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ESTABLISHMENT OF A FAST AND HIGH-THROUGHPUT PROTOCOL FOR RNA ISOLATION FROM BARLEY LEAVES

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Abstract

The tissues of barley contain high levels of polysaccharides, making most RNA extraction methods unsuitable for handling the excess polysaccharides. The present study compared various RNA extraction methods suitable for extracting RNA from barley leaves at two different growth stages. Intriguingly, found that using an extraction buffer containing guanidium thiocyanate to directly grind barley leaf tissue with 4 µL of 97.4% β-mercaptoethanol produces good-quality RNA from barley leaves at flowering and milky dough stage. Guanidium thiocyanate (GTC) is a RNase activity throughout the extraction process. The high-quality RNA extracted was further used in the downstream process, and the result of RT-PCR clearly showed the expression of the chitinase and β-1,3-glucanase genes. The suggested modifications to the RNA extraction procedure resulted significantly improved RNA quality extracted from the barley leaf tissue.

Introduction

The isolation and purification of RNA is a critical step for subsequent molecular experiments involving real-time polymerase chain reactions (RT-PCR) for the analysis of specific gene expression. The methods of RNA isolation vary from plant to plant, as well as the type of tissue within the same plant.

Barley (*Hordeum vulgare* L.) is an important crop in the world, ranking after wheat, rice, and maize (Jacobsen *et al.* 2006). The primary physiologically active ingredients that have been separated from barley are phenolics, flavonoids, alkaloids, β-glucan, polysaccharides, amino acids, minerals and dietary fiber (Hagiwara and Ueyama 2001, Yan *et al.* 2022). Purification and isolation of high-quality RNA from cells containing high amounts of polysaccharides, polyphenolic compounds, fibres, and proteins can be challenging due to their tendency to interact with nucleic acids and form insoluble complexes (Reddy *et al.* 2015). Low yield is caused by a large number of polysaccharide fractions that physically hold RNA, entice it during centrifugation, and then discard it during phase separation. Furthermore, during phase separation, tiny polysaccharide particles may partition into the aqueous phase and co-precipitate with RNA, further reducing RNA yield and making it less useful for downstream applications (Liu *et al.* 2018). Polysaccharides have similar physical and chemical properties to RNA, which causes them to co-precipitate with RNA during the nucleotide purification process. However, polysaccharides, polyphenols, and other secondary metabolites can also interfere with the quality of RNA, leading to its degradation (Birtic *et al.* 2006, Shu *et al.* 2014). Extraction techniques vary due to the unique characteristics of different plants. Additionally, because plants have diverse compositions, it isn't easy to ensure the effectiveness of any single technique. Extracting high-quality RNA from plants with high levels of polysaccharides and polyphenols is often a challenging task. Therefore, the primary challenge is to find a method for removing polysaccharides and other secondary metabolites before extracting RNA. Extracting RNA from various parts of barley is a laborious process due to the high content of phenolics, flavonoids, alkaloids, β-glucans, and polysaccharides (Shu *et al.* 2014).

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The aim of this study was to develop a reliable RNA extraction method for obtaining high-quality RNA from barley plant tissues at two different growth stages. To achieve the objective, four RNA extraction methods were employed to compare the quantity and quality of RNA extracted from mature barley leaves collected from field-grown plants under natural conditions.

Materials and methods

The barley genotype Geetanjali was planted in the experimental field of the Bihar Agricultural University farm in Sabour, Bhagalpur, India. Samples were collected at the flowering stage and the milky dough stage. Plants with uniform growth and colour were randomly selected from each plot and then washed with a 0.9% NaCl solution. The upper two leaves were taken and collected in a falcon tube with liquid nitrogen, and the samples were stored at -80°C for further use.

Conventional Trizol method, SV total RNA isolation kit (Promega, USA), and Guanidium thiocyanate (GTC) buffer-based method were used for extracting RNA. The detailed protocol is outlined in Fig. 1.

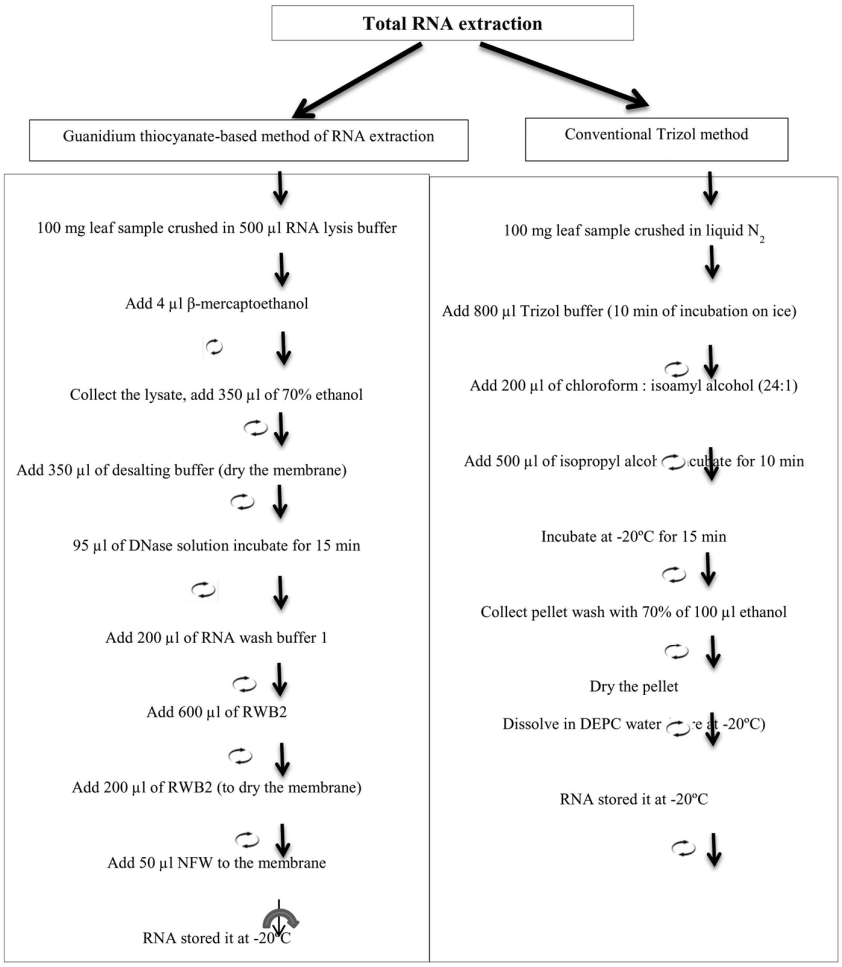

Fig. 1. Schematic outline and comparison between conventional trizol method of purification and modified guanidium thiocyanate-based method of RNA extraction.

Leaf samples weighing 100 mg were crushed in 500 µl of RNA lysis buffer (RLB: 4 M GTC). Next, 4 µl of 97.4% β-mercaptoethanol (BME) was added to the crushed samples and vortexed for 10-20 seconds. The mixture was then applied to an RNA sure shredder in a collection tube and centrifuged at 15000 rpm for 6 min. The resulting filtrate was carefully transferred to a new sterilized tube. Chilled ethanol (70%) was mixed thoroughly by vortexing for 10-15 sec. Subsequently, the plant column is placed in a collection tube and centrifuged at 14000 rpm for 3 min. Following centrifugation, 350 µl of desalting buffer (1.3 M guanidium salt + 8% ethanol) is added, and the mixture is centrifuged again as described earlier. DNase solution was freshly prepared by mixing 20 µl of DNase with 80 µl of DNase reaction buffer. The mixture was incubated for 15 min at room temperature. Next, 200 µl of RNA wash buffer 1 (containing 3.4 M Guanidium salt, Tris buffer at pH 7.6, and 70% ethanol) was added, and the solution was centrifuged at 14,000 rpm for 3 min. Following this, 600 µl of RNA wash buffer 2 was applied and centrifuged. Again 200 µl of RWB 2 (3.4 M Guanidium salt, tris buffer at pH 7.6, and 96% ethanol) was added and centrifuged. Finally, 50 µl of nuclease-free water was added to the membrane and centrifuged at 14,000 rpm for 1 min. The extracted RNA can be stored at -20ºC for future use. The concentration of RNA samples was analyzed using the nanodrop Total RNA 6000 Nano Kit (Vers. II), which is specifically designed for total RNA analysis with the Agilent 2100 Bioanalyzer and the RNA 6000 Nano Lab Chip (Agilent Technologies, CA, USA). The RNA samples were loaded on 1% agarose gel to check the purity of the RNA bands. Gel images were captured using a UV transilluminator (Alphalabs, UK).

The RNA extracted from the leaf samples collected at the flowering and milky dough stages underwent first-strand cDNA synthesis. The synthesis was carried out using the GoScript Reverse Transcriptase First Strand cDNA Synthesis Kit (Promega, USA) following the manufacturer's instructions. For RT-qPCR analysis, the following reference and gene-specific primers were used: RT-qPCR analysis was performed using the GoTaq RT-qPCR kit (Promega, USA), with Actin (Forward: 5'-GCCATGTACGTCGCAATTCA-3', Reverse: 5'-AGTCGAGAACGATACCAGTAGTACGA-3') serving as the internal control. For the RT-qPCR analysis, three replicates were used. The RT-qPCR was carried out using a reaction mixture consisting of 5 µl of SYBR Green master mix, one µl each of forward and reverse primers (10 pmol), and 20 ng of cDNA (2 µl) to make a final reaction volume of 10 µl. The RT-qPCR process was conducted in the AriaMx Real-Time PCR system (Agilent Technologies, USA) at 95°C for 4 min followed by 40 cycles of 95°C for 30 seconds and 58°C for 30 seconds. A melt curve profile was included to verify the specificity of the RT-qPCR reaction, ensuring that there was no gDNA contamination, no primer dimers, and no non-specific amplification. The relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen (2001). The expression of the chitinase and β-1,3-glucanase genes (Table 1) was normalised using actin as an internal control.

Table 1. Sequence of primer used for the analysing the specific gene expression of chitinase.

Gene		Primer sequence	Annealing temp.	Ref.
Chitinase (PR-3)	Forward primer	ACGGTGTGATCACCAACATC		
	Reverse Primer	CAGTCCAGGTTGTCACCGTA	60°C	Shetty *et al.* 2009
β-1,3-glucanase (PR-2)	Forward primer	AACGACCAGCTCTCCAACAT		
	Reverse Primer	GTATGGCCGGACATTGTTCT	60°C	

Results and Discussion

Crushing of barley leaf samples directly in GTC-based RNA lysis buffer leads to better disruption of cells. Using liquid nitrogen for grinding samples to convert the tissue into fine powder is one of the most common methods. However, it was found that the frozen powder resulting from liquid nitrogen grinding became recalcitrant to the buffer even after prolonged and vigorous vortexing and centrifugation; the grinding process was slow and adds a

freeze/thaw to the procedure that could degrade RNA. Rashid *et al*. (2016) also reported a similar observation, where they extracted RNA from the sprouted seeds of barley. The barley leaf samples were directly ground in pre-chilled mortar and pestle on ice in 500 µl of RNA lysis buffer containing GTC. Although the quality and yield of RNA resulting from the prescribed vs. GTC-based method were significantly better after purification (Table 2), the protocol led to the isolation of good-quality RNA (Fig. 2). The presence of BME in the buffer, used for tissue grinding without liquid N_2, successfully inhibited RNase, can be observed by RNA integrity (Fig. 2).

Table 2. Comparative quantification of mature wheat seed RNA yield evaluated by Nanodrop ND-1000UV spectrophotometer.

Sample	Trizol method	Promega kit	Guanidium thiocyanate-based method of RNA extraction
Barley leaf (flowering stage)	10 ng/µl	30 ng/µl	100 ng/µl
Barley leaf (milky dough stage)	3 ng/µl	15 ng/µl	90 ng/µl

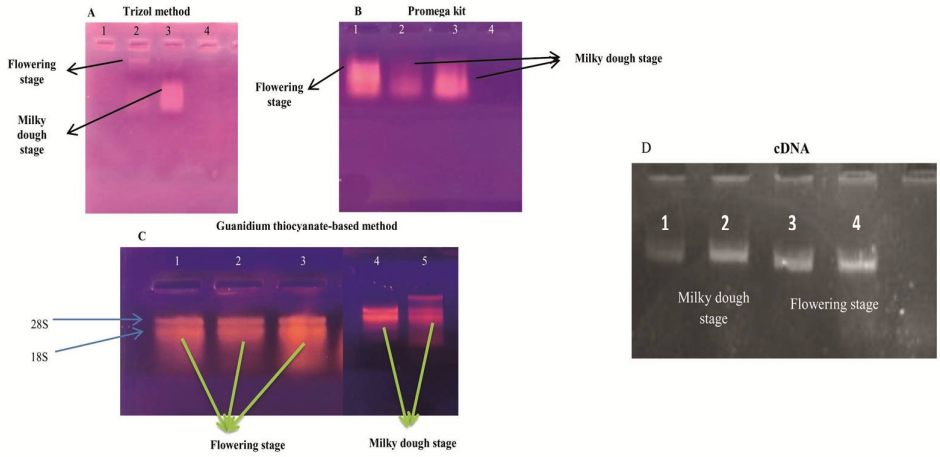

Fig. 2. Agarose gel electrophoresis image of RNA extracted from barley leaf using 3 different RNA extraction methods. A: Trizol method: gel labeled 2 and 3 represent samples from the flowering stage, and milky dough stage respectively, B: Promega kit: gel labeled in the lane 1 and 2, 3 represent samples from the flowering stage, and milky dough stage, respectively, C: Guanidium thiocyanate-based method: gel labeled in the lanes 1, 2 and 3 represent samples from the flowering stage, and 4 and 5 represent samples from milky dough stage of barley leaf, D: Agarose gel image showing cDNA synthesized from RNA extracted from barley at the milky dough stage and flowering stage by guanidium thiocyanate-based method. Bands labeled in the lanes 1 and 2 represent cDNA from the sample of milky dough stage, while bands in the lanes 3 and 4 represent the flowering stage.

The maximum concentration of RNA was obtained from the modified extraction method, which was 100 and 90 ng/µl from the leaf samples of barley collected at flowering stage and milky dough stage, respectively as compared to conventional trizol method where the concentration of RNA obtained was 10 and 3 ng/µl at flowering and milky dough stage, and SV total RNA isolation kit (30 and 15 ng/µl) (Table 2). The extracted RNA was then subjected to cDNA synthesis (Fig. 2) to observe the expression of a particular gene. The cDNA obtained from RNA extracted by using the GTC-based method is of good quality. The RT-qPCR results of total RNA quantification showed a tendency to obtain lower Ct values for the synthesized cDNA from RNA samples extracted by the GTC-based method from the barley leaves. Examined the expression of the chitinase which was (PR-3) and β-1,3-glucanase (PR-2) gene in barley at both the stages (Fig. 3). According to the result obtained the fold expression of PR-2 gene in healthy leaves of barley was higher at milky dough stage as compared to the flowering stage, same trend was also observed in case of PR-3 where the

fold expression of gene was higher at milky dough stage but among both the genes the expression of PR-2 is higher than PR-3 at both the stages. These results clearly represent the fact that the healthy leaves of barley show the expression β-1,3-glucanase and chitinase at some level which help the plant to fight against the initial stressful conditions. A graphical abstract illustrating the key steps of the entire experiment is presented in Fig. 4. The guanidium thiocyanate-based method of RNA extraction by direct grinding in buffer without liquid nitrogen are the simplest and fastest procedure for mature barley leaf samples at different growth stages.

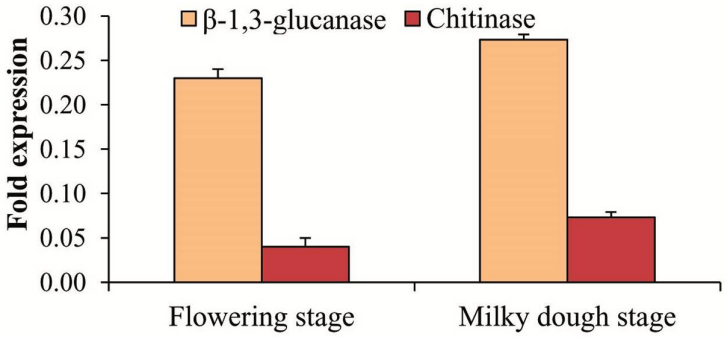

Fig. 3. Expression analysis of specific gene chitinase and β-1, 3-glucanase from the RNA extracted by guanidium thiocyanate-based method ($p \leq 0.05$).

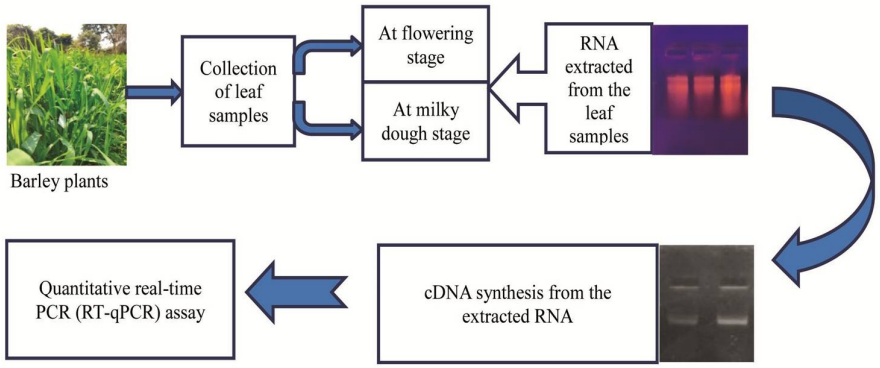

Fig. 4. Brief representation of all the key steps of the experiment.

The GTC-based method of RNA extraction was primarily developed for extracting RNA from barley leaves containing phenolics, flavonoids, alkaloids, β-glucan, and polysaccharides to obtain high-quality RNA. The presence of salt in extraction facilitated the precipitation of the protein through salting out, preventing the simultaneous precipitation of polysaccharide with RNA. As a result, the RNA remained in the solution (El-Ashram *et al.* 2016, Liao *et al.* 2023). GTC is a potent RNase inhibitor that efficiently suppresses the activity of RNase throughout the extraction process (Suzuki *et al.* 2001, Liao *et al.* 2023).

The current method using GTC by direct grinding in buffer without liquid nitrogen is the simplest and rapid procedure for isolation of RNA from mature barley leaves at different growth stages. The GTC-based RNA isolation protocol has rendered quality yield of RNA from the barley leaves at flowering and milky dough stages. Under this modified method, the resulting RNA samples were of sufficient quality for expression analysis of genes by RT-PCR via the synthesis of cDNA.

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