′ end to qualify such structures and sequences as most probable mirtrons although the presence of A/U at these positions is not an essential criteria (see Supplementary Fig. 1). For this work the presence of A/U at the first and/or tenth position was not considered. The MirtronPred algorithm allowed rapid identification of 377 putative mirtrons from the repertoire of rice intronic sequence data, of which 203 sequences followed the A/U residue criteria (Fig. 1).

2.3. Prediction of mirtrons from rice

MirtronPred was applied to the fully sequenced rice genome (*Oryza Sativa* Japonica, release 5.0) for identification of novel putative mirtrons. From the available rice intronic sequence data, at TIGR website (http://rice.plantbiology.msu.edu/) 65,744 introns of 60–110 nt in length were extracted. MirtronPred pipeline's first step filtered 17,664 introns which were predicted to have proper hairpin loop by RNAfold. After removing the redundant sequences, the subsequent steps of MirtronPred pipeline were subjected to each filtered sequence which resulted in 377 introns capable of behaving as putative mirtrons. This list was further filtered on the basis of free energies to obtain a list of 70 putative mirtrons.

In order to arrive at a minimal list of the most probable mirtrons for *in vitro* verification, the best folded structures for each of the putative 70 mirtrons were further analyzed for a minimum number of mismatches in the stem region of the putative mirtronic miRNA. This retained 46 candidate mirtrons. From this pool of 46 introns, 16 sequences having the best hairpin structures with lowest free energies were selected (see Supplementary Fig. 2).

To determine the validity of the MirtronPred results the 377 predicted sequences were searched in the Indica rice small RNA database available in the lab (see Supplementary Table 1). At least 106 of the 377 sequences and 41 of the 70 sequences matched with the small RNA sequences in the NGS data. Subsequently the 46 putative mirtrons were filtered for minimum number of mismatches in the stem region of the mirtronic miRNA and 28 of these sequences had significant matches in the NGS small RNA database (see Supplementary Table 1). It was observed that increasing numbers of small RNA

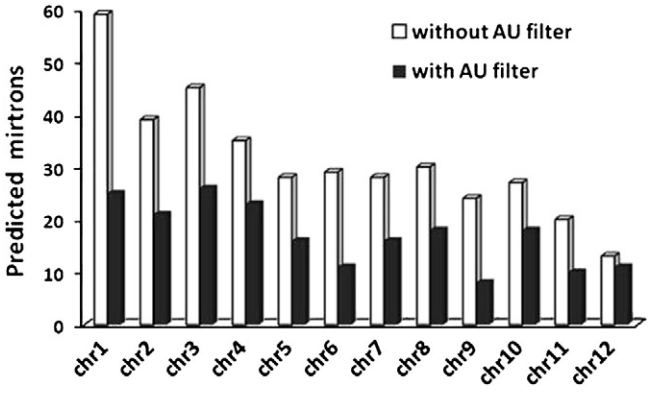

Fig. 1. Distribution of the predicted rice mirtronic sequences across chromosomes. The graph shows comparison of chromosome wise distribution of putative mirtrons predicted using the MirtronPred server pipeline. The differences obtained with or without using the A/U filter are shown.

sequences from the rice NGS data were recognized as the stringency for mirtron predictions was lowered. When the intron length parameter was relaxed to consider intronic sequences between 110 and 500 nt 651 mirtrons could be predicted with 432 sequences sharing significant matches in the NGS database (see Supplementary Table 1).

Among the 16 stringently selected sequences nine exhibited significant similarity with the sequences present in the small RNA database (see Supplementary Table 2). From these sequences the structure of a putative mirtronic sequence, derived from chromosome 9, locus AP005904; LOC_Os09g23220.1|12009.m50224|intron_2 (Os-mirtron-109), was deemed as the best-fit stem–loop structure and selected for further experimental validations (Fig. 2).

2.4. Northern blot analyses

The putative mirtron, Os-mirtron-109 (locus AP005904) was found to express in rice leaves only. Oligonucleotides complementary to the 5′ and 3′ stem region were used for probing RNA isolated from leaves of 15-day-old rice seedlings. The primer complementary to the loop region was used as a negative control. Signals corresponding to the 21 nt-mature miRNA were clearly visible with the oligonucleotide probe complementary to the 5′ arm of the predicted mirtron (Fig. 2A), while a very low intensity signal was detectable with the oligonucleotide probe complementary to the 3′ arm in the sample from normal leaf (Fig. 2B). However no signal was obtained with the loop primer (Fig. 2C).

2.5. Differential expression of Os-mirton-109

To follow the biogenesis of Os-mirtron-109, its expression profile was studied across salt-stressed and cold-stressed tissues using northern analysis (Fig. 3A). The expression in leaf and root tissues from 15-day-old seedlings grown under normal (unstressed) conditions was compared against the respective tissues obtained from seedlings exposed to 250 mM salt-stress or cold-stress (4 °C) for 1 h. It was observed that the Os-mirtron-109 was present in the leaves of both the unstressed and stressed rice seedling but was not present in the roots.

Further detection and expression profiling of the putative mirtron was carried out by stem–loop RT PCR (see Supplementary Fig. 3) as it has been demonstrated as a fast yet sensitive method for detection and quantification of a specific miRNA [32]. The RNA samples from leaf tissues of 15-day-old seedlings grown under normal (unstressed) conditions were compared with those obtained from seedlings stressed for 1 h with salt or low temperature. The expected band size (~100 bp) for Os-mirtron-109 was obtained using the stem–loop primer (Fig. 3B). In this experiment amplification of Glyoxylase I (GlyI), a known salt stress upregulated gene was used as molecular marker for the stress treatments [33].

For further confirmation, the 109 bp PCR amplified band of Os-mirtron-109 was excised from the gel and cloned into pGEMT vector. Five independent colonies were sent for sequencing. The determined sequence of Os-mirtron-109 from the cloned PCR product matched exactly with the sequence of Os-mirtron-109 predicted using the MirtronPred tool.

2.6. Predicted targets of Os-mirton-109

Considering the fact that mirtrons resemble the miRNAs in structure and function, we used the psRNATarget (http://bioinfo3.noble.org/psRNATarget; *Oryza sativa* release 17) to predict targets of Os-mirtron-109 and the other predicted mirtrons. The targets could be predicted for all 16 putative mirtrons. The predicted list of targets is available in Supplementary Table 3. The targets were also searched in the publicly available rice degradome databases using starBase (http://starbase.sysu.edu.cn/degradomeSearch.php) and targets for 9 putative mirtrons were found to be present (Supplementary Table 2). The targets include several important genes including strictosidine synthase, *WRKY* domain containing protein, Receptor-like protein kinase (*OsWAK22*) and so on. The putative Os-mirtron 109 was predicted to target strictosidine synthase. It is a key enzyme in alkaloid biosynthesis and it catalyzes the condensation of tryptamine with secologanin to form strictosidine [34]. Its putative target in the degradome database is a calcium binding and coiled-coil domain containing protein. The putative miR* sequence of this mirtron was predicted to target phosphatidylinositol-4-phosphate 5-kinase. It is therefore, likely that the mirtrons may play a pivotal role in plant development by targeting essential transcripts.

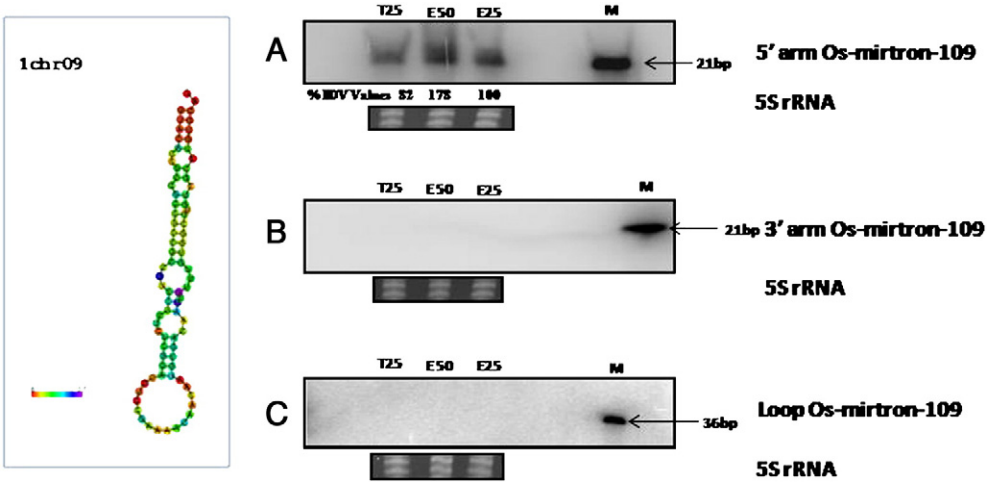

Fig. 2. Structure and Northern blot analysis of predicted Os-mirtron 109. Different concentrations (25 μg and 50 μg) of Enriched (En25 and En 50) and Total (T25) RNA isolated from leaves of 15-day-old seedlings were used for northern analysis. Hybridization was performed using (A) oligonucleotide complementary to the 5′ arm, (B) oligonucleotide complementary to the 3′ strand and (C) oligonucleotide complementary to the loop. 5S rRNA stained by ethidium bromide shown as loading control. A radiolabelled 21 nt oligo was used as the size marker (M) in all three blots. The inset shows the predicted stem–loop structure of the mirtron.

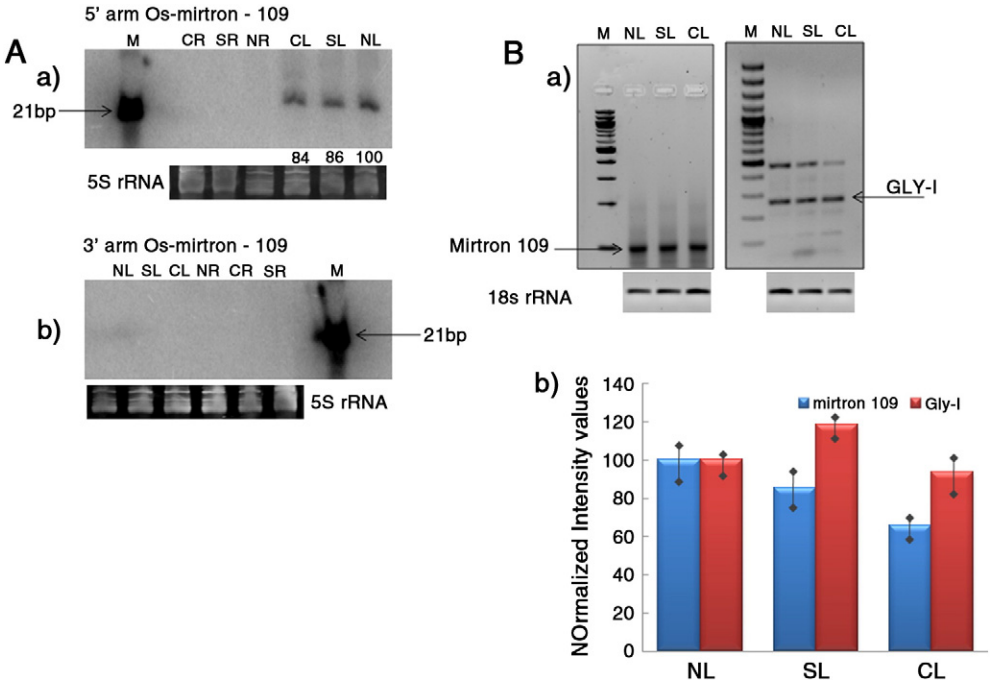

Fig. 3. Expression profiling of Os-mirtron-109 by northern analysis and stem–loop RT-PCR. Total RNA isolated from leaves (L) and roots (R) of 15-day-old seedlings grown under normal (unstressed, NL and NR), 1 h 250 mM salt-stress (SL and SR) and 1 h cold stress (CL and CR) was used for northern analysis and stem–loop RT-PCR. (A) Hybridization was performed using (a) oligonucleotide complementary to the 5′ arm and (b) oligonucleotide complementary to the 3′ strand. 5S rRNA stained by ethidium bromide shown as loading control. (B) The amplification products obtained with stem–loop PCR using specific primers (a) representing the 5′ arm of Os-mirtron-109 and Gly-I and analyzed on 3% agarose gel. 18S RNA was used as an internal control. (b) The plot of densitometric values normalized with respect to unstressed samples is shown. Gly-I was used as the molecular marker of stress treatment.

3. Discussion

In the work presented here, we have developed and validated a computational method for the prediction of mirtrons in a pool of plant introns. The MirtronPred algorithm predicts and filters probable intronic miRNAs, based on distinct structural and sequence features of known plant miRNAs and mirtrons. The algorithm developed by us is available as a publicly available web server MirtronPred (http://bioinfo.icgeb.res.in/mirtronPred). The server screens mirtronic features in the RNAfold predicted secondary structures in the input sequences and gives the output of the probable miRNA/miRNA* sequences along with its secondary structures. The MirtronPred server reliably predicted 70 putative mirtronic sequences from rice intronic sequences on the basis of known characteristics of mirtrons and plant miRNAs. After subsequent analysis and stringency filtration 16 sequences were selected as having the best fit structures as putative mirtrons.

Recently, the structural determinants of precursors of animal and plant miRNA fold backs have been determined [7,30,35]. It has been shown that animal pri-miRNAs are processed in a loop-to-base manner to produce a structure containing 21 base pair miRNA/miRNA* duplex, and a terminal loop at the distal end [16]. In plants, the miRNA-containing foldbacks are much more diverse in length and structure, with the miRNA/miRNA* duplex being at variable positions [30,36,37]. Recently Werner and colleagues performed an extensive *in vivo* structure–function analysis of *Arabidopsis thaliana* pri-miRNA 172a. They identified that a defined distal structure is important for miR172a processing although deleting almost the entire distal portion, leaving only a single-paired base pair beyond the miRNA/miRNA* and a predicted 4 nucleotide terminal loop, was sufficient for miRNA overexpression [30]. The structures of the predicted rice mirtrons were in accordance with the reported structural parameters for other plant miRNAs. Therefore, MirtronPred may be used to predict mirtrons in other plant genomes.

Subsequently one of the best-fit structures was selected for experimental confirmation of the prediction. The cloning and expression analysis by northern and RT-loop PCR verified the prediction of MirtronPred. In northern blots the mature 21 nt miRNA arising from of the 5′ arm of Os-mirtron-109 can be detected at higher levels as compared to the corresponding 21-nt from the 3′ arm, while probe corresponding to the loop region does not give any signal. This clearly indicates that the 21-nt duplex is originating from the stem region of the predicted structure. These findings were validated by isolation and cloning of the mature 21-nt molecule arising from the 5′ arm of Os-mirtron-109. The mature mirtronic miRNA was observed to have a differential expression across tissues. It's expression is more in leaves as compared to the roots. In root tissues the expression could be observed only after amplification using the stem–loop RT PCR and not by regular northern blotting. Moreover the mature mirtronic miRNA appears to be down-regulated under salt and cold stress in the leaves.

The present work thus describes the functionality of MirtronPred as a new web server for predicting plant mirtrons and also demonstrates the prediction and validation of rice mirtrons. In addition, the study also provides information on the target genes that suggests a possible role of these mirtrons in plant development and stress response.

4. Materials and methods

4.1. Plant materials and growth conditions

Seeds of rice *var. Pusa Basmati* were grown on the germinating sheets under controlled conditions, temperature (28 ± 2 °C), relative air humidity (70%) and 16/8-h light/dark cycle. For further analysis the 15 day old seedlings were harvested.

4.2. Stress treatments

For providing salt stress 15-day-old rice seedlings were transferred to 250 mM NaCl solution for 1 h. The low temperature stress

was provided by transferring the 15-day-old rice seedlings to 4 °C for a period of 1 h. Leaves and roots were harvested separately for RNA isolation.

4.3. RNA isolation

Total RNA was extracted from leaf and root tissues of 15-day-old rice seedlings using an extraction method described earlier. The small RNA's enrichment was then performed as previously described [38].

4.4. Northern blot analyses

Increasing concentration of total RNA (15 and 25 μg) and enriched small RNA (15 and 25 μg) was resolved on 15% denaturing Urea-polyacrylamide gels and subsequently transferred to Hybond-N Nylon membranes (Amersham Pharmacia Biotech, UK) by use of a trans-blot semidry transfer cell (Bio-Rad). To generate specific probes, DNA oligonucleotides that are perfectly complementary to the stem region of the 3′ and 5′ arms were end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase. The membranes were hybridized with a ^{32}P-labeled probe in a solution of 5× SSC, 0.01% heat-denatured salmon sperm DNA, 5× Denhart's solution, 5% dextran sulfate, 0.05 M sodium phosphate, 2.5 mM EDTA and 0.4% SDS at 37 °C for 24 h. The membranes were finally washed twice in 2× SSC, 0.5% SDS at 37 °C, then exposed to phosphor screens for 24 h and was visualized with a Typhoon PhosphoImager.

4.5. Stem–loop RT-PCR

Considering the small size of mature mirtron, specific stem–loop primer was designed to amplify it from the total RNA pool. The stem–loop primer 5′ CGAGATCTGGTACCGTTCATTTCCTCTTCTTCGGTTTTTTTTTTTTCC-CAAGAAGAGGAAATGAACGGTACCAGATCTCGACGGCG 3′ was 86 nucleotides long. The sequence of the stem–loop adaptor was randomly generated and checked for cross reactivity within the rice genome sequence. The specificity of stem–loop RT primers to the predicted putative mirtron is conferred by a six nucleotide extension at the 3′ end (underlined); this extension is a reverse complement of the last six nucleotides at the 3′ end of the miRNA (Supplementary Fig. 3). The forward primer [5′ GTACGCCGCCGCCGCCGCCGT 3′] was specific to the putative mirtron sequence while the reverse primer [5′ CGAGATCTGGTACCGTTCAT 3′] was specific to the stem–loop sequence. The putative mirtron was reverse transcribed into cDNA by MMLV reverse transcriptase (Fermentas). 2 μg of the total RNA template and 0.5 μl of 1.0 μg/μl stem–loop RT primer were mixed and the final volume was adjusted to 15 μl and heated at 70 °C for 5 min. The mixture was immediately placed on ice for 2 min. Then 5.0 μl of MMLV 5× reaction buffer, 1.0 μl of 10 mmol/l dNTPs and 1.0 μl of MMLV reverse transcriptase were added and mixed. The 25 μl of reaction volume was incubated for 60 min at 42 °C, 15 min at 70 °C and then held at 4 °C. All reverse transcriptions as well as no-template controls were run at the same time. 2 μl of the sample was used as template for further PCR reactions. The amplified PCR product was eluted and purified by QIAquick gel extraction kit (Qiagen). It was ligated into pGEMT easy vector (Promega USA). The ligation product was transformed into *E. coli* DH5α and cultured on LB agar media including ampicillin (100 mg/ml), IPTG (1 mM), and X-GAL (1 mM). Then plasmid extraction was achieved from white colonies and cloning was verified by digestion with restriction enzymes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2012.04.002.

Authors' contributions

PKJ carried out the molecular studies and participated in sequence alignment. NSM participated in sequence alignment, design of the study and drafted the manuscript. SKM conceived of the study, participated in the design of the study and helped draft the manuscript. DG, UKN and YK carried out the major component of the bioinformatics analysis. DG also helped in writing the manuscript. All authors read and approved the final manuscript.

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References

[1] J.F. Palatnik, E. Allen, X. Wu, C. Schommer, R. Schwab, J.C. Carrington, D. Weigel, Control of leaf morphogenesis by microRNAs, Nature 425 (2003) 257–263.
[2] N. Sanan-Mishra, S.K. Mukherjee, A peep into the plant miRNA world, The Open Plant Sci. J. 1 (2007) 1–9.
[3] B. Zhang, X. Pan, G.P. Cobb, T.A. Anderson, Plant microRNA: a small regulatory molecule with big impact, Dev. Biol. 289 (2006) 3–16.
[4] B. Zhang, Q. Wang, X. Pan, MicroRNAs and their regulatory roles in animals and plants, J. Cell. Physiol. 210 (2007) 279–289.
[5] G. Tang, X. Tang, V. Mendu, X. Jia, Q.J. Chen, L. He, The art of microRNA: various strategies leading to gene silencing via an ancient pathway, Biochim. Biophys. Acta 1779 (2008) 655–662.
[6] P. Brodersen, L. Sakvarelidze-Achard, M. Bruun-Rasmussen, P. Dunoyer, Y.Y. Yamamoto, L. Sieburth, O. Voinnet, Widespread translational inhibition by plant miRNAs and siRNAs, Science 320 (2008) 1185–1190.
[7] J. Winter, S. Jung, S. Keller, R.I. Gregory, S. Diederichs, Many roads to maturity: microRNA biogenesis pathways and their regulation, Nat. Cell Biol. 11 (2009) 228–234.
[8] O. Voinnet, Origin, biogenesis, and activity of plant microRNAs, Cell 136 (2009) 669–687.
[9] Y. Kurihara, Y. Takashi, Y. Watanabe, The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis, RNA 12 (2006) 206–212.
[10] Z. Dong, M.H. Han, N. Fedoroff, The RNA-binding proteins HYL1 and SE promote accurate *in vitro* processing of pri-miRNA by DCL1, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 9970–9975.
[11] Y. Lee, C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Rådmark, S. Kim, V.N. Kim, The nuclear RNase III Drosha initiates microRNA processing, Nature 425 (2003) 415–419.
[12] B. Yu, Z. Yang, J. Li, S. Minakhina, M. Yang, R.W. Padgett, R. Steward, X. Chen, Methylation as a crucial step in plant microRNA biogenesis, Science 307 (2005) 932–935.
[13] K.M. Bollman, M.J. Aukerman, M.Y. Park, C. Hunter, T.Z. Berardini, R.S. Poethig, HASTY, the *Arabidopsis* ortholog of exportin 5/MSN5, regulates phase change and morphogenesis, Development 130 (2003) 1493–1504.
[14] N. Baumberger, D.C. Baulcombe, DC, *Arabidopsis* ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 11928–11933.
[15] J.C. Carrington, V. Ambros, Role of microRNAs in plant and animal development, Science 301 (2003) 336–338.
[16] A.C. Mallory, H. Vaucheret, ARGONAUTE 1 homeostasis invokes the coordinate action of the microRNA and siRNA pathways, EMBO Rep. 10 (2009) 521–526.
[17] S. Griffiths-Jones, R.J. Grocock, S. vanDongen, A. Bateman, A.J. Enright, miRBase: microRNA sequences, targets and gene nomenclature, Nucleic Acids Res. 34 (2006) D140–D144.
[18] K. Okamura, J.W. Hagen, H. Duan, D.M. Tyler, E.C. Lai, The mirtron pathway generates microRNA-class regulatory RNAs in *Drosophila*, Cell 130 (2007) 89–100.
[19] J.G. Ruby, C.H. Jan, D.P. Bartel, Intronic microRNA precursors that bypass Drosha processing, Nature 448 (2007) 83–86.
[20] E. Berezikov, W.J. Chung, J. Willis, E. Cuppen, E.C. Lai, Mammalian mirtron genes, Mol. Cell 28 (2007) 328–336.
[21] A.M. Denli, B.B. Tops, R.H. Plasterk, R.F. Ketting, G.J. Hannon, Processing of primary microRNAs by the Microprocessor complex, Nature 432 (2004) 231–235.
[22] R.I. Gregory, K.P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch, R. Shiekhattar, The Microprocessor complex mediates the genesis of microRNAs, Nature 432 (2004) 235–240.
[23] J. Han, Y. Lee, K.H. Yeom, J.W. Nam, I. Heo, J.K. Rhee, S.Y. Sohn, Y. Cho, B.T. Zhang, V.N. Kim, Molecular basis for the recognition of primary microRNAs by the Drosha–DGCR8 complex, Cell 125 (2006) 887–901.
[24] T. Du, P.D. Zamore, microPrimer: the biogenesis and function of microRNA, Development 132 (2005) 4645–4652.
[25] V.N. Kim, J.W. Nam, Genomics of microRNA, Trends Genet. 22 (2006) 165–173.
[26] L. Zhu, Y. Zhang, W. Zhang, S. Yang, J.Q. Chen, D. Tian, Patterns of exon–intron architecture variation of genes in eukaryotic genomes, BMC Genomics 10 (2009) 47.
[27] R. Martin, P. Smibert, A. Yalcin, D.M. Tyler, U.S. Schafer, T. Tuschl, E.C. Lai, A *Drosophila* pasha mutant distinguishes the canonical microRNA and mirtron pathways, Mol. Cell. Biol. 29 (2009) 861–870.

[28] S.P. Chan, F.J. Slack, And now introducing mammalian mirtrons, Dev. Cell 13 (2007) 605–607.
[29] J.W.S. Brown, D.F. Marshall, M. Echeverria, Intronic noncoding RNAs and splicing, Trends Plant Sci. 13 (2008) 335–342.
[30] S. Werner, H. Wollmann, K. Schneeberger, D. Weigel, Structure determinants for accurate processing of miR172a in *Arabidopsis thaliana*, Curr. Biol. 20 (2010) 42–48.
[31] A.R. Gruber, R. Lorenz, H. Stephan, S.H. Bernhart, R. Neuböck, I.L. Hofacker, The Vienna RNA websuite, Nucleic Acids Res. 36 (2008) W70–W74.
[32] E. Varkonyi-Gasic, R.P. Hellens, qRT-PCR of small RNAs, Methods Mol. Biol. 631 (2010) 109–122.
[33] S.L. Singla-Pareek, M.K. Reddy, S.K. Sopory, Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 14672–14677.
[34] Y. Lu, H. Wang, W. Wang, Z. Qian, L. Li, J. Wang, G. Zhou, G. Kai, Molecular characterization and expression analysis of a new cDNA encoding strictosidine synthase from *Ophiorrhiza japonica*, Mol. Biol. Rep. 36 (2009) 1845–1852.
[35] A. Tsutsumi, T. Kawamata, N. Izumi, H. Seitz, Y. Tomari, Recognition of the pre-miRNA structure by *Drosophila* Dicer-1, Nat. Struct. Mol. Biol. 18 (2011) 1153–1158.
[36] S. Raffaele, F. Vailleau, A. Leger, J. Joubes, O. Miersch, C. Huard, E. Blee, S. Mongrand, F. Domergue, D. Roby, A MYB transcription factor regulates very-long-chain fatty acid biosynthesis for activation of the hypersensitive cell death response in *Arabidopsis*, Plant Cell 20 (2008) 752–776.
[37] M.J. Axtell, D.P. Bartel, Antiquity of microRNAs and their targets in land plants, Plant Cell 17 (2005) 1658–1673.
[38] N. Sanan-Mishra, V. Kumar, S.K. Sopory, S.K. Mukherjee, Cloning and validation of novel miRNA from Basmati rice indicates cross-talk between abiotic and biotic stresses, Mol. Genet. Genomics 282 (2009) 463–474.