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Original Article

Comparison of Different Methods for RNA Extraction from Floral Buds of Tree Peony (*Paeonia suffruticosa* Andr.)

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Abstract

Tree peony (*Paeonia suffruticosa* Andr.), a species native to China, is one of the most important ornamental and medicinal plants. Like other tree species in temperate and boreal zones, the dormancy-activity transition of floral buds is critical for blooming time and fruit production. However, floral buds contain high levels of secondary metabolites, making the isolation of high quality RNA difficult. To obtain a method suitable for extracting RNA from floral buds of tree peony, we evaluated five different methods, including the cetyltrimethylammonium bromide (CTAB) and Sodium dodecyl sulfate (SDS)-based methods, a modified SDS-TRNzol protocol, and two commercial kits (TRNzol and Qiagen RNeasy Plant Mini Kit). The modified SDS-TRNzol method was capable of efficiently removing polyphenols and other metabolites in floral buds. The isolated RNA was of high purity and integrity, as demonstrated by the the $A_{260/280}$ ratio of approximately 2.0, and RIN values of more than 9.0. Gel electrophoresis analysis indicated that the extracted RNA had clear 28S and 18S ribosomal RNA bands without DNA contamination. The RNA isolated by this protocol was successfully used for downstream manipulations, such as RT-PCR, RACE, and real-time PCR. Together, the modified SDS-TRNzol protocol is an easy, efficient, and highly reproducible method for RNA isolation from floral buds rich in secondary metabolites.

Keywords: expression analysis; RNA isolation; secondary metabolites

Introduction

The floral industry has comprised an important part of the global economy, featured as cultural and high-added values. Tree peony (*Paeonia suffruticosa* Andr.) is a species native to China, being one of the most important ornamental and medicinal plants worldwide (Wister, 1995). Like other tree species native to temperate and boreal zones, the dormancy-activity transition of floral buds is important for blooming time and fruit production (Cooke *et al.,* 2012). The dormancy of floral buds is a major bottleneck for the seasonal flowering of tree peony. Technically, the dormant buds can be effectively induced to bloom by chilling treatment (Horvath, 2009). However, the mechanism underlying the dormancy-activity transition of floral buds by chilling treatment is largely unknown. Thus, it is attractive and important to investigate the developmental profiles of floral buds at molecular level.

The extraction of high quality RNA is a prerequisite step for biological research at transcriptional level. However, floral buds often contain high levels of carbohydrates, anthocyanidin, polyphenols, hormones, and other secondary metabolites (Mornya *et al.,* 2011; Li *et al.,* 2012). These rigid and complicated compounds make the isolation of high quality RNA from floral buds extremely difficult. So far, a bunch of different RNA extraction protocols have been developed to isolate intact RNA from samples with abundant polysaccharides, polyphenols and secondary metabolites. To obtain a method suitable for extracting high quality RNA from secondary metabolites-rich floral buds of tree peony, we evaluated five different methods, including the cetyltrimethylammonium bromide (CTAB) (Chang *et al.,* 1993) and Sodium dodecyl sulfate (SDS)-based methods (Zhou *et al.,* 1999; Wu *et al.,* 2006), a modified SDS-TRNzol protocol (Wang *et al.,* 2012), and two commercial kits (TRNzol and Qiagen RNeasy Plant Mini Kit). Here, we present an effective and rapid protocol based on the combination of SDS and TRNzol, allowing isolating high quality total RNA from floral buds of tree peony.

Materials and Methods

Plant material

Tree Peony (*Paeonia suffruticosa* Andr.) cultivar 'Fengdan'

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plants were cultivated in the field in Shanghai Botanical Garden, Shanghai, China. Floral buds undergoing the dormancy-activity transition were collected at the end of November. After cutting, the buds were harvested and frozen in liquid nitrogen, and stored at -80 °C for RNA isolation.

Reagents and solutions

All reagents used in this study were of analytic purity. Plastic materials (tubes and pipette tips) were pretreated overnight in 0.1% diethyl pyrocarbonate (DEPC) water and then autoclaved. The mortar, pestle and ironware were baked for 3-6 h at 180 °C. RNeasy Plant Mini Kit (Qiagen, Germany), TRNzol (TIANGEN, Beijing, China), Phenol solution (Sigma, P4682, Saturated with 0.1 M citrate buffer, pH 4.3 ± 0.2). The following buffers and solutions were prepared:

- CTAB extraction buffer: 2% (w/v) CTAB, 100 mM Tris-HCl (pH8.0), 25 mM EDTA (pH8.0), 2% (w/v) polyvinyl pyrrolidone (PVP), 2 M NaCl, add 1% (v/v) β-mercaptoethanol before use.
- SSTE washing buffer: 10 mM Tris-HCl (pH8.0), 1 mM EDTA (pH8.0), 0.5% (w/v) SDS, 1 M NaCl.
- SDS extraction buffer: 100 mM Tris-HCl (pH 9.0), add 2% β-mercaptoethanol (v/v) before use.
- 20% SDS (Sodium dodecyl sulfate) (w/v), add 0.1% DEPC, autoclave and store at room temperature.

Protocols

The following protocols were used:

◆ CTAB method (adapted from Chang *et al.,* 1993 with modifications)

(1) Floral buds were ground into fine power in liquid nitrogen by using mortar and pestle.
(2) A volume of 6 mL CTAB extraction buffer was added to the powder (1 g) in an RNase-free 15 mL tube; the sample was mixed thoroughly using a vortexer and incubated at 65 °C for 15 min with occasional inversion.
(3) An equal volume of chloroform: isoamyl alcohol (24:1, v/v) was added and mixed thoroughly; the mixture was centrifuged at 12,000 rpm for 10 min at room temperature.
(4) The supernatant was transferred to a new 15 mL tube and then repeated step (3).
(5) The aqueous phase was carefully transferred to a fresh tube, 1/3 volume of 8 M LiCl (2 mL) was added, mixed gently by inversion and incubated at 4 °C for 8 h.
(6) After centrifugation at 12, 000 rpm for 20 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 0.5 mL SSTE buffer.
(7) The sample were transferred into a new 1.5 mL tube, an equal volume of phenol: chloroform: isoamyl alcohol (25: 24:1) was added and mixed thoroughly.
(8) The mixture was centrifuged at 12. 000 rpm for 10 min at 4 °C, the supernatant was collected in a new 1.5 mL tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly.
(9) After centrifugation at 12, 000 rpm for 10 min at 4 °C, the supernatant was transferred into a fresh 1.5 mL tube, 1/10 volume of 3 M sodium acetate (pH 4.8) and 2 volume of ice-cooled ethanol was added and mixed by inversion several times, and precipitated at -80 °C for 30 min.
(10) After centrifugation at 12, 000 rpm for 20 min at 4 °C, the supernatant was removed using a pipette and 500 μL of pre-cooled 70% ethanol was added.
(11) The sample was centrifuged at 12,000 rpm for 5 min at 4 °C, the supernatant was discarded and the pellet was allowed to dry at room temperature for 10 min.
(12) An appropriate volume of DEPC-treated H_2O was used to dissolve the pellet, and then stored at -80 °C for further use.

◆ *SDS method* (according to Zhou *et al.,* 1999; Wu *et al.,* 2006)

(1) The powder (1 g) was suspended into 6 mL SDS extraction buffer, mixed thoroughly using a vortexer and incubated at room temperature for 15 min with occasional inversion.
(2) A volume of 300 μL 20% SDS was added into the mixture, and inverted gently for 5-8 times and incubated at room temperature for 5 min.
(3) After centrifugation at 12, 000 rpm for 10 min at 4 °C, the supernatant was transferred into a new tube and 1/3 volume of 8 M LiCl (2 mL) was added, mixed gently by inversion and incubated at 4 °C for 8 h.
(4) The following steps are the same as the CTAB method step 6-12.

◆ *SDS-TRNzol method* (adapted from Wang *et al.,* 2012)

(1) Approximately 0.2 g powder was suspended into 0.4 mL SDS extraction buffer, mixed thoroughly and incubated at room temperature for 15 min with occasional inversion.
(2) A volume of 20 μL 20% SDS was added into the suspension inverted gently for 5-8 times and incubated at room temperature for 5 min.
(3) After centrifugation at 12, 000 rpm for 10 min at 4 °C, the supernatant was transferred into a new tube and 2 volume of TRNzol (0.8 mL) was added, mixed thoroughly and incubated at room temperature for 10 min.
(4) 1/5 volume of chloroform (240 μL) was added and mixed thoroughly, then centrifuged at 12, 000 rpm for 10 min at 4 °C.
(5) The supernatant (0.7 mL) was carefully transferred to a fresh tube, an equal volume of isopropanol was added, and mixed by inversion several times, and precipitated at -20 °C for 20 min.
(6) The following steps are the same as the CTAB method step 6-12.

◆ *TRNzol method (TIANGEN, Beijing, China)*

(1) Approximately 0.4 g powder was suspended into 1 mL TRNzol, mixed thoroughly and incubated at room temperature for 10 min.
(2) 1/5 volume of chloroform (200 μL) was added and mixed thoroughly, then centrifuged at 12, 000 rpm for 10 min at 4 °C.
(3) The supernatant (0.5 mL) was carefully transferred to a fresh tube, an equal volume of isopropanol was added, and mixed by inversion several times, and precipitated at room temperature for 10 min.
(4) The following steps are the same as the CTAB method step 10-12.

◆ *RNeasy Plant Mini Kit (Qiagen, Germany)*

(1) Approximately 0.1 g powder was suspended into 450 μl

Buffer RLT or Buffer RLC 1 mL TRNzol, mixed thoroughly and transferred the lysate to a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at full speed.

(2) The supernatant of the flow-through was carefully transferred to a new microcentrifuge tube, 1/2 volume of ethanol (96–100%) was added to the cleared lysate, and mix immediately by pipetting, and the mixture was transferred to an RNeasy spin column placed in a 2 ml collection tube, and centrifuged for 15 s at ≥8000 g (or ≥10,000 rpm).

(3) A volume of 700 µl Buffer RW1 was added to the RNeasy spin column, and centrifuged for 15 s at ≥8000 g (≥10,000 rpm) to wash the spin column membrane.

(4) An volume of 500 µl Buffer RPE was added to the RNeasy spin column, and centrifuged for 15 s at ≥8000 g (≥10,000 rpm) to wash the spin column membrane, then repeated this step.

(5) The RNeasy spin column was placed in a new 1.5 ml collection tube, and an appropriate volume of DEPC-treated H_2O was used to dissolve the pellet, and then stored at -80 °C for further use.

Evaluation of RNA quantity and quality

The quantity and purity of the isolated total RNA was determined through the ratios of $A_{260/280}$ and $A_{260/230}$, using the NanoDrop™ 1000 spectrophotometer (Thermo Scientific). RNA integrity was evaluated by electrophoresis on a 1.0% native agarose gel at 5 V/cm, with EtBr (Ethidium Bromide) staining. RIN (RNA integrity number) values were calculated using the Eukaryote Total RNA Nano assay on an Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip (Agilent Technologies, CA, USA).

Reverse transcription and downstream applications

The remnant amount of DNA contamination was removed by using RNase-free DNase I (Takara) treatment. For RT-PCR, first strand cDNA was synthesized using 2 µg total RNA with oligo $d(T)_{18}$ primer using AMV reverse transcriptase XL (Takara). The PCR reaction of 20 µl contained 2.0 µl 10×PCR buffer (with Mg^{2+}), 0.4 µl 10 mM dNTP mixture, 0.4 µl 10 µM each degenerated primers of *CYCLOIDEA*, a TCP transcriptional factor (forward primer: 5'-AAAGAYCGGCACAGCAARAT-3'; reverse: 5'-CCTTTCCCTYGCYCTYGCYCTYGC-3'), 0.2 µl Taq polymerase (2.5 U/µl, TIANGEN, China), and 1.0 µl 10-fold diluted cDNA. The cycling conditions were as follows: predenatutation at 94 °C for 5 min, then 35 cycles at 94 °C for 20 s, annealing at 56 °C for 20 s and extension at 72 °C for 30 s, and a final extension for 10 min at 72 °C. The PCR product was analyzed on 1% (w/v) agarose gel electrophoresis.

The 5′ RLM-RACE of Tree Peony *APETALA1* gene was obtained using the FirstChoice® RLM-RACE Kit (Ambion) with the following primer (5'-TTGGAGTATTCTAGGGACCAGTTT-3'), according to the manufacturer's instructions. The opening reading frame (ORF) of the *PsAP1* was amplified using KOD Plus Polymerase (Toyobo), with the primers AP1-F (5′-ATGGGAAGAGGCAGGGTTCA-3′) and AP1-R (5′-TGCACCAAAGCACCCAAGGT-3′).

Results and Discussion

In perennial woody plants, floral bud initials generate between the end of July and middle of September, and continue to grow till the first chilly days. The differentiation of floral buds is the most critical biological process to anthophyta, which is accompanied with extensive gene expression shift (Horvath 2009). The extraction of high-quality RNA is the first step to perform such kind assay. Floral buds are rich in polysaccharides, polyphenols, and other secondary metabolites, which thus interfere with RNA extraction and downstream applications.

The method based on Trizol has been widely used for RNA isolation from various tissues and species (Kalinowska *et al.,* 2012). We first attempted to use TRNzol reagent to isolate RNA from floral buds of tree peony. However, the TRNzol buffer could not effectively remove bud pigments, making the aqueous phase red after chloroform purification. Moreover, the polyphenols abundant in floral buds were readily oxidized to quinones that could tightly bind proteins and nucleic acids and finally result in brown and insoluble RNA pellets (Muoki *et al.,* 2012). As shown in Fig. 1 (lane 1 and 2), only the smeared 5.8 S rRNA band could be detected in the well. Thus, the extracted RNA using the TRNzol method is unsuitable for downstream analysis.

RNeasy Plant kit is designed specifically to extract RNA from plant tissues rich in secondary metabolites, such as conifer needles. Indeed, a small amount of RNA was successfully isolated using this kit (Fig.1 lane 3 and 4). However, the buds powder was easy to form a sticky, glue-like gel in Buffer RLT, making the separation of two phases by centrifugation extremely difficult. Moreover, this mixture seriously decreased the selective binding of RNA to the RNeasy membrane. The RNA yield was very low, about 44.5 µg/g fresh weight, and the $A_{260/280}$ ratio was only 1.29 (Table 1).

Since the above two guanidine-based RNA extraction protocols could not give satisfactory results, we tried to use a CTAB-based method to isolate RNA from Tree Peony floral buds. The CTAB method has been widely used for RNA isolation from polyphenols and polysaccharides-rich plants, including pine needles (Chang *et al.,* 1993), a variety of tissues and organs of Chinese fir (Wang *et al.,* 2007, 2011), cotton (Zhao *et al.,* 2012), Spurge (Xu *et al.,* 2010), and tea (Muoki *et al.,* 2012). Unexpectedly, it failed to extract high quality RNA from floral buds of tree peony. The isolated RNA appeared degraded, featured as smear in the lane and bright 5.8 S rRNA band (Fig. 1 lane 5 and 6). The yield was as low as 94.4 µg/g fresh weight, and the purity was also not well (Table 1). This was probably caused by the cross reaction between LiCl and the complicated secondary metabolites in floral buds, which thus highly impaired RNA precipitation efficiency.

Next, we attempted to use a SDS-based method, which can effectively remove polysaccharides and has been successfully used to isolate RNA from onion epidermis (Zhou *et al.,* 1999) and lily floral organs (Wu *et al.,* 2006). Importantly, a LiCl precipitation step was used before the phenol/chloroform purification and the isopropanol precipitation, which could minimize gel formation in the homogenate by dissolution of the gel-forming materials (Zhou *et al.,* 1999). It could successfully isolate RNA with high yield (186.5 µg/g fresh weight), but the $A_{260/280}$ ratio was very low (Fig. 1, lane 7 and 8;

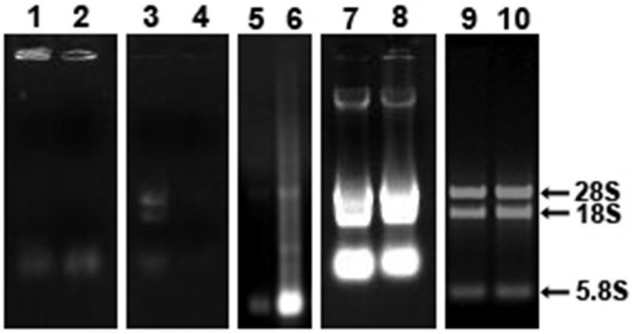

Fig. 1. Agarose gel electrophoresis of total RNA from floral buds of Tree Peony. Lane 1-2: RNA isolated using TRNzol reagent (TIANGEN). Lane 3-4: RNA isolated using RNeasy Plant kit (Qiagen). Lane 5-6: RNA isolated using the CTAB-based method. Lane 7-8: RNA isolated using the conventional SDS protocol. Lane 9-10: RNA isolated using the modified SDS/TRNzol method. All lanes were loaded about 1 μg total RNA

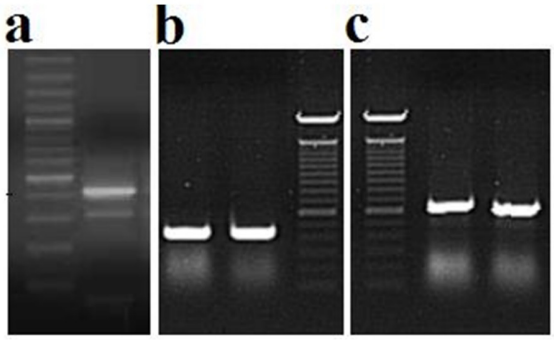

Fig. 3. Downstream application of RNA isolated from floral buds of Tree Peony using the modified SDS/TRNzol method. (a) Homolog fragment of *CYCLOIDEA* transcriptional factor obtained from Tree peony. (b) 5′ RACE product of *APETALA1* gene. (c) The ORF product of *APETALA1*. DNA Ladder: 100 bp

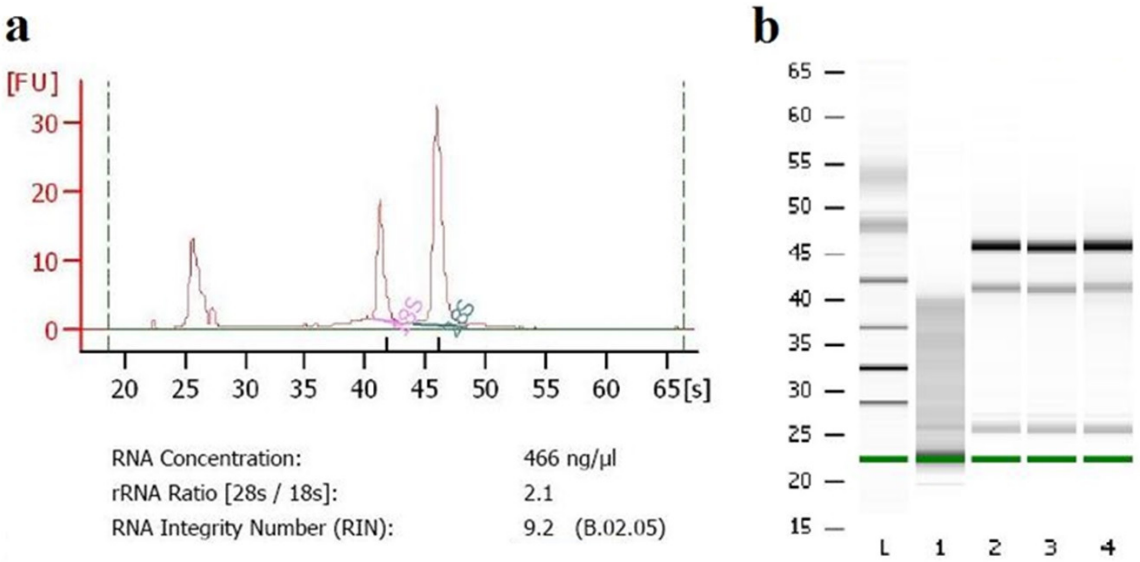

Fig. 2. Analysis of total RNA isolated from floral buds of Tree Peony using the Eukaryote total RNA nano assay on the Agilent 2100 bioanalyzer. (a) Microcapillary electrophoresis of RNA extracted using the modified SDS/TRNzol method present on the right panel with detailed parameters: concentration 466 ng/μl, rRNA ratio 2.1, and RNA integrity number 9.2. (b) The corresponding gel-like image generated by the bioanalyzer. L, RNA ladder; 1: RNA isolated using TRNzol reagent, as a negative control. 2-4: RNA isolated using the modified SDS/TRNzol method

Table 1). There was also considerable amount of genomic DNA contamination in the extracted RNA. Additionally, the entire procedure was time-consuming and labor-intensive.

A recently published SDS/TRNzol RNA extraction protocol was then applied to isolate RNA from floral buds of tree peony. The method is suitable for isolating RNA from samples containing high levels of starch (Wang *et al.,* 2012). Two critical steps were performed before the conventional TRNzol extraction. First, the alkaline condition (pH 9.0) in the suspension buffer was feasible to lyse cells and inhibit endogenous ribonuclease. Second, a high concentration of SDS was used to promote cell lysis and to inhibit homogenate solidification by effectively dissolving polysaccharides in samples. In addition, we added an SSTE washing step that could cleanup contaminants co-precipitated with RNA. The result showed that the modified SDS/TRNzol method could effectively isolate high quality RNA from floral buds of tree peony.

Table 1. Yield and purity of total RNA extracted by the five methods from floral buds of tree peony

Method	NanoDrop™ 1000			Bioanalyzer	
	$A_{260/280}$	$A_{260/230}$	Yield (μg/g fresh weight)	RIN	rRNA ratio (28S/18S)
TRNzol	1.53 ± 0.15	0.59 ± 0.10	32.9 ± 5	2.2	0.0
RNeasy	0.90 ± 0.05	0.42 ± 0.08	44.5 ± 3	nd	nd
CTAB	1.58 ± 0.02	1.47 ± 0.05	94.4 ± 5	nd	nd
SDS	1.69 ± 0.05	1.18 ± 0.03	186.5 ± 10	nd	nd
SDS/TRNzol	1.95 ± 0.03	2.06 ± 0.03	165.7 ± 8	9.2	2.1

Values represent the mean ± SD of at least five replicate samples; RIN, RNA integrity number; 'nd', not detect.

The yield of total RNA obtained by this method was 165.7 μg/g fresh floral buds (Table 1), which was comparable to that of the conventional SDS method. The ratios of $A_{260/280}$ and $A_{260/230}$ were near to 2.0 and above 2.0, respectively (Table 1). This indicated that the extracted RNA is of high purity without detectable contamination of proteins or other secondary metabolites. Electrophoresis on 1% agarose gel demonstrated that the total RNA is of good integrity, illustrated by the bright and sharp 28S and 18S rRNA bands and no sign of degradation (Fig. 1 lane 9 and 10). We further analyzed the extracted RNA on an Agilent 2100 Bioanalyzer. Microcapillary electrophoresis displayed three clearly peaks, corresponding to 28S, 18S and 5.8S, respectively (Fig. 2a). The RNA Integrity Number (RIN) value was 9.2, and the rRNA (28S/18S) ratio was 2.1. The schematic gel-like image also showed that the total RNA isolated from floral buds is of high quality (Fig. 2b).

To confirm that the RNA isolated by the modified SDS/TRNzol protocol is suitable for further molecular assays, we reverse transcribed the prepared RNA into cDNA for RT-PCR and RACE analyses. A homolog fragment of the *CYCLOIDEA* transcriptional factor was obtained from tree peony using the degenerated primers. It was about 450-bp in-length and was validated by DNA sequencing (Fig. 3a). To isolate the full-length cDNA of *APETALA1*, a clear 400-bp PCR product was obtained by performing 5′ RACE procedure (Fig 3b). Moreover, the opening reading frame (ORF) of *APETALA1* was amplified from the cDNAs reverse transcribed from the RNA extracted by our method (Fig. 3c). Taken together, these results indicated that the RNA isolated by the modified SDS/TRNzol protocol is of high quality and is suitable for further gene expression analysis.

Conclusions

In summary, we systematically compared five commonly used protocols for RNA isolation, and reported a modified SDS/TRNzol method for isolation RNA from floral buds of tree peony. This protocol is an easy, efficient, and highly reproducible method for RNA isolation from floral buds rich in secondary metabolites, and could be potentially widely applied for other ornamental plants.

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