Physiologia Plantarum 128: 1–7. 2006

TECHNICAL FOCUS

Evaluation of different RNA extraction methods for small quantities of plant tissue: Combined effects of reagent type and homogenization procedure on RNA quality-integrity and yield

Mary Portillo, Carmen Fenoll† and Carolina Escobar†*

Facultad de Ciencias del Medio Ambiente, Universidad de Castilla-La Mancha, Campus de la Real Fábrica de Armas, E-45071 Toledo, Spain

Correspondence
*Corresponding author,
e-mail: carolina.escobar@uclm.es

Received 27 July 2005; revised 14 February 2006

doi: 10.1111/j.1399-3054.2006.00716.x

Highly sensitive techniques for transcriptome analysis, such as microarrays, complementary DNA-amplified fragment length polymorphisms (cDNA-AFLPs), and others currently used in functional genomics require a high RNA quality and integrity, as well as reproducibility among extractions of replicates from the same tissue. There are, however, few technical papers comparing different homogenization techniques and reagents to extract RNA from small quantities of plant tissue. We extracted RNA from tomato seedlings with the three different commercial reagents TRIZOL LS®, TRIZOL®, and TRI Reagent® in combination with pulverization, homogenization-maceration in a mortar, and homogenization with mild vibration plus glass beads, and evaluated total RNA integrity-quality and yield. Pulverization under liquid nitrogen combined with TRIZOL LS® as extraction reagent and homogenization-maceration in mortar with TRI Reagent®, are the procedures that rendered higher RNA yield, integrity and quality, as well as reproducibility among independent RNA extractions. In contrast, short mild vibration pulses (4500 r.p.m. for 5 s) mixed with glass beads, rendered low extraction efficiency and caused, in most cases, partial RNA degradation.

Introduction

RNA quality and integrity, as well as reproducibility among extractions of replicates from the same tissue, are critical for correct transcriptome analysis and some physiological and biochemical studies of plants. Partial degradation and loss during RNA preparation are important parameters to examine before analyses such as microarray hybridization or cDNA-AFLPs are performed. Differences in RNA levels observed among treatments or samples might result from differences in extraction efficiency and/or RNA quality, rather than from biological variations. Therefore, it is important to assess methods that produce high RNA recovery, RNA integrity and reproducibility among replicates.

Total plant RNA has been isolated with different solutions involving cetyltrimethylamonium bromide, dodecyl sulphate salts, phenol/chloroform, guanidine hydrochloride, benzyl chloride, and guanidine isothiocyanate (Chomczynski and Sacchi 1987, Logemann et al. 1987, Verwoerd et al. 1989, Suzuki et al. 2001, Kolosova et al. 2004). Modifications of some of these procedures are being routinely used to avoid insoluble polysaccharides and/or secondary metabolites often present in high amounts in plant tissues and/or species (Puissant and Houdebine 1990, Shirzadegan et al. 1991, Chomczynski and Mackey 1995, Gesteira et al. 2003, Suzuki et al. 2003). Several commercial reagents such as

†Both authors have contributed equally to this work.

TRIZOL® and TRIZOL LS® (Invitrogen Life Technologies, Carlsbad, CA, USA) and TRI Reagent® (Molecular research Center, INC, Ohio, USA) are based in the guanidine isothiocyanate method (developed by Chomczynski and Sacchi 1987), a highly reliable extraction technique that performs well with small quantities of plant tissues. Although manufacturers do not disclose the exact composition of these reagents, all three are based on the strong denaturant guanidinium thiocyanate which is a potent chaeotropic agent more effective than guanidine hydrochloride or phenol in denaturing RNases, as confirmed also in animal tissues (Chirgwin et al. 1979, Seeburg et al. 1977).

Three procedures are often used for RNA extraction from plant material: pulverization, homogenization and maceration. These three extraction procedures have not been systematically evaluated in combination with different extraction reagents. In this paper, we compare three current commercial total RNA extraction reagents in combination with different pulverization and/or homogenization methods, paying special attention to extraction efficiency, quality and integrity of RNA from tomato seedlings.

Materials and methods

Plant material

Tomato seeds (*Lycopersicon esculentum* Mill, cv. Moneymaker) were surface sterilized by soaking for 30 min in 20% commercial bleach with 0.1% v/v Triton® X-100. Seeds were germinated in vitro in Petri dishes (125 mm) containing Gamborg B5 medium (Gamborg et al. 1968) supplemented with 20 g l^{-1} sucrose and 1.5% Daishin agar (Duchefa Biochemie), pH 6.2. Plates were tilted vertically and kept in the dark at 4°C for 48 h. Germination and growth were at 28°C and 65% humidity for 7 days in darkness. Each plate contained 8–10 seedlings. Hypocotyls and cotyledons were collected and frozen in liquid nitrogen for further processing.

RNA extraction procedures

Mortars, pestles and spatulas used to manipulate and homogenize the tissue were treated for 20 min with 0.5*M* NaOH, rinsed in abundant distilled water and autoclaved to avoid RNase activity. Glass beads were also treated with sulphuric acid 1/10 (v/v) for 30 min, washed in abundant water and autoclaved.

Three different procedures were used to grind plant tissue:

(1) *Pulverization:* 100 mg of plant tissue were ground with a mortar and pestle under liquid nitrogen for 3 min. The fine powder was transferred to a 1.5 ml Eppendorf tube with a spatula and mixed with each one of the three different reagents for total RNA isolation.

(2) *Mortar homogenization-maceration:* 100 mg of plant tissue were directly placed in a liquid nitrogen precooled mortar. The RNA isolation reagent was then added, resulting in both tissue and reagent freezing. Homogenization with a precooled pestle continued at 25°C and the tissue gradually defrosted. This homogenization-maceration step was stopped when the solution changed from a thick paste to liquid, which took 4–6 min. Thus, tissue maceration started while tissue and reagent were defrosting. The homogenate was transferred to an Eppendorf tube to complete the extraction procedure.

(3) *Homogenization:* 100 mg of plant tissue were placed in a 1.5 ml Eppendorf tube, precooled in liquid nitrogen. Glass beads (425–600 μm, Sigma-Aldrich Co., Germany) were added into the Eppendorf tube (1/3 of the tube volume) followed by the appropriate reagent. The Eppendorf tube was firmly attached to a SILAMAT® S5 vibrator (Ivoclar Vivadent, Schaan, Liechtenstein) to homogenize the plant tissue. SILAMAT is a universal mixer for amalgam, glass ionomer cements and other predosed dental materials, and it operates at a mixing speed of 4.500 r.p.m. Vibration lengths of 5, 60, 120, 180, 240 and 300 s were tested.

Three different reagents, TRIZOL LS®, TRIZOL® and TRI Reagent®, that contain guanidine isothiocyanate in a mono-phase solution to facilitate RNase activity inhibition and that are improvements of the single-step RNA isolation method, developed by Chomczynski and Sacchi (1987), were used to extract intact RNA. Equivalent amounts of tissue (100 mg) were used for the different extraction procedures. For TRI Reagent® or TRIZOL®, 1 ml of reagent per 100 mg of plant tissue was used, while for TRIZOL LS® the ratio was 0.7 ml/100 mg of tissue. Extraction was always followed by a 5 min maceration step at 25°C to ensure complete dissociation of nucleoprotein complexes. Protocol recommendations from the different companies for samples with high protein, fat and/or polysaccharides content were followed. After extraction, a further centrifugation step was performed at 12.000 xg for 10 min at 4°C prior to phase separation with chloroform. The precipitation step was also modified (Chomczynski and Mackey 1995) to remove polysaccharides. Approximately 0.25 ml of isopropanol followed by 0.25 ml of a high salt precipitation solution (0.8 *M* sodium citrate and 1.2 *M* NaCl) was added to the aqueous phase. The last salt wash step with 75% ethanol was repeated twice in all treatments, as we detected traces of precipitated salts

when, as recommended by all three manufacturers, only one wash step was used.

At least three independent replicates of the same extraction procedure were performed to obtain representative data. RNA samples were dissolved in 20 µl of Milli-Q water.

Concentration and quality of total RNA

Concentration and purity of total RNA were assessed in a NanoDrop® -ND 1000 UV-Vis Spectrophotometer (NanoDrop Technologies, New Zealand), using a 1.2 µl aliquot of the total RNA solutions. RNA purity was estimated from the A260/A280 absorbance ratio, which is an estimation of contamination mainly by proteins and phenol. Putative DNA contamination was not directly assessed. However, no high molecular weight bands indicative of DNA presence were visible after gel electrophoresis of 10–30 µg of total RNA. Special care was taken during aqueous phase recovery to avoid contamination from the interphase and the phenol phase where DNA molecules should be present. However, a very low DNA contamination cannot be ruled out.

RNA integrity

Size and distribution of the extracted RNA molecules were evaluated using an Agilent 2100 Bioanalyser with the RNA 6000 Nano LabChip® kit (Agilent technologies, Palo Alto, California, USA). 1 µl of total RNA solution extracted through the different procedures was used to obtain the electropherograms. The Agilent 2100 Bioanalyser uses an intercalating fluorescent dye that interacts with nucleic acids. The size of the 28 S rRNA molecule is more or less twice that of the 18 S (Ivell 1998). Therefore, the ratio of the peak areas (28 S/18 S) corresponding to the 28S and 18S ribosomal RNAs was used as a reference for RNA degradation, as in intact RNA samples 28S peak area should double that of the 18S.

Statistical analysis of data

The mean values for RNA yield of the different treatments were compared between and within groups by one-way ANOVA and the Tukey HSD and Dunnett's T3 as posthoc tests using the SPSS program (Statistical Package for the Social Sciences, produced by SPSS, Inc. in Chicago, Illinois). The level of significance was set to $P \leq 0.05$.

Results and discussion

Differences in RNA yield within reagent types and within homogenization procedures were statistically significant between pulverization and SIMALAT® S5 ($P = 0.03$) and between homogenization-maceration and SIMALAT® S5 ($P = 0.01$). Homogenization in the SILAMAT® S5 for 5 s resulted in a very low RNA yield as compared to the pulverization and mortar homogenization-maceration using all three different chemicals, although there is a tendency for a higher efficiency when using TRIZOL LS® (Fig. 1). The pulverization and mortar-homogenization methods gave similar results across all the combinations of method and extraction reagent. The TRI Reagent® combined with mortar homogenization-maceration gave significantly

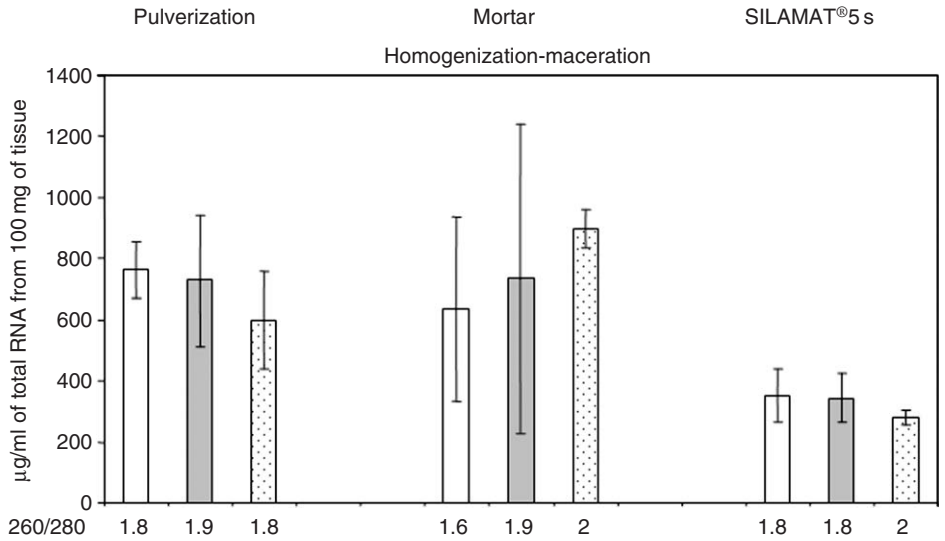

Fig. 1. RNA extraction efficiency for different homogenization procedures and extraction reagents. *Y*-values represent µg μl^{-1} of total RNA, extracted from 100 mg of tissue and dissolved in 20 µl of Milli-Q water. The 260/280 ratio below the graphic is an indication of RNA contamination, mostly by proteins. Data represent means of three independent experiments for each treatment. Standard errors are indicated by bars. Extractions were performed with □ TRIZOL LS®, ▩ TRIZOL®, and ⁙ TRI Reagent®.

higher yields than any combination of reagent with the SIMALAT® S5. TRIZOL LS® with pulverization gave higher yields than any combination of reagents with the SIMALAT® S5, but the differences were slightly above the threshold of statistical significance of $P < 0.05$. Hence, higher yields were obtained with pulverization combined with TRIZOL LS®, and with mortar homogenization-maceration combined with TRI Reagent® (Fig. 1).

The nature of RNA contaminants was not systematically addressed in this work. Phenol is present in all three reagents and special care was taken during phase separations to avoid contamination, so it is not probable that phenol accounts for the contamination differences found among the different procedures. In addition, DNA contamination is very low or null, as judged from gel electrophoresis analyses (see Materials and methods). Therefore, the differences in A260/280 ratios after extraction must be mainly due to differences in protein contamination (see Materials and methods) that were very low in most procedures. However, RNA extracted by mortar homogenization-maceration with TRIZOL LS®, showed more protein contamination than the rest of the extraction methods (Fig. 1). Thus, TRIZOL LS® possibly had a higher efficiency of nucleoprotein complexes dissociation and protein extraction as compared to TRIZOL® and TRI Reagent®. In contrast, the best RNA quality was obtained with TRI Reagent® when mortar homogenization-maceration or SILAMAT® S5 for 5 s was used (Fig. 1).

Regarding RNA integrity, all three reagents maintained an intact RNA when pulverization was used, as inferred from the proportion of 28S/18S RNA peak areas in the electropherograms, which is around 1.8, and the gel-like images (Fig. 2A, B, C). When mortar homogenization-maceration was used, most of the samples extracted with TRIZOL® showed good quality RNA as did all the samples extracted by TRI Reagent® (Fig. 2E, F). Only occasionally partial degradation was observed with TRIZOL® (data not shown). In contrast, the quality of RNA extracted with TRIZOL LS® was variable (data not shown) and some samples showed a distinct 28S RNA degradation pattern, with a 28S/18S peaks area ratio below 1.1 (Fig. 2D). This is probably reflected in the high variation among yields of independent extractions, which was much higher with TRIZOL® and TRIZOL LS® than with TRI Reagent®, as shown from the standard errors in Fig. 1 (a higher absorbance at 260 nm would be expected in partially degraded RNA samples, as single nucleotides show higher absorbance than polymerized RNA). When tissue was homogenized for 5 s in SILAMAT® S5, TRIZOL LS® or TRIZOL®, treatments showed partial degradation and reproducibility of samples was lower than with the TRI Reagent®, as indicated from the higher standard errors of TRIZOL LS® and TRIZOL® (Fig. 1, 2G, H, I). In addition, the 28S/18S ratio was lower by TRI Reagent® combined with SILAMAT® for 5 s than by TRI Reagent® combined with pulverization or mortar homogenization-maceration methods (1.26 ± 0.235 from 4 independent extractions as compared to 1.8 ± 0.2–values when pulverization or homogenization-maceration was used).

SILAMAT® S5 extractions of 2, 4, 6, 8 and 10 cycles of 30 s each (corresponding to 60, 120, 180, 240 and 300 s in total) caused RNA degradation with all three chemicals, as denoted by the bioanalyser patterns obtained (28S/18S peaks area ratio was below 1.1 in all procedures from the 60 s vibration treatment on). As cycle number and length increased, degradation also increased, as shown by the rise in low molecular weight degradation products and the decrease in the 28S/18S peaks area ratios (Fig. 3). In addition, RNA seemed to be more labile with the TRIZOL LS® chemical: the 28S peak sharply decreased from 2 to 4 cycles, as compared to TRIZOL® and TRI Reagent®, where the 28S peak decreased with a more linear tendency. It is important to point that, with all three chemicals, RNA partial degradation was already observed at 2 cycles. The reasons for this effect are unclear and they were not addressed in this work. Possible explanations include: (i) the glass beads in the Eppendorf tube generated mechanical shearing and increasing heat with repeated cycling, resulting in molecular degradation. (ii) a slow mechanical disruption of the tissue in the SILAMAT® S5, which might delay access for the reagents that inactivate endogenous RNases. The differences encountered in RNA stability with TRIZOL LS® as compared to TRIZOL ® and TRI reagent® might stem from differential ability of the three reagents to dissociate the nuclear complexes. Different combinations of the former explanations are certainly possible.

Our systematic evaluation was performed in aerial organs from dark-grown tomato seedlings because this material was easier to collect. However, root tissue and galls from nematode-infected roots were also tested in SILAMAT® S5 and mortar homogenization-maceration procedures. Two different extraction reagents were compared, TRIZOL LS® and TRI Reagent®. Results were similar to those obtained with aerial tissue (data not shown), suggesting that efficiency, quality and integrity of RNA are maintained with the same procedure in different tomato tissues.

To summarize, we have found that when a high RNA quality-integrity from small quantities of plant tissues is required, it is crucial to reach a compromise between extraction efficiency and RNA quality-integrity. TRIZOL LS® is probably the reagent with higher and quicker extraction capacity, as indicated by the highest yield obtained

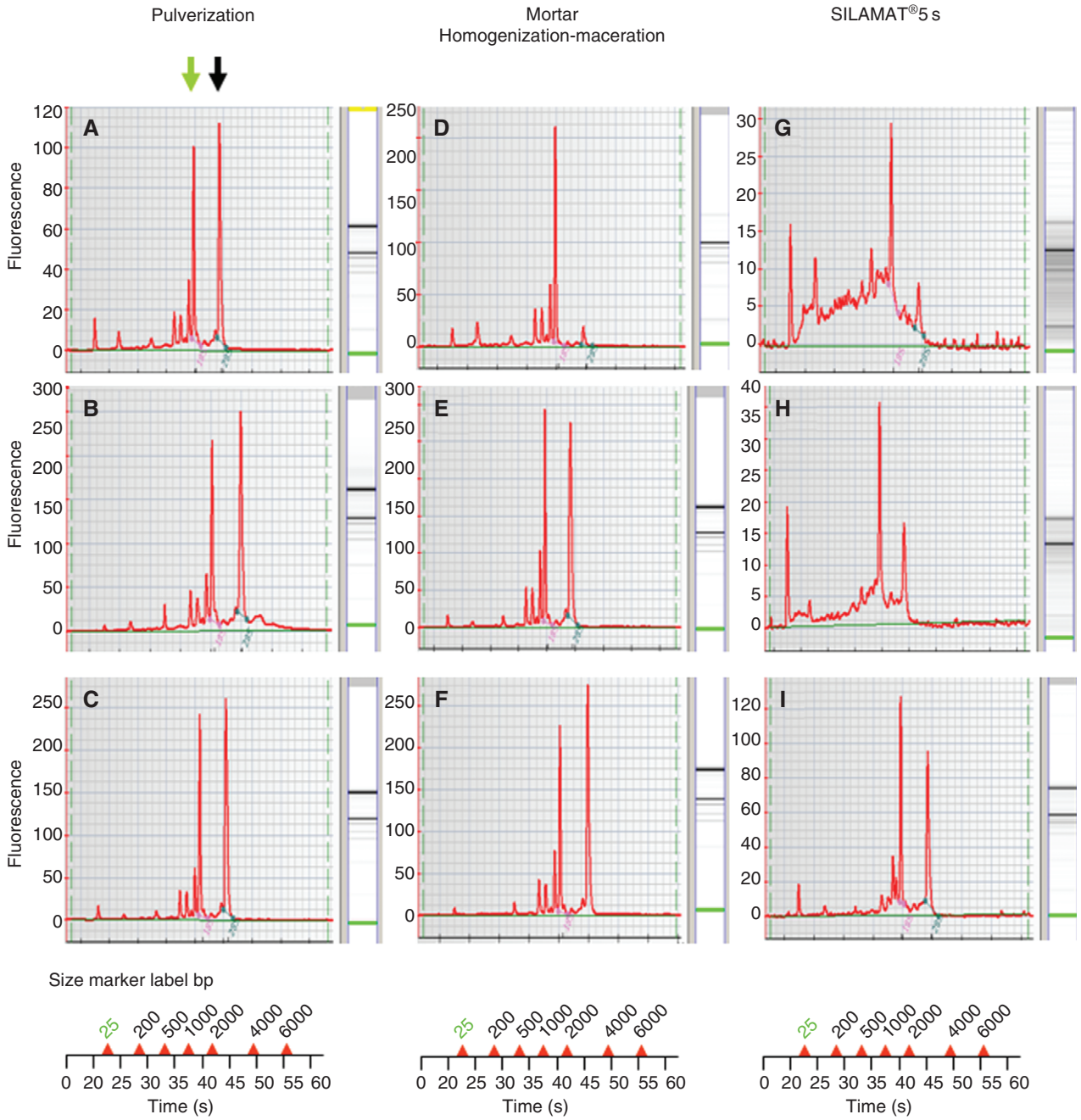

Fig. 2. RNA integrity assessment by bioanalyser analysis. Electropherograms of RNA samples for the different treatments are shown (A, D G: TRIZOL LS®, B, E, H: TRIZOL® and C, F, I: TRI Reagent®). Standard size marker labels in pb is shown at the bottom of the figure. Gel-like images for each treatment, generated from the electropherograms, are included for clarity on the right sides. Positions of 28S and 18S RNAs are indicated at the top of the figure by a black and a green arrow, respectively.

after a brief 5 s homogenization in SILAMAT® S5 and after only pulverization, as compared with the rest of the treatments (Fig. 1). However, RNA quality-integrity is not maintained so well as with the TRI Reagent® with most homogenization procedures, except for pulverization (Figs 1 and 2). Therefore, our recommended methods are: (1) TRI Reagent® combined with the mortar homogenization-maceration procedure, which shows the highest RNA yield and quality-integrity (Figs 1 and 2F), and (2) as a quicker choice, TRIZOL LS® combined with the pulverization procedure, with also a high yield and good quality-integrity (Figs 1 and 2A). If many different samples

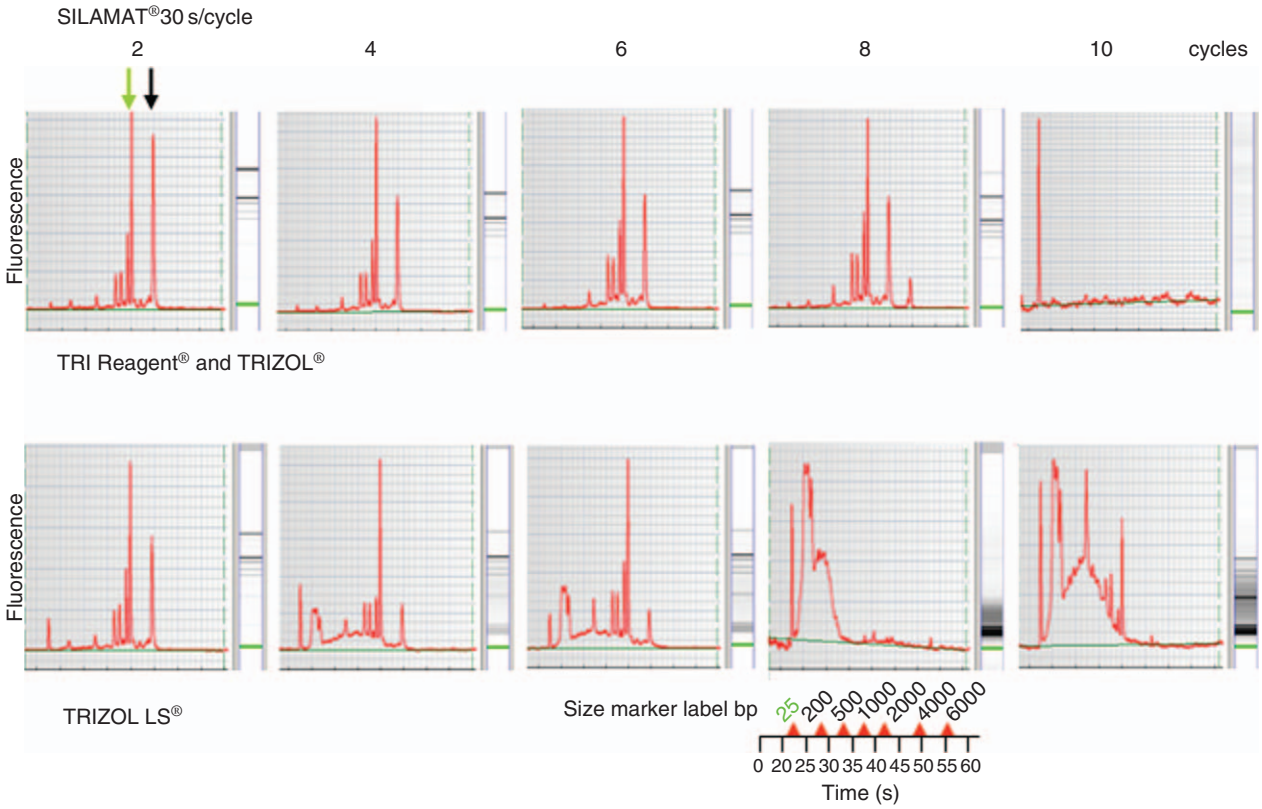

Fig. 3. Combined effects of reagent type and SILAMAT® S5 cycle number on RNA integrity. Electropherograms for samples processed with different RNA extraction reagents and homogenization cycles in the SILAMAT® S5 homogeniser are show. Standard size marker labels in pb is shown at the bottom of the figure. Positions of 28S and 18S RNAs are indicated at the top of the figure by a black and a green arrow, respectively.

need to be processed, laboratories often use SILAMAT® S5 or similar equipments with a short vibration cycle, such as 5 s, combined with different reagents plus glass beads. However, in our hands and combined with TRI Reagent®, RNA integrity was not completely preserved and, more important, RNA extraction efficiency was significantly lower than in homogenization-maceration procedures (only 46% recovery). In addition, the 5 s homogenization procedure with TRIZOL LS® showed a significantly lower yield than pulverization and, like with TRIZOL®, it might produce partial degradation. Therefore, variability in sample quality would be expected.

To our knowledge, the present work is the first systematic assessment of different homogenization techniques in combination with different commercial extraction reagents. All these reagents are based in the guanidine isothiocyanate method (developed by Chomczynski and Sacchi 1987), appropriate to extract RNA from small quantities of plant tissues. We have also failed to find any reference describing performance of different RNA extraction methods specifically from tomato plants. These comparisons are, however, an asset for Solanaceous functional genomics projects, since many of the transcriptome analysis techniques, such as microarray hybridization (Noordewier and Warren 2001) need high integrity, quality and RNA yield as well as a high reproducibility, often from very small quantities of tissue (Kerk et al. 2003).

Acknowledgements – We thank Fernando Carrasco and Eloisa Sanz for their technical support with the bioanalyser (Servicio de Genomica, CBM, CSIC, UAM). This work was supported by grants from the European Commission (QLK5- 1999–01501) and the Castilla-La Mancha Autonomous Government (JCCM, GC-02–011) to CF, and from the Fundación Ramón Areces and the Ministerio de Educación (AGL-2004–08103-C02-02) to CE. MP was a recipient of fellowships from Fundación Ramón Areces and from the European Commission.

References

Chirgwin J, Przybyla A, MacDonald R, Rutter W (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 27: 5294–5299

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159

Chomczynski P, Mackey K (1995) Short technical reports. Modification of the TRI Reagent® procedure for isolation of RNA from polysaccharide and proteoglycan rich sources. Biotechniques 19: 942–945

Gamborg OL, Miller RA, Rajima K (1968) Nutrient requirement of suspensions cultures of soybean root cells. Exp Cell Res 50: 151–158

Gesteira AS, Micheli F, Ferreira CF, Cascardo JC (2003) Isolation and purification of functional total RNA from different organs of cacao tree during its interaction with the pathogen *Crinipellis perniciosa*. Biotechniques 35: 494–500

Ivell R (1998) A question of faith-or the philosophy of RNA controls. J Endocrinol 159: 197–200

Kerk NM, Ceserani T, Tausta L, Sussex I, Nelson TM (2003) Laser capture microdissection of cells from plant tissue. Plant Phys 132: 27–35

Kolosova N, Miller B, Ralph S, Ellis BE, Douglas C, Ritland K, Bohlmann J (2004) Isolation of high-quality RNA from gymnosperm and angiosperm trees. Biotechniques 36: 821–824

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem 163: 16–20

Noordewier MO, Warren PV (2001) Gene expression microarrays and the integration of biological knowledge. Trends Biotechnol 19: 412–415

Puissant C, Houdebine LM (1990) An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Biotechniques 8: 148–149

Seeburg PH, Shine J, Martial JA, Ullrich A, Baxter JD, Goodman HM (1977) Nucleotide sequence of part of the gene for human chorionic somatomammotropin: purification of DNA complementary to predominant mRNA species. Cell 12: 157–165

Shirzadegan M, Christie P, Seemann JR (1991) An efficient method for isolation of RNA from tissue cultured plant cells. Nucleic Acids Res 19: 6055

Suzuki Y, Hibino T, Kawazu T, Wada T, Kihara T, Koyama H (2003) Extraction of total RNA from leaves of Eucalyptus and other woody and herbaceous plants using sodium isoascorbate. Biotechniques 34: 988–993

Suzuki Y, Makino A, Mae T (2001) An efficient method for extraction of RNA from rice leaves at different ages using benzyl chloride. J Exp Bot 52: 1575–1579

Verwoerd TC, Dekker BM, Hoekema A (1989) Small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res 17: 2362

Edited by D. Campbell