Isolation of Quality Total RNA from the Aromatic Plant
Origanum onites
I˙smail Poyraz*, Emel Sözenb, and Muhittin Arslanyolu

a Bilecik University, Pazaryeri Vocational School, Pazaryeri, 11800, Bilecik, Turkey
b Anadolu University, Science Faculty, Biology Department, Yunusemre Campus, 26470, Eskisehir, Turkey. E-mail: esozen@anadolu.edu.tr
* Author for correspondence and reprint requests

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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 chaotropic 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...
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 *O. 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**

*Oreganum 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*. 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.

**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 dT18 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 MgCl₂, 0.1 mM of each dNTP, 2.5 μM forward and reverse primers (forward: 5'-ATGGCYGABRCTGABGACATTCA-CC-3'; reverse: 5'-GAAGCAYTTYCTGTGRA-CAATBSMTGGACC-3'), and 1 U of Taq DNA polymerase (Fermentas). Amplifications were performed in a Progene thermocycler (Technne Inc., Burlington, USA) programmed as follows: 2 min denaturation at 94 ºC and 35 cycles of 40 s each denaturation at 94 ºC, 50 s annealing at 50 ºC and a 50-s extension at 72 ºC, followed by a final extension at 72 ºC 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/μl 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 (A₂₆₀/₂₈₀) 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 A₂₆₀/₂₈₀ ratio of all RNA samples was in the range of 1.9–2.02 indicating the absence of protein contaminants. The absorbance ratio A₂₆₀/₂₃₀ 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-
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.

