Isolation of Quality Total RNA from the Aromatic Plant *Origanum onites*

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We successfully used the guanidine isothiocyanate method for isolation of total RNA from leaf, stem, and root tissues of the aromatic plant *Origanum onites*. The RNA was extracted with TRI Reagent® at room temperature and was recovered by isopropanol precipitation. The isolated RNA was capable of reverse transcription. The extraction method described here does not require ultracentrifugation, and it is fast, simple, and effective. The procedure can be completed within 3 hours and may be applicable to other aromatic medicinal plants containing high amounts of phenolic compounds.

Key words: RNA Isolation, *Origanum onites*, Secondary Metabolites

Introduction

Molecular investigations of many interesting phenomena in plants require a high RNA quality and integrity, as well as reproducibility among extractions of replicates from the same tissue. This is not easy to accomplish when working with aromatic plants that contain large amounts of polysaccharides and polyphenolic compounds (Gehrig *et al.*, 2000; Kiefer *et al.*, 2000). Phenolic compounds are readily oxidized to form covalently linked quinones and interact irreversibly with nucleic acids leading to their oxidation and degradation (Salzman *et al.*, 1999). This renders RNA not suitable for fundamental procedures such as cDNA library construction, reverse transcription-polymerase chain reaction (RT-PCR) and Northern hybridization (Chomczynski and Sacchi, 1987; Liu *et al.*, 1998; Sharma *et al.*, 2003).

Several commercial reagents such as TRIZOL® and TRIZOL LS® (Invitrogen Life Technologies, Carlsbad, CA, USA) and TRI Reagent® (Sigma, St. Louis, MO, USA) based on the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987) have been developed for RNA isolation. The strong denaturant guanidine thiocyanate, a potent chaeotropic agent, has been known to be more effective than guanidine hydrochloride or phenol in denaturing RNases and performs well in RNA extractions from small quantities of plant tissues (Portillo *et al.*, 2006). However, the use of the guanidine isothiocyanate method is very restricted to aromatic plants, and it was reported that this method yields either poor-quality RNA or no RNA at all in plants with high levels of phenolic compounds and/or polysaccharides (Gehrig *et al.*, 2000; Ghangal *et al.*, 2009).

Various protocols for RNA isolation from plant species rich in polyphenolics or polysaccharides exist (Dong and Dunstan, 1996; Geuna *et al.*, 1998; Gehrig *et al.*, 2000; Wang *et al.*, 2005; Provost *et al.*, 2007; Rubio-Pina and Vazquez-Flota, 2008; Muge *et al.*, 2009). However, these methods have been developed for specific plant tissues and are generally time-consuming. Moreover, modern PCR techniques like RT-PCR, used for transcript quantitations, often cannot be successfully applied to such RNA preparations. In addition, the chemotypic heterogeneity among species may not allow optimal RNA yield from one isolation protocol and, perhaps, even closely related species may require different isolation protocols (Sharma *et al.*, 2003).

Origanum onites L. (Labiatae) is a perennial species with woody stems; it can be distinguished from other *Origanum* species by the form of inflorescence (spikes arranged in false corymbs) and one-lipped calyces (Ietswaart, 1982). *O. onites* grows naturally in Aegean and Mediterranean regions of Turkey, and is also widely cultivated due to its economical importance. *O. onites* is one of the main medicinal and aromatic plants exported

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from Turkey (Başer, 2001). It is known to have antiseptic, antibacterial, and antispasmodic properties due to its volatile oils and terpenic materials (Başer *et al.*, 1993; Aligiannis *et al.*, 2001; Daferera *et al.*, 2000, 2003). Crop improvement studies were undertaken with this species to obtain different chemotypes rich in carvacrol or thymol. Production of transgenic *O. onites* plants in which the thymol and carvacrol contents are altered is also the future aim of several research institutes in Turkey. However, in order to begin an extensive study on producing transgenic *O. onites* plants, the finding of suitable RNA isolation methods is necessary to clone genes, *e.g.* terpene synthases from this plant, and conduct gene expression analyses.

In the present study, the guanidine isothiocyanate method using TRI Reagent® was evaluated to isolate good-quality RNA from leaves and roots of *Origanum onites*. Reverse transcription of the RNA followed by PCR amplification was used to confirm that the RNA produced is able to generate cDNA. The protocol described here is simple, fast and does not require ultracentrifugation.

Material and Methods

Plant material

Origanum onites plants were kindly provided by Atatürk Research Institute of Garden Cultivars, Medicinal and Aromatic Plants Division, Yalova, Turkey. Leaf tissues were frozen in liquid nitrogen and stored at –20 °C for future use.

RNA isolation

TRI Reagent® (Sigma), containing guanidine isothiocyanate, for improvement of the single-step RNA isolation method developed by Chomczynski and Sacchi (1987) was used to extract total RNA from *O. onites* plants.

All equipments such as mortars, pestles, Eppendorf tubes, and tips were DEPC (diethylene pyrocarbonate)-treated and autoclaved. Approx. 100 mg of leaf, stem or root tissues were ground in liquid nitrogen to a fine powder and transferred to an 1.5-ml Eppendorf tube, and 1 ml of TRI Reagent® was added. The tube was inverted several times and incubated at room temperature for 5 to 10 min. After centrifugation at 12000 × *g* for 10 min at 4 °C, the supernatant was carefully transferred to a new tube and left at room temperature for 5 min. In the following step, 0.25 ml chloroform was added and the mixture shaken vigorously for 15 s. The mixture was then incubated at room temperature for 5 to 15 min and centrifuged at 12000 × *g* at 4 °C for 15 min. The mixture was separated into a lower phenol/chloroform phase, interphase, and the colourless upper aqueous phase. The aqueous phase was transferred to a new 1.5-ml tube. If the transferred solution was cloudy, the chloroform extraction step was repeated. Approx. 0.5 ml of isopropanol per 1 ml of TRI Reagent® was used to precipitate RNA. After centrifugation at 12000 × *g* for 10 min at 4 °C, a white and gel-like RNA pellet was formed. The supernatant was discarded and 1 ml 75% ethanol was added to the tube. The tube was centrifuged shortly to collect the RNA pellet after 30–60 min of incubation at –20 °C. Although one salt wash step with 75% ethanol is recommended by the manufacturer, we have repeated this step twice to make sure that there was no precipitated salt trace in the pellet. RNA samples were dissolved in 50 μl DNase RNase-free water. The quantity and purity of the RNA sample was determined using a Nanodrop® ND-1000 spectrophotometer (Wilmington, DE, USA). The RNA was also examined by electrophoresis on 1% agarose gels in TBE (1× Tris-borate-EDTA buffer: 90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0).

RT-PCR

To test the quality of the RNA obtained, total RNA was treated with DNase I (Fermentas, Glen Burnie, MD, USA), then cDNA was prepared using RevertAid First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. The reaction mix contained 3 μg total RNA and 0.5 μg oligo dT_{18} primer, 4 μl reaction buffer, 1 μl ribonuclease inhibitor (20 U/μl), 2 μl dNTP mix (10 mM) and 1 μl RevertAid™ M-MvLV reverse transcriptase in a final volume of 20 μl. The thermal cycling conditions were: 5 min at 70 °C, 5 min at 37 °C, 60 min at 42 °C, 10 min at 70 °C, and a final hold at 4 °C. The resulting cDNA was used as template to amplify the actin gene from *Origanum onites*.

PCR amplifications were carried out using degenerate primer pairs designed for the gene encoding actin. The total reaction volume of 25 μl

contained 20 ng of total RNA, 1X Taq polymerase reaction buffer, 3 mM $MgCl_2$, 0.1 mM of each dNTP, 2.5 μM forward and reverse primers (forward: 5'-ATGGCYGABRCTGABGACATTCARCC-3'; reverse: 5'-GAAGCAYTTYCTGTGRACAATBSMTGGACC-3'), and 1 U of Taq DNA polymerase (Fermentas). Amplifications were performed in a Progene thermocycler (Techne Inc., Burlington, USA) programmed as follows: 2 min denaturation at 94 °C and 35 cycles of 40 s each denaturation at 94 °C, 50 s annealing at 50 °C and a 50-s extension at 72 °C, followed by a final extension at 72 °C for 7 min. Amplification products were separated on 1.4% agarose gel containing ethidium bromide (0.5 μg/ml). Gels were visualized under UV light and digitally photographed with an UVIpro gel documentation system (UVItec, Cambridge, UK).

Results and Discussion

Today, many research groups focus on using transgenic plants for the production of industrial enzymes and other materials, and also boosting the levels of pharmacologically active compounds, changing the essential oil composition in medicinal and aromatic plants by using the recombinant DNA technology. For this reason, isolating of good-quality RNA is the first requirement to achieve these goals.

Although RNA isolation is considered a routine protocol, the extraction from aromatic plants could be critical, due to the presence of large amounts of polysaccharides, polyphenols, and other secondary metabolites which limit the RNA extraction yield and purity (Wang *et al.*, 2005; Provost *et al.*, 2007). There are many published RNA isolation protocols, of which the single-step method using guanidine isothiocyanate (Chomczynski and Sacchi, 1987) has proved to be useful in model plants, *i.e.* rice and *Arabidopsis*. However, this method was reported to be unsuccessful in isolating quality RNA from plants rich in secondary metabolites (Ghangal *et al.*, 2009).

In the present study, we have shown that TRI Reagent®, based on the guanidine isothiocyanate method, can be used successfully to extract RNA from leaves, stems, and roots of *Origanum onites* plants. Typical yields of total RNA obtained by using this method ranged from 15–100 μg/g fresh weight of tissue, which compares favourably with yields reported for other methods designed for plants containing high amounts of phenolic compounds (Gehrig *et al.*, 2000, and references therein). The total RNA yield was high in young leaves (2 μg/μl) and significantly low in both stems (0.3 μg/μl) and roots (0.4 μg/μl). This is not surprising since last two tissues contain differentiated xylem.

The quality of RNA isolated by this method was shown by spectrophotometric methods. The ratio of absorbance at 260 and 280 nm ($A_{260/280}$) was taken as a measure of purity, with a value of 2.0 for pure RNA (Kiefer *et al.*, 2000; Sharma *et al.*, 2003). The $A_{260/280}$ ratio of all RNA samples was in the range of 1.9–2.02 indicating the absence of protein contaminants. The absorbance ratio $A_{260/230}$ was also in the range of 1.9–2.0 indicating little or no polyphenol contamination.

The RNA integrity was also assessed by visualization of ribosomal RNA bands on 1% agarose gels (Fig. 1). For all RNA samples tested, distinct 28S and 18S rRNA bands were observed. The 28S rRNA bands appeared equal to or more abundant than the 18S rRNA bands, thereby indicating that little or no RNA degradation occurred during extraction (Fig. 1).

Effective cDNA synthesis is also another indicator of high-quality RNA, because reverse transcription is sensitive to impurities and therefore is a relevant test of RNA quality for diverse applications, including full-length cDNA isolation and gene expression analysis (Tang *et al.*, 2007; Vasanthaiah *et al.*, 2008). The RNAs isolated from *Origanum* served as robust templates for reverse transcription which was indicated by PCR amplifi-

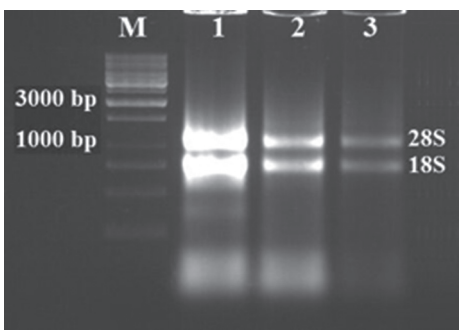

Fig. 1. Agarose gel electrophoresis of total RNA from leaf (1), root (2), and stem (3) tissues of *Origanum onites* isolated using the guanidine isothiocyanate method. M, 1-kb DNA ladder (Fermentas).

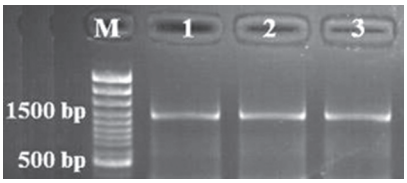

Fig. 2. Agarose gel electrophoresis of RT-PCR products obtained from leaf (1), root (2), and stem (3) total RNA for actin. M, 100-bp DNA ladder plus (Fermentas).

cation from cDNA for actin which is an abundant gene in plant systems (Fig. 2). We have also successfully cloned a full-length cDNA of a mitogen-activated kinase kinase (*Oo*MAPKK1, GenBank No. EF558371) from *Origanum onites* by using same RNA samples (data not shown).

In conclusion, the TRI Reagent® based on the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987) allowed the isolation of intact, high-yield and -quality RNA from *Origanum* leaves, roots, and stems and can be used successfully for RT-PCR and library constructions of this species. To our knowledge, the present work is the first assessment for RNA extraction based on the guanidine isothiocyanate method from the aromatic plant *Origanum onites*. We have also failed to find any reference describing different RNA extraction methods specifically for *Origanum* plants. The guanidine isothiocyanate method is effective, simple, and can be completed within 3 hours and does not require ultracentrifugation. This method may also be useful for other aromatic plant species with high contents of phenolic compounds and polysaccharides.

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