Full Length Research Paper

Extraction of low molecular weight RNA from *Citrus trifolita* tissues for microRNA Northern blotting and reverse transcriptase polymerase chain reaction (RT-PCR)

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The study of microRNA (miRNA), a component of low molecular weight RNA (LMW RNA), has received increasing attention in recent years. A critical prerequisite in miRNA studies is acquisition of high quality LMW RNA. LMW RNA is generally obtained from total RNA or from total nucleic acids solutions. Most traditional methods for LMW RNA isolation involve many steps and chemical reagents which upon degradation may negatively affect results. We employed a simple and quick method involving trizol for total RNA extraction from citrus tissues, then generation of LMW RNA using 4M LiCl, which have been successfully utilized in studies in our laboratory. Compared with traditional methods, this method is less expensive and produced high RNA yields while avoiding the use of phenol or other toxic reagents. In addition, the entire procedure can be completed within 4 hours with many samples being processed simultaneously. Therefore, this is a practical and efficient method for LMW RNA extraction from woody fruit crops containing high levels of polysaccharide and polyphenolics. Using the extracted LMW RNA, miRNAs were successfully detected and characterized by reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blotting.

Key words: Citrus, low molecular weight RNA, trizol reagent, 4 M LiCl, microRNAs.

INTRODUCTION

Low molecular weight RNA (LMW RNA) consists of several components with microRNAs (miRNAs) being among the important ones. miRNAs are endogenous 19-24nt RNAs that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression. In recent years, miRNAs and their functions in particular, have attracted increasing attention among plant scientists. More and more evidence demonstrates that miRNAs play critical functions in many biological and metabolic processes, including tissue identity, developmental timing and response to environmental stresses (Jones-Rhoades et al., 2006; Zhang et al., 2007; Wang et al., 2009; Wu et al., 2009). miRNAs usually control gene expression by targeting protein coding gene for cleavage, or repressing protein translational control gene expression instead of directly controlling plant growth and development (Carrington and Ambros, 2003; Bartel, 2004; Carthew and Sontheimer, 2009). They regulate gene expression by binding specific sequences in target genes and thus suppressing their expression. In a study focusing on miRNAs regulatory functions, it was found that in 70% of the cases, the target genes were transcription factors mainly related to plant developmental processes. Therefore, isolation of high quality LMW RNA in a safe and economical way is essential for miRNAs molecular research.

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Abbreviation: **LMW RNA,** Low molecular weight RNA; **miRNA,** microRNA; **RT-PCR,** reverse transcriptase PCR; **DEPC,** diethyl pyrocarbonate; **DTT,** dithiothreitol; **EtBr,** ethidium bromide.

LMW RNA can be used for small RNA library construction, miRNAs cloning, Northern blotting, high throughput sequencing and miRNAs expression analysis. For further molecular research in plant development, extraction of high quality low molecular weight RNA is important. *Citrus* is by far the most economically important evergreen fruit crop in the world. *Citrus trifoliata* (*Poncirus trifoliate* in the NCBI taxonomy) has been an important rootstock species used in the citrus industry, and has even been used widely as a model species for citrus molecular biology and genomics. Efficient extraction of high quality RNA from citrus is crucial in gene expression studies and cDNA library construction. Generally, LMW RNA isolation heavily relies on total RNA extraction (Llave et al., 2002; Wang et al., 2004; Lu et al., 2007). The traditional methods of total RNA extraction involve so many steps (Lopez-Gomez and Gomez-Lim, 1992; Liu and Goh, 1998; Asif et al., 2000), which may lead to degradation or loss of most LMW RNAs during total RNA extraction. Therefore, the method of total RNA extraction can be a limiting factor for good LMW RNA isolation, especially for perennial plant materials. Another major challenge in isolating high-quality RNA is the products of secondary metabolism that accumulate in many higher plants, especially in mature tissues, therefore complicating the isolation of good-quality RNA. Efforts have been made to develop methods for RNA isolation from different recalcitrant materials. For example, Carra et al. (2007) developed a method for LMW RNA isolation from polysaccharide-rich plant tissue which however is not popular as it is time-consuming and has a high probability of LMW RNA degradation due to the need for overnight precipitation and dissolution of RNA in water before getting LMW RNA.

Use of trizol is convenient and gives high quality end products in a time and cost effective way. With this protocol, we successfully isolated high-quality LMW RNA from root, stem, leaf, flower and fruit tissues of *C. trifolita* different tissues. Additionally, many samples could be processed simultaneously in centrifuge tubes. Using the isolated LMW RNA samples, miRNAs were then successfully detected and characterized by reverse transcriptase polymerase chain reaction (RT-PCR), Northern blotting and deep sequencing (Song et al., 2009; 2010). In order to do some help to similar researches on citrus in other laboratories and make this method to be a referable one, we presented the detailed procedures of this efficient LMW RNA isolation method here in this report.

MATERIALS AND METHODS

Plant material and reagents

Different tissues namely: Root, stem, leaf, flower and fruit (diameter 1 cm) were collected from five-year-old trifoliate orange (*C. trifoliata*) trees at the Tree Fruit Research and Extension Center, Suzhou Fruit Tree Research Institute, People's Republic of China in 2009. After collection, all the samples were immediately frozen in

liquid nitrogen and stored at -80°C until use.

Solutions and reagents used include trizol (Invitrogen, Life Technologies, Carlsbad, CA), isopropanol, absolute ethanol, 4 M LiCl and 0.1% diethyl pyrocarbonate (DEPC) v/v treated autoclaved double-distilled water. Mortars and pestles were baked for 6 h at 180°C, while pipette tips and centrifuge tubes were all treated with 0.1% DEPC water and autoclaved.
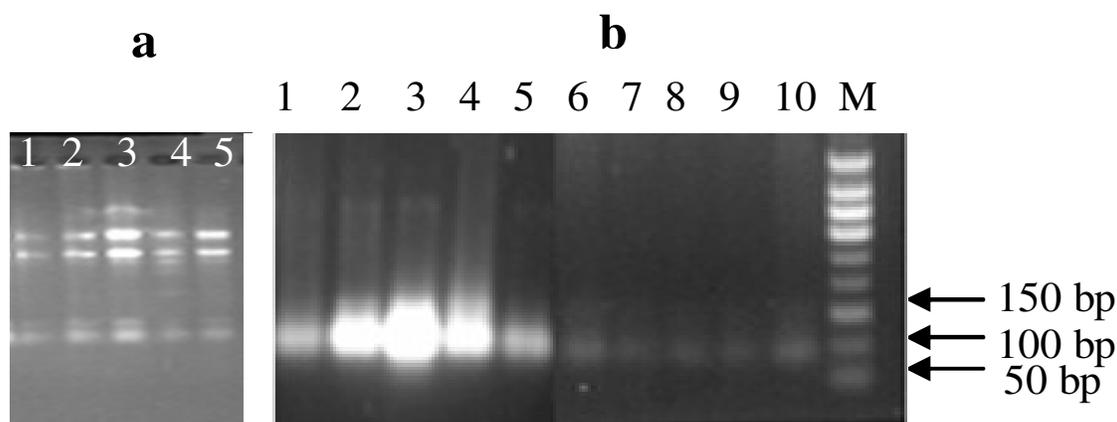
LMW RNA isolation

100 mg of *C. trifoliate* tissues were weighed and ground in liquid nitrogen followed by thorough mixing with 1 ml trizol and transferred to a centrifuge tube. After placing on ice for 30 min, the mixtures were centrifuged for 10 min at 11,000 ×g at 4°C. The upper aqueous phase was transferred into a new centrifuge tube and 500 μl of extraction reagent was added and thoroughly mixed on ice for 5 min, followed by centrifuging for 10 min at 11,000 ×g and 4°C. The upper aqueous phase was further transferred into another centrifuge tube and 500 μl of isopropanol was added to each sample and the samples were left to precipitate for 10 min at 37°C. The mixtures were then centrifuged for 10 min at 12,000 ×g and 4 °C. The upper aqueous phase was again removed and the remaining RNA pellet was dried. The RNA pellet was then washed using 700 μl 75% ethanol and total RNA was precipitated by centrifuging for 5 min at 11,000 ×g and 4°C. After this, RNA pellets were completely air dried for 5 min, then dissolved in 500 μl of 4 M LiCl, and set at –20°C for 1 h, before centrifuging for 10 min at 13,000 ×g at 4°C. An equal volume of isopropanol was added to the upper aqueous part, set in –20°C for 30 min and then centrifuged for further 10 min at 13,000 ×g at 4°C, the upper aqueous phase was removed, and then the precipitate was washed in 700 μl 75% ethanol. After that, the LMW RNA pellets were completely dried and LMW RNA was dissolved in 30 μl of RNase free water, and finally stored in at -80°C until use.

Yield and quality analysis

A small amount of the LMW RNA solution and solute of the pellet obtained using the protocol above were subjected to a quality test by electrophoresis on 1.0% agarose gels (Figure 1). The quantity and quality of LMW RNA were assessed spectrophotometrically at 260 and 280 nm as described by Sambrook and Russell (2001). The results of the yield and quality analysis are shown in Table 1.

Further evaluation of protocol on *C. trifoliata* by miRNAs detection, sequencing and Northern blotting

To further evaluate the protocol on *C. trifoliata*, we detected miRNA from the isolated LMW RNA according to the methods of Fu et al. (2005). Small RNAs were polyadenylated at 37°C for 60 min in a 50 μl reaction mixture with 1.5 μg of total RNA, 1 mM ATP, 2.5 mM $MgCl_2$ and 4 U poly(A) polymerase (PAP, Ambion, Austin, TX). Poly(A)-tailed small RNA was recovered by phenol/chloroform extraction and ethanol precipitation. Reverse transcription was performed using 1.5 μg of small RNA and 1 μg of $(dT)_{30}$ RT primer (ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}$ (A, G, or C) (A, G, C, or T)) with 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Poly(A)-tailed small RNA (10 μl total volume) was incubated with 1 μl of (dT)30 RT primer and 1 μl dNTP mix (10 mM each) at 65°C for 5 min to remove any RNA secondary structure. The reactions were chilled on ice for at least 2 min, then the remaining reagents [5 × buffer, dithiothreitol (DTT), RNaseout, SuperScript III] were added as specified in the SuperScript III manual, and the reaction was allowed to proceed for 60 min at

Figure 1. Ethidium bromide stained 1.0% agarose gel of pellet solute in step 6 and LMW RNA from different *C. trifoliata* tissues. a. Lanes 1, 2, 3, 4 and 5: Solute of pellet in step 6 of the protocol for LMW RNA isolation from root, stem, leaf, flower, and fruit tissues, respectively. b. Lanes 6, 7, 8, 9 and 10: LMW RNA of root, stem, leaf, flower and fruit tissues, respectively; poly (A)-tailed LMW RNA; M: 50 bp DNA ladder.

Table 1. LMW RNA yield and quality by spectrophotometric evaluation.

Sample	$A_{260}/_{280}$	Yield (µg/gFW)
Root	1.82±0.035	10.2±0.40
Stem	1.81±0.024	12.5±0.52
Leaf	1.86±0.045	18.6±0.38
Flower	1.95±0.038	20.3±0.47
Fruit	1.84±0.036	15.5±0.56

Results are expressed in l mg/g fresh weight tissue as mean ± standard deviation for three determinations.

50°C. Finally, the reverse transcriptase was inactivated by incubation for 15 min at 70°C. The cDNA was amplified for 30 cycles at an annealing temperature of 58°C using the primer RT (5'-ATTCTAGAGGCCGAGGCGGCCGACATG-3') combined with primers of Primer319 and Primer171 (Table 2), respectively. The PCR products were analyzed on a 2.5% agarose gel with ethidium bromide (EtBr) staining. The gel slices containing DNA with a size of about 80 bp were excised and the DNA fragments were purified using an agarose gel DNA purification kit (Takara, Japan), according to the manufacturer's instructions. The DNA fragment was directly sub-cloned with the TOPO TA cloning Kit (Invitrogen, USA) into T vector and sequenced.

The miRNA expression was analyzed by RT-PCR by the method described previously (Xie et al., 2007). The amplification of miRNAs was carried out for 30 cycles at a final annealing temperature of 60°C using the corresponding miRNA sequences as sense primers and antisense adaptor primer (5'-CGAACATGTACAGTCCA-TGGATAG-3'). The PCR product was analyzed on 2.5% agarose gel with ethidium bromide (EtBr) staining. Expression of citrus 5s rRNA (sense, 5'-CTCGGCAACGGATATCTCGGCTCT-3'; antisense, 5'-CTAATGGCTTGGGGCGCAACTTG -3') was performed for cDNA normalization.

For Northern blotting, twenty micrograms of LMW RNA of roots, stems and leaves as well as mixtures from diffentent *C. trifoliata* tissues were used. The LWM RNA from different tissues were loaded per lane on a 15% denaturing polyacrylamide gel, resolved and transferred electrophoretically to Hybond Nt membranes (Amersham, Piscataway, NJ) using a TransBlot-SD apparatus (Bio-

Rad). Membranes were UV cross-linked at 1200 µjoules × 100 in a Stratalinker 1800 (Stratagene, La Jolla, CA). DNA oligonucleotides complementary to small RNA sequences were end-labeled with γ-^{32}P-ATP using T4 polynucleotide kinase (Invitrogen). The membranes were pre-hybridized for 1 h and hybridized for approximately 16 h using Perfect Hyb^{TM} Plus buffer (Sigma, St. Louis, MO) at 38°C. Thereafter, the membranes were washed 4 times (2 times with 1 × SSC and 0.1% SDS for 20 min and 2 times with 0.5 × SSC and 0.1% SDS for 50 min at 50°C). The washed membranes were air-dried for a few minutes and then exposed to BIOMAX X-ray film for 48 h using an intensifying screen (Sunkar and Zhu, 2004).
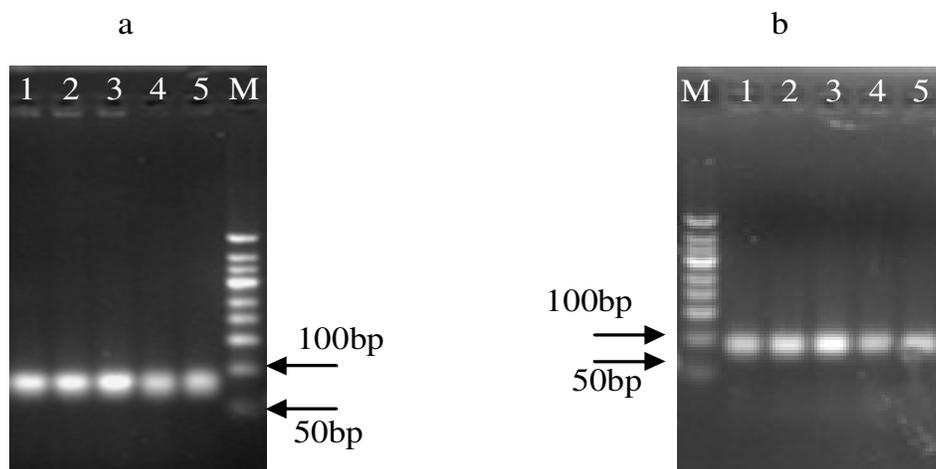
RESULTS AND DISSCUSSION

Size of LMW RNA obtained

After isolation, the pure LMW RNA of different citrus tissues obtained using the protocol described above was tested on 1.0% agarose gel as shown in Figure 1a. Whereby, it is discernible that the LMW RNA was less than 150 bp in length. Using the LMW RNA isolated from citrus as the template, miR319 and miR171 were detected by poly(A)-tailed RT-PCR. The PCR products were then verified by sequencing, revealing that the LMW RNA contained small RNAs of about 20 bp in length and thus could be used in microRNA identification (Figures 2 and 3 and Table 2). Moreover, the expression patterns of miR171 and miR319 are similar: strong expression in leaves, stems and roots, high expression in leaves, flowers and fruits of trifoliate orange (Figures 2a, 2b 3a and 3b) by Northern blotting and RT-PCR. Accordingly, the LMW RNA could be used for small RNA library construction using many methods (Berezikov et al., 2006) as well as Northern blotting, and PCR for miRNAs detection and identification (Fu et al., 2005). Compared with other studies (Chun et al., 2008), the LMW RNAs obtained here were slightly larger, possibly because isolated LMW RNA contains the precursors of the

Table 2. Primer combinations for poly (A)-tailed RT-PCR and sequence of amplified product.

miRNA	Primer combination	Product size(bp)	Sequence of product (5'-3')
MiR319	RT+Primer319	77	<u>TTGGACTGAAGGGAGCTCCC</u>AAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAACATGTCGGCCGCCTCGGCCTCTAGAAT
MiR171	RT+Primer171	78	**TGATTGAGCCGTGCCAATATC**AAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAACATGTCGGCCGCCTCGGCCTCTAGAAT

Bases denoted in bold present the mature miRNA sequences, miR319 and miR171, respectively. Underlined bases represent primer 319 and 171 sequences, respectively.

a   b

Figure 2. Expression analysis of miR319 and miR171 in different tissues by RT-PCR from poly (A)- tailed LMW RNA. a: cDNA amplified with RT and primer 319. Lanes 1, 2, 3, 4, and 5 are root, shoot, leaf, flower and fruit, respectively; b: cDNA amplified with RT and primer 319. Lanes 1, 2, 3, 4, and 5 are root, shoot, leaf, flower and fruit, respectively. M: 50 bp DNA ladder.

Figure 3. Expression patterns of miR319 and miR171 by Northern blotting. RNA gel blots of total RNA isolated from different tissues were probed with labeled oligonucleotides. The samples are leaf (L), stem (S), root (R) and mixture (M). The tRNA and 5S rRNA bands were visualized by ethidium bromide staining of polyacrylamide gels and served as loading controls.

miRNAs. Therefore, it is necessary to get the sequence information of the LMW RNA to clone the precursors of miRNAs.

Yield and quality of LMW RNA

About 10 to 20 μg of LMW RNA was obtained from 1 g of fresh citrus root, stem, leaf, lower or fruit tissues (Table 1). Then it was analyzed by UV spectrophotometry (Table 1), and it showed that the ratio of A_{260}/A_{280} of LMW RNA solution ranged from 1.9 to 2.0, which suggested that it has good quality. When the total RNA was analyzed on

agarose gel by electrophoresis, two obvious rRNA bands, 28S and 18S rRNA, were observed as shown in lanes 1 to 5 in Figure 1a. The 28S rRNA band looked twice as bright as the 18S rRNA band suggesting that the RNA in the extraction mixture used for LMW RNA isolation was intact and not comprised of degradation products indicating that the procedure is good for LMW RNA isolation. Therefore, the protocol described can be used not only to get total RNA which can also be saved for further isolation of LMW RNA, but also, that this method is applicable in different tissues of citrus including leaves and fruit.

Several established methods were initially used to

isolate RNA. These included standard and modified LiCl precipitation, nonphenolic extractions and precipitations with various salts (Schuler and Zielinski, 1989; Chang et al., 1993; Bugos et al., 1995). The results however were not always positive since RNA appeared to be partially degraded, and the RNA bands were not distinct. Yield and purity problems are very common in RNA isolation, which can bind irreversibly to nucleic acids and coprecipitate with RNA (Loomis, 1974; Chang et al., 1993). We reported a quick and simplified method for isolating RNA from citrus leaves, young shoots, roots, flowers and fruits. Distinct RNA bands were apparent, and the isolated LMW RNA was pure, as judged by a good A_{260}/A_{280} ratio (approximately 2) as well as hybridization to a specific RNA.

When compared to reported methods which have several steps in the whole process of isolating RNA that can make miRNAs in the LMW RNA unstable, the advantage of our protocol is that it has fewer steps and takes less time, therefore minimizing the chance for RNA degradation thus saving miRNAs with ultra-low abundance in the samples. Another important benefit of using our method is that one can work with several samples at the same time, therefore decreasing the differences caused by degradation of miRNAs whilst LMW RNA from other samples are stored to await parallel expression analysis. Owing to the few and simple steps, the whole procedure can be completed within 6 h and this protocol utilizes common chemical reagents. Therefore, this procedure can be effortlessly applied as a common method in the laboratory.

In summary, according to the results presented in this study, the method we described is efficient and reliable for isolation of LMW RNA from various *C. trifolita* tissues and other citrus cultivars (data not shown). With high quality LMW RNA, it is suitable for downstream applications such as small RNA library construction, high throughput deep sequencing, RT-PCR expression analysis of miRNAs and Northern blotting. Lastly, this developed protocol may be applied in other plant species that contain high levels of polysaccharides, thus facilitating small RNA research in such plants.

REFERENCES

Asif MH, Dhawan P, Math P (2000). A simple procedure for the isolation of high quality RNA from ripening banna fruit. Plant Mol. Biol. Rep. 18: 109-115.

Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116: 281-297.

Berezikov E, Cuppen E, Plasterk RHA (2006). Approaches to microRNA discovery. Nat. Genet. 38: 2-7.

Bugos RC, Chiang VL, Zhang XH, Campbell ER, Podila GK, Campbell WH (1995). RNA isolation from plant tissues recalcitrant to extraction in guanidine. Biotech. 19: 734-737.

Carra A, Gambino G, Schubert A (2007). A cetyltrimethylammonium bromide-based method to extract low molecular weight RNA from polysaccharide-rich plant tissues. Anal. Biochem. 360: 318-320.

Carrington JC, Ambros V (2003). Role of microRNAs in plant and animal development. Science, 301(5631): 336-338.

Carthew RW, Sontheimer EJ (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell, 136: 642-655.

Chang S, Pryear J, Cairney J (1993). A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11: 113-117.

Chun J, Zheng YF, Wang SH, Chen F (2008). A RNA isolation method suitable for a wide range of materials. Prog. Inorg. Biochem. Biophys. 35: 591-597.

Fu H, Tie Y, Xu C, Zhang Z, Zhu J, Shi Y (2005). Identification of human fetal liver miRNAs by a novel method. FEBS Lett. 579: 3849-3854.

Jones-Rhoades MW, Bartel DP, Bartel B (2006). MicroRNAs and their regulatory roles in plants. Annu. Rev. Plant Biol. 57: 19-53.

Liu JJ, Goh CS (1998). A method for isolation of total RNA from fruit tissues of banana. Plant Mol. Biol. Rep. 16: 1-6.

Llave C, Kasschau KD, Rector MA, Carrington JC (2002). Endogenous and silencing-associated small RNAs in plants. Plant Cell, 14: 1605-1619.

Loomis WD (1974). Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Meth. Enzymol. 31: 528-544.

Lopez-Gomez R, Gomez-Lim MA (1992). A method for extracting intact RNA from fruits rich in polysaccharides using ripe mango mesocarp. Hort. Sci. 27: 440-442.

Lu C, Meyers BC, Green PJ (2007). Construction of small RNA cDNA libraries for deep sequencing. Methods, 43: 110-117.

Sambrook J, Russell DW (2001). Molecular cloning: A laboratory manual (3rd ed). New York: Cold Spring Harbor Press.

Schuler MA, Zielinski RE (1989). RNA isolation from light- and dark-grown seedlings In: Schuler MA and Zielinski RE (eds). Academic Press, San Diego. Methods in Plant Molecular Biology. pp. 89-96.

Song C, Fang J, Li X, Liu H, Chao C (2009). Identification and characterization of 27 conserved microRNAs in citrus. Planta, 230: 671-685.

Song C, Wang C, Zhang C, Nicholas KK, Yu H, Ma Z, Fang J (2010). Deep sequencing discovery of novel and conserved microRNAs in trifoliate orange (*Citrus trifoliata*). BMC Genomics, 11: 431.

Sunkar R, Zhu JK (2004). Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. Plant Cell, 16: 2001-2019.

Wang JW, Czech B, Weigel D (2009). miR156 regulated *SPL* transcription factors define and endogenous flowering pathway in *Arabidopsis thaliana*. Cell, 138: 738-749.

Wang JF, Zhou H, Chen YQ, Luo QJ, Qu LH (2004). Identification of 20 microRNAs from *Oryza sativa*. Nucleic Acids Res. 32: 1688-1695.