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Research article

Evaluation of suitable reference genes for normalization of microRNA expression by real-time reverse transcription PCR analysis during longan somatic embryogenesis

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ABSTRACT

Accurate profiling of microRNAs (miRNAs) is an essential step for understanding both developmental and physiological functions of miRNAs. Real-time quantitative PCR (qPCR) is being widely used in miRNA expression studies, but choosing a suitable reference gene is a crucial factor for correct analysis of results. To date, there has been no systematic evaluation of qPCR reference genes for the study of miRNAs during somatic embryogenesis (SE) in the longan tree (*Dimocarpus longan*). Here, the most stably expressed miRNAs in synchronized longan tree embryogenic cultures at different developmental stages were determined using the geNorm and NormFinder algorithms. Validation qPCR experiments were performed for 24 miRNAs together with a snRNA (*U6 snRNA*), a rRNA (*5S rRNA*), and three housekeeping genes. It was found that small RNAs had better expression stability than protein-coding genes, and *dlo-miR24* was identified as the most reliable reference gene, followed by *dlo-miR168a**, *dlo-miR2089*-1* and *5S rRNA*. *dlo-miR24* was recommended as a normalizer if only a single reference gene was to be used, while the combination of *dlo-miR156c*, *dlo-2089*-1* and *5S rRNA* was preferred to normalize miRNA expression data during longan SE.

1. Introduction

Plant somatic embryogenesis (SE) resembles zygotic embryogenesis and is an integral part of many biotechnological tools for genetic improvement, and is crucial for studying plant cell differentiation and embryo development. MicroRNAs (miRNAs), a group of endogenous small RNA molecules, generally 20–25 nt in length, are essential regulators of many important developmental and physiological events, such as morphogenesis, polarization, organogenesis, reproduction and plant SE [1–3]. miRNAs in embryonic development have been mostly studied in rice [4], *Arabidopsis* [5,6], *Liriodendron chinense* [7], *Citrus sinensis* [8], loblolly pine [9] or larch [10] embryos. The expression of miRNAs during embryogenic development is stage and tissue-specific [4,7–10]. They prevent precocious gene expression to enable pattern formation during plant embryogenesis [5], and to regulate the timing of embryo maturation [6].

miRNA gene expression profiling is crucial for investigating gene function in plant biology. Compared with northern blot and microarray technologies, real-time quantitative RT-PCR (qPCR) is one of the most powerful techniques for analyzing miRNA expression because of its sensitivity, specificity, wide dynamic range and low template concentration requirements [11]. To produce accurate relative qPCR data, a suitable internal control gene is a crucial factor. In previous studies of plant embryos, well known housekeeping genes, including *eIF-4A1* [5], *actin* [8] and *5S rRNA* [9,10], have been used as reference genes to normalize miRNA expression data without systematic selection. To our knowledge, use of unreliable reference genes for normalization may lead to large errors and is therefore inappropriate [12,13]. To date, two studies in plants, in soybean [14] and wheat [15], have selected suitable reference genes for qPCR analysis of miRNAs and demonstrated that miRNA expression levels were much more stable than the most commonly used protein-coding genes. Kou et al. [16] tested three miRNAs and eight non-coding RNAs as reference candidates in developing citrus SE tissues; the best single references were snoR14 or snoRD25 and the optimal multiple references were snoR14+U6 or snoR14+U5. Currently, there is no consensus on reference miRNAs for qPCR analysis of plant miRNAs.

The longan tree (*Dimocarpus longan* Lour.) is a tropical/subtropical fruit tree in the family Sapindaceae. Its SE system has been used as a model system for investigating regulation of *in vitro* and

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in vivo embryogenesis in woody plants [17–19]. In our previous study, we reported suitable reference genes for mRNA qPCR in longan SE tissues and the optimal multiple references, *Fe-SOD*, *eIF-4A* and *EF-1a*, were identified [20]. However, they may be not suitable internal controls for miRNA qPCR analysis. We have previously revealed important roles of miRNAs in longan SE [21]; therefore, to investigate the expression patterns of miRNAs during longan SE, suitable internal controls for miRNA qPCR need to be identified. In this study, to identify optimal reference genes for miRNA qPCR studies of longan SE, we compared the stability among miRNAs, snRNA, rRNA and mRNA by selecting 24 miRNAs, *U6 snRNA*, *5S rRNA* and three common mRNAs (*Fe-SOD*, *eIF-4A* and *EF-1a*) [20]. NormFinder [12] and geNorm [13] were then used to evaluate the stability of these genes to enable selection of the most reliable controls for miRNA qPCR analysis of longan embryo development.

2. Results

2.1. PCR efficiency and qPCR amplification specificity

A set of nine samples from synchronized longan embryogenic cultures at different developmental stages were studied in this experiment. High quality total RNAs from the different samples were isolated and reverse transcribed. A total of 29 candidate genes were selected for normalization of gene expression. The amplification efficiency for each primer pair of the 29 candidate genes was determined in a qPCR assay using a ten-fold dilution series from a pooled cDNA template. The PCR efficiency values for all candidate genes ranged from 1.844 for *miR168a** to 2.187 for *miR390a*.1*, as listed in Table 1. Furthermore, the correlation coefficient (R^2) was calculated, and all primer pairs showed an $R^2 > 0.99$, indicating a strong relationship between the detected *Cq* values and the corresponding relative amount of template in all amplification reactions. Melting curve analysis and agarose gel electrophoresis indicated that all primers used to amplify the candidate reference genes generated a single PCR product of the desired size, indicating good qPCR specificity for all of the primer sets and enabling all candidate genes to be used in the next assay.

2.2. Expression stability of the candidate reference genes

Transcripts of all genes were detected in all nine samples by qPCR. According to the raw *Cq* values, all candidate genes displayed a wide range of expression levels during longan SE. Among the genes, nine miRNAs, including *miR159b**, *miR160a*, *miR166c**, *miR167a*, *miR390a*-1*, *miR398b.1*~*3 and miR26*, were expressed in a tissue-specific manner. Naturally, only those genes expressed in all samples were further evaluated for their expression stability. Therefore, the above nine miRNAs with tissue-specific expression, as well as *miR2089*-2*/3/5/6, which were expressed at low levels (*Cq*: 30.74–36. 48), were excluded from further analysis. Furthermore, to reduce the risk of selected genes that were putatively co-regulated, a number of miRNAs encoded by the same gene family were discarded and only one representative miRNA per family was retained. Consequently, a total of eleven miRNAs, along with *U6 snRNA* and *5S rRNA*, were retained for further analysis, also *Fe-SOD*, *eIF-4a* and *EF-1a* have been retained (see Tables 2 and 3).

The sixteen candidate genes displayed a wide range of expression levels with *Cq* values from 14.47 to 33.35. Mean *Cq* values per sRNA and the range of *Cq* values for each sRNA are shown in Table 2. *dlo-miR4a*, *-159f*, *-5077*, *U6 snRNA* and *5S rRNA* showed relatively high levels of expression with mean *Cq*s of 16.74, 18.93, 20.05, 16.47 and 15.82, respectively. While *miR156c*, *miR168a**, *miR390a*.1*, *miR397a*, *miR808*, *miR17* and *miR24* were moderately expressed with mean *Cq*s of between 23.51 and 27.89. Only *miR2089*-1* had a

Table 1
microRNAs, primer sequences, PCR efficiencies and annealing temperatures.

Number	miRNA name	Primer sequence (5′–3′)	PCR efficiency (E)	Annealing temperature (°C)
1	*dlo-miR156a*	TCCTGACAGAAGAGAGTGAGCAC	2.026	59
2	*dlo-miR156c*	GATTTGACAACGAGAGAGAGCAC	2.1	59
3	*dlo-miR159a.1*	GATTTGGATTGAAGGGAGCTCTA	2.001	59
4	*dlo-miR159a.2*	GCTTTGGATTGAAGGGAGCTTA	2.097	59
5	*dlo-miR159c*	CTTTGGATTGAAGGGAGTCCTA	1.95	59
6	*dlo-miR159f*	ACTTGGATTGAAGGGAGCTCTA	1.932	57
7	*dlo-miR159b**	CGAGCTCCTTGAAGTCCAATAG	1.911	62
8	*dlo-miR160a*	CTGGCTCCCTGTATGCCA	2.09	59
9	*dlo-miR166c**	GAATGTTGTCTGGCTCGAGG	1.98	62
10	*dlo-miR167a*	AGAAGCTGCCAGCATGATCTA	2.061	59
11	*dlo-miR168a**	ACGCCTTGCATCAACTGAAT	1.844	59
12	*dlo-miR390a.1*	GCGCTATCCATCCTGAGTTTC	2.09	59
13	*dlo-miR390a*.1*	GCCGCTATCTATCCTGAGTTTCA	2.187	60
14	*dlo-miR397a*	CATTGAGTGCAGCGTTGATG	2.062	59
15	*dlo-miR398b.1*	AAGTTCTCAGGTCGCCCCT	1.965	59
16	*dlo-miR398b.2*	TGTTCTCAGGTCGCCCC	1.93	59
17	*dlo-miR398b.3*	GTGTTCTCAGGTCGTCCCTG	2.086	59
18	*dlo-miR808*	CGAGATCAGTGGGAAAAGAAGAA	2.139	59
19	*dlo-miR2089*-1*	AGGAGTGGTGAATAGGTACATGAA	2.1	59
20	*dlo-miR5077*	TTCACGTCGGGTTCACCA	2.007	60
21	*dlo-miR4a*	ACGGGTTCAAAGGTTGACAGA	2.043	60
22	*dlo-miR17*	TGCAGCACAAAATACAGTCTGG	1.934	56
23	*dlo-miR24*	AAATGATTTCGGACCAGGCT	2.091	59
24	*dlo-miR26*	TGTGAATGATGCGGGAGATAA	2.047	59
25	*U6 snRNA*	CGATACAGAGAAGATTAGCATGG	1.954	59
26	*5.8S RNA**	ACGTCTGCCTGGGTGTCACAA	1.999	62
27	*Fe-SOD*	F: GGTCAGATGGTGAAGCCGTAGAG R: GTCTATGCCACCGATACAACAAACCC	2.0	58
28	*eIF-4a*	F: TTGTGCTGGATGAAGCTGATG R: GGAAGGAGCTGGAAGATATCATAGA	2.01	58
29	*EF-1a*	F: GATGATTCCCACCAAGCCCAT R: GGGTCCTTCT TCTCAACACTCT	2.0	58

*Primers cited from Shi and Chiang (2005) [11].