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EFFECT OF DIFFERENT REVERSE TRANSCRIPTION APROACHES IN Pru p 3 TRANSCRIPTS SEMIQUANTITATIVE AMPLIFICATION

Jana Žiarovská, Matúš Kyseľ, Lucia Zeleňáková, Eloy Fernández Cusimamani

ABSTRACT
Reverse transcriptase transcribes the cDNA based on its previous extraction and standardization. Reverse transcription step is considered to be critical in the workflow of quantification of transcribed genes. The aim of the study was to extract total RNA by different methods and to analyse the results of the subsequent reverse transcription reaction when different commercial RT kits were used to process RNA extracted from pulp of matured peach fruit. Mature peach pulp was used in the study. The fruit of variety Vistarich was collected in summer 2017 in the orchard of Dvory nad Žitavou. Two RNA extraction methods, TRIzol® Reagent and GeneJET Plant RNA Purification Kit, were tested in to determine the suitable method for peach fruit RNA extraction. Three different cDNA reagent sets were used to transcribe 115 ng/500 ng total RNA or 11 ng/115 ng, respectively. Both variants of the primers, random hexamers as well as oligo (dT) 18, were used to anneal the target mRNA of Pru p 3 allergen following the manufacturer instructions. No specific effect was obtained in the case of peach fruit when using ethanol-extracted tissue treatment and the effect of the used extraction method was more significant. The A260/230 ratios were similar for three from four tested methods. In the case of these three methods, the A260/A230 ratios for all the extracted samples were higher than 1.9 which indicates high purity without contamination by polyphenols and polysaccharides. The specificity of obtained amplicons was proved by restriction cleavage using Tse I restriction endonuclease. This provided the cleavage of the 179 bp long product in all amplicons. Working with mature fruit meet a specific situation in the field of RNA extraction and subsequently all the downstream applications. That is, why choosing the most fitting methods and kits is a crucial step. Here, the method for the semi-quantitative analysis of the Pru p 3 allergen expressions was set up in the way that will be directly applicable for Pru p 3 expression analyses.

Keywords: reverse transcription; peach; RNA extraction; Pru p 3; semiquantitative amplification

INTRODUCTION
The variable types of specific analytical procedures are used to describe plant genome variability and plant transcriptomic characteristics actually. Different DNA markers are used for the purpose of the genome mapping and revealing their natural variability **(Vivodík et al., 2015; Ražná et al., 2016)**. Quantifying of gene expression is one of the well establishing methods that are a part of a research in many different area of interest **(Kačániová et al., 2012; Žiarovská et al., 2013)**. RT-PCR (reverse transcriptase polymerase chain reaction) transcribes the cDNA based on its previous extraction and standardization. Reverse transcription step is considered to be critical in the workflow of quantification especially for the low copy transcribed genes **(Sanders et al., 2014)**. The process of reverse transcription optimizing comprises from a several steps (Figure 1) that conditioned the final efficiency of the analysis.

The research strategy based on the RT method is a very reproducible one, gives a very high precision and allows amplification of different types of mRNA **(Nicot et al., 2005)**.

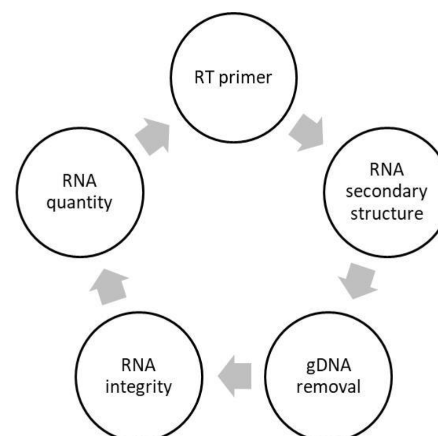

Figure 1 Components of reverse transcription process optimization.

The aim of the study was to extract total RNA by different methods and to analyse the results of the subsequent reverse transcription reaction when different commercial RT kits were used to process RNA extracted from pulp of matured peach fruit.

Scientific hypothesis

Here, two premises were set up for the experiments.
1) The secondary metabolites content in the peach fruit is well-drained by water content that allow use the standard extraction method, even those of commercially available.
2) Effectivity of reverse transcription will be different for the same peach extracted RNA for different cDNA synthesis kits used to process it.

Statistical analysis

The primary testing for both of the hypothesis data was based on the qualitative analysis by resolution through an agarose gel. Statistical evaluation of the results was used for data obtained for RNA extraction method and for results of reverse transcription. It was realized by ezANOVA software for Windows (http://www.cabiatl.com/mricro/ezanova/) Measurements of repeating of samples were expressed as means ± standard deviation. The data were subjected to the one factorial ANOVA pairwise comparisons with Tukey HSD with the level of significance associated to the statistical test 0.01. The null hypothesis was tested that a difference exists among the amounts of extracted RNA depending on the extraction method used as well as in effectivity of reverse transcription.

MATERIAL AND METHODOLOGY

Biological material

Mature peach pulp was used in the study. The fruit of variety Vistarich was collected in summer 2017 in the orchard of Dvory nad Žitavou. Collected fruit were stored in -20°C until the processing.

RNA extraction method and quality/quantity checking

Two RNA extraction methods, TRIzol® Reagent (Invitrogen) and GeneJET Plant RNA Purification Kit – (Thermo Fisher Scientific), were tested in to determine the suitable method for peach fruit RNA extraction. Both of the methods were tested in two ways – without any change of the manufacturer´s instruction and with the initial step of ethanol-extracted method of the peach tissue preparation following the protocol according the **Asif et al. (2006)**. The samples were signed as determined in the table 1. Extracted RNA quantity was analysed by Nanodrop spectrophotometer (Thermo Scientific) with absorbances at 230 nm, 260 nm and 280 nm. Contamination level of the extracted RNA by protein and polysaccharides and phenolic compounds was determined as the ratio of the A260/A280 and A260/A230 absorbances. Integrity of the extracted RNA was analysed in 1% agarose gel stained with GelRed™ (Biotium).

Reverse transcription

Three different cDNA reagent sets were used to transcribe 115 vs. 500 ng of total RNA or 11 vs. 115 ng, respectively as follows: Tetro cDNA Synthesis Kit (Bioline), Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific) and AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies). Both variants of the primers, random hexamers as well as oligo (dT) 18, were used to anneal the target mRNA of Pru p 3 allergen following the manufacturer instructions. The reverse transcription reactions were performed at the time and temperature settings recommended by the supliers, too. A half of the obtained transcription product was cleaned by AgencourtAMPure XP purification system (Beckman Coulter) following the manufacturer´s instructions, dissolved in water subsequently and measured for the quantity and quality by NanodropNanophotometer™. The second half of the transcription product was subjected to semi-quantitative amplification.

Table 1 Codes of samples used in the RNA extraction method testing.

RNA extraction method	Codes of 10 samples extracted in total
GeneJET Plant RNA Purification Kit without change	A1 – A10
GeneJET Plant RNA Purification Kit with ethanol-extracted step	B1 – B10
TRIzol® Reagent method without change	C1 – C10
TRIzol® Reagent with ethanol-extracted step	D1 – D10

Semi-quantitative amplification and product specificity checking

Amplification of Pru p 3 allergen transcripts were performed by Combi PPP Master Mix (Top-Bio) using the 300 nmoL × dm^{-1} of the specific primers and 100 ng of transcribed cDNA. Primers for the amplification of Pru p 3 allergen were designed by Primer3web version 4.0.0 (http://primer3.ut.ee/) on the base of sequence from NCBI under the accession AY620230.1. Thermal profile of PCR reactions was as follows: 94 °C, 1 minute, 35 x (94 °C for 20 seconds; 60 °C for 20 seconds; 72 °C for 30 seconds) and final 72 °C 7 minutes. PCR products specificity was checked using the 2% AGE and confirmed subsequently by Tse I (NEB Enzymes) restriction cleavage.

RESULTS AND DISCUSSION

RNA isolation is often the most serious difficulty to solve in the workflow of gene expression analysis during fruit development and ripening. This obstacle is caused by the biochemical nature of secondary metabolite concentrations in fruit and its changes that occur during the process of ripening. That is, what affect both the quantity and quality of isolated RNA **Gudenschwager et al. (2012)**.

Here, four protocols were used to extract total RNA from the pulp of peach that is known to contain high levels of polysaccharides and polyphenolic compounds **(Gil et al., 2002; Hu et al., 2002)**.

The A260/230 ratios were similar for three from four tested methods (Table 2).

Table 2 Purity and yield analysis of total extracted RNA from peach pulp using different methods.

Method	Quantity and quality parameters			
	A260/A208 ±SD	A260/A230 ±SD	RNA yield ng.µL^{-1}±SD	Number of samples
A	2.00 ±0.22	2.12 ±0.10	340 ±71	10
B	1.98 ±0.13	1.88 ±0.37	400 ±20	10
C	1.84 ±0.25	1.86 ±0.16	35 ±28	10
D	1.95 ±0.18	1.98 ±0.03	18 ±7	10

Note: A – GeneJET Plant RNA Purification Kit without change; B – GeneJET Plant RNA Purification Kit with ethanol-extracted step; C – TRIzol® Reagent method without change; D – TRIzol® Reagent with ethanol-extracted step

Table 3 ANOVA analysis of the yield of RNA extraction methods used.

Descriptive details	Extraction method			
	A	B	C	D
Mean	340	400	35	18
StDev	71	20	28	7
SE	40.99	11.55	16.17	4.04
Var	5041	400	784	49
CI95%	52.73	52.73	52.73	52.73
N	10	10	10	10
Skew	0	0	0	0
zSkew	0	0	0	0

PAIRWISE COMPARISONS [Q=TukeyHSD: **=p <0.01]
[A] vs [B] t(4) = 1.41 p <0.2317 Q = 2.6240
[A] vs [C] t(4) = 6.92 p <0.0023 Q = 13.3388**
[A] vs [D] t(4) = 7.82 p <0.0014 Q = 14.0823**
[B] vs [C] t(4) = 18.37 p <0.0001 Q = 15.9629**
[B] vs [D] t(4) = 31.22 p <0.0001 Q = 16.7064**
[C] vs [D] t(4) = 1.02 p <0.3653 Q = 0.7435

Note: A – GeneJET Plant RNA Purification Kit without change; B – GeneJET Plant RNA Purification Kit with ethanol-extracted step; C – TRIzol® Reagent method without change; D – TRIzol® Reagent with ethanol-extracted step.

Table 4 Influence of the priming method on the cDNA yield using different kits.

Variant of the reverse transcription	Amount of transcribed product in 1 µL
Tetro cDNA Synthesis Kit/ oligo dT(18) primers/ 115 ng RNA in reverse transcription	311
Tetro cDNA Synthesis Kit/ random primers/ 115 ng RNA in reverse transcription	353
Tetro cDNA Synthesis Kit/ oligo dT(18) primers/ 500 ng RNA in reverse transcription	1589
Tetro cDNA Synthesis Kit/ random primers/ 500 ng RNA in reverse transcription	1568
Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase/ primer mix/ 115 ng RNA in reverse transcription	1587
Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase/ primer mix/ 500 ng RNA in reverse transcription	5847
AccuScript High Fidelity 1st Strand cDNA Synthesis Kit / oligo dT(18) primers/ 11 ng RNA in reverse transcription	1571
AccuScript High Fidelity 1st Strand cDNA Synthesis Kit / random primers/ 11 ng RNA in reverse transcription	1469
AccuScript High Fidelity 1st Strand cDNA Synthesis Kit / oligo dT(18) primers/ 115 ng RNA in reverse transcription	14870

Table 5 ANOVA analysis of the yield obtained by different transcriptions.

Descriptive details	cDNA synthesis kit		
	Tetro	**Maxima**	**Accu**
Mean	322	1539.75	14603.75
StDev	22.88	50.7	432.85
SE	11.44	25.35	216.42
Var	523.33	2570.92	18358.92
CI95%	284.97	284.97	284.97
N	4	4	4
Skew	0.992	-0.005	-1.687
zSkew	0.81	-0.005	-1.378

PAIRWISE COMPARISONS [Q = TukeyHSD: ** = p <0.01]
[Tetro] vs [Maxima] t(6) = 43.78 p <0.0001 Q = 9.6662**
[Tetro] vs [Accu] t(6) = 65.90 p <0.0001 Q = 113.3647**
[Maxima] vs [Accu] t(6) = 59.95 p <0.0001 Q = 103.6986**

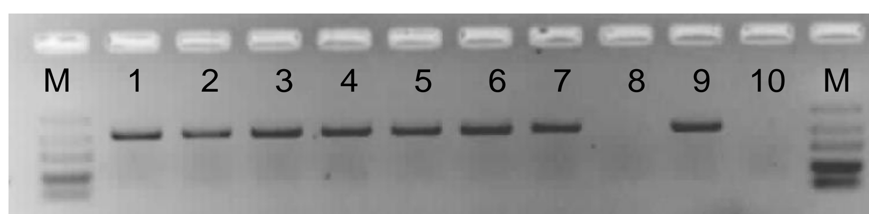

Figure 2 Amplification of Pru p 3 transcripts in the tested transcribed cDNA.

Note:

Code in the electrophoreogram	Variant of the reverse transcription
1	Tetro cDNA Synthesis Kit/ oligo dT(18) primers/ 115 ng RNA in reverse transcription
2	Tetro cDNA Synthesis Kit/ random primers/ 115 ng RNA in reverse transcription
3	Tetro cDNA Synthesis Kit/ oligo dT(18) primers/ 500 ng RNA in reverse transcription
4	Tetro cDNA Synthesis Kit/ random primers/ 500 ng RNA in reverse transcription
5	Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase/ primer mix/ 115 ng RNA in reverse transcription
6	Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase/ primer mix/ 500 ng RNA in reverse transcription
7	AccuScript High Fidelity 1st Strand cDNA Synthesis Kit / oligo dT(18) primers/ 11 ng RNA in reverse transcription
8	AccuScript High Fidelity 1st Strand cDNA Synthesis Kit / random primers/ 11 ng RNA in reverse transcription
9	AccuScript High Fidelity 1st Strand cDNA Synthesis Kit / oligo dT(18) primers/ 115 ng RNA in reverse transcription
10	AccuScript High Fidelity 1st Strand cDNA Synthesis Kit / random primers/ 115ng RNA in reverse transcription

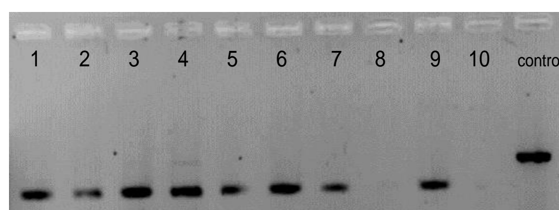

Figure 3 Restriction analysis of Pru p 3 amplicons.
Note: Codes of the samples correspond to the codes in the Figure 2.

In the case of these three methods, the A260/A230 ratios for all the extracted samples were higher than 1.9 which indicates high purity without contamination by polyphenols and polysaccharides. Here, the A260/A280 ratios varied between 1.88 and 2.12 for the extracted samples with the lack of contamination by proteins. In contrast, the samples extracted by TRIzol® Reagent method without change showed protein contamination indicated by the lower A260/280 ratios. Extraction protocols tested in the study resulted in much higher RNAyield in the case of GeneJET Plant RNA Purification Kit with/without change in the manufacturer´s workflow when compared to the TRIzol® Reagent method.

Ethanol-extracted step was added to the protocol because of the removal of water and carbohydrates from fruit were critical for obtaining high-quality and sufficient quantities of RNA **(Davis et al., 2006)**. Here, no specific effect was obtained in the case of peach fruit when using ethanol-extracted tissue treatment and the effect of the used extraction method was more significant (Table 3). Setting of the RNA extraction protocol efficiency differ highly for the individual plant species, because **Da Luz et al. (2016)** reported, that TIzol® Reagent/ice protocol is preferred for extracting of *P. edulis* RNA. This method eliminates polyphenols very effectively and a high amount of extracted RNA was obtained for the reported species.

Extracted RNA with the best parameters of quality and quantity was processed by different reverse transcription strategies further. All the transcriptomic reactions actually used are very dependent on the reliability of the reverse transcription and the accuracy of this steps both, in the experiments as well as in the diagnostics **(Mannonen et al., 2011; Huggett and Bustin, 2011)**. The reverse transcription is still not completely understood **(Ståhlberg et al., 2004)** and in spite of its importance, it is considered as an uncertain step of the transcriptomic analysis. Reverse transcriptases possess a much higher error rates when comparing them to other DNA polymerases **(Roberts et al., 1988)**. The sucsess here a mix of the effect of secondary and tertiary structure of mRNA, priming variability and effectivity, and finally the characteristics of reverse transcriptase that is used. All this is strongly affected by inhibitors that can persist in minor after RNA extraction, especially in plant biological material **(Lekanne et al., 2002; Polumuri et al., 2002)**. Actually, no unified method exists for plant species.

Three different cDNA synthesis kits were used to transcribe 500 ng, 115 ng or 11 ng of extracted RNA respectively. All of them are suitable for the RNA extracted from plants and possess a certain range of the starting amount of RNA. Tetro cDNA Synthesis Kit and Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase were used in the 500 ng and 115 ng of RNA and AccuScript High Fidelity 1st Strand cDNA Synthesis Kit was used with the 115 ng and 11 ng of RNA, because the manufacturer declares a lower amount of RNA that is needed for the reverse transcription. Tetro cDNA Synthesis Kit and AccuScript High Fidelity 1st Strand cDNA Synthesis Kit was tested in the both, random hexamers as well as oligo (dT) 18 primers. Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase provides a primer mix that is prepared and mixed by the supplier.

First, the sensitivity of the reverse transcription kits was analysed. When comparing all three cDNA synthesis kit, the starting amount specific differences were obtained dependent in the amount of transcribed product among variants of different amount of RNA used for the transcription (Table 4). Further, the differences among the individual kits were obtained. The lowest amount of transcribed product measured in the case of Tetro cDNA Synthesis Kit / 115 ng of RNA and the highest for the Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase / 500 ng. All the three cDNA synthesis kits are suitable for further processing of the transcribed product with gene-specific primers. Tetro cDNA Synthesis Kit contains MMLV reverse transcriptase and is designed to be used with the range of RNA from 100 pg up to the 2 µg. Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase contains *in vitro* derivate of MMLV reverse transcriptase and is designed to be used with the range of DNA from 5 ng up to the 0.5 fg. AccuScript High Fidelity 1st Strand cDNA Synthesis Kit contains a derivate of MMLV reverse transcriptase, too and is designed to be used with a range of 10 ng up to the 5 µg. When comparing data from the reverse transcription from 115 ng that was realized by all the three tested cDNA kits, statistical differences exist in the obtained amounts of transcribed product (Table 5).

Second, the applicability and the incorporation of the cDNA protocol to the workflow of Pru p 3 semiquantitative PCR were analysed. Individual transcribed products were diluted and unified to the 100 ng.µL^{-1} and the semiquantitative reactions were performed. The PCR resulted in the negative amplification only in the case of using the random primers for both of the tested different starting amount of RNA with AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Figure 2).

The specifity of obtained amplicons was proved by restriction cleavage using Tse I restriction endonuclease. This provided the cleavage of the 179 bp long product in all amplicons (Figure 3).

Reverse transcription PCR is accepted as a very sensitive and specific approach that is used widely for the transcripts detection and their subsequently quantification. Despite the accuracy of absolute or quantitative techniques, semi-quantitative methods are still widely used and appropriate for many purposes **(Marone et al., 2001)**, when the specific transcripts quantification and detection of any variation in their expression levels under different experimental conditions is needed. Semi quantitative approach was applied previously successful in the expression analysis of the genes in different plant species **(Hirose and Terao, 2004; Zou et al., 2008)**. The expression patterns of different starch synthase genes **(Hirose and Terao, 2004)** and nine heat shocks protein genes **(Zou et al., 2008)** were obtained by semi quantitative RT–PCR analysis in *Oryza sativa*, L.

Quantifying of plant allergen expression is still limited mainly to its analysis of the presence/absence in the food matrix and only a few studies exist where the methods for RNA extraction or RT-PCR can be found **(Žiarovská and Zeleňáková, 2016)**. **Knoteková and Žiarovská (2017)** used the semi quantitative approach to analyse the Mal d 1.03 allergen in the varieties Golden and Spartan during

the ripening. This technique was proved to be sensitive and effective in all of these studies.

CONCLUSION

Working with mature fruit meet a specific situation in the field of RNA extraction and subsequently all the downstream applications. That is, why choosing the most fitting methods and kits is a crucial step. Here, the initial step of ethanol-extracted method of the peach tissue preparation was not proved as a statistical significant in the workflow with the p values $p < 0.0023$ and $p < 0.3653$. Subsequently, the method for the semi-quantitative analysis of the Pru p 3 allergen expression was set up in the way that will be directly applicable for Pru p 3 expression analysis with the amplicon specificity analysis with Tse I restriction endonuclease.

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Contact address:
doc. Ing. PaedDr. Jana Žiarovská, PhD., Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: jana.ziarovska@uniag.sk

Ing. Matúš Kyseľ; Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: xkysel@is.uniag.sk

doc. Ing. Lucia Zeleňáková, PhD. Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail:lucia.zelenakova@uniag.sk

prof. Dr. Ing. Eloy Fernández Cusimamani; Czech University of Life Sciences Prague, Faculty of Tropical AgriSciences, Kamýcká 961/129, 165 00, Prague-Suchdol, Czech Republic