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Computational Identification of MicroRNAs and Their Targets in Perennial Ryegrass (*Lolium perenne*)

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Abstract MicroRNAs (miRNAs) are small non-coding RNA molecules of 22 nucleotides in length that have been characterized as regulators of messenger RNA (mRNA) regulating a number of developmental processes in plants and animals by silencing genes using multiple mechanisms. miRNAs have been extensively studied in various plant species; however, few information are available about miRNAs in perennial ryegrass, animal feed, and industrial raw materials. In this study, the 12 potential perennial ryegrass miRNAs were identified for the first time by computational approach. Using the newly identified miRNA sequences, the perennial ryegrass mRNA database was further used for BLAST search and detected 33 potential targets of miRNAs. Prediction of potential miRNA target genes revealed their functions involved in various important plant biological processes. Our result should be useful for further investigation into the biological functions of miRNAs in perennial ryegrass. The selected miRNAs representing four families were verified by RT-PCR experiment, indicating that the prediction method that we used to identify the miRNAs was effective.

Keywords MicroRNA · Computational approach · Perennial ryegrass · Target genes

Introduction

MicroRNAs (miRNAs) are a small single-stranded endogenous non-coding RNAs with a length of about 22 nt, which cause transcriptional cleavage or translational repression through binding their targets, depending on the extent of complementarity between miRNA and mRNA [1–3]. So far, an increasing number of plant miRNAs have been discovered, and the functions of a small number of them have been elucidated [4, 5]. Increasing evidence showed

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that plant miRNAs target a large number of genes with functions that are in a range of development processes, including meristem cell identity, leaf organ morphogenesis and polarity, floral differentiation, development, and responses to biotic and environmental stresses [6–10]. Most miRNAs are conserved in animals and plants and from animals to plants [11, 12]. The sequence conservation of miRNAs has laid a sound foundation for predicting and studying miRNAs in non-model plants whose whole genome sequences are unknown, such as expressed sequence tags (ESTs), genome survey sequences (GSS), and nucleotide database [13, 14]. The computational screening of potential miRNAs in plant species has been proved to be successful and more effective for the discovery of new miRNAs that usually cannot be detected by the direct cloning, particularly of those miRNAs at low expression level and/or spatiotemporal expression. As of now, computational approaches have been successfully developed and have accurately predicted miRNA genes in *Arabidopsis*, rice, and other plant species [15–20]. Moreover, many miRNAs in miRBase have been contributed through computational approach only [21].

Perennial ryegrass is one of the high quality grasses, which was grown and utilized widely in China, especially in southern areas. It can be utilized as fresh, hay, and silage [22]. The wide utilization of perennial ryegrass played an important role in the adjustment of agriculture production system. Meanwhile, perennial ryegrass is grown as a forage crop in many parts of the world [23]. To date, a large number of miRNAs have been reported in many species, but none for perennial ryegrass. In this study, we used the characteristic features of previously known other plants miRNAs to efficaciously prediction novel perennial ryegrass miRNA in the publicly available GSS, EST, and nucleotide database. A total of 12 novel miRNAs were firstly identified belonging to 11 miRNA families, to help us understand the biological processes in which they might be involved, and the miRNA potential targeted genes were also predicted. These findings will be useful for further functional analysis of perennial ryegrass miRNA during development. Novel predicted miRNAs were further verified by RT-PCR experiment, an effective and widely-used method for detecting miRNAs.

Materials and Methods

Referenced Sequence Data

To search for potential perennial ryegrass miRNAs, the known plant miRNAs (http://www.mirbase.org/; Version 20, released June 2013) were used as nucleotide reference set of miRNA sequences. To avoid the redundant or overlapping miRNAs, the repeat mature miRNA sequences were removed, and the remaining sequences were used as query sequences for BLAST search. The 29,293 ESTs, 308 GSSs, and 1,768 nucleotide sequences of perennial ryegrass were obtained from NCBI database (http://www.ncbi.nlm.nih.gov/).

Computational Prediction of miRNAs

A stable version of the BLAST tool was downloaded from NCBI database. BLASTN parameters were the same as that described in the previous papers [24, 25]. The procedure of search for potential miRNAs in the perennial ryegrass was showed in Fig. 1. Four criteria were used to distinguish miRNAs and pre-miRNAs from other kinds of RNAs were as follows: (a) predicted mature miRNAs were allowed to have only 0–4 nucleotide mismatches in sequence with all previously known plant mature miRNAs; (b) pre-miRNAs sequence can fold into an appropriate hairpin secondary structure that contains the ~22 nt mature miRNA

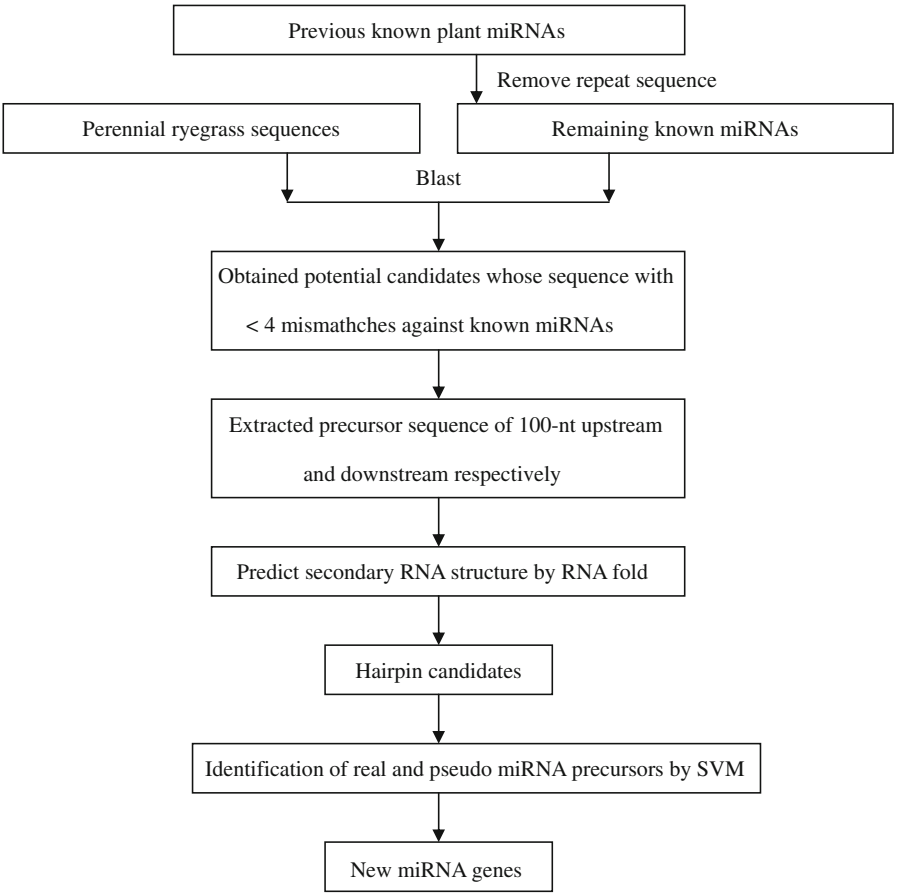

Fig. 1 Procedure of potential perennial ryegrass miRNA gene search by identifying homologs of previously known miRNA gene

sequence within one arm of the hairpin; (c) miRNA precursors with secondary structures had higher negative minimal free energies (MFEs) and minimal free energy index (MFEIs) than other different types of RNAs by RNA-fold prediction; and (d) pre-miRNA had 15–70 % contents of A+U by SVM (support vector machine) since the unstable structures of pre-miRNAs are needed to produce mature single-stranded miRNAs [26].

Conservation and Phylogenetic Analyses of miRNAs

The candidate miRNAs were analyzed for conservation with their orthologues. The newly identified miRNA156k and the well-known other plant miRNAs orthologues were selected for conservation and were done with the help of publically available web logo: a sequence logo generator [27]. The phylogenetic analyses were investigated for their evolutionary relationships (www.clustal.org/). Evolutionary distances were calculated neighbor-joining (NJ) method following 1,000 bootstrapped replicates. All the analyses were performed using the MEGA 4.0 software [28].

Experimental Verification of Predicted miRNAs

The efficiency of the computational strategy was tested by biological experiments to validate the predicted miRNA genes. The four predicted miRNAs genes were randomly selected from perennial ryegrass for RT-PCR validation. The small RNA samples from perennial ryegrass young leave were isolated using mirVana^{TM} miRNA isolation kit (Ambion) according to the manufacturer's instruction. The cDNAs were synthesized from small RNAs by using miRNA specific stem–loop primers according to criteria mentioned as described previously [29, 30]. The stem–loop RT primers and gene specific primers were listed in Table S1. The DNA fragments were directly subcloned into pMD18-T vector (Takara) and sequenced.

The analysis of mature miRNA expression by RT-PCR was carried out. The cDNAs were diluted ten times to perform PCR for expression confirmation and expression pattern analysis. PCRs were performed, respectively, in 20 μl mixture containing 1 μl cDNA, 0.5 μM forward and reverse primers, 10×PCR buffer, 0.25 μM each of dNTPs (Takara), and 2∪*Taq* polymerase (Takara), under the following parameters: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The PCR products were detected by electrophoresis with 3 % agarose gel containing ethidium and photographed under UV light.

Prediction of Potential miRNA Target Genes

In plant, it has been reported that most miRNAs bind to the protein coding region of mRNA targets with perfect or nearly perfect sequence complementarities [31, 32]. The mRNA database of the perennial ryegrass downloaded from NCBI database. The targets were predicted with a plant miRNA potential target by the RNAhybrid program (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) [33]. The parameters employed were described as follows: *P* value cutoff of 0.05, target duplex free energy$^{\triangle}$G≤−24 kcal/mol. The following criteria were set for predicting the potential perennial ryegrass miRNA target genes: (a) not more than four mismatches between identified miRNA; (b) one mismatch was allowed between position 2nd and 12th; and (c) not more than two consecutive mismatches.

Results and Discussion

Identification of Potential miRNAs

In the plant kingdom, a substantial number of miRNAs are conserved in different plant species. After screening, a total of 12 potential perennial ryegrass miRNAs were predicated for the first time (Table 1). All of the precursors for those mature miRNAs fold into the typical secondary structure of miRNAs, and they are postulated to be important validation parameters for the miRNA genes predicted (Fig. 2). The lengths of the precursors vary in a larger range from 61 to 148 nt with an average of 112 nt. The diversity of the identified miRNAs could be also found in the location of mature miRNA sequences. The sequences of miRNA396h, miRNA156i, miRNA845a, miRNA5021, miRNA6245, and miRNA2937 were located at the 3′ end of the miRNA precursors; whereas, the rest of the sequences of miRNAs were all found at the 5′ end. Predicated miRNAs belong to 11 miRNA families and every miRNA family only has

Table 1 The 12 novel identified miRNAs in perennial ryegrass

miRNAs	Source	Mature sequence (5′ to 3′)	Side	N M (nt)	Strand	L P (nt)	A+U (%)	MFEs
miRNA3980b-3p	GR512615	GUGGCCGAGGCC GUCGCCGUG	5′	3	Plus	61	18.6	−33.1
miRNA5817	GR515766	GGAAAUUUGAAA GAAAAAAAUUG	5′	3	Plus	130	61.5	−23.9
miRNA5636	GR521279	AUAGCUUGCAGA GCUUGACGG	5′	3	Minus	82	34.1	−34.9
miRNA396h	GR509680	UCCACAGGCUUU CUUGAACGG	3′	0	Plus	88	36.3	−32.5
miRNA156i	GR521537	UGACAGAAGAGA GUGAGCAC	3′	1	Plus	137	48.9	−67.2
miRNA5543	GR513826	UAUGAAUGGUAU AUUUGUUGG	5′	3	Plus	84	66.8	−18.8
miRNA845a	GR513146	GGGCUCUGAUAC CAAUUGAAA	3′	3	Plus	142	47.1	−50.6
miRNA5075	GR523733	UUCUCGUCGC CGCCGUCCGU	5′	2	Plus	123	26.8	−64.2
miRNA5021	GR515553	GGAGAAGAAGAA GAAGAAGA	3′	2	Minus	117	62.3	−30.0
miRNA156k	GR521537	UGACAGAAGAGA GUGAGCACA	5′	1	Plus	148	39.8	−83.9
miRNA6245	GR524151	GGUAUAGGUGUC GGCUAAGCA	3′	3	Plus	89	47.2	−31.6
miRNA2937	GR516193	GCCAGAGCUGUU GAAGGAGGG	3′	3	Plus	143	41.9	−45.7

NM, number of mismatch; LM, Length of mature miRNAs; LP, Length of precursor; MFEs, minimal folding free energy (kcal/mol)

one member, but miRNA156 family has two members. These perennial ryegrass miRNAs were also evaluated for their A + U content, and the results showed that the A + U contents ranged from 18.6 % to 66.8 % in the perennial ryegrass miRNA precursors, which was consistent with previous studies on other plants [24, 34–36]. The identified potential perennial ryegrass miRNAs also have highly negative minimal fold energies (MFEs) between −64.2 and −18.8 kcal/mol. These results showed that the predicted miRNAs were taken with strict screening criteria.

Conservation and Phylogenetic Studies of miRNAs

The perennial ryegrass miRNA156k (*Lolium perenne* miRNA156K, lpe-MIR156K) conservation and phylogenetic studies were conducted with their orthologues in *Oryza sativa* (osa), *Zea mays* (zma), *Populus trichocarpa* (ptc), *Glycine max* (gma), *Malus domestica* (mdm), *Manihot esculenta* (mes), and *Solanum tuberosum* (stu). These findings suggest conservation of these miRNAs among dicotyledon and monocotyledon as shown in (Fig. 3). The phylogenetic analysis of the same miRNA (miRNA156k) sequences suggested that *L. perenne* is more closed to Solanum tuberosum (Fig. 4).

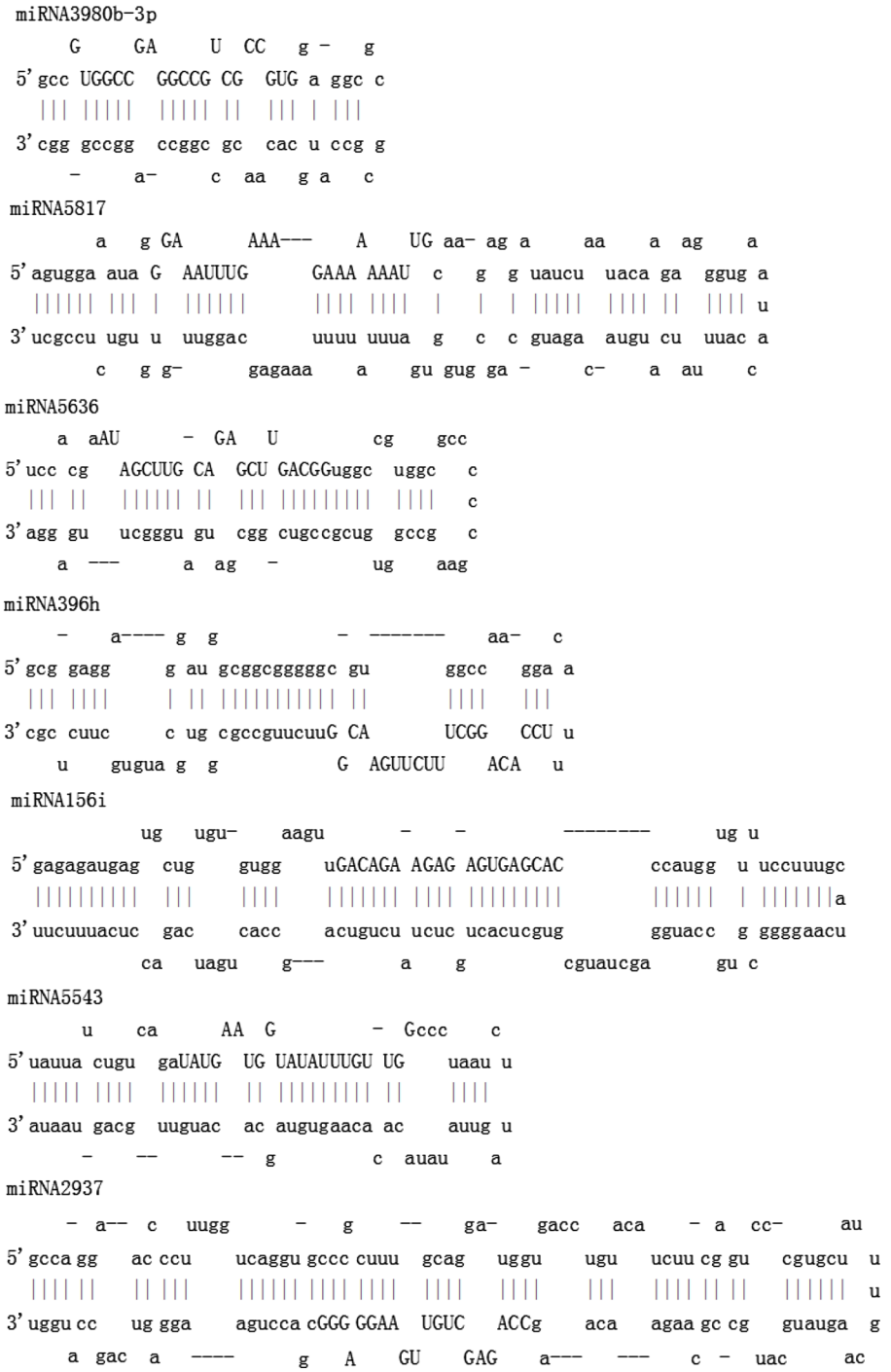

Fig. 2 Predicted stem–loop structures of newly identified perennial ryegrass precursor miRNAs, osa-miRNA156k, and perennial ryegrass miRNA156k had similar precursor sequence of stem–loop structure. The mature miRNAs are indicated by the *capital letters*

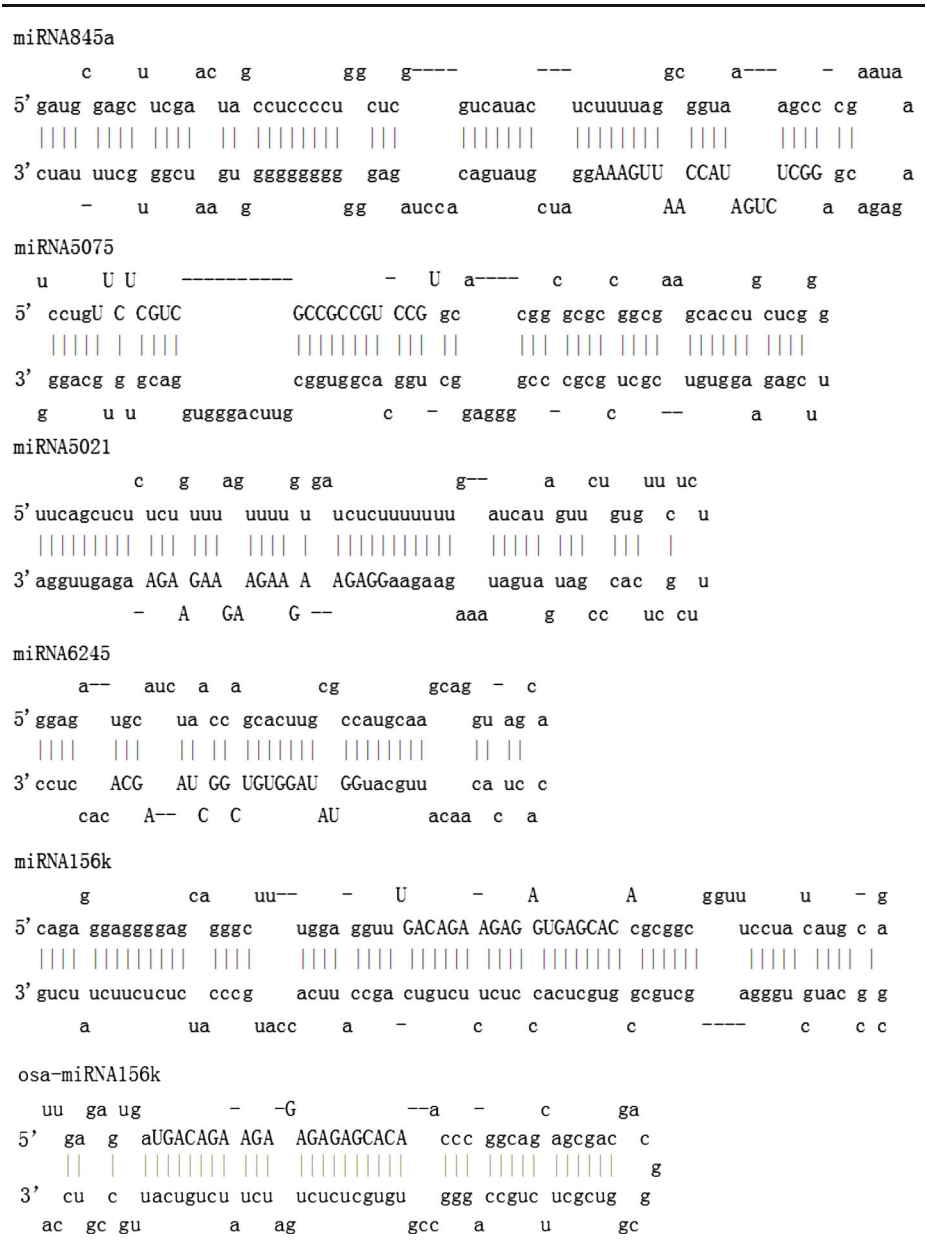

Fig. 2 continued.

Experimental Verification of Predicted miRNAs

To further validate the expression of the identified miRNAs in perennial ryegrass, RT-PCR analyses were carried out using total RNA isolated from young leave, an effective and widely-used method for detecting miRNAs [37]. The randomly selected four miRNAs, miRNA5021, miRNA396h, miRNA156k, and miRNA5075, were subjected to experimental studies. Results show that the transcripts of the four miRNA genes

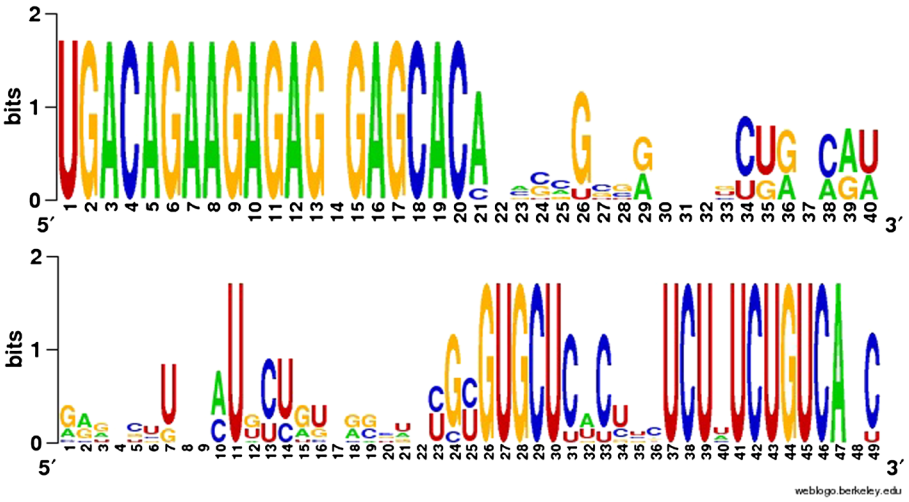

Fig. 3 Alignment of miRNA156k of perennial ryegrass and conservation studies

were successfully detected, demonstrating a high accuracy rate for the computational miRNAs identification (Fig. 5).

Prediction of Potential Targets of miRNAs

The miRNAs target identification is an interesting and demanding step for the new identified miRNAs [33]. The knowledge on target function of the identified perennial ryegrass miRNA will help us gain insight into the important function and regulation of miRNAs in this plant. In our study, a total of 33 targets were identified for the 12 newly identified perennial ryegrass miRNAs (Table 2). Our prediction of target genes for the perennial ryegrass miRNAs revealed that more than one gene was regulated by individual miRNA. This result was similar to the findings in other plant species which suggested that miRNA research should be focused on networks rather than individual connections between miRNA and strongly predicted targets [38, 39]. These targets of perennial ryegrass miRNAs can be separated into several groups. The first group contains targets that are predicted to encode

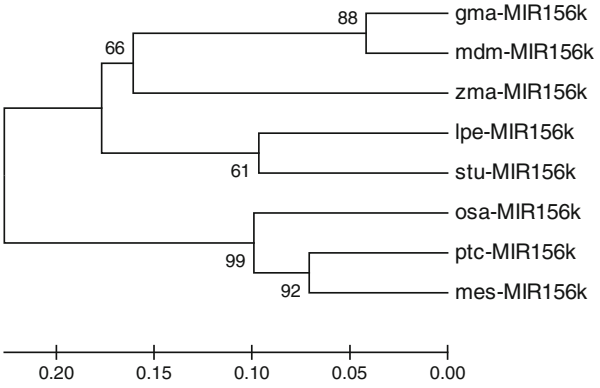

Fig. 4 The perennial ryegrass miRNA156k phylogenetic analysis

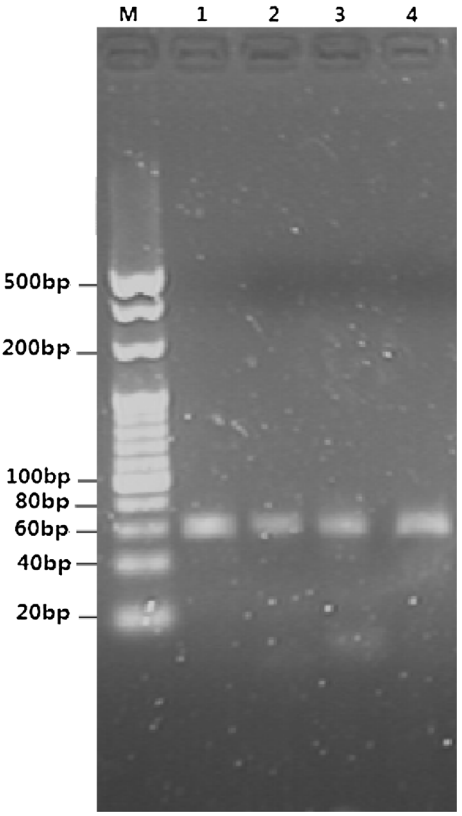

Fig. 5 Experimental validation of perennial ryegrass miRNAs. *M* 20 bp ladder maker, *1* miRNA156k, *2* miRNA396h, *3* miRNA5021, *4* miRNA5075

transcription factors. Another group contains miRNA targets encoding a range of different proteins which may play important roles in the aspects of metabolisms, stress response, and signal transduction. The transcription factors are the famous and well-known class of proteins targeted by miRNAs in almost all plant and animal species [40, 41]. The novel identified perennial ryegrass miRNAs also target this class of proteins. For example, the predicted perennial ryegrass targets for miRNAs, 2937 and 3980b-3p, are CONSTANS-like protein, MYB transcriptional regulator. Overall, these findings made us clear that perennial ryegrass miRNAs targeted both transcription factors as well as others specific genes.

Conclusion

With the availability of sequence resources in public databases, computer-based miRNA identification methods have been focused more and more in the recent years due to its advantages of low cost and high efficiency. In the present study, with a computational approach, 12 miRNAs were identified from the EST and GSS databases of perennial ryegrass, which belong to 11 families where miRNA156 family has 2 members and the rest has a single member in each. A total of 33 potential targets were identified, and we found that most of the genes are involved in transcriptional regulation and metabolism, suggesting their essential role

Table 2 Potential targets of the identified miRNAs in perennial ryegrass

miRNA	Targeted protein	Target function	Targeted genes
miRNA156i	Cinnamyl alcohol dehydrogenase	Metabolism	KC442297
	Myo-inositol phosphate synthase	Signal transduction	AY154382
	Fructosyltransferase-like protein	Metabolism	DQ073970
	NADH dehydrogenase	Metabolism	JX438155
miRNA156k	Galactinol synthase 1	Metabolism	AY154380
	Fructosyltransferase	Metabolism	AY082350
	Elongation factor 1-alpha	Transcription factor	EU168438
	Putative heat shock cognate 70 kDa protein	Signal transduction	JF747497
	ADP-ribosylation factor-like protein	Transcription factor	JF747487
	Oxalate oxidase 2	Metabolism	AJ492380
miRNA845a	CRT-binding factor	Transcription factor	AB258392
	Putative zinc finger protein ID1	Transcription factor	DQ328600
	Ice recrystallization inhibition protein	Signal transduction	EU680848
miRNA396h	Thioredoxin-like protein	Signal transduction	FJ663045
	Methyl pectinesterase	Metabolism	AY165036
	SnRK1b protein kinase	Signal transduction	JF747456
miRNA3980b-3p	Nucleoside diphosphate kinase	Signal transduction	AF271362
	CONSTANS-like protein	Transcription factor	AY600919
	Herbicide-binding protein D1	Transcription factor	AF363674
miRNA2937	MYB protein MYB3	Transcription factor	AF515725
	Disease resistance like-protein	Stress response	EU054386
	Pathogenesis-related protein 2	Stress response	HQ229923
miRNA5817	Pyrroline-5-carboxylate synthetase	Metabolism	KC896627
	Coronatine insensitive 2-like protein	Signal transduction	JF747499
miRNA5636	Victorin-binding protein	Unknown	JF747467
	C-repeat/DRE-binding factor 3	Transcription factor	AY960831
miRNA5543	Caffeic acid O-methyltransferase	Metabolism	AF033538
	Putative NADPH HC toxin reductase	Signal transduction	JF747360
	Small GTP-binding protein	Metabolism	JF747370
miRNA5075	UDP-sugar pyrophospharylase	Metabolism	JF747494
miRNA5021	Cinnamoyl-CoA reductase	Metabolism	KC442296
	Glycine-rich RNA-binding protein	Transcription factor	AB207971
miRNA6245	Phospoenolpyruvate carboxylase	Metabolism	JF747406

in biological processes of perennial ryegrass. The 4 miRNAs out of the 12 that were randomly selected were verified by RT-PCR. Taken together, the knowledge gained from this research will provide an understanding of the essential roles of miRNAs in perennial ryegrass growth and development, stress response, and other biological processes. Our study findings also considerably broaden the scope of understanding the function of miRNA in near future in perennial ryegrass.

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