International Journal of Horticultural Science 2009, 15 (1–2): 101–104
Agroinform Publishing House, Budapest, Printed in Hungary
ISSN 1585-0404

Optimization of RNA isolation from stone fruits at different ripening stages

Pfeiffer, P., Halász, J., György, Z., Pedryc, A. & Hegedűs, A.

Corvinus University of Budapest, Faculty of Horticultural Science, Department of Genetics and Plant Breeding, 1118 Budapest, Ménesi út 44., Hungary; E-mail: hegedus.attila@uni-corvinus.hu

Summary: This study was conducted to select the most appropriate RNA isolation method that can be used successfully in case of stone fruits. The changing pattern of gene expression during the ripening process of stone fruits may elucidate the molecular background of several phenotypical or phytochemical alterations present among different genotypes. Our laboratory aims to study the expression of genes encoding for enzymes that catalyze crucial steps in the flavonoid biosynthesis pathway. RNA isolation from fruit mesocarp is a challanging task due to high levels of sugars and polyphenolics accumulating during fruit development. Therefore, at first, the optimal techniques eligible for RNA isolation from fruit tissues at different ripening stages must be selected. Our study compares three different RNA isolation protocols and describes their potential applicability according to different fruit species and ripening stages.

Key words: apricot, peach, *Prunus*, RNA isolation, sour cherry, stone fruits

Introduction

Fruits and vegetables are rich sources of antioxidant compounds and efficiently inhibit the aetiology and pathogenesis of many degenerative diseases (*Block* et al., 1992; *Dauchet & Dallongeville*, 2008; *Terry* et al., 2001). Health-prompting effects of fruits including stone fruits are widely attributed to the polyphenolic, flavonoid compounds showing potent antioxidant capacity (*Ruiz* et al., 2005, *Serrano* et al., 2005). Even if flavonoids are devoted to metal chelation they retain their radical scavenging capacity (*Lugasi* et al., 2003). In berry species known to be rich in antioxidants, the total radical scavenging activity was well-correlated with the total phenolic content having a correlation coefficient of 0.681 confirming that phenolics have a crucial contribution to the radical scavenging capacity (*Hegedűs* et al., 2008a).

Different fruit crops are characterized by quite different polyphenol contents (*Cevallos-Casals* et al., 2006; *Prior* et al., 1998; *Wu* et al., 2006). Our preliminary results confirmed published data that huge differences can be also detected in the total polyphenolic content and antioxidant capacity of various varieties belonging to the same species (*Hegedűs* et al., 2008; *Scalzo* et al., 2005). Furthermore, a range of factors may also influence the total polyphenolic contents of fruits including maturity and geographic region of cultivation (*Dragovic-Uzelac* et al., 2007; *Prior* et al., 1998).

Genes encoding for key enzymes in flavonoid biosynthesis pathways have been isolated and characterized from only a limited number of fruit species in the *Rosaceae* family including apple, pear or strawberry (*Fischer* et al., 2003, 2007; *Pfeiffer* et al., 2006; *Davies*, 1993; *Kim* et al., 2003). A long-term research project was established in the Department of Genetics and Plant Breedig, Corvinus University of Budapest to identify and characterise the genes participating in the flavonoid biosynthesis pathways of stone fruits. The first step of our work was to isolate high quality RNA for further examinations. Usually RNeasy Plant Mini Kit (Qiagen, Budapest, Hungary) and cetyltrimethylammonium bromide (CTAB)-based methods are used for RNA-isolation from plant tissues. However, problems may be encountered during the isolation process due to the large amounts of secondary plant metabolites, such as carbohydrates or polyphenolic compounds (*Schneiderbauer* et al., 1991). Protocols worked out for RNA-isolation from apple (*Gasic* et al., 2004) and bilberry fruit (*Jaakola* et al., 2001) and several plant tissues (*Kiefer* et al., 2000) are based on a pine tree RNA-extraction method (*Chang* et al., 1993), because pine tissues are also very rich in polyphenolic compounds.

Materials and methods

Plant material

Seven apricot (*Prunus armeniaca* L.) genotypes including three commercial cultivars ('Aurora', 'Gönci magyarkajszi', and 'Orange red') and four hybrids ('Preventa', 7/1, 18/61 and 18/79) resulting from a breeding program (conducted in the Department of Genetics and Plant Breeding, Corvinus University of Budapest) have been used in the experiments. Two peach [*Prunus persica* (L.) Batsch] genotypes with red-fleshed fruit and two sour cherries (*Prunus cerasus* L.) were also used for the analyses. The

apricot samples have been collected from the Experimental and Research farm of the Department of Genetics and Plant Breeding, CUB, Szigetcsép, Hungary. Fruits were harvested at five different developmental stages (1. growing green fruits, 2: full-sized green fruits, 3: colouring, full-size semi-mature fruits, 4: fully coloured full-size mature fruits, 5: overripe fruits). The sour cherry fruits were harvested in the Research and Extension Center for Fruit Growing, Újfehértó, Hungary. Fruits were cut into pieces and immediately frozen in liquid nitrogen. RNA extraction was carried out from different fruit parts including skin, flesh and whole slices (skin and flesh together) of apricot. For peaches, white and red parts of the fruit flesh and slices (skin and flesh together) were used as samples. All samples were stored at –80°C.

RNA extraction and cDNA preparation

Three different methods were used for RNA-isolation. The RNeasy Plant Mini Kit (Qiagen) uses silica membrane to bind the RNA molecules selectively. A CTAB-based protocol was decribed for apple fruit rich in polyphenols and polysaccharides (*Gasic* et al., 2004) and another optimized for RNA extraction from bilberry, which uses more purifying steps than the apple protocol (*Jaakola* et al., 2001). The cDNA was synthesized from 1 µg of total RNA using First-strand cDNA Synthesis Kit (Fermentas, Hungary) with oligo(dT)$_{18}$ primers according to the manufacturer's instructions.

Quality control of the isolated RNA

We controlled the quality of the RNA solutions with electrophoresis in 1% agarose gel in 1× TBE-buffer. PCR amplification with specific primers after reverse transcription was also carried out. The amplicones were separeted on 1% agarose gels.

Results and discussion

The RNeasy Plant Mini Kit provides a relatively rapid and clear RNA-isolation method, free from poisonous or toxic agents like phenol or chloroform. The RNeasy Plant Mini Kit yielded RNA of adequate quality and quantity from some apricot samples including all ripening stages. From other samples, especially from ripe and overripe fruits only low quality (degraded) RNA could have been extracted or the RNA extraction was not successful at all (*Figure 1*). RNA extraction with the RNeasy Plant Mini Kit proved to be successful typically in case of fruits with relatively low total polyphenolic contents even at ripe stages (18/61 or 7/1). The skin of the apricot fruit is usually richer in polyphenolic compounds than its flesh. It is interresting to observe that RNA extraction was successful from fruit flesh but not from skin of the fruits at the same ripening stage (e.g. 7/1 and 18/61). The RNA extraction by using the kit was not successful from the red part of ripe peach fruits. This fruit contains high levels of anthocyanins and other polyphenols.

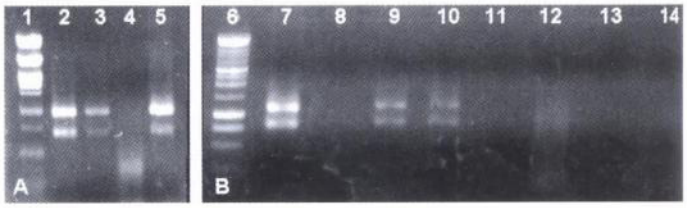

Figure 1: Agarose gel electrophoresis of RNA isolated from fruits at different ripening stages of apricot genotypes and red-fleshed peaches with RNaesy Plant Mini Kit (A) or RNeasy Plant Mini Kit and apple protocol (B). **1:** DNA ladder (Promega Bench Top Ladder 1 kb); **2:** 18/61 hybrid, ripening stage 4, fruit flesh; **3:** 18/79 hybrid, ripening stage 5, fruit flesh; **4:** 'Orange red', ripening stage 2, fruit skin; **5:** 'Orange red', ripening stage 3, fruit skin; **6:** DNA ladder (Promega Bench Top Ladder 1 kb); **7:** 18/61 hybrid, ripening stage 4, fruit flesh; **8:** red-fleshed ripe peach; **9:** 7/1 hybrid, ripening stage 5, fruit flesh; **10:** 'Aurora', ripening stage 2, fruit flesh; **11:** 18/61 hybrid, ripening stage 4, fruit skin; **12:** red-fleshed ripe pech; **13:** 7/1 hybrid, ripening stage 5, fruit skin; **14:** 'Auróra', ripening stage 2, fruit flesh.

RNA-isolation method has not yet been described for apricot fruit flesh until now, so the protocols previously described for other plant tissues rich in polyphenolics or sugars had to be tested and modified if needed. The apple protocol was desribed for RNA isolation from apple bud, internodal shoot, flower and fruit tissues with high levels of polyphenolics and polysaccarides (*Gasic* et al., 2004). This CTAB-based RNA-isolation method differs in many steps from the RNeasy Plant Mini Kit protocol. In contrast to our expectations, we could not isolte RNA from apricot fruits and only degraded RNA was obtained from peach tissues with this method.

Therefore, another RNA-isolation protocol was tested, which is described for bilberry fruit containing outstanding levels of polyphenols, much more than the apple tissues contain (*Jaakola* et al., 2001). The extraction buffer of the protocol is the same as in the apple protocol, but contains more steps for extraction and purification. The longer and more labour-intensive method resulted in successful RNA isolation from all samples (mature apricot, blood peach and sour cherries at all developmental stages) for which the peviously described protocols failed (*Figure 2*).

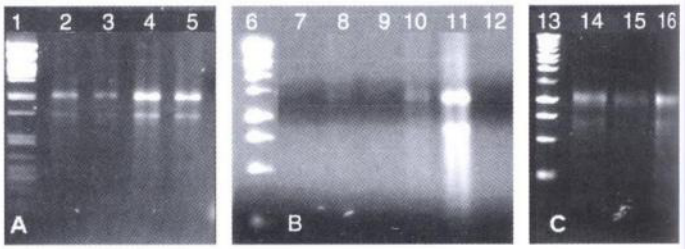

Figure 2: Agarose gel electophoresis of RNA isolated with the bilberry protocol from apricot fruits at different ripening satges (A and B), red-fleshed peaches (A, B and C) and sour cherries (C). **1:** DNA ladder (Promega Bench Top Ladder 1 kb); **2:** Preventa, ripening stage 1, fruit flesh; **3:** 'Aurora', ripening stage 3, fruit skin; **4:** red-fleshed ripe peach; **5:** 18/61 hybrid, ripening stage 5, fruit flesh; **6:** DNA ladder (Promega Bench Top Ladder 1 kb); **7:** 'Gönci magyarkajszi', ripening stage 1, fruit flesh; **8:** Preventa, ripening stage 5, fruit flesh; **9:** 'Orange red', ripening stage 1, fruit skin; **10:** 'Gönci magyarkajszi', ripening stage 4, fruit flesh and skin; **11:** 'Gönci magyarkajszi', ripening stage 4, fruit flesh; **12:** 'Gönci magyarkajszi', ripening stage 5, fruit skin; **13:** DNA ladder (Gene Ruler™ DNA Ladder, Low range), **14:** 'Pipacs 1' sour cherry, ripening stage 2; **15:** 'Korai pipacs', ripening stage 3; **16:** 'Korai pipacs', ripening stage 4.

Table 1: Orthologues gene sequences retrieved from the NCBI GenBank database showing high sequence similarity with the newly isolated apricot flavonoid biosynthesis genes

Gene	Species	Identities (%)	Accession number
chs 18/61 hybrid	*Prunus cerasifera*	94	DQ856583
	Rubus idaeus	87	AF400566
	Sorbus aucuparia	87	DQ286037
	Pyrus communis	87	DQ901397
	Malus domestica	87	EU872155
chs Preventa	*Prunus cerasifera*	96	DQ856583
	Malus domestica	87	EU872155
	Sorbus aucuparia	87	DQ286037
	Malus domestica	86	AY786996
	Pyrus communis	86	AY786998
dfr Aurora	*Prunus persica*	98	AB095030
	Pyrus communis	90	AY227732
	Malus domestica	90	AY227729
	Rosa hybrida	85	ROZD4R
	Fragaria × ananassa	85	AF029685
dfr Preventa	*Prunus persica*	97	AB095030
	Pyrus communis	90	AY227732
	Malus domestica	90	AY227729
	Rosa hybrida	86	ROZD4R
	Fragaria × ananassa	85	AF029685
ans Aurora	*Prunus cerasifera*	98	EF683132
	Prunus persica	97	AB097216
	Malus domestica	91	AF117269
	Pyrus communis	91	DQ230994
	Fragaria × ananassa	85	AY695817

It must be emphasized that this method was also successful for RNA extraction from ripe fruits of the apricot hybrid 'Preventa' which accumulates huge amounts of polyphenolics in its flesh and skin (*Hegedűs* et al., 2008b). The purity, quality and quantity of these RNA samples were controlled with PCR amplifications using the synthesized cDNAs. Degenerate PCR primers were designed for some of the flavonoid biosynthesis genes based on sequence homology to their apple and pear orthologs (*Fischer* et al., 2003, 2007; *Kim* et al., 2003). Genes encoding for the chalcone synthase (CHS; EC 2.3.1.74), dihydroflavonol 4-reductase (DFR; EC 1.1.1.219), anthocyanidin synthase (ANS; EC 1.14.11.19) and flavanone 3-hydroxylase (F3H; EC 1.14.11.9) enzymes were chosen for the quality control PCRs. Two forward and one reverse primer was designed for the *chs*, *dfr* and *ans* genes, which resulted in successful amplification in both combinations. In case of the *f3gt* gene, 3 forward primers were designed and each was used in combination with the oligo$(dT)_{18}$ primer, but we couldn't detect any amplification product.

All amplified fragments were cloned into pGEM-T Easy plasmid vector (Promega, Madison, Wis.) and sequenced. For *chs* (18/61, Preventa, 'Aurora'), *dfr* (18/61, Preventa, 'Aurora') and *ans* ('Aurora', 18/61 and sour cherry), fragments of 760 bp, 667 bp and 853 bp long have been sequenced, respectively. These partial gene sequences were blasted in the NCBI GenBank database, which confirmed that the isolated fragments indeed represent the targeted genes *(Table 1)*. The apricot sequences showed the highest similarity with the corresponding ortholog genes from several *Prunus* species. The degree of identity was lower when sequences were aligned with orthologs from species belonging to the *Maloideae* and *Rosoideae* subfamilies. The expression of the examined genes was detectable at all ripening stages from unripe to mature fruits, which explains the high quantities of polyphenolic compounds present even in unripe, green fruits (*Dragovic-Uzelac* et al., 2007).

Our results led us to the conclusion that stone fruits with lower polyphenol contents or unripe fruits, which have also lower levels of polyphenols in many cases the RNA-isolation was successful with RNeasy plant Mini Kit. From other samples (mainly apricot, red-fleshed peach or sour cherries with extremely high polyphenol content), RNA of appropriate quality could have only been isolated with the protocol decsribed for bilberry. For detailed molecular examinations, an adequate yield and quality of RNA can be achieved by this RNA-isolation method adapted for stone fuits.

Acknowledgements

This work was funded by the NKTH-OTKA K68921 project and also supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

References

Block, G., Patterson, B. & Subar, A. (1992): Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence. Nutr. Cancer, 18: 1–9.

Cevallos-Casals, B.A., Byrne, D., Okie, W.R. & Cisneros-Zevallos, L. (2006): Selecting new peach and plum genotypes rich in phenolic compounds and enhanced functional properties. Food Chem., 96: 273–280.

Chang, S., Puryear, J. & Cairney, J. (1993): A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep., 11: 113–116.

Dauchet, L. & Dallongeville, J. (2008): Fruit and vegetables and cardiovascular disease: epidemiological evidence from the non-Western world. Brit. J. Nutr., 99: 219–220.

Davies, K.M. (1993): A cDNA clone for flavanone 3-hydroxylase from *Malus*. Plant Physiol., 103: 291.

Dragovic-Uzelac, V., Levaj, B., Mrkic, V., Bursac, D. & Boras, M. (2007): The content of polyphenols and carotenoids in three apricot cultivars depending on stage of maturity and geographical region. Food Chem., 102: 966–975.

Fischer, T.C., Gosch, C., Pfeiffer, J., Halbwirth, H., Halle, C., Stich, K. & Forkmann, G. (2007): Flavonoid genes of pear (*Pyrus communis*). Trees, 21: 521–529.

Fischer, T.C., Halbwirth, H., Meisel, B., Stich, K. & Forkmann, G. (2003): Molecular cloning, substrate specifity of the functionally expressed dihydroflavonol 4-reductases from *Malus domestica* and *Pyrus communis* cultivars and the consequences for flavonoid metabolism. Arch. Biochem. Biophys., 412: 223–230.

Gasic, K., Hernandez, A. & Korban, S.S. (2004): RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Mol. Biol. Rep., 22: 437a–437g.

Hegedűs, A., Balogh, E., Engel, R., Sipos, B.Z., Papp, J., Blázovics, A. & Stefanovits-Bányai, É. (2008a): Comparative nutrient element and antioxidant characterization of berry fruit species and cultivars grown in Hungary. HortScience, 43: 1711–1715.

Hegedűs, A., Tordai, E., Engel, R., Stefanovits-Bányai, É. & Pedryc, A. (2008b): Antioxidant characterization of apricot fruits: genotype affected variability and correlations among different antioxidant assays. XIV International Symposium on Apricot Breeding and Culture, June 16–20, 2008, Matera, Italy. Book of Abstracts and Symposium Programme, oral 3.3.

Jaakola, L., Prittilä, A.M., Halonen, M. & Hohtola, A. (2001): Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. Mol. Biotechnol., 19: 201–203.

Kiefer, E., Heller, W. & Ernst, D. (2000): A simple and efficient protocol for isolation of functional RNA from plant tissues rich in secondary metabolites. Plant Mol. Biol. Rep., 18: 33–39.

Kim, S.H., Lee, J.R., Hong, S.T., Yoo, Y.K., An, G. & Kim, S.R. (2003): Molecular cloning and analysis of anthocyanin biosynthesis genes preferentially expressed in apple skin. Plant Sci., 165: 403–413.

Lugasi, A., Hóvári, J., Sági, K.V. & Bíró, L. (2003): The role of antioxidant phytonutrients in the prevention of diseases. Acta Biol. Szeged., 47: 119–125.

Pfeiffer, J., Kühnel, C., Brandt, J., Duy, D., Nimal Punyasiri, P.A., Forkmann, G. & Fischer, T.C. (2006): Biosynthesis of flavan 3-ols by leucoanthocyanidin 4-reductases and anthocyanidin reductases in leaves of grape (*Vitis vinifera* L.), apple (*Malus x domestica* Borkh.) and other crops. Plant Physiol. Biochem., 44: 323–334.

Prior, R.L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G., & Mainland, C.M. (1998): Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J. Agric. Food Chem., 46: 2686–2693.

Ruiz, D., Egea, J., Gil, M.I. & Tomás-Barberán, F.A. (2005): Characterization and quantitation of phenolic compounds in new apricot (*Prunus armeniaca* L.) varieties. J. Agric. Food Chem., 53: 9544–9552.

Scalzo, J., Politi, A., Pellegrini, N., Mezzetti, B., & Battino, M. (2005): Plant genotype affects total antioxidant capacity and phenolic contents in fruit. Nutrition, 21: 207–213.

Schneiderbauer, A., Sandermann, H. & Ernst, D. (1991): Isolation of functional RNA from plant tissues rich in phenolic compounds. Anal. Biochem., 197: 91–95.

Serrano, M., Guillén, F., Martínez-Romero, D., Castillo, S. & Valero, D. (2005): Chemical constituents and antioxidant activity of sweet cherry at different ripening stages. J. Agric. Food Chem., 53: 2741–2745.

Wu, X., Beecher, G.R., Holden, J.M., Haytowitz, D.B., Gebhardt, S.E. & Prior, R.L. (2006): Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. J. Agric. Food Chem., 54: 4069–4075.