Australian Journal of Crop Science AJCS
AJCS 7(7):911-916 (2013) ISSN:1835-2707

Isolation of high quality RNA from plant rich in flavonoids, *Melastoma decemfidum* Roxb ex. Jack

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Abstract

Chalcone synthase (CHS) is a plant-specific enzyme that synthesises naringenin chalcone, an essential precursor of the flavonoid biosynthetic pathway. Naringenin and kaempferol are two flavonoids that have been demonstrated to inhibit the proliferation of HeLa cells. To study chalcone synthase gene regulation in *Melastoma decemfidum*, we developed a high-yield total RNA isolation method to assemble a partial putative *CHS* cDNA sequence. Our results indicated that a modified CTAB method produced the highest total RNA yield (8.26±3.99 µg/gFW) compared to other methods. Thus, we used this method to isolate total RNA from different types of tissues from this plant. Our improved protocol produced high-quality total RNA from different tissues, including the mature leaf (7.02±2.60 µg/gFW), stem (4.27±1.72 µg/gFW), flower bud (37.54±10.61 µg/gFW), flower (21.31±5.20 µg/gFW), and root (3.38±1.89 µg/gFW). The total RNA was then converted into cDNA, and a putative *CHS* gene product (~1049 bp fragment) was amplified using degenerate primers. A partial *CHS* gene sequence shared 80% homology with an *Anthurium andraeanum CHS* gene sequence (AY232492) and 92% homology with the amino acid sequence of the *Acer maximowiczianum CHS* gene (AEK80412.1), as determined using BlastN and BlastX, respectively. This study shows that our modified CTAB method allows for the isolation of high-quality and high-yield total RNA from various tissues of *M. decemfidum.* A partial putative *CHS* gene was amplified,thus confirming that the modified CTAB method is suitable for RT-PCR and gene isolation.

Keywords: *Melastoma decemfidum*; chalcone synthase; flavonoid biosynthesis; RNA extraction; CTAB.
Abbreviations: *CHS*, chalcone synthase; C:I, chloroform:isoamyl alcohol (24:1, v/v); CTAB, hexacetyltrimethyl ammonium bromide; DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; LiCl, lithium chloride; NaOAc, sodium acetate; PVP-10, polyvinyl pyrrolidone (MW: 10,000); PVPP, polyvinyl polypyrrolidone.

Introduction

Melastoma decemfidum, popularly known as Rhododendron in local Malaysian communities, is commonly grown in waste sites and open fields. In traditional medicine, the leaves and roots of this plant have been used as remedies for diarrhea, dysentery, gastric ulcers, epilepsy and rheumatism (Lohezic-Le et al., 2002). Recently, four flavonoids (naringenin, kaempferol, kaempferol-3-*O*-D-glucoside and kaempferol-3-*O*-[2',6'-di-*O*-*p*-trans-coumaroyl] glucoside) were identified from the floral tissues of *M. decemfidum.* Naringenin and kaempferol-3-*O*-(2',6'-di-*O*-*p*-trans-coumaroyl) glucoside have been shown to inhibit the proliferation of MCF7, a human breast cancer cell line (Susanti et al., 2007). The chalcone synthase enzyme (CHS, EC.2.3.1.74) is a member of the CHS superfamily, also known as type III polyketide synthases, and is present in all plant species. CHS enzymes condense the phenylpropanoid CoA ester with three acetate units of malonyl-CoA molecules to produce naringenin chalcone (Tian et al., 2006; Koduri et al., 2010), which is an essential precursor in the phenylpropanoid biosynthetic pathway (Heller and Hahlbrock, 1980; Han et al., 2006). The induction of *CHS* gene expression results in the accumulation of flavonoid and isoflavonoid phytoalexins and can occur under stress conditions, such as exposure to UV light and bacterial or fungal infection (Schijlen et al., 2007; Dao et al., 2011). Several *CHS* gene sequences have been isolated recently from such species, including *Anthurium andraeanum* (AY232492.1), *Vitis vinifera* (FQ379425.1), *Rhododendron simsii* (AJ413277.1) and *Nelumbo nucifera* (FJ999627.1). High-quality RNA is essential to produce cDNA for gene expression studies, such as RT-PCR, qPCR, northern blot and microarray analyses (Noor Adila et al., 2007).Various methods are used to extract total RNA from plants; however, RNA extraction by acid guanidine thiocyanate is the most common technique (Wang et al., 2010). The following factors affect RNA extraction: enzymatic degradation by RNAse; the presence of high levels of phenolic compounds, polysaccharides and secondary metabolites (Kansal et al., 2008);the formation of phenol-quinone complexes that bind to nucleic acids; and the presence of polysaccharides that co-precipitate with RNA in low-ionic strength buffers (Birtic and Kranner, 2006; Manickavelu et al., 2007). Thus, the difference in the levels of polysaccharides, phenols and secondary metabolites in various plant tissues significantly alters the efficiency of nucleic acid extraction and purification procedures. Accordingly, a reliable RNA

Table 1. Quality and quantity of total RNA extracted by the modified CTAB method and other methods using different *M. decemfidum* tissues.

Method	Tissue	Absorbance ratio		RNA yield
		$A_{260/230}$	$A_{260/280}$	(µg/gFW)
Modified CTAB	Young leaf	2.13±0.04	2.16±0.09	8.26±3.99
	Mature leaf	1.86±0.18	1.98±0.24	7.02±2.60
	Stem	1.82±0.21	1.89±0.19	4.27±1.72
	Flower bud	1.99±0.04	2.04±0.09	37.54±10.61
	Flower	1.84±0.03	1.79±0.12	21.31±5.20
	Root	1.90±0.10	1.87±0.08	3.38±1.89
CTAB (Smart and Roden, 2010)	Young leaf	2.41±0.11	2.11±1.02	4.78±2.98
Sokolovsky et al.(1990)	Young leaf	1.54±0.03	1.93±0.12	2.57±0.25
Sharma et al.(2003)	Young leaf	1.69±0.17	0.33±0.09	1.07±0.50
Wang *et al.* (2010)	Young leaf	0.54±0.17	2.59±1.10	2.46±1.04
RNeasy Plant Mini Kit (Qiagen)	Young leaf	0.06±0.02	2.52±0.23	1.39±0.14

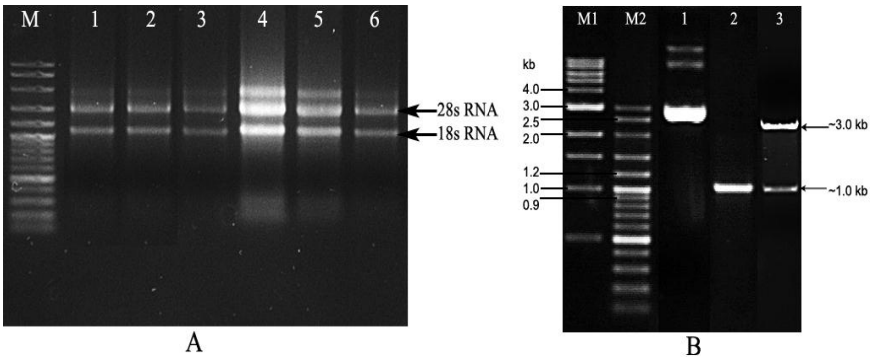

Fig 1. RNA extracted from various plant tissues. (A) RNA extracted from various plant tissues using our modified CTAB method was resolved on a 1.3% (w/v) agarose gel. Lane 1: young leaf, Lane 2: mature leaf, Lane 3: stem, Lane 4: flower bud, Lane 5: flower, Lane 6: root. (B) Recombinant plasmid, pGEM-T-*chs*, as resolved by 0.8% (w/v) gel electrophoresis. M1: 1 kb DNA ladder, M2: VC100bp Plus Ladder, Lane 1: undigested recombinant plasmid, Lane 2: positive control (RT-PCR product of the putative *CHS* gene), Lane 3: digested recombinant plasmid, pGEM-T-*chs*, using *Eco*RI produces two bands of 3.0 kb (pGEM-T Easy) and 1.0 kb (putative *CHS* gene). The arrows indicate the size of the vector backbone (~3.0 kb) and insert (~1.0 kb).

extraction method is the first step to study gene regulation in *M. decemfidum*. The total RNA must be of high quality, intact and pure, as contamination by other compounds can affect the downstream applications. To further understand the flavonoid biosynthetic pathway in *M. decemfidum*, we isolated a *CHS* gene using an RT-PCR method. However, isolation of total RNA is challenging due to the high flavonoid content in *M. decemfidum*. Many RNA isolation methods for plants with high levels of secondary metabolites have been published recently; whereas several of these protocols are species-specific or suitable for specific groups of plants (Sokolovsky et al., 1990; Yao et al., 2009), some have been reported to be applicable in a wide range of plant species and tissues (Sharma et al., 2003; Wang et al., 2008; Smart and Roden, 2010). However, not all of these methods have been successful in isolating high-quality RNA from *M. decemfidum*, which contains high levels of flavonoids, particularly in its leaves and roots. Thus, an efficient method for the isolation of high-quality total RNA from *M. decemfidum* tissues was developed through the comparison and manipulation of several published and commercially available RNA extraction methods. Modifications in the extraction buffer and incubation temperature in the hexacetyltrimethyl ammonium bromide (CTAB) method produced the desired result, and this modified CTAB method was then applied to various vegetative tissues of *M. decemfidum*. The total RNA obtained was then converted into cDNA for the amplification of the *CHS* gene using degenerate primers.

Results and Discussion

Quantity and quality of the total RNA

Melastoma decemfidum is a medicinal plant with a high content of secondary metabolites, including flavonoids, which are polyphenolic compounds that tend to contaminate nucleic acids during extraction (Wang et al., 2010). To overcome this challenge, several RNA extraction methods were evaluated to isolate high-quality and high-yield total RNA from *M. decemfidum*. Table 1 shows the yield and quality of the RNA extracted from young leaves using six different methods. The results indicated that our modified CTAB method produced a higher RNA yield (8.26±3.99 µg/gFW) and higher RNA purity compared to other evaluated methods. The method proposed by Sokolovsky et al. (1990) produced RNA that was free of polyphenolic compounds or polysaccharides (Asif et al., 2000; Wang et al., 2010); however, the RNA yield was very low (2.46±1.04 µg/gFW). In comparison, the RNA extracted using the methods of Sharma et al. (2003) and Wang et al. (2009) and the RNeasy Plant Mini Kit were contaminated with protein, polysaccharides and polyphenolic compounds; the absorbance ratios at $A_{260/280}$ and $A_{260/230}$ were less than 2.0. In this study, a few parameters, such as the incubation

Table 2. Sequence identity analysis of the putative *CHS* gene fragment relative to other sequences in GenBank using Blast.

Analysis	Accession no.	Source	Identity	Total score	Max indent	Putative function of the gene
BlastN	FJ999634.1	*Nelumbo nucifera*	810/1049	913	91%	1. A key enzyme involved in flavonoids biosynthetic pathway. 2. Variation in expression of the *CHS* gene might affect the colour of the flowers and the berry skin due to the different contents of flavonoids (Goto-Yamamoto et al., 2002). a. e.g.,*N. nucifera, A. andraeanum, V. vinifera, A. manihot, C. chekiangoleosa*. 3. Flavonoids are involved in pigmentation, plant pathogen defence and protection against UV light (Hahlbrock 1981). Hence, the expression of the gene encoding *CHS* gene can easily be monitored by colour formation in various tissues including leaves, flowers, fruits and berry. The expression level can also differ under stress conditions (Niesbach-Klosgen, 1987).
	AY232492.1	*Anthurium andraeanum*	842/1049	958	80%	
	AF315345.1	*Hypericum androsaemum*	839/1049	949	80%	
	XM002263983.1	*Vitis vinifera*	830/1049	904	79%	
BlastX	AEK80412.1	*Acer maximowiczianum*	323/349	660	92%	
	ACE60221.1	*Abelmoschus manihot*	322/349	655	92%	
	XP002264019.1	*Vitis vinifera*	322/349	653	92%	
	ADW11243.1	*Camelia chekiangoleosa*	322/349	653	92%	

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1     P  D  Y  Y  F  R  I  T  N  S  E  H  K  A  E  L  K  E  K  F  Q  R  M  C  D  K  S
1    CCGGACTACTACTTCCGGATCACCAACAGCGAGCACAAGGCCGAGCTCAAGGAGAAGTTCCAGCGCATGTGCGATAAGTCC
28    M  I  K  K  R  Y  M  Y  L  T  E  E  I  L  K  E  N  P  N  V  C  A  C  E  A  P  S
82   ATGATCAAGAAGAGGTATATGTACTTGACAGAGGAGATCCTGAAGGAGAACCCGAATGTCTGTGCTTGTGAGGCCCCGTCG
55    L  D  A  R  Q  D  M  V  V  V  E  V  P  K  L  G  K  E  A  A  A  K  A  I  K  E  W
163  CTGGATGCGAGGCAGGATATGGTCGTGGTTGAGGTCCCAAAACTCGGCAAGGAAGCCGCCGCCAAGGCTATCAAGGAGTGG
82    G  Q  P  K  S  K  I  T  H  V  V  F  C  T  T  S  G  V  D  M  P  G  A  D  Y  Q  L
244  GGTCAGCCTAAGTCCAAGATCACCCATGTTGTCTTCTGCACGACCAGTGGGGTTGACATGCCTGGCGCGGATTACCAGCTC
109   T  K  L  L  G  L  R  P  S  V  K  R  L  M  M  Y  Q  Q  G  C  F  A  G  G  T  V  L
325  ACCAAGCTCCTCGGTCTTCGTCCCTCTGTGAAGCGTCTCATGATGTACCAGCAGGGCTGTTTTGCTGGAGGCACGGTCCTC
136   R  V  A  K  D  L  A  E  N  N  K  G  A  R  V  L  V  V  C  S  E  I  T  A  V  T  F
406  CGTGTCGCCAAGGACCTCGCTGAGAACAACAAGGGGGCTCGCGTCCTCGTCGTCTGCTCCGAAATCACTGCTGTCACCTTC
163   R  G  P  S  E  S  H  L  D  S  L  V  G  Q  A  L  F  G  D  G  A  G  A  I  I  M  G
487  CGAGGCCCTAGCGAGAGCCACCTTGACAGCCTCGTCGGCCAGGCTCTGTTTGGAGATGGTGCCGGCGCCATCATCATGGGA
190   S  D  P  I  P  G  V  E  K  P  M  F  E  L  V  S  A  A  Q  T  I  L  P  D  S  D  G
568  TCTGACCCCATCCCGGGGGTCGAGAAGCCCATGTTCGAGCTCGTTTCCGCTGCCCAGACCATCCTCCCTGACTCCGATGGT
217   A  I  D  G  H  L  R  E  V  G  L  T  F  H  L  L  K  D  V  P  G  L  I  S  K  N  I
649  GCCATAGATGGCCACCTCCGTGAAGTCGGCCTGACCTTCCACCTCCTGAAAGACGTTCCCGGGCTCATCTCCAAGAACATC
244   E  K  S  L  V  E  A  F  Q  P  L  G  I  S  D  W  N  S  I  F  W  I  A  H  P  G  G
730  GAGAAGAGCCTCGTCGAGGCCTTCCAGCCGCTCGGCATCTCGGACTGGAACTCCATCTTCTGGATTGCTCATCCCGGCGGA
271   P  A  I  L  D  Q  V  E  E  K  L  G  L  K  P  E  K  M  R  A  T  R  Q  V  L  S  D
811  CCGGCCATCCTCGACCAGGTGGAGGAGAAACTCGGCCTCAAGCCCGAGAAGATGCGGGGCCACCAGGCAAGTGCTCAGCGAC
298   Y  G  N  M  S  S  A  C  V  L  F  I  L  D  E  M  R  R  S  S  K  Q  N  G  F  K  T
892  TACGGGAACATGTCGAGCGCCTGCGTCCTGTTCATCCTGGACGAGATGAGGAGGAGCTCCAAGCAGAACGGTTTCAAGACA
325   T  G  E  G  L  E  W  G  V  L  F  G  F  G  P  G  L  T  V  E  T  V  V  L  H
973  ACAGGGGAAGGGCTCGAATGGGGGGTCCTGTTCGGTTTCGGTCCGGGGCTTACCGTTGAGACTGTTGTCCTCCACAG
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Fig 2. Gene sequence of the putative *CHS* gene fragment isolated from *Melastoma decemfidum* Roxb. (ex. Jack leaf).

temperature, centrifugation period and precipitation method, were optimised. The incubation temperature was increased from 42°C to 65°C to increase the efficacy of cell wall disruption, and the incubation time was shortened from 90 min to 25 min. The use of PVPP in the extraction buffer often demonstrated a moderate effect and required multiple extraction steps, resulting in a correspondingly low yield of nucleic acids (Japelaghi et al., 2011). The substitution of soluble PVP-10 for the PVPP allowed soluble PVP to bind competitively to the polyphenolic compounds that often co-precipitated during extraction, thereby affecting both the quality and quantity of the isolated nucleic acids (Salzman et al., 1999; Asif et al., 2000; Jaiprakash et al., 2003). The PVP-bound polyphenolic compound was then easily eliminated using LiCl and isopropanol precipitation. Based on these results, the modified CTAB method was later adopted to isolate total RNA from different tissues of *M. decemfidum* (Fig 1-A). The total RNA concentration obtained from flower buds (37.54±10.61 µg/gFW) was higher than that obtained from young leaves (8.26±3.99 µg/gFW), mature leaves (7.02±2.60 µg/gFW), stems (4.27±1.72 µg/gFW), flower (21.31±5.20 µg/gFW), and roots (3.38±1.89 µg/gFW). Pure RNA will exhibit $A_{260/280}$ ratios of 2.0±0.1 and $A_{260/230}$ ratios between 2.0 to 2.4; an $A_{260/280}$ ratio below 2.0 indicates protein contamination, whereas an $A_{260/230}$ ratio less than 2.0 indicates contamination by organic compounds or polysaccharides (Farrell, 2010). The ribosomal RNA isolated from the different plant organs is shown in Fig 1-A. Variation in the intensity of ribosomal RNA bands was due to the different total RNA concentrations. Our improved CTAB protocol demonstrated that the interfering molecules (including polyphenols, polysaccharides, protein and salts) were efficiently eliminated, thereby producing a high yield of total RNA compared to the other methods (Sharma et al., 2003; Sokolovsky et al., 1990; Wang et al., 2009) and the commercial kit.

Amplification of a partial putative *CHS* gene

To confirm the applicability of the methods, the cDNA generated was later used to identify the putative *CHS* gene. The total RNA from young and mature leaf tissues was treated with DNase I (Fermentas) to eliminate any genomic DNA contamination. The total RNA was used as a template for RT-PCR amplification of the putative *CHS* gene using degenerate primers. As shown in Fig 1-B, the RT-PCR amplification produced cDNA fragments with an approximate size of 1049 bp. The cDNA fragments were excised from the gel, purified, cloned into the pGEM-T Easy vector (Promega) and sequenced. Fig 1-B shows two bands at 3.0 kb and 1.0 kb that were obtained from the *Eco*RI digestion of the plasmid; the ~3.0 kb and ~1.0 kb bands represent the vector backbone and *CHS* gene fragment, respectively.

Sequence analysis of the putative *CHS* gene

Sequence analysis showed that the DNA fragment obtained was a partial *CHS* gene (1049 bp) (Fig 2). The average sizes of other *CHS* gene deposited in GenBank were between 1.0 kb - 1.5 kb, for example, *Arabidopsis thaliana* (1188 bp), *Populus trichocarpa* (*chs*5, 1176 bp; *chs*7, 1014 bp), *Vitis vinifera* (1182 bp) and *Ricinus communis* (1164 bp). The partial *CHS* gene had a high similarity (up to 91%) with the other *CHS* gene in GenBank when analysed by BlastN (Table 2). Interestingly, the amino acid identity of the sequence (BlastX) was also high, up to 92%.

Materials and methods

Plant materials

All of the *M. decemfidum* plants were grown and maintained in a greenhouse at the Faculty of Biosciences and Medical Engineering, UTM. We used six different plant tissues of *M. decemfidum* for the RNA extraction: young leaf (third leaf of the main shoot), mature leaf (fourth leaf of the main shoot), stem, flower bud, flower and root.

RNA extraction

Modified CTAB method

The CTAB-based method used in this study was a slight modification of the previous method reported by Smart and Roden (2010). The fine tissue powder was suspended in 1 mL of pre-warmed extraction buffer (65°C) (100 mM Tris (pH 8.0), 2% [w/v] CTAB, 30 mM ethylenediaminetetraacetic acid [EDTA], 2 M NaCl, 2% polyvinyl pyrrolidone [PVP-10], and 2% β-mercaptoethanol [addedfresh]) and mixed thoroughly. The mixture was then incubated at 65°C for 25 min with vortex for every 5 min. After the mixture was chilled on ice, an equal volume of C:I was added. The mixture was shaken gently until the two phases were fully emulsified, followed by centrifugation at 12 000×g for 5 min at 4°C. The aqueous phase was then transferred into a new microcentrifuge tube, the extraction was repeated using an equal volume of C:I, and the resulting solution was re-centrifuged. The aqueous solution was collected and precipitated with 0.1 volume of 3 M NaOAc (pH 5.2) and an equal volume of isopropanol at -80°C for 45 min. The tube was inverted several times to mix the solution. After centrifugation for 30 min, the pellet was dissolved in 200 µL of DEPC-treated water. LiCl (8 M) was added to the RNA-containing solution to a final concentration of 2 M before incubation at 4°C overnight. The RNA pellet was recovered by a 30 min centrifugation and washed twice with 1 mL of chilled 70% ethanol. The pellet was air-dried and dissolved in 20 µL DEPC-treated water and stored at -80°C for further analysis. The deviations from the published method (Smart and Roden, 2010) included the replacement of the PVPP with soluble PVP-10 in the extraction buffer, a higher incubation temperature (65°C instead of 42°C), a shorter centrifugation period (25 min instead of 90 min) and the use of isopropanol and NaOAc for only 45 min at -80°C followed by 2 M LiCl overnight to precipitate the RNA; the two precipitation steps were introduced to increase the total RNA yield. The RNA pellet was then washed with 1 mL 70% EtOH twice to eliminate the isopropanol and salt residues. Lastly, the duration of the centrifugation was shorter for the extraction stage (5 min instead of 15 min) but longer for the precipitation (30 min instead of 20 min) to increase the precipitation efficiency.

Other RNA isolation methods

The RNA was also isolated using different extraction methods: the RNeasy Plant Mini Kit (Qiagen, guanidine thiocyanate based); Sokolovsky et al. (1990) (SDS based); Sharma et al. (2003) (guanidine hydrochloride based); and Wang et al. (2010) (guanidine isothiocyanate based). All of the RNA extraction methods were performed according to the manufacturer's instructions or previously published reports.

Quantitative and qualitative analyses

The quantity and quality of the total RNA extracted was measured at a specific optical density using a NanoDrop1000 Spectrophotometer V3.7. An aliquot (5 µL) of each total RNA sample was examined by electrophoresis (85 V, 40 min, 240 mA) on a 1.3% (w/v) agarose gel and stained with EtBr, followed by visualisation under UV light to assess the integrity of the ribosomal RNA bands.

cDNA synthesis and RT-PCR

The isolated RNA was treated with DNAse I (Fermentas) prior to the RT-PCR with the Sensiscript RT Kit (Qiagen); the DNase treatment and cDNA synthesis procedures were performed according to the manufacturer's instructions. The RT-PCR was performed using a pair of degenerate primers that were designed based on the conserved sequence of the *CHS* gene from six different plants. The sequences of the degenerate CHS primers were: dCHS-F, 5'-CCKGACTACTACTTCCGSATC-3' and dCHS-R, 5'-CTGTGSAGGACRACAGTCTC-3'. The thermocycling conditions were 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min. A final extension at 72°C for 5 min was included after the 30th cycle. The amplification of the PCR products was analysed by electrophoresis using a 1% (w/v) agarose gel stained with EtBr and visualised under UV light.

Cloning the PCR products into pGEM-T Easy vectors and sequence analysis

The amplified putative *CHS* gene fragment was cloned into the pGEM-T Easy Vector (Promega) according to manufacturer's instructions. The plasmid was then transformed into competent *Escherichia coli* strain DH5α cells. Positive clones were collected, and the recombinant plasmid was digested with *Eco*RI and sequenced (FirstBase Laboratories Sdn Bhd). A homology analysis of the putative *CHS* gene with other plant genes was performed using bioinformatics software available in publicly accessible databases. The sequence was used as a query in a Basic local alignment search tool (Blast) sequence analysis.

Conclusion

In conclusion, our modified CTAB method offers a high-yield and high-quality extraction of total RNA from various tissues of *M. decemfidum.* The total RNA extracted is suitable for molecular applications, such as RT-PCR and cloning. Our sequence analysis indicates that the fragment cloned was indeed a partial sequence of the *CHS* gene. For further characterisation of this partial *CHS* gene of *M. decemfidum*, the presence of the *CHS* gene in chromosomal DNA will be confirmed using Southern blot analysis, and real-time qRT-PCR will be used to analyse the expression.

Acknowledgements

This research was funded by the Fundamental Research Grant Scheme (Vote 3F597). The Ministry of Higher Education, Malaysia and Universiti Teknologi Malaysia is gratefully acknowledged.

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