

EVALUATION OF THREE RNA EXTRACTION METHODS FROM THREE CULTIVARS OF MALYSIAN UPLAND RICE

Shahkila Mohd Arif^a, Nyuk Ling Ma^b, Alina Wagiran^a, Zaidah Rahmat^{a,c*}

^aDepartment of Biotechnology and Medical Engineering, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia

^bSchool of Fundamental Science, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

^cInnovation Center in Agrotechnology for Advanced Bioprocessing (ICA), Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia

Article history

Received

14 May 2015

Received in revised form

12 July 2016

Accepted

15 December 2015

*Corresponding author
zaidahrahmat@utm.my

Abstract

Rice (*Oryza sativa* L.) can be divided into two major categories, which is upland rice and lowland rice. Apart from being the staple food for more than half of the world population, it is also known as model plant for functional genomics study. However, it possess high amount of starch and polysaccharide that makes the isolation of good quality RNA for downstream purposes often a difficult task. While there are many studies being carried out for lowland rice extraction, none has been reported for upland rice. This study is the first to report on evaluation of three RNA extraction methods for three Malaysian upland rice cultivars in order to determine the best method to isolate high grade RNA. The result obtained demonstrated that good quality RNA in terms of integrity, purity and quality can be isolated from young leaves of these cultivars by using guanidine isothiocyanate based extraction method that is fast, simple and efficient and had been proven suitable for further downstream applications.

Keywords: Malaysian upland rice, RNA extraction, guanidine isothiocyanate, purity, downstream applications

Abstrak

Padi (*Oryza sativa* L.) boleh dibahagikan kepada dua kategori utama, iaitu padi bukit dan padi sawah. Selain daripada menjadi makanan rui untuk lebih daripada separuh penduduk dunia, ia juga dikenali sebagai tumbuhan model bagi kajian genomik fungsian. Walau bagaimanapun, ia mempunyai jumlah kanji dan polisakarida yang sangat tinggi yang menyebabkan kesukaran pengasingan RNA berkualiti bagi tujuan proses hiliran. Walaupun terdapat banak kajian pengekstrakan RNA dijalankan bagi padi sawah, tiada yang dilaporkan untuk padi bukit. Kajian ini adalah yang pertama melaporkan penilaian tiga kaedah pengekstrakan RNA dari tiga kultivar padi bukit Malaysia bertujuan menentukan kaedah terbaik bagi penghasilan RNA bergred tinggi. Hasil kajian yang diperolehi menunjukkan bahawa RNA yang berkualiti dari segi integriti, ketulenan dan kualiti boleh diperolehi dari daun muda kultivar –kultivar ini dengan menggunakan kaedah pengekstrakan berasaskan guanidine isothiocyanate yang pantas, mudah dan efisien dan terbukti sesuai untuk tujuan aplikasi hiliran selanjutnya.

Kata kunci: Padi bukit Malaysia, Pengekstrakan RNA, guanidine isothiocyanate, ketulenan, aplikasi hiliran

© 2016 Penerbit UTM Press. All rights reserved

1.0 INTRODUCTION

Rice or its scientific name *Oryza sativa* L. is one of the most important members of the family Poaceae. It

can be grown in two ecosystems; 1) lowland or wetland and 2) upland or dryland. Classification of the better known rice is the lowland version which includes irrigated lowland rice, rainfed lowland rice,

deep water rice and tidal or coastal wetland. The lowland rice survives under adequate water supply with some of them able to survive certain level of drought and some could survive flooding condition. Although not as well-known as its lowland counterpart, upland rice thrives with minimum water requirement or rainfed and is drought tolerant. This staple food is grown commercially in many parts of the world, particularly in tropical and subtropical areas such as Asia and Latin America that occupied 150 million hectares of land for more than 10, 000 years [1] and also widely used as model plant for genomics studies [2-4]. However, this plant possesses very high amount of starch and polysaccharide [5] that makes the isolation of good quality ribonucleic acid (RNA) for downstream applications such as polymerase chain reaction (PCR), complementary deoxyribonucleic acid (cDNA) library construction and northern blotting often a difficult task. This is mainly because this substance could give significant impact to the quality and quantity of the RNA produced [6-7] by binding or co-precipitate with RNA and inhibit enzyme activity [5, 8]. With the advancement of molecular technology, various numbers of studies has been carried out in order to determine the best method to obtain good quality RNA from lowland rice [9, 10]. However, thus far, there is no report on RNA extraction from upland rice. Therefore, in this study, three different RNA extraction protocols namely PUREzol reagent (Bio-Rad), QIAGEN RNeasy Plant Mini Kit (QIAGEN) and modified CTAB method described by [11] were evaluated based on integrity, purity and quality of the RNA obtained in order to determine the best method to isolate good quality RNA from young leaves of Malaysian upland rice.

2.0 EXPERIMENTAL

2.1 Plant Material

Three cultivars of upland rice locally named Wai, Hitam and Bario obtained from Sarawak, Malaysia were cultivated in the glasshouse in Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, Malaysia. After a month, the young leaves were harvested, immersed in liquid nitrogen and stored at -80°C prior to use.

2.2 RNA Extraction Protocols

Three different RNA extraction protocols were carried out. The first protocol is RNA extraction by using PUREzol RNA isolation reagent from Bio-Rad. It was done as per manufacturer's instruction. The second is by using QIAGEN RNeasy Plant Mini Kit from QIAGEN and the procedure was performed according to manufacturer's handbook. The third protocol is

modified CTAB method that was carried out as described by [11].

2.3 RNA Analysis

The integrity of the RNA was assessed by electrophoresis on 1.2% (w/v) agarose gel stained with SYBR Safe (Invitrogen) followed by visualization under the UV light by using Gel documentation unit (Fisher Scientific) and the purity was measured at absorbance ratio of A_{260}/A_{280} using NanoDrop 1000 spectrophotometer (ThermoFisher). The quality of RNA was analysed by performing cDNA synthesis and reverse transcription polymerase chain reaction (RT-PCR). The isolated RNA was treated with DNase I (Promega) as per manufacturer's instruction to remove any traces of DNA and cDNA was synthesized using iScript Select cDNA Synthesis Kit (Bio-Rad). Reference gene known as 18S was used for RT-PCR and the amplification was carried out in a master cycler (Eppendorf) for 30 cycles. The thermocycling profiles were predenaturation at 94°C for 3 minutes, denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, extension at 72°C for 2 minutes and final extension at 72°C for 10 minutes. The amplicon was evaluated via gel electrophoresis with 1.2 % (w/v) agarose SYBR Safe-stained gel.

3.0 RESULTS AND DISCUSSION

3.1 RNA Quality of Malaysian Upland Rice

Good RNA extraction method is of paramount importance in molecular studies as it is one of the key factors that will determine the success of the downstream applications. In this study, RNA from three cultivars of Malaysian upland rice namely Wai, Bario and Hitam were isolated using three different methods and evaluated based on the integrity, purity and quality. This is due to the fact that the successful RNA isolation is largely determined by these parameters [12]. The first and the second method (PUREzol reagent and QIAGEN RNeasy Plant Mini Kit) are based on chaotropic agent, guanidine isothiocyanate to recuperate the RNA. On the contrary, the other method is based on surfactants agent, Cetyltrimethylammonium bromide (CTAB), with the aid of antioxidant compound, β -mercaptoethanol and polyvinylpyrrolidone (PVP) [13] and lithium chloride (LiCl) to recover the RNA. Figure 1 shows the integrity of RNA that has been assessed in 1.2% (w/v) agarose gel electrophoresis for all three methods. Figure 1 shows a variation of 28S and 18S ribosomal RNA bands which implies the different concentration of the samples.

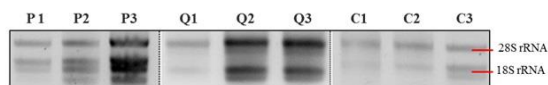


Figure 1 RNA of three different cultivars on 1.2 % (w/v) agarose gel extracted using three different methods. RNA products in panel P were extracted by PUREzol reagent, cultivar **P1**: Wai, **P2**: Hitam, and **P3**: Bario respectively; RNA products in panel Q were extracted by QIAGEN RNeasy kit, cultivar **Q1**: Wai, **Q2**: Hitam, and **Q3**: Bario respectively; RNA products in panel C were extracted from modified method of CTAB, cultivar C1: Wai, C2: Hitam, and C3: Bario respectively

All three methods produced intact band for 28S and 18S ribosomal RNA. However, both ribosomal RNA for modified CTAB method is slightly faint comparing with the other two methods. The purity of RNA was determined by absorbance ratio of A_{260}/A_{280} where the desired range is within 1.8 – 2.1, which indicates the RNA is high in purity and free from contaminants like protein [14, 11]. Table 1 shows purity of RNA isolated from young leaves of three upland rice cultivars by using the aforementioned methods. The result indicated that all of the method used could produce RNA with acceptable purity value. Even though all three protocols could give acceptable results, guanidine isothiocyanate based method has advantages in terms of short duration of time needed and simplicity, thus could process large number of sample. Meanwhile, the CTAB based method is time-consuming, cumbersome and laborious [15, 16]. Moreover, it involved the use of LiCl to precipitate the RNA which could interfere with downstream reactions [17] such as the amplification of rare transcripts where large amount of RNA template and high numbers of cycles is used [18]. Additionally, between two guanidine isothiocyanate based methods, PUREzol RNA isolation reagent gives better result in terms of RNA concentration and yield to compare with QIAGEN RNeasy Plant Mini Kit. There have been reports that guanidine isothiocyanate based method such as QIAGEN RNeasy Plant Mini Kit and TriZOL reagent (Invitrogen) produced low quality RNA in *Fritillaria unibracteata* [19], Mangrove [20] and *Lycium barbarum* [21] in different plant tissues. However, there is no universal RNA extraction method exists yet, which is due to the chemical and biological diversities of plants and tissues used [22, 23]. Nevertheless, guanidine isothiocyanate based method has been proven to be effective for isolating RNA from young tissues [24] of crop like rice and barley and model plant, *Arabidopsis* [12, 25].

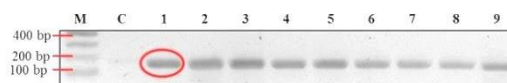


Figure 2 RT-PCR amplification products from RNA of three different cultivars extracted using three different methods. The size of each PCR product was consistent at approximately 150 bp. **M** (Promega 100 bp ladder), **C** (negative control using distilled water), **1-9** represented **cDNA products**. **1** (Wai), **2** (Hitam), **3** (Bario) isolated using QIAGEN RNeasy Plant Mini Kit. **4** (Wai), **5** (Hitam), **6** (Bario) isolated using PUREzol reagent. **7** (Wai), **8** (Hitam), **9** (Bario) isolated using modified CTAB method

Table 1 Purity of RNA isolated from three upland rice cultivars using three different methods. Concentration was based on actual Nanodrop spectrometry readings and yield was calculated against 100 mg fresh weight (FW) of starting material

Method	Sample	Concentration		Yield ($\mu\text{g}/100$ mgFW)
		A_{260}/A_{280}	(ng/ μL)	
PUREzol Reagent	Wai	1.84 \pm 0.03	348.83 \pm 9.47	13.95 \pm 0.38
	Hitam	1.96 \pm 0.08	616.7 \pm 6.96	24.67 \pm 0.28
	Bario	2.05 \pm 0.03	702.53 \pm 12.45	28.10 \pm 0.5
RNeasy Plant Mini Kit	Wai	2.05 \pm 0.02	261.57 \pm 14.13	10.46 \pm 0.57
	Hitam	2.09 \pm 0.01	540.7 \pm 3.3	21.62 \pm 0.14
	Bario	2.1 \pm 0.02	523.77 \pm 5.95	20.95 \pm 0.24
Modified CTAB [9]	Wai	2.18 \pm 0.07	278.33 \pm 12.59	11.13 \pm 0.5
	Hitam	2.13 \pm 0.06	286.4 \pm 3.66	11.46 \pm 0.15
	Bario	2.11 \pm 0.04	314.5 \pm 7.85	12.58 \pm 0.31

Values represents the mean \pm SEM (n=3)

3.2 Evaluation of RNA in Downstream Application

Reverse transcription is very sensitive to impurities [26] as reverse transcriptase enzyme could be inhibited by exonucleases, endonucleases, photosynthetic pigments and polysaccharides [27, 28]. Therefore, in order to ensure the RNA isolated is high in quality and suitable for subsequent downstream applications, the first strand cDNA was synthesized and RT-PCR analysis was carried out by using 18S primer. As shown in Figure 2, successful bands corresponding to the expected size of the 18S housekeeping gene from each method and varieties of rice proved the quality of the RNA for further downstream utilization. Better

band intensity was also observed from rice varieties isolated using guanidine isothiocyanate methods.

4.0 CONCLUSION

It was demonstrated that good quality RNA can be isolated from three Malaysian upland rice cultivars using guanidine isothiocyanate based extraction method in a fast, simple and efficient way. Supported by evaluation of the RNA obtained via RT-PCR, the result indicates that this method is reliable and suitable for young leaves tissues of the aforementioned Malaysian upland rice cultivars for further downstream analysis.

Acknowledgement

We thank the Universiti Teknologi Malaysia (UTM) and Ministry of Higher Education (MOHE) for funding this project (Flagship Research University Grant vote: 00G65), Prof. Dr. Roji Sarmidi (project leader) and Zamalah for sponsoring the graduate student scholarship.

References

- [1] Khush, G. S. 1997. Origin, Dispersal, Cultivation and Variation of Rice. *Plant Molecular Biology*. 35(1-2): 25-34.
- [2] Shahsavari, E., Maheran, A., Akmar, A. S. N., Hanafi, M. M. 2010. The Effect of Plant Growth Regulator on Optimization of Tissue Culture System in Malaysian Upland Rice. *African Journal of Biotechnology*. 9(14): 2089-2094.
- [3] Bajaj, S., Mohanty, A. 2005. Recent Advances in Rice Biotechnology Towards Genetically Superior Transgenic Rice. *Plant Biotechnology Journal*. 3: 275-307.
- [4] Tyagi, A. K., Mohanty, A. 2000. Rice Transformation for Crop Improvement and Functional Genomics *Plant Science*. 158: 1-18.
- [5] Song, G. S., Zha, H. L., Wei, G., Zhu, Z. 2007. A Simple and Inexpensive Total RNA Extraction Method from the Rice Immature Seeds Rich in Polysaccharides. *Journal of Agricultural Science and Technology*. 9(4): 111-115.
- [6] Japelaghi, R. H., Haddad, R., Garoosi, G. A. 2011. Rapid and Efficient Isolation of High Quality Nucleic Acids from Plant Tissues Rich in Polyphenols and Polysaccharides. *Molecular Biotechnology*. (49): 129-137.
- [7] Asif, M. H., Dhawan, P., Nath, P. 2000. A Simple Procedure for the Isolation of High Quality RNA from Ripening Banana Fruit. *Plant Molecular Biology Reporter*. (18): 109-115.
- [8] Fang, G., Hammar, S., Grumet, R. 1992. A Quick and Inexpensive Method for Removing Polysaccharides from Plant Genomic DNA *Biotechniques*. 13(1): 52-56.
- [9] Jhala V. M., M., V. B., Thaker V. S. 2015. Simple and Efficient Protocol for RNA and DNA Extraction from Rice (*Oryza sativa* L.) for Downstream Applications. *International Research Journal of Biological Sciences*. 4(2): 62-67.
- [10] Suzuki, Y., Makino, A., Mae, T. 2001. An Efficient Method for Extraction of RNA from Rice Leaves at Different Ages Using Benzyl Chloride. *Journal of Experimental Botany*. 52(360): 1575-1579.
- [11] White, E. J., Venter, M., Hiten, N. F., Burger, J. T. 2008. Modified Cetyltrimethylammonium Bromide Method Improves Robustness and Versatility: The Benchmark for Plant RNA Extraction. *Biotechnology Journal*. (3): 1424-1428.
- [12] Wang, G., Wang, G., Zhang, X., Wang, F., Song, R. 2011. Isolation of High Quality RNA from Cereal Seeds Containing High Levels of Starch. *Phytochemical Analysis*. 23: 159-163.
- [13] Codeiro, M. C. R., Silva, M. S., Oliveira-Filho, E. C., Gayoso de Miranda, Z. D. J., Aquino, F. D. G., Fragoso, R. D. R., Almeida, J., Roveinia, L., Andrade, M. D. 2008. Optimization of a Method of Total RNA Extraction from Brazilian Native Plants Rich in Polyphenols and Polysaccharides. *Simposio Internacional Savanas Tropicais ParlaMundi, Brazil*. 12-17 October 2008.
- [14] Zhang, Y., Qin, Y., Guo, L., Zhou, Z., Liang, Z., Zhang, C., Guo, H. 2012. Isolation of High Quality RNA from *Polyporus umbellatus* (Pers.) Fries. *Molecular Biology and Genetics*. 15(5): 10.
- [15] Yu, D., Tang, H., Zhang, Y., Du, Z., Yu, H., Chen, Q. 2012. Comparison and Improvement of Different Methods of RNA Isolation from Strawberry (*Fragaria ananassa*). *Journal of Agricultural Science*. 4(7): 51-56.
- [16] Zarei, A., Zamani, Z., Mousavi, A., Fatahi, R., Alavijeh, M. A., Dehsara, B., Salami, S. A. 2012. An Effective Protocol for Isolation of High-Quality RNA from Pomegranate Seeds. *The Asian and Australasian Journal of Plant Science and Biotechnology*. 6(1): 32-37.
- [17] Rosato, E. 2007. *Circadian Rhythms: Methods and Protocols*. Volume 362. Totowa, NJ: Humana Press Inc.
- [18] Del Aguila, E. M., Dutra, M. B., Silva, J. T., Paschoalin, V. M. F. 2005. Comparing Protocols for Preparation of DNA-free Total Yeast RNA Suitable for RT-PCR. *BMC Molecular Biology*. 6(9): 9-14.
- [19] Hou, P., Xie, Z., Zhang, L., Song, Z., Mi, J., He, Y., Li, Y. 2011. Comparison of Three Different Methods for Total RNA Extraction from *Fritillaria unibracteata*: A Rare Chinese Medicinal Plant. *Journal of Medicinal Plants Research*. 5(13): 2834-2838.
- [20] Rubio-Pina, J., and Zapata-Perez, O. 2011. Isolation of Total RNA from Tissues Rich in Polyphenols and Polysaccharides of Mangrove Plants. *Electronic Journal of Biotechnology*. 14(5).
- [21] Tao, N., Gao, Y., Liu, Y., Zhang, J. 2010. Extraction of High-quality RNA and Construction of a cDNA Library from Fruits of *Lycium barbarum* Linnaeus (Fructus lycii). *Biotechnology & Biotechnological Equipment*. 1569-1572.
- [22] Liao, Z., Chen, M., Guo, L., Gong, Y., Tang, F., Sun, X., Tang, K. 2004. Rapid Isolation of High-Quality Total RNA from *Taxus* and *Ginkgo*. *Preparative Biochemistry & Biotechnology*. 34(3): 209-214.
- [23] Jones, C. S., Iannetta, P. M., Woodhead, M., Davies, H. V., McNicol, R. J., Taylor, M. A. 1997. The Isolation of RNA from Raspberry (*Rubus idaeus*) Fruit. *Molecular Biotechnology*. 8: 219-221.
- [24] Gambino, G., Perrone, I., Gribaudo, I. 2008. A Rapid and Effective Method for RNA Extraction from Different Tissues of Grapevine and Other Woody Plants. *Phytochemical Analysis*. 19: 520-525.
- [25] Wang, T., Zhang, N., Du, L. 2005. Isolation of RNA of High Quality and Yield from *Ginkgo biloba* Leaves. *Biotechnology Letters*. 27: 629-633.
- [26] Ghangal, R., Raghuvanshi, S., Sharma, P. C. 2009. Isolation of Good Quality RNA from a Medicinal Plant Seabuckthorn, Rich in Secondary Metabolites. *Plant Physiology and Biochemistry*. 47:1113-1115.
- [27] Talyshinsky, M. M., Souprun, Y. Y., Huleihel, M. M. 2000. Antiviral Activity of Red Microalgal Polysaccharides Against Retrovirus. *Cancer Cell International*. 2: 2-8.
- [28] Lau, A. F., Siedlecki, J., Anleitner, J., Patterson, G. M. L., Caplan, F. R., Moore, R. E. 1993. Inhibition of Reverse Transcriptase Activity by Extracts of Cultured Blue-green Algae (Cyanophyta). *Plant Medica*. 59: 148-151.