Research Article

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High quality RNA from hydroponically grown grapevine roots suitable for gene expression studies

Gen Anlatımı Çalışmaları İçin Uygun Özellikte Hidroponik Olarak Yetiştirilmiş Asma Köklerinden Yüksek Kalitede RNA Eldesi

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Abstract

Objectives: Grapevine root system plays a great role in sensing and adapting to abiotic and biotic stresses. Identification of candidate genes involved in the tolerance to abiotic stress is becoming a crucial strategy to select and breed resilient genotypes. However, obtaining high quality RNA from grapevine roots under hydroponic culture is difficult. Hence, we have developed a new extraction procedure to improve RNA quality for root gene expression studies.

Methods: Conventional RNA extraction methods using CTAB are not suitable for gene expression studies and need to be improved. Here we report the application of a CTAB- based method for RNA extraction using an additional clean-up purification step.

Results: The RIN value of the resulting RNA indicated that our procedure allowed the purification of high RNA quality and quantity. Hence, the clean-up purification step efficiently eliminated contaminants which inhibit downstream applications. Derived RNA was successfully used for differential gene expression analysis in salt stressed grapevine by Northern Blot hybridizations.

Conclusion: In this study, we developed an efficient RNA isolation protocol from hydroponic cultivated grapevine roots which yielded RNA suitable for gene expression studies. This will open large perspectives in grapevine functional genomics with the identification of pertinent genes of agronomic interest.

Keywords: Grapevine roots; Hydroponic culturel; RNA purification; Gene expression; Genomics.

Özet

Genel Bilgiler: Asma kök sistemi abiyotik ve biyotik stres etkenlerini algılama ve bu etkenlere adapte olmada büyük rol oynar. Kökler üzerine genomik çalışmalar yetersiz olsa da, abiyotik stres toleransıyla ilişkili aday genlerin tanımlanması, dirençli genotipleri seçmek ve ıslaha kazandırmak için önemli bir strateji haline gelmektedir.

Amaç: Kökler çevre ile ilk temas kuran organdır. Ancak, hidroponik kültür altında asma köklerinden yüksek kaliteli RNA elde etmek zordur. Bu nedenle, gen ekspresyon çalışmaları için kök RNA kalitesini artırmak amacıyla yeni bir izolasyon yöntemi geliştirdik.

Yöntem: CTAB kullanılarak gerçekleştirilen klasik RNA izolasyon yöntemleri gen ekspresyon çalışmaları için uygun değildir ve geliştirilmeye ihtiyacı vardır. Burada ek bir temizlik saflaştırma adımı kullanarak RNA izolasyonu için CTAB tabanlı bir yöntem uygulaması sunduk.

Bulgular: Elde edilen RNA RIN değeri, prosedürümüzün yüksek kalitede ve kantitede RNA saflaştırmayı sağladığını gösterdi. Bu nedenle, temizlik saflaştırma adımı downstream uygulamalarını engelleyen kirlilikleri etkili bir

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şekilde ortadan kaldırmıştır. Elde edilen RNA Northern blot hibridizasyon yöntemiyle tuz stresi altındaki asmanın diferansiyel gen ekspresyon analizi için başarıyla kullanılmıştır.

Sonuç: Bu çalışmada, gen ekspresyon çalışmalarında uygun RNA eldesi için hidroponik kültür asma köklerinden etkili bir RNA izolasyon protokolü geliştirdik. Bu çalışma, tarımsal özellikler ile ilgili genlerin tanımlanmasıyla asma fonksiyonel genomiğine geniş bir perspektif açacaktır.

Anahtar Kelimeler: Asma kökleri; Hidroponik kültür; RNA izolasyonu; Gen ekspresyonu.

Introduction

Grapevine is one of the important economic crops and is widely distributed throughout the world. However, it is particularly sensitive to salinity. Surface viticulture land area is expected to decrease by 73% in the major grapevine producing areas of the world by 2050 [1]. In order to promote its sustainability, grapevine physiology has been extensively investigated [2]. However, a better understanding of the molecular mechanisms involved in grapevine stress tolerance, would be helpful for molecular breeding programs, especially to improve the resistance of cultivars to biotic and abiotic stresses [3].

The great importance of the root systems and their role in mediating shoot responses to environmental stress such as salinity, were recently emphasized [4]. Grapevine roots were found to express more tissue-specific transcripts than leaves [5] which is consistent with findings reported by Tillett et al. [6] who compared large-scale EST libraries from roots and shoots of Cabernet Sauvignon and identified 135 root enriched transcripts more, compared to shoots. These findings indicate that shoot expression analyses of grapevine, while useful, do not give a complete picture of root gene expression patterns. Therefore, studies related to abiotic stress response of roots are required [7] in order to better understand the plant development under stressful conditions [4]. Hydroponic system remains the desired cultivation method for controlled plant growth and has the advantage to easily access all plant tissues especially roots. This experimental system also allows better control of the nutrients compared to the use of soil where complex interaction of ions with soil particles takes place. RNA isolation is a prerequisite for carrying out several molecular biology studies such as reverse transcription (RT), real-time quantitative PCR (RT-qPCR), construction of cDNA libraries, microarrays or next generation sequencing analyses. However, obtaining high-quality RNA from grapevine roots, necessary to investigate gene expression profiles, is challenging. Any contamination of the isolated RNA affects the downstream applications and requires extra cleaning procedures resulting in a reduced RNA yield. In grapevine plants, isolation of high quality intact RNA can be a challenge due to the presence of contaminants such as secondary metabolites, polysaccharides and polyphenols [8–10]. It is even more challenging in the roots which is the first tissue in contact with the salty environment and where high amounts of secondary metabolites accumulate. Secondary metabolites are intensively synthesized in hydroponically grown roots under abiotic stress conditions and possess considerable potential to protect molecular cell structures against oxidative stress [11].

Polyphenolic compounds, particularly tannins can irreversibly bind proteins and nucleic acids to form high molecular complexes [12]. Therefore, these intrinsic contaminants severely affect RNA integrity and quantity [13, 14] rendering it unsuitable for gene expression studies. Hence, RNA isolation from these tissues often requires modifications of existing protocols for developing tissue-specific procedures [15] including the removal of polysaccharides and polyphenols. Here, we developed an improved protocol efficient for RNA isolation of high quality and quantity from salt stressed grapevine roots grown in hydroponic conditions. A CTAB (Cetyltrimethylammoniumbromid) based RNA extraction protocol initially developed for woody plants rich in polyphenols and polysaccharides [16], was used with an additional cleaning step using RNA clean-up protocol of the RNeasy Mini kit (Qiagen) resulted in high-quality total RNA from salt stressed roots of grapevine. The major improvement of the obtained RNA is clearly demonstrated by the elimination of high molecular contaminants in Northern Blots and the improvement of the RIN (RNA integrity number) value. Our research study involves gene profiling of roots under salt stress and cloning the relevant genes.

Materials and methods

Plant growth

Cuttings of *V. vinifera* L. cv. Razegui were obtained from our vineyard in the Center of Biotechnology of Borj Cedria, in Tunisia, cultured in perlite for 6 months. Single plants were then transferred into pots filled with continuously aerated hydroponic culture medium (Figure 1) and grown

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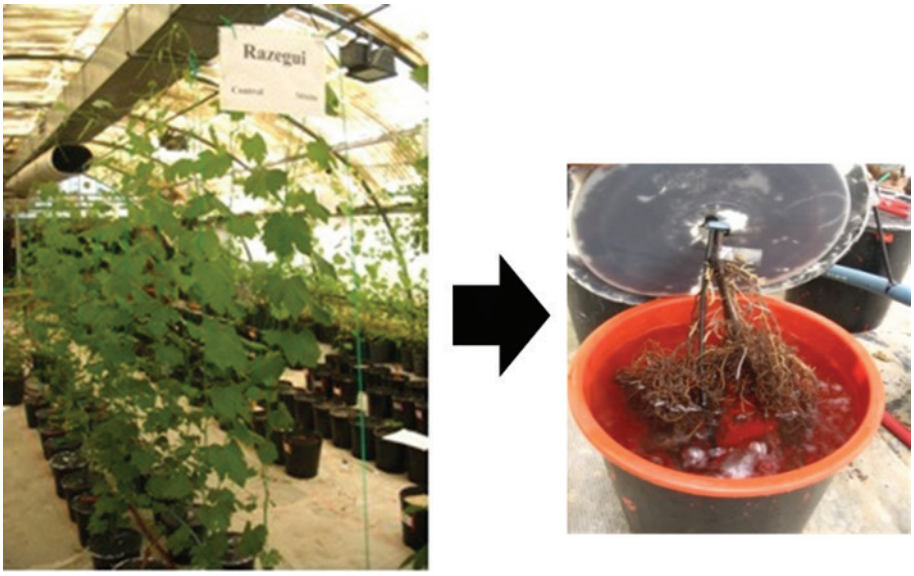


Figure 1: Hydroponic culture of salt stressed grapevine.

under greenhouse conditions (16 h light/8 h dark period at a photosynthetic active radiation of at least 300 $Em^{-2}\,s^{-1}$, a temperature between 25°C and 28°C and relative humidity of 50% to 70%). The nutrient solution [17] was composed of 3.5 mM $(CaNO_3)_2$, 3 mM KNO_3, 2 mM $NH_4\,NO_3$, 0.6 mM K_2HPO_4, 1.5 mM $MgSO_4$, 2.8 mM $(NH_4)_2SO_4$, 1.6 mM KH_2PO_4, 0.43 mM KI, and the micronutrients, 90 M Fe-EDTA, 9.1 M $MnCl_2$, 0.76 M $ZnSO_4$, 0.7 M $CuSO_4$, 46.3 M H_3BO_3 and 0.21 M $(NH_4)_6MO_7O_{24}$ with pH 6.0.

Stress application

Gradually increased salt concentrations were applied 2 weeks after the transfer which allowed to grapevine plants to adapt to hydroponic culture conditions. NaCl concentrations were raised up to 100 mM by increments of 25 mM per 3 days. For control plants, the electrical conductivity (ECe) values were 2 dSm^{-1}while they increased linearly up to 10 dSm^{-1} for 100 mM NaCl treated plants. Plant material was harvested after 3 and 6 h exposure to 100 mM NaCl.

RNA isolation

Grapevine root RNA was isolated using the protocol of Zeng et al. [16] combined with additional clean-up purification (Figure 2). All the reagents and materials were DEPC-treated and autoclaved.

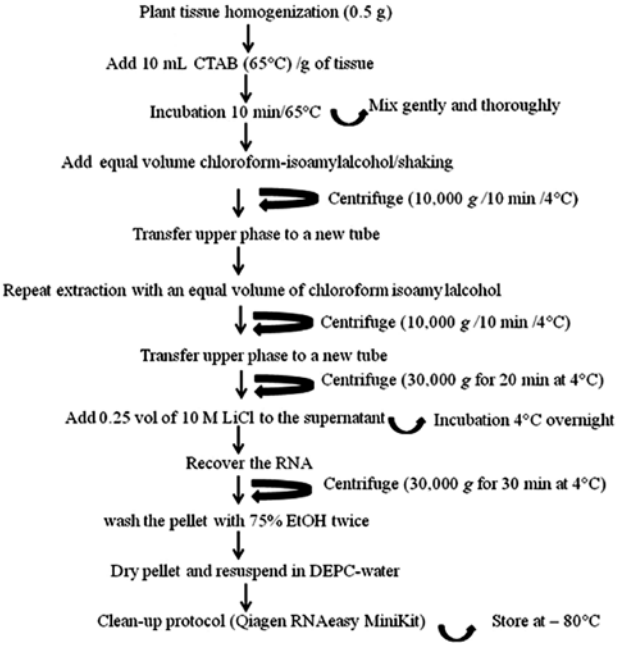


Figure 2: Flow diagram of the principal steps of total RNA isolation with the modified CTAB protocol.

Reagents

- Extraction buffer: 2% CTAB, 2% polyvinylpyrrolidone (PVPmolwt 25,000), 100 mMTris-HCl (pH 8), 25 mM ethylenediaminetetraacetic acid (EDTA), 2 M NaCl, 0.05% spermidine trihydrochloride, 2% β-mercaptoethanol
- Chloroform-isoamylalcohol (24 : 1)
- 10 M LiCl

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RNA quality analysis

The quality and the purity of the isolated RNAs were monitored by measuring the A260/280 and A260/230 ratios using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). RNA Integrity was tested on 1.2% agarose gel electrophoresis and ethidium bromide staining. Images were recorded by a GelDoc BIS 303 PC gel documentation system version 3.0.1 (Biostep, Jahnsdorf, Germany). Extracted RNA was further evaluated on a RNA 6000 Nano LabChip using Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) [18]. RNA was further quantified by RiboGreen RNA assay (Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions.

Northern blot analysis

Ten micrograms of total RNA were glyoxylated and separated by size in MOPS (3-(N-morpholino-propanesulfonic acid) agarose gel in MOPS buffer, and transferred to nylon membranes (Hybond N^+, GE Healthcare Europe GmbH, Munich, Germany) by capillary transfer. Hybridisations were performed at 65°C in a phosphate-SDS-EDTA buffer (Church and Gilbert, 1984) using α-[^{32}P]-dCTP (3000 Ci/mmole) labelled cDNA-probes:[EF1α: accession number (TIGR plant transcript assembly TA36627 29760); Aquaporin *VvPIP2.1* (GenBank: EF364438.1); *VvDHN*: (GenBank: AY634281); *Vv-α-gal/SIP*: (GenBank: EU543561)].

Labelling was carried out with the HexaLabel DNA Labelling Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. After overnight hybridisation, membranes were washed with decreasing stringency up to 0,2X SSC (Salt Sodium Citrate) and 1% (w/v) SDS (Sodium Dodecyl Sulfate) at 65°C. Membranes were then exposed to either Kodak BioMax MS X-ray films (Sigma Aldrich, Munich) or to BAS-MS IP screens (Fuji Film GmbH, Düsseldorf, Germany), accordingly. Non-saturated IP-screens were scanned at a resolution of 100 µm by means of a Pharos-FX Plus fluorescence imager and images were analyzed using Image J software [19].

Results and discussion

In this study, we used a CTAB based RNA isolation protocol adopted by Zeng et al. [16] in combination with RNA clean-up protocol of the commercial RNeasy Mini kit. The CTAB extraction buffer contained PVP which helps the dissociation of polysaccharides, phenols and other compound complexes [20], which can be removed later by chloroform extraction. PVP and β-mercaptoethanol were used as reducing agents to prevent the oxidation of phenolic compounds [21]. Pre-warmed extraction buffer was used in order to facilitate the RNase inactivation which was high in mature tissues. In addition, the purification steps used the lithium chloride which was described by Rubio-piña et al. [22] is the best choice for selective RNA precipitation. In fact, lithium chloride has the function to selectively retain DNA [21] as well as proteinaceous substances. The detergent CTAB preserves the integrity of nuclear membrane and organelle membranes yielding total RNA with lower concentrations of unspliced heteronuclear transcripts (hnmRNA) as well as a higher RNA-to-DNA ratio [23].

The integrity and size distribution of the extracted RNA without a clean-up purification step were verified with analytical agarose gels and Bioanalyzer (Figure 3). The corresponding RNA integrity values were about 7.3 indicating the presence of some organic contaminations in the extracted RNA which were confirmed by Northern Blot hybridizations (Figure 4). In addition, a smeary background was also observed during the run on agarose gel (Figure 3C). When we compared total RNA isolated from root samples using the CTAB protocol without any cleaning step (Figure 4) with the total RNA receiving an additional purification step (Figure 4+), we obtained purer RNA and better hybridization patterns as a result of the supplementary clean-up step (Figure 4).

In this study, we showed that high-quality RNA can be obtained from grapevine roots grown in hydroponics using the CTAB-based method in combination with the RNA clean-up protocol of the RNeasy Mini kit (Qiagen). The additional clean-up purification step improved the root RNA quality by eliminating the molecular smear when hybridizing the extracted RNA before and after Qiagen clean-up with the EF1α probe in Northern Blot (Figure 4). The observed smear could be due to the polyphenolic compounds which can strongly bind proteins and nucleic acids to form high-molecular-weight complexes [12]. Cleaning steps with Qiagen purification successfully removed these contaminant complexes.

The electrophoregram of the RNA samples run on the Bioanalyzer (Figures 3A and 5A) helped to determine the intensity of each band on the gel. A successful ladder run with well resolved peaks was observed reflecting a good run process (Figures 3D and 5D). To evaluate RNA purity derived from the combined protocol, absorbance levels at 280 nm, 260 nm, and 230 nm were determined. Low A260/A230 and A260/A280 absorbance ratios indicate,

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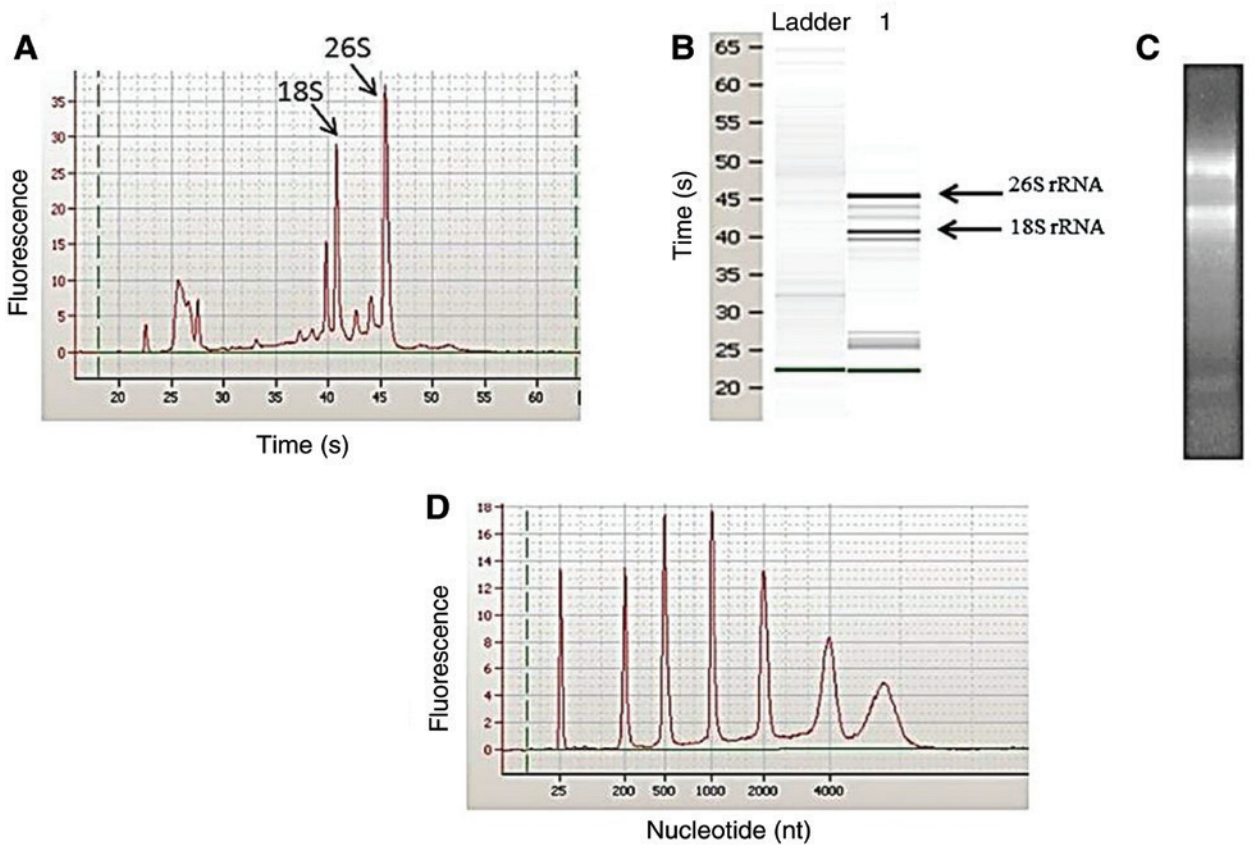


Figure 3: (A) Electropherogram used to assess the RNA integrity number (RIN) for root stressed RNA without Qiagen clean-up purification. The arrows indicate 18S and 26S ribosomal bands. (B) Total RNA of the tested sample electrophoresed on RNA 6000 Nano Lab Chip using Agilent 2100 Bioanalyzer. RIN = 7.3 (Lane 1). (C) Electrophoresis on agarose gel of non-cleaned RNA. (D) Electropherogram for the eukaryote Total RNA Nano Series II ladder. Results are expressed as the averages of three samples ± standard errors.

respectively potential contamination with polysaccharides and polyphenols, and potential contamination with proteins [24]. A_{260}/A_{230} ratio was 2.4 ± 0.15 for all root RNA samples indicating that the RNA is of high purity and without polyphenols and polysaccharides contamination. A_{260}/A_{280} ratio of root RNA was 2.2 ± 0.18 indicating the presence of only trace amounts of proteins. Bioanalyzer-based analysis suggested high integrity of RNA. In fact, the RNA integrity number (RIN) was of 8.4. A RIN value above eight indicates very low RNA degradation. Qiagen clean-up step was able to improve the removal of contaminants in the RNA extracts. The integrity of RNA was assessed by the sharpness of ribosomal RNA bands visualized on denaturing agarose gel (Figure 5C). Well-resolved 26S and 18S rRNA bands were observed with no visible signs of degradation.

The RNA yield was of 79 ± 6.2 µg per g of fresh sample. Several protocols showed that young leaves reached the highest RNA yield and roots generally had the lowest amount of RNA [25, 26].

Expression analysis of salt stress responsive candidate genes

Suitability of the extracted RNA for gene expression studies was confirmed by Northern Blot. The effect of salt stress treatment was further investigated for root-derived RNA from the salt tolerant cultivar Razegui [27]. Transcript levels of three candidate genes, known to be regulated by salt stress (The alkaline alpha galactosidase/seed imbibition protein gene: *Vv-α-gal/SIP*, GenBank accession no. EU543561 [28]; The Dehydrin gene: *VvDHN*, GenBank accession no. AY634281 [29], and the aquaporin gene: *VvPIP.2.1* GenBank accession no. EF364438.1) were evaluated by RNA blot hybridization (Figure 6).

mRNA levels of elongation factor one alpha (EF1-α), a housekeeping gene commonly used as internal control, were clearly not affected by salt treatments. When control and salt stressed plants were compared; an increase in *Vv-α-gal/SIP* transcripts was noticed 6 h after salt treatment with a 2.4-fold increase compared to

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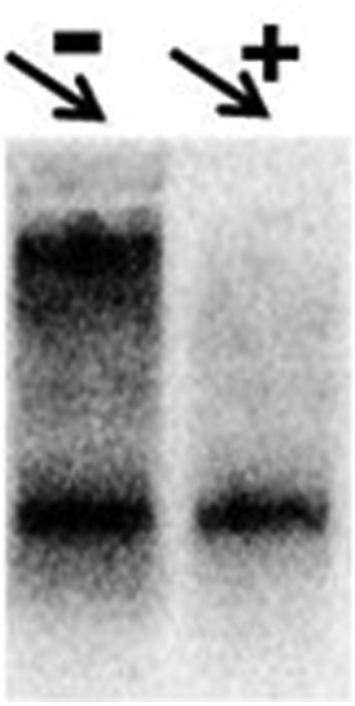


Figure 4: Northern blot hybridization using RNA extracted before (–) and after (+) Qiagen clean-up purification. Probe used correspond to *EF1α*.

the control. *Vv-α-gal/SIP* gene was found to be expressed starting from the first stages of berry formation and peaked at veraison stage. Furthermore, this gene was also induced after 6 and 24 h of salt stress treatment in grapevine leaves [28] suggesting that *Vv-α-gal/SIP* might be involved in the enhancement of the availability of specific carbohydrates, such as raffinose under abiotic stress to help the plant to survive under adverse conditions [30].

Similarly, a dehydrin gene (*VvDHN*), known to be induced by osmotic stress [29–31], was up-regulated in grapevine root tissues under salt-stress conditions. The corresponding mRNA was mostly expressed after 3 and 6 h of exposure to 100 mM NaCl with a 2.4 and 4 fold increase, respectively. Concerning the aquaporin *VvPIP.2*.1 gene, which was previously reported to be involved in abiotic stress response in grapevine [32], the very early response was detected in roots at 3 h after salt treatment with an induction factor of 2.6. This ratio was reduced to 1.4 at 6 h of salt treatment. At 3 h, the *VvPIP.2*.1 gene is induced to enhance water permeability as a very early response [33]. At 6 h, salt stress is already installed, *VvPIP.2*.1 gene expression decreases. Our speculation is that at this time point, plants use other mechanisms

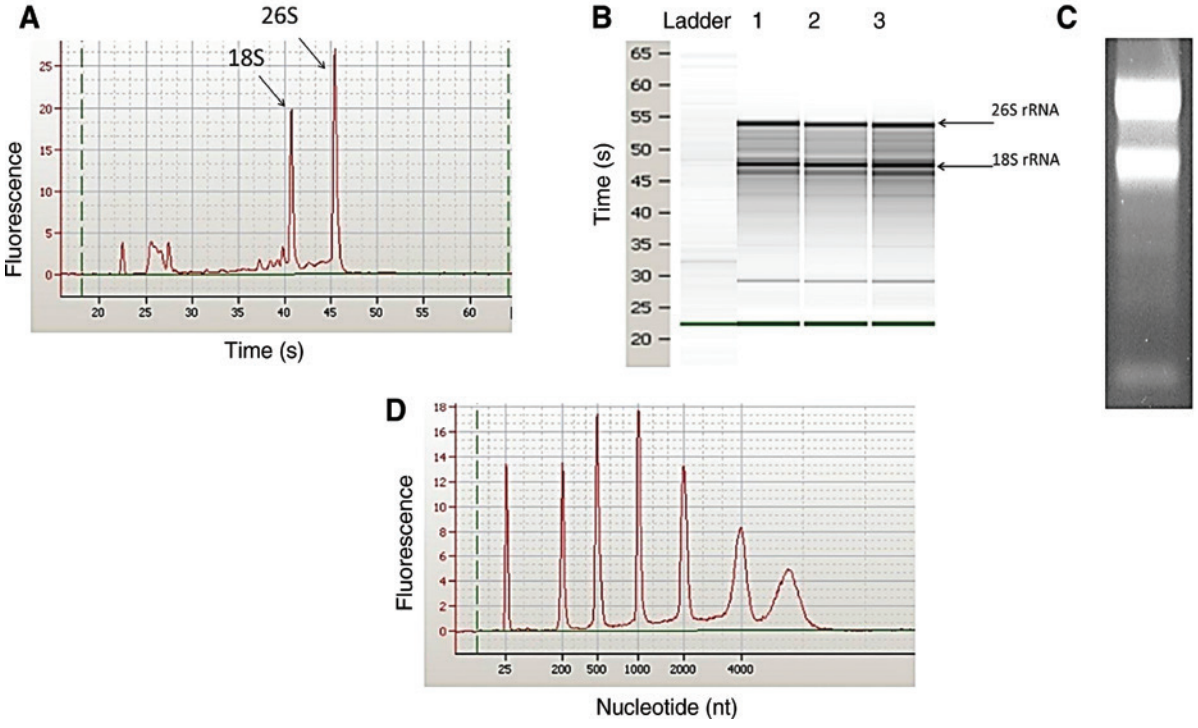


Figure 5: (A) Electropherogram used to assess the RNA integrity number (RIN) for root stressed RNA with Qiagen Clean-up purification. The arrows indicate 18S and 26S ribosomal bands. (B) Total stressed RNA roots electrophoresed on RNA 6000 Nano LabChip using Agilent 2100 Bioanalyzer. RIN = 8.4 (lanes 1,2,3). (C) Electrophoresis on agarose gel of cleaned RNA. (D) Electropherogram for the eukaryote Total RNA Nano Series II ladder. Results are expressed as the averages of three samples ± standard errors.

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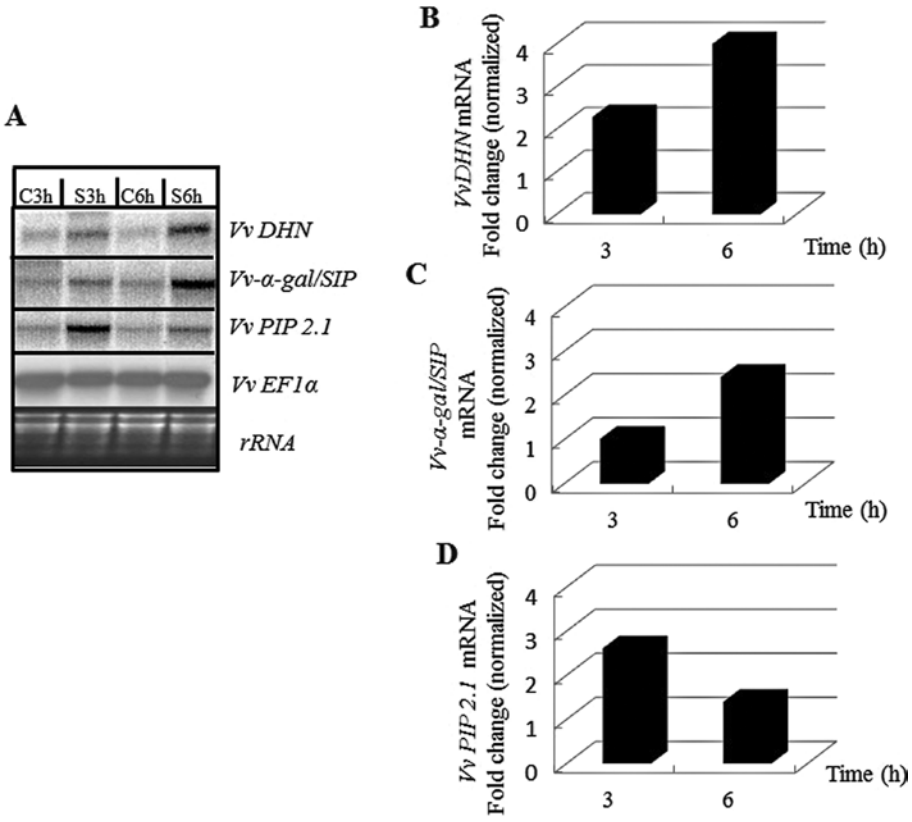


Figure 6: (A) Northern blot analysis of total Root RNA using cDNA probes for *VvDHN, Vv-α-gal/SIP, VvPIP.2.1* and *VvEF1-α*. RNA gel blots were prepared from control (c) and salt stressed (S) plants after 3 h and 6 h of NaCl treatment. The intensities of the bands were calculated using ImageJ and normalized to the value of the *EF1α* gene as internal control. (B, C and D) showed fold change ratios (mRNA stress/mRNA control) of the *VvDHN, Vv-α-gal/SIP* and *VvPIP.2.1* transcript respectively for each time point.

such as the osmotic adjustment strategy to maintain their water status [33].

Conclusions

Our optimized RNA extraction protocol allowed the isolation of high quantity and quality RNA from hydroponically cultivated grapevine roots which are considered to be highly difficult for purification and molecular studies. The isolated RNA can be used for various downstream applications and therefore could help the identification of stress-specific genes.

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Conflict of interest: The authors have no conflict of interest.

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