Agr. Nat. Resour. 55 (2021) 349–358

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Efficient mini-prep RNA extraction from *Dendrobium* floral tissues rich in polysaccharides for validation of reference genes during flower development

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Article Info

Article history:
Received 8 October 2020
Revised 14 April 2021
Accepted 22 April 2021
Available online 18 June 2021

Keywords:
Dendrobium,
Polysaccharide-rich tissue,
Real-time polymerase chain reaction (PCR),
Reference gene,
RNA extraction

Abstract

RNA extraction from polysaccharide-rich *Dendrobium* Sonia 'Bom' flowers always yields low RNA quantity and quality. Four different standard extraction methods were compared to investigate the most effective RNA extraction method suitable for validation of reference genes during flower development. Quantity analysis of total RNA extracted from petal tissues of flowers at three developmental stages using LiCl-based, hexadecyltrimethylammonium bromide-based, spin column-based and Ribozol™ Reagent-based methods showed that the RNA yield was dependent on the flower developmental stage and the extraction method. The LiCl-based extraction method gave the highest total RNA yield from fully opened flowers. Different LiCl concentrations for the total RNA precipitation step in this method also affected the total RNA yield, which gradually increased from the standard 4 M LiCl and reached a maximum yield at 10 M of 8.29 µg/100 mg fresh weight. However, the addition of 12 M LiCl significantly reduced the total RNA yield compared to adding 10 M LiCl. The ratios of absorbance of 260 nm to 280 nm for protein contamination and of 260 nm to 230 nm for polysaccharide contamination were 1.97–2.13 and 1.5–2.14, respectively. Four different software packages were used to validate the suitable reference genes for gene expression analysis during flower development based on eight candidate genes of *Dendrobium*: *Actin* (*ACT*), *Cyclophilin* (*CYP*), *Eukaryotic initiation factor* (*EIF*), *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), 18S ribosomal RNA (*18S*), *Elongation factor 1 alpha* (*EF1α*), *Gamma-secretase subunit APH1-like* (*APH1L*) and *Cyclic nucleotide-gated cation channel protein* (*SAND*). *ACT* was the most stable reference gene, followed by *EF1α*, *CYP*, *EIF*, *SAND*, *18S*, *GAPDH* and *APH1L*, respectively.

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https://doi.org/10.34044/j.anres.2021.55.3.04

Introduction

Isolation of a sufficient quantity of high-quality total RNA is a crucial first step for gene expression studies such as northern blot hybridization or reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and conventional methods for total RNA isolation from plant tissues usually use the cetyltrimethylammonium bromide (CTAB)-based and sodiumdodecyl sulfate (SDS)-based methods (Tan and Yiap, 2009). However, isolation of total RNA from certain plant tissues containing high levels of polysaccharides always yields low amounts of poor-quality RNA or no RNA at all, as polysaccharides co-precipitate with RNA in low ionic strength buffers (Salzman et al., 1999).

Commercial RNA isolation kits based on the use of an acidic guanidinium thiocyanate-phenol-chloroform extraction reagent also yield low amounts of poor quality RNA because in general, large polysaccharide fragments released after sample digestion can trap nucleic acids, which are then discarded during phase separation (MacRae, 2007), while small polysaccharide fragments co-precipitate with RNA in the RNA precipitation step interfering with subsequent applications (Kansal et al., 2008). A silica-membrane spin column is another widely used method for RNA isolation based on a guanidinium thiocyanate lysis buffer; however, polysaccharide contamination significantly decreases spin-column efficiency (Gambino et al., 2008).

Therefore, for tissues containing relatively high levels of polysaccharides it is necessary to selectively remove the polysaccharide contamination or to precipitate the RNA (Wilkins and Smart, 1996). Although RNA precipitation with alcohol and a monovalent cation such as sodium or ammonium ions is widely used, LiCl has been frequently used to precipitate RNA because it does not efficiently precipitate DNA, proteins or carbohydrates (Barlow et al., 1963; Barman et al., 2017). It is also a method of choice for removing inhibitors of translation or cDNA synthesis from RNA preparations and a number of modifications of protocols based on the CTAB, SDS and LiCl methods for RNA isolation from plants rich in polysaccharides have been reported (Verwoerd et al., 1989; Wanqian et al., 2005; Falcão et al., 2008; Kumar et al., 2011; Christou et al., 2014).

Dendrobium orchids contain high levels of polysaccharides in all tissues, particularly in mature organs (Xing et al., 2013). Thus, extensive modifications of the total RNA isolation protocol must be considered to obtain high yields of good quality RNA for quantitative gene expression analysis. Quantitative analysis of gene expression requires reliable reference genes for any specific condition, and there are many reference genes that have been validated for gene expression analysis in orchids with the most commonly used being: *Actin* (*ACT*), *Cyclophilin* (*CYP*), *Eukaryotic initiation factor* (*EIF*), *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), 18S ribosomal RNA (*18S*), *Elongation factor 1 alpha* (*EF1α*), *Gamma-secretase sub unit APH1-like* (*APH1L*) and *Cyclic nucleotide-gated cation channel protein* (*SAND*) (Yuan et al., 2014; An et al., 2016; Jiang et al., 2017). However, validation of suitable reference genes is still necessary for specific gene expression conditions, as several studies have reported that the expression levels of reference genes might vary across different organisms, tissues, developmental stages or experimental conditions (Vandesompele et al., 2002; Souček et al., 2017).

The objectives of the current study were to demonstrate alternative methods that are effective for total RNA extraction from *Dendrobium* Sonia 'Bom' floral tissues containing different levels of polysaccharides, and to demonstrate their applicability by validating reference genes for expression analysis of genes related to flower development.

Materials and Methods

Plant material

D. Sonia 'Bom' plants with inflorescence were obtained from commercial orchid farms in Thailand. Flowers were collected for total RNA isolation at different developmental stages from young flower buds to fully opened flowers (stage 1 = less than 2 cm, stage 3 = 2.8–3 cm, stage 4 = 3.3–3.5 cm, stage 7 = fully opened flower) according to Ratanasut et al. (2015).

Total RNA isolation

Samples (100 mg) of fresh *D*. Sonia 'Bom' petal or sepal tissues were powdered in liquid nitrogen and the total RNA was extracted using four different methods;

Method 1: LiCl-based method (modified from Verwoerd et al. (1989))

Powdered petal or sepal tissues were mixed with 650 µL extraction buffer (0.1 M Tris pH 8.0, 0.1 M LiCl, 10 mM ethylene-diamine-tetraacetic acid (EDTA), 1% (weight per volume; w/v) sodium dodecyl sulfate (SDS), 1% (w/v) Na_2SO_3). Phenol extraction was performed by adding 500 µL

phenol, followed by chloroform: isoamyl alcohol (24:1 volume per volume; v/v). RNA precipitation was carried out using an equal volume of different LiCl concentrations (4 M, 6 M, 8 M, 10 M or 12 M) at 4°C overnight. The RNA was collected using centrifugation at 12,470×g for 10 min and dissolved in 200 µL RNase-free water. The RNA solution was mixed with 2.5 volumes of absolute ethanol and 20 µL 2.5 M CH_3COONa (pH 6.0). The RNA was precipitated at -80°C for 30 min and recovered using centrifugation (12,470×g for 10 min at 4°C). The RNA pellets were washed with 70% (v/v) ethanol, air dried for 5–10 min and resuspended in 20–30 µL of RNase-free water.

Method 2: Hexadecyltrimethylammonium bromide-based method (modified from Doyle and Doyle (1987))

Powdered petal tissues were mixed with the buffer containing 2% (v/v) β-mercaptoethanol, 2% (w/v) hexadecyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl (pH 7.5), 20 mM EDTA, 2 M NaCl and 1% (w/v) polyvinylpyrrolidone (MW 40,000). The homogenate was incubated at 65°C for 15 min with occasional shaking. After centrifugation (12,470×g for 15 min at 4°C), chloroform: isoamyl alcohol (24:1, v/v) extraction was performed twice. The total RNA was precipitated by adding 0.25 volumes of 10 M LiCl and incubation at 4°C overnight and then centrifugation at 12,470×g for 15 min at 4°C. The RNA pellet was washed twice with 70% (v/v) ethanol, air-dried and then dissolved in 20 µL of RNase-free water.

Method 3: Spin column-based method

Powdered petal tissues were used for total RNA extraction using the RBC Total RNA Extraction Kit Mini (Plant) (RBC Bioscience; Taiwan) according to the manufacturer's instructions.

Method 4: RiboZol™ Reagent-based method

Powdered petal tissues were used for total RNA extraction using the Ribozol™ RNA Extraction Reagent (Amresco; USA) according to the manufacturer's instructions.

RNA analysis and cDNA synthesis

The total RNA quantity was determined by measuring the absorbance at 260 nm using NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific; USA). The RNA purity was determined by the ratios of absorbance at 260 nm to 280 nm ($A_{260/280}$) for protein contamination and at 260 nm to 230 nm ($A_{260/230}$) for polysaccharide contamination. The integrity of total RNA was analyzed by running samples in an agarose bleach gel (Aranda et al. 2012), modified by the addition of 1.5% (w/v) of agarose and 1% (v/v) Haiter bleach (6% sodium hypochlorite) (Kao Industrial; Thailand) and the gel was stained using RedSafe™ (iNtRON Biotechnology; Korea).

The extracted total RNA was treated with RNase-free DNase I (Thermo Fisher Scientific; USA) according to the manufacturer's instructions. Subsequently, first strand cDNA was reverse transcribed from 1 µg of DNase I-treated RNA using a qScript XLT cDNA SuperMix Kit (Quantabio; USA) according to the manufacturer's recommendation.

Extraction of crude polysaccharides

Crude polysaccharides were extracted using a minor modified method according to Obsuwan et al. (2019). Sepal and petal tissues were oven-dried at 60°C and then ground using a mortar and pestle. A sample (1 g) was extracted by adding 40 mL of distilled water and the mixture was incubated at 70°C for 1 hr with regularly vortexing every 2 min. The mixture was vacuum-filtered and then the residue was re-mixed with 70% ethanol and incubated at room temperature for 12 hr. The mixture was centrifuged at 2,656×g for 10 min and the sediment was washed again with 70% ethanol. Then, the final mixture was vacuum-filtered and the residue was air-dried before weight measurement. The percentage of the polysaccharide yield was calculated by dividing the weight of the crude polysaccharide by the weight of the initial sample.

Quantitative polymerase chain reaction amplification and expression stability analysis

Eight gene-specific primer pairs (for *ACT*, *CYP*, *EIF*, *GAPDH*, *18S*, *EF1α*, *APH1L* and *SAND*) were designed for evaluation of potential reference genes based on the sequences in the NCBI database. Quantitative polymerase chain reaction (qPCR) amplification was performed using the Maxima SYBR Green qPCR Master Mix Kit (Thermo Fisher Scientific; USA) according to the manufacturer's instructions; data were analyzed using the ECO™ Study software (PCRmax Limited; UK). PCR amplification efficiency was analyzed using a standard curve assay with 5-fold serial dilutions of cDNA. The amplification conditions were set up using uracil-DNA glycosylase incubation at 50°C for 2 min and polymerase activation at 95°C for 10 min, followed by 40 cycles of

denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. The specificity of primers was analyzed after the 40 cycles based on a melting curve analysis generated by increasing the temperature from 55°C to 95°C. All qPCR assays were carried out in three biological and three technical replications. The expression stability of candidate reference genes from petal and sepal tissues at stages 1, 4 and 7 were determined in this experiment. Raw cycle threshold (Ct) values of qPCR were analyzed using the RefFinder software (Xie et al., 2012).

Statistical analysis

All experiments were performed in a completely randomized design with at least three biological replicates. The collected data were subjected to one-way analysis of variance followed by a comparison of the mean using Duncan's multiple range test in the SPSS software package (version 16; SPSS Inc.; USA). Differences were considered significant at $p < 0.05$.

Results and Discussion

Total RNA yields from different extraction methods

The yields of total RNA isolated from the petal tissues varied with the flower developmental stage and the extraction method. Younger flower tissues provided relatively high total RNA yields with all extraction methods (Fig.1). The RiboZol™ Reagent-based method was highly effective for total RNA extraction from young flower tissues but was very ineffective for the fully opened flower stage. The CTAB-based method could be used for all flower stages but was significantly less effective than the LiCl-based method. The spin column-based method produced the lowest total RNA yields compared to the other extraction methods tested and did not yield any total RNA from the fully opened flower stage (Fig.1). Like the total RNA, the contents of the crude polysaccharides from the different flower stages were significantly different. The percentages of polysaccharides gradually increased in the older flowers. This result showed a negative correlation between the yields of total RNA and the polysaccharide contents. The RNA was harder to extract from samples containing higher polysaccharide contents. This was consistent with another study that have suggested that the high polysaccharide content of the mature flower causes the RNA to co-precipitate with the polysaccharide and clog the silica membrane of the spin column during the flow-through process (Barman et al., 2017). The current results showed that the LiCl-based method was the most effective method amongst the methods tested for isolating high RNA yields from petal tissues at all tested flower developmental stages, particularly for the fully opened flower, while the RiboZol™ Reagent-based method was highly effective for total RNA extraction from the petal tissues of the early flower stages. Therefore, the LiCl method was considered the method of choice for total RNA extraction from the mature flowers of *D.* Sonia 'Bom'.

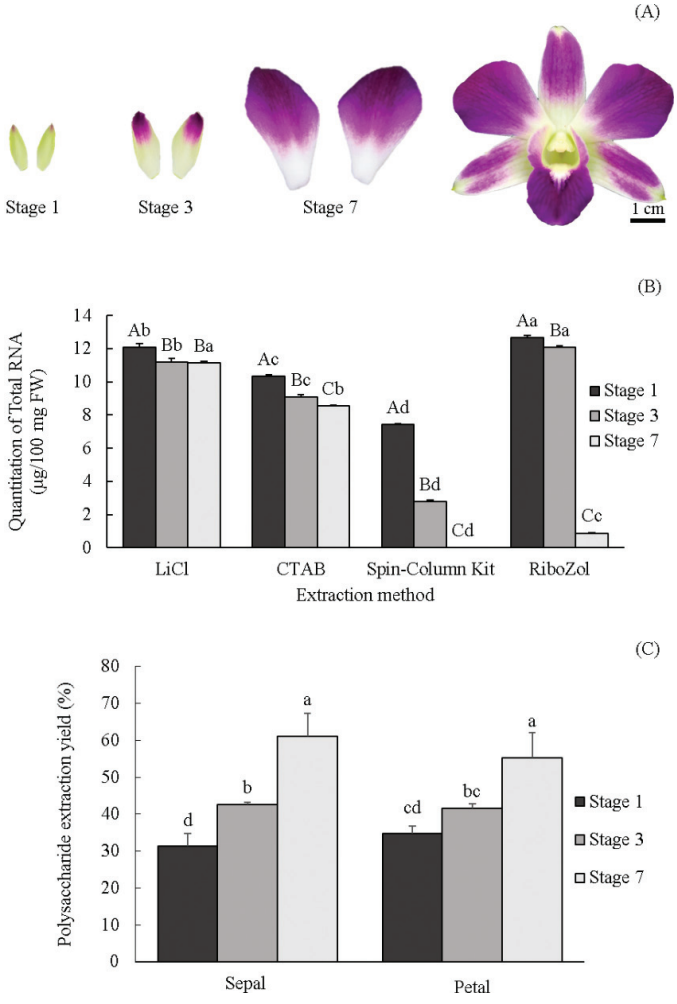

Fig. 1 *Dendrobium* Sonia 'Bom' floral tissues: (A) petals of flowers at three different developmental stages and fully opened flower; (B) total RNA yield from petal tissues at three different flower developmental stages using four different extraction methods, where same uppercase letter indicates non-significant difference between different stages in each method and same lowercase letter indicates non-significant difference between the same stage of different methods ($n = 3$; $p < 0.05$) and CTAB = cetyltrimethylammonium bromide; (C) crude polysaccharide yield from sepal and petal tissues at three different flower developmental stages, where same lowercase letter indicates non-significant difference ($n = 3$; $p < 0.05$)

Yields and qualities of total RNA precipitated with different LiCl concentrations in LiCl-based method

The use of different LiCl concentrations for RNA precipitation in the LiCl-based method has been reported for many plants including high secondary metabolite trees such as pine (Smart and Roden, 2010) as well as high polysaccharide plants such as brown seaweed (Sim et al., 2013) and yam (Barman et al., 2017). To determine the effect of LiCl concentration on the RNA yield and quality from floral tissues of the fully opened flowers rich in polysaccharides, LiCl concentrations at 4 M, 6 M, 8 M, 10 M or 12 M were used for the RNA precipitation step of the LiCl-based method. The total RNA quality was defined as the combination of RNA purity and RNA integrity (Imbeaud et al., 2005).

The yields of total RNA isolated from the sepal and petal tissue samples of the fully opened flowers varied in the ranges 5.36–7.56 µg/100 mg fresh weight (FW) and 3.94–8.29 µg/100 mg FW, respectively (Table 1). The yield of total RNA gradually increased with the addition of LiCl concentrations above 4 M before reaching a maximum yield at 10 M, above which the yield decreased. Contrary to the finding by Chan et al. (2004), the current results showed that higher concentrations of LiCl increased the total RNA yield, but did not increase RNA impurities. Although the original protocol of the LiCl-based method used 4 M LiCl for RNA precipitation (Verwoerd et al., 1989), RNA precipitation using 10 M LiCl maximized the yield of total RNA extracted from floral tissues of *D.* Sonia 'Bom'. This indicated that optimization of the LiCl concentration in the RNA precipitation step of the LiCl-based method is a critical point for maximizing the RNA yield of individual polysaccharide-rich tissues.

The purity of total RNA can be evaluated by the ratios $A_{260/A280}$ and $A_{260/A230}$. The ratio $A_{260/280}$ typically indicates protein contamination from proteins such as RNases, DNases and proteases. These proteins are major factors in the failure of RNA preparations in downstream applications, for example, cDNA synthesis will be unsuccessful in the presence of a protease because the reverse transcriptase can be degraded, while in the presence of a DNase the first-strand cDNA might be degraded. Theoretically, pure RNA has an $A_{260/280}$ ratio of 2, but values higher than 1.8 are generally considered as acceptable for many molecular protocols (Imbeaud et al., 2005; Udvardi et al., 2008; Die and Román, 2012). The current results had $A_{260/280}$ ratios in the range 1.97–2.13 (Table 1), indicating that the total RNA precipitated from all LiCl concentrations tested was relatively free of protein contamination and suitable for qPCR applications.

Another important RNA purity indicator is the $A_{260/230}$ ratio that can be used to determine the presence of organic contaminants such as polyphenols and polysaccharides. Polysaccharide-rich tissues such as the fully opened orchid flower tend to produce a viscous solution when mixed with extraction buffer due to the high mucilage and this causes inefficient mixing and low recovery of RNA in the supernatant after centrifugation (Khairul-Anuar et al., 2019). The addition of a phenol extraction step in the LiCl-based method improved this process, resulting in better RNA recovery compared to the other methods. However, this did not make the isolated RNA samples totally free from organic contaminants, as indicated by the $A_{260/230}$ ratio of samples which varied from 1.58 (considered as contaminated) up to 2.14 (considered as pure RNA), as shown in Table 1. A recalcitrant gel-like RNA pellet that was hard to dissolve occurred even when the $A_{260/230}$ ratio was higher than 1.8 in the preparations based on the fully opened flower, but it did not appear in preparations from the young flower buds even when the $A_{260/230}$ ratio was as low as 1.5 (data not shown). Since there is no clear consensus of the minimum requirement for the $A_{260/230}$ ratio in a qPCR application, an actual application test of this RNA on qPCR was considered necessary.

Table 1 Yield and purity of total RNA from sepals and petals of fully opened flowers precipitated in different concentrations of LiCl

LiCl concentration	Sample	RNA yield (µg/100g FW)	Absorbance ratio	
			$A_{260\ nm}/A_{280\ nm}$	$A_{260\ nm}/A_{230\ nm}$
4 M	Sepal	5.36±0.46^{bc}	2.05±0.05^{b}	1.58±0.06^{d}
	Petal	3.94±0.59^{d}	2.07±0.04^{ab}	1.63±0.14^{cd}
6 M	Sepal	5.73±0.48^{bc}	2.13±0.02^{a}	2.14±0.06^{a}
	Petal	5.56±1.04^{bc}	2.10±0.04^{ab}	1.90±0.16^{abc}
8 M	Sepal	6.27±1.00^{b}	2.07±0.09^{ab}	1.88±0.35^{abc}
	Petal	6.41±0.90^{b}	2.08±0.04^{ab}	1.83±0.23^{bcd}
10 M	Sepal	7.56±1.04^{a}	2.06±0.05^{ab}	1.90±0.15^{abc}
	Petal	8.29±0.96^{a}	2.02±0.10^{bc}	1.78±0.28^{bcd}
12 M	Sepal	5.49±1.29^{bc}	2.09±0.04^{ab}	1.98±0.16^{ab}
	Petal	4.86±0.44^{d}	1.97±0.04^{c}	1.64±0.13^{cd}

FW = fresh weight

Mean values ± SD in a column superscripted with different lowercase letter are significantly ($p < 0.05$) different ($n = 5$).

RNA integrity is one of the most important aspects of RNA quality assessment for qPCR applications. Since purity and integrity are unrelated measures, independent assessment of each aspect is required. In the current experiment, the RNA integrity was assessed using agarose bleach gel electrophoresis. This type of gel is less expensive and safer than a microfluid electrophoretic device or formaldehyde-based gels (Aranda et al., 2012). Even though the electrophoresis-based assay is less sensitive compared to newer methods, it is still the simplest and most widely available method for most laboratories.

The electrophoresis results showed a clear and visible separation of 18S and 28S rRNA without any major degradation both before and after the DNase I treatment used to eliminate genomic DNA contamination (Fig. 2). This result indicated good quality RNA for qPCR applications. With the assumption that degradation of RNA would happen equally in all types of RNA, assessment of rRNA integrity on the gel could show the relative mRNA integrity that later on would be converted to cDNA and measured using qPCR. However, the assessment through electrophoresis gels was subjective and was unable to detect the minor degradation of the samples, with an issue being the acceptable level of degradation.

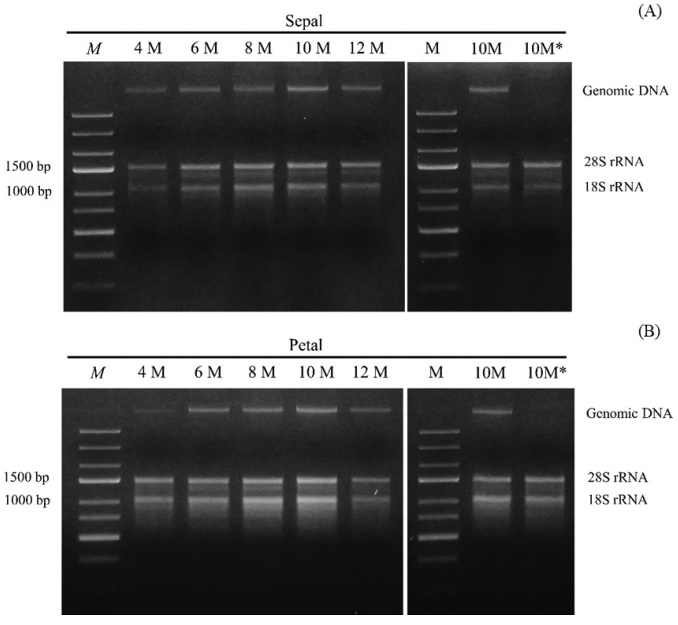

Fig. 2 RNA integrity of total RNA extracted from *Dendrobium* Sonia 'Bom' fully-opened flowers using the LiCl-based method: (A) sepals; (B) petals, where total RNA was precipitated with different LiCl concentrations (4 M, 6 M, 8 M, 10 M or 12 M), *M* = O'GeneRuler Express DNA ladder and * = purified RNA after DNase I treatment

Test of RNA purity for real-time reverse transcriptase quantitative polymerase chain reaction

To examine whether the total RNA extracted using the LiCl-based method had sufficient purity for RT-qPCR, eight candidate reference genes were selected and their expression stability during flower development was tested. A standard curve assay was conducted using 5-fold serial dilutions of an equal volume of cDNA samples from flower stages 1, 4 and 7. The amplification efficiencies (E) and the correlation coefficients (R^2) were 91.55–105.11% and 0.990–0.998, respectively (Table 2). These are acceptable conditions for qPCR applications which are recommended to have values for E and R^2 in the ranges 90–110% and 0.99–1, respectively (Taylor et al., 2011; Stephan et al., 2019).

The melting curve analysis of each candidate reference gene showed single peaks without any amplification detected from no template controls, indicating that specific PCR products were generated by each primer pair (Fig. 3A). The single expected band of each gene was also confirmed as shown in Fig. 3B. No PCR product was detected from amplification of the *18S* gene using DNase I-treated RNA as a template, indicating the complete digestion of genomic DNA (Fig. 3B).

Validation of reference genes for floral gene expression analysis

The expression profiles of the candidate reference genes for floral gene expression analysis in *D.* Sonia 'Bom' are shown in Table 2. Among all tested genes, *18S* was the most expressed, indicated by its lowest average Cq value (6.28) while *EIF*, *ACT*, *GAPDH* and *CYP* were moderately expressed with average Cq values in the range 20.67–23.96, while *APH1L*, *SAND* and *EF1α* were the least expressed with average Cq values in the range 26.71–27.50. The expression levels of reference genes were determined on the basis of cycle quantification (Cq) values, where Cq is defined as the cycle at which the fluorescence from amplification exceeds the threshold value (Freitas et al., 2017).

The expression stabilities of the candidate reference genes were determined using RefFinder, which is web-based software integrating the algorithm of the major computational software for validating reference genes, using four different software programs, namely comparative Delta Ct (Silver et al., 2006), BestKeeper (Pfaffl et al., 2004), Normfinder (Andersen et al., 2004) and geNorm (Vandesompele et al., 2002). Summarizing information from the relevant software

references above, these software programs calculate the expression stabilities in different ways as a consequence of the specific algorithm used and can generate different end results. The NormFinder algorithm calculates the overall variation of the candidate reference genes and the variation between sample subgroups of the sample set. The geNorm algorithm assumes that similar changes in the expression of two different genes represent technical differences rather than treatment-caused changes, thus taking genes with similar expression patterns into consideration. Furthermore, the BestKeeper algorithm is based on assessing the standard deviation and correlation coefficients of each individual reference gene with the geometric mean of all genes (Stephan et al., 2019). RefFinder specifies an appropriate weight to an individual gene based on ranking from each algorithm and calculates the geometric mean for overall final ranking (Xie et al., 2012). However, overall, there is no general agreement in the scientific community regarding the most appropriate algorithm for reference gene analysis.

For the eight candidate reference genes in this study, the RefFinder comprehensive ranking showed *ACT* as the most stable reference gene followed by *EF1α*, *CYP*, *EIF*, *SAND*, *18S*, *GAPDH* and *APH1L*, respectively (Table 3). The Comparative Delta Ct, Normfinder and geNorm results generated from RefFinder were comparable. The RefFinder comprehensive ranking showed two of the three most stable and least stable reference genes in a similar way. In contrast, *18S* (the least stable reference gene generated from these algorithms) became the most stable reference gene in BestKeeper (Table 3). Contrary results from BestKeeper to the other algorithms have also been reported by Paim et al. (2012) and Stephan et al. (2019).

Accurate normalization of RT-qPCR data requires the optimal number of reference genes, which were analyzed through the pairwise variations (V) calculated by the geNorm software. If the Vn/Vn+1 (n is the number of reference genes) value is below or equal to 0.15, the number of suitable reference genes is equal to n. The pairwise variation analysis showed that pairwise variation values for V3/4 were below the cut-off value (Fig. 4), indicating that the first three reference genes (*CYP*, *EF1α* and *ACT*) ranked by geNorm would be sufficient to normalize RT-qPCR measurements and the addition of a fourth reference gene would not improve the results substantially.

RT-qPCR is a very sensitive measurement procedure with the potential to detect the presence of a single template molecule. However, this sensitivity has no impact on the precision of the measurement without a proper normalization strategy. The goal of normalization is to minimize the technical variations as much as possible and to determine true biological variations. It has been suggested to use three or more validated reference genes (Derveaux et al., 2010), since the use of a single reference gene could lead to relatively large errors. Furthermore, the use of a single non-validated reference gene contributes to significant bias, ranging from 3-fold in 25% of the results up to 6-fold in 10% of the results (Vandesompele et al., 2002).

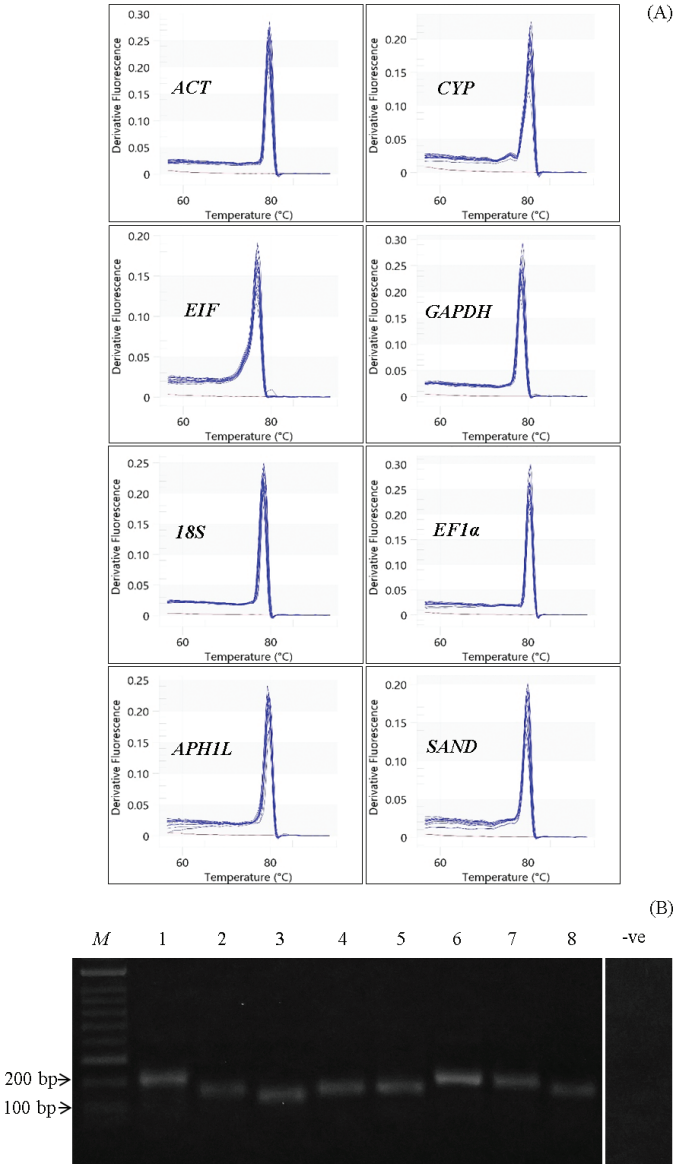

Fig. 3 Melting curve analysis of each candidate reference gene: (A) reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) melting curves of eight candidate reference genes. The single peak of the blue curve indicates single amplification of the gene and the black curve indicates no detection on the non-template control; (B) RT-qPCR products of each candidate reference gene, where *M* = Hyperladder™ 50 bp, Lanes 1–8 = *ACT*, *CYP*, *EIF*, *GAPDH*, *18S*, *EF1α*, *APH1L*, *SAND*, respectively, and -vc = *18S* amplification using DNase I-treated RNA as template

Table 2 Amplification efficiencies (E), correlation coefficients (R^2) and amplicon characteristics of candidate reference genes

Gene Symbol	Primer sequence (forward / reverse, 5'→3')	Amplicon length (bp)	Amplicon Tm (°C)	E (%)	R^2	Cq mean
ACT	CCACTACTGCAGAGCGTGAA / GCTGGAAAAGAACTTCTGGGC	189	79.3	99.15	0.998	21.24±1.21
CYP	CCAACGGGTCCCAGTTCTTC / TCCGTCCAGACGAAGATCCA	135	80.5	101.71	0.997	23.96±1.29
ELF	GTTGAAGAAAGAGTACAGTAAATC / CACCTTGGAGTTGAATGAC	116	76.9	91.64	0.997	20.67±1.18
GAPDH	CGATCCGTTCATCACCACTGA / GGTTCCTGACGCCAAAGACT	149	78.4	100.31	0.998	23.80±1.37
18S	GGGGGCATTCGTATTTCATA / TTTATGGTTGAGACTAGGACG	153	78.1	105.11	0.998	6.28±0.32
EF1α	CTCGATTGCCACACCTGTCAC / GACCGCAAACCTTCCAAGCG	198	80.2	104.62	0.990	27.50±1.21
APH1L	TGCCTGCGTACTTTGCATTG / GTACCAAAGGAGGGGCCAAA	181	79.6	98.23	0.990	26.71±0.96
SAND	TTGTAGTTGGGTGCGGGAAG / TCCATCAAGTTTGGAACCACCA	148	79.6	91.55	0.996	26.72±0.83

Amplicon Tm = temperature of 50% double-stranded DNA melted to a single chain.
Cq mean = mean ± SD of triplicate values in three biological replicates, with Cq threshold manually set up at optimal condition of each primer

Table 3 Rankings of reference genes generated by RefFinder

Rank Position	Comprehensive ranking	Delta Ct	BestKeeper	Normfinder	geNorm*
1	*ACT*	*ACT*	*18S*	*ACT*	*CYP* / *EF1α*
2	*EF1α*	*EF1α*	*SAND*	*EF1α*	–
3	CYP	CYP	APH1L	GAPDH	ACT
4	EIF	EIF	ACT	EIF	EIF
5	SAND	GAPDH	EIF	SAND	GAPDH
6	18S	SAND	EF1α	CYP	SAND
7	GAPDH	APH1L	CYP	APH1L	APH1L
8	APH1L	18S	GAPDH	18S	18S

* The geNorm software normally analyzes the two most stable genes as the first rank and the second rank will not be given.

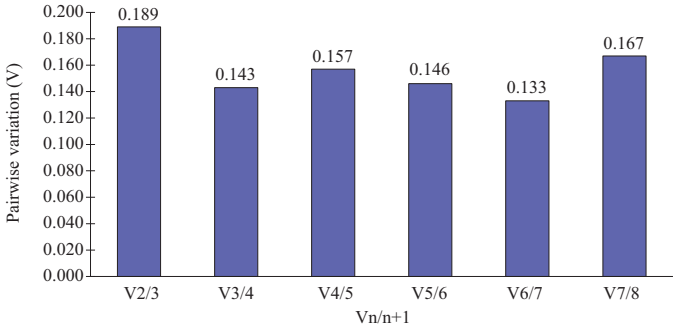

Fig. 4 Determination of optimal number of reference genes for accurate normalization based on geNorm pairwise variation analysis, where Vn/Vn+1 (n is the number of reference genes) values equal or lower than 0.15 indicate n as the optimal number of reference genes

In summary, the LiCl-based total RNA extraction method was the most effective RNA mini-prep for the fully opened flowers of *D.* Sonia 'Bom' which are rich in polysaccharides, whereas the RiboZol™ Reagent-based and spin column-based methods were comparatively ineffective. The LiCl concentration in the RNA precipitation step of the LiCl-based method directly affected the total RNA yield. Total RNA precipitated with 10 M LiCl was the optimal condition resulting in the highest total RNA yield. The A_{260}/A_{280} and A_{260}/A_{230} ratios of the total RNA extracted using the LiCl-based method were within the acceptable ranges for protein and polysaccharides contamination, respectively. The isolated RNA could serve as templates for reverse transcription, as indicated by good amplification efficiencies, correlation coefficients and melting curve analysis on the RT-qPCR assay for the eight candidate reference genes for expression analysis of the flower development related genes. The expression stabilities of the eight candidate reference genes calculated using the

RefFinder software showed *ACT* as the most stable reference gene, followed by *EF1α*, *CYP*, *EIF*, *SAND*, *18S*, *GAPDH* and *APH1L*, respectively. The pairwise variation analysis showed the first-three most stable reference genes would be sufficient to normalize RT-qPCR measurements.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was partially supported by research grant R2559C159, Naresuan University, Thailand. Professor Dr Duncan R. Smith (Institute of Molecular Biosciences, Mahidol University, Thailand) assisted with English editing and scientific proofreading of an earlier version of the manuscript.

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