Characterization of flavonoid biosynthesis in apricot and sour cherry fruits

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1. INTRODUCTION

Epidemiological studies suggest that increased fruit consumption decreases the risk of several degenerative diseases. Health promoting effects of stone fruits are increasingly attributed to their polyphenolic, flavonoid compounds of antioxidant capacity. Fruits of different species show great variations in their polyphenolic composition. In addition, former studies revealed that huge intraspecific variations (differences in polyphenolic composition in fruits of different cultivars of the same species) can be also detected in polyphenolic contents and antioxidant capacity. A healthy diet can be based on fruits with outstanding antioxidant capacity, which can be also processed into functional foods. Although many compounds (ascorbic acid, carotenoids, betalains, etc.) contribute to the antioxidant capacity, the role of polyphenolic compounds seems to be of crucial importance.

In plant tissues, the formation of flavonoids and phenolic (and other polyphenols) is associated with the phenylpropanoid pathway. The biosynthesis of such compounds starts with the conversion of phenylalanine aromatic amino acid into cinnamic acid, than to *p*-coumaric acid and *p*-coumaroyl-CoA. The molecule *p*-coumaryl-CoA will be used for the biosynthesis of stilbenes, aurones, lignins, cinnamic acid and benzoic acid derivatives. Derivatives of cinnamic acid and benzoic acid play a crucial role in forming the antioxidant capacity of several fruit crops. The first enzymes of the flavonoid biosynthesis pathway catalyse the formation of flavonoid sceleton from *p*-cumaroil-CoA. Subsequent enzymes of the flavonoid biosynthesis pathway help the formation of different flavonoid groups.

Currenly, the flavonoid biosynthesis is examined in many plant species, e.g. petunia, strawberry, blueberry, rose, grape, orange, apple, pear and peach. The modification of the anthocyanin biosynthesis in ornamental plants represents huge economical potential, since cultivars with unique flower colours can be produced artificially. An example for this is the ‘Moonlite’ carnation cultivar with blue flower colour, the only transgenic plant licensed in the EU. The carotenoid or flavonoid biosynthesis pathway was also modified in some edible plants (golden rice, potato with high carotenoid content). The whole genome sequencing projects of several plant species – like *Arabidopsis*, grape, apple or peach – will help to understand the biosyntheesis pathway and its regulation in details. However, information on the flavonoid biosynthesis of other stone fruits (e.g. apricot or sour cherry) was not available at the beginning of our work.

The study conducted at the Department of Genetics and Plant Breeding, Corvinus University of Budapest, was carried out to determie and characterize some flavonoid

biosynthesis genes of apricot and sour cherry. The apricot breeding program in the department aims to provide new apricot cultivars with high polyphenolic contents in fruit coupled with other valuable agronomic properties (e. g. canopy shape, fruit size, taste, resistance against microorganisms etc.). We examine the fruit inner contents of apricot cultivars grown at the germplasm collection the department in collaboration with the Department of Applied Chemistry. Hungary is an important country at an international scale for sour cherry production and available genetic sources. During the last decades numerous cultivars and cultivar candisates were produced with selective breeding. Among these, many cultivars or cultivar candidates are kown representing the whole range from high anthocyanin content to low anthocyanin content fruits. Previous studies showed that light coloured fruits of some cultivars with low anthocyanin content may have outstanding antioxidant capacity.

The inheritance of some properties (e.g. anthocyanin content of sour cherry fruit mesocarpium) in a breeding program can be evaluated just 4-6 years after crossing. Such long time is necessary for a seedling to become a productive tree. The purpose of a breeding program is to produce a new cultivar with improved agronomic properties. The improvement of several agronomic traits requires several crossings, which will significantly prolong the breeding period. The development of a DNA-based marker associated with the target properties would provide a huge advance. The application of such kind of marker assisted selection (MAS) would result in reducing cost and energy waste for the breeding programmes. However, detailed information on the flavonoid biosynthesis is needed for the development of such a marker.

2. AIMS OF THE STUDY

Our study was carried out to reach the following objects:

1. To design suitable primers for the identification of genes encoding the key enzymes of the flavonoid biosynthesis of apricot and sour cherry fruits.
2. Determination and identification of the DNA sequences of the amplified bands.
3. Based on the sequences, to design adequate primers for real-time PCR experiments.
4. Optimization of the real-time PCR for fruit sample analysis (RNA-extraction, examination of the effect of residual genomic DNA, choosing the optimal reference gene, optimization of reaction conditions).
5. To estimate the alterations in antioxidant capacity of apricot fruits through ripening, in different fruit parts and genotypes.
6. To characterize the expression of the genes encoding the main enzymes of the flavonoid biosynthesis at different ripening stages of fruits of two apricot and sour cherry genotypes with differing antioxidant acapity.
7. To identify the genes that are responsible for the alterations in antioxidant capacity.
8. To identify the main flavonoid components in apricot with HPLC-ESI-(Q)TOF MS hyphenated technique and compare the corresponding data with the results of the gene expression analyses.

3. MATERIALS AND METHODS

3.1. Plant material and sample preparation

We examined six apricot (‘Auróra’, ‘Ceglédi arany’, ‘Gönci magyarkajszi’, ‘Harcot’, ‘Preventa’ és 18/61-es hybrid) and three sour cherry (‘Pipacs 1’, ‘Újfehértói fürtös’ and ‘VN-1’ hybrid) genotypes. Apricot fruits were harvested in the germplasm collection of the Department of Plant Breeding and Genetics, Corvinus University of Budapest, at Szigetcsép and Soroksár. Sour cherry cultivars were grown in the Research and Extension Center for Fruit Growing, Újfehértó. Apricot and sour cherry fruits were harvested at five different ripening stages.

The preparation of the samples included the separation of the different parts of the fruits and an inmediate freezing in liquid nitrogen. We used the skin and flesh of fruits, stored in -80°C until analysis.

3.2. Determination of the parameters characterizing the ripening process

A photo was taken of fruits at all ripening stages with a Canon PowerShot S5 IS type digital camera. We determined size (height, diameters perpendicular or paralell to suture), weight and colour indices of 35 fresh fruits. Size was measured with a digital caliber and was recorded in mm, weight was recorded in g. Both parameters were recorded with the accuracy of two decimal points. The soluble carbohydrate content (brix value) was measured from the pressed fruit juice using a digital refractometer (ATAGO Corporation, Tokió, Japán) with 3-10 parallel measurements.

3.3. DNA- and RNA isolation, cDNA synthesis

Genomic DNA was extracted from sour cherry leaves using DNeasy Plant Mini Kit. RNA was extracted from ‘Gönci magyarkajszi’, ‘Preventa’, ‘VN-1’ and ‘Pipacs 1’ fruit skin and flesh tissues applying the hot borate method. For the reverse transcription of the purified RNA, an equal quantity of RNA, 1000 ng, was used.

3.4. PCR analisys with newly designed primers

Genes encoding the following enzymes were designed based on the sequences downloaded from the NCBI GeneBank and EST databases: *PAL* (*phenylalanine ammonia lyase*), *C4H* (*cinnamate 4-hydroxylase*), *4CL* (*4-coumaroyl CoA-ligase*), *CHS* (*chalcone synthase*), *CHI* (*chalcone isomerase*), *F3H* (*flavanone 3-hydroxylase*), *F3'H* (*flavonoid 3'-hydroxylase*), *F3'5'H* (*flavonoid 3'5'-hydroxylase*), *DFR* (*dihydroflavonol 4-reductase*), *ANS* (*anthocyanidin synthase*), *ANR* (*anthocyanidin reductase*), *LAR* (*leucoanthocyanidin reductase*), *UFGT* (*UDP-glucose:flavonoid 3*-O-*glucosyltransferase*), *FLS* (*flavonol synthase*) és *GAPDH* (*glyceraldehyde 3-P-dehydrogenase*). We also used the primer pair designed for the examination of sweet cherry *PAL* gene. PCR products with adequate size were isolated from the agarose gel, cloned and sequenced.

3.5. Real-time PCR

We designed primers optimized for real-time PCR experiments based on new sequences and sequences downloaded from databases. Real-time PCR experiments were carried out using a Rotor Gene 6000 machine from Corbett Research (QIAGEN), which, in addition to the quantitative analyses, is also equiped with a melting point analysis („Melting Analysis") function of a good resolution. The EvaGreen® non sequence specific flourescent dye was used in the experiments. We designed primer pairs for PCR and real-time PCR analyses in order to characterize the influence of genomic DNA. The expression stability of six different reference genes was measured during fruit ripening. The optimization of the experimental conditions of PCR and real-time PCR was carried out applying a gradient PCR and a dilution matrix. Dilution matrix was used in order to determinate the optimal concentration of the PCR components. For the evaluation of data supplied by the real-time PCR experiments, we used TOP and PCR-efficiency values. Data were evaluated with the REST© („Relative Expression Software Tool") 2009 V2.0.13 software. Through the evaluation we applied a data pooling technique, so all relative expression values can be compared to each other.

3.6. Determining the antioxidant capacity

The antioxidant capacity of fruit samples was determined using three different assays; total antioxidant capacity (FRAP), total phenolic content (TPC) and trolox equivalent antioxidant capacity.

3.7. Determination of polyphenolic compounds using LC-MS

Determination of polyphenolic compounds of ‘Gönci magyarkajszi’ and ‘Preventa’ fruit skin and flesh tissues was carried out using an HPLC-ESI-(Q)TOF MS hyphanated technique. Qualitative and half-quantitative results were presented. We carried out a preliminary analysis, and hence there was no opportunity for the identification of all possible flavonoid components and the precise determination of the quantities of the components. The m/z values could reveal the most flavonoid components, at least the aglycones. According to earlier experiments, we focused on specific components (quercetin- and kaempferol derivatives).

4. RESULTS AND DISCUSSION

4.1. Characterisation of the ripening process of apricot fruits

The ripening process of ‘Gönci magyarkajszi’ and ‘Preventa’ apricot fruits was characterized by the change in colour, size, weight and soluble corbohydrate content.

4.1.1. Changes in fruit colour through ripening

In case of ‘Gönci magyarkajszi’, the *a** and *b** indices of fruit skin colour precisly monitored the colour change ranging from green → greenish yellow → yellow → pale orange → orange. A similar tendency was shown in fruit flesh with the exception that higher *a** and lower *b** values indicated the occurrence of red overcolour on fully ripen fruits.

The fruits of ‘Preventa’ showed very similar changes in *a** and *b** colour parameters for skin, overcolour and flesh as ‘Gönci magyarkajszi’. In different tissues of fruits of both genotypes, *a**, *b** and hue angle (*C**) values increased, while chroma (H^o) decreased through ripening.

4.1.2. Other parameters of fruit development at different ripening stages

Early in the ripening process higher growth rates were detected for both genotypes; however, the growth rates were slightly different at later stages. The soluble solid content of apricot fruits showed an increasing tendence throughout the ripening process.

4.1.3. Changes in the antioxidant capacity and total phenolic content during fruit development

Three different assays to determine the antioxidant capacity – FRAP, TEAC és TPC (Folin-Ciocalteu)– in fruits were used. Samples covered five ripening stages and two parts (skin and flesh) of fruits of both genotypes, ‘Gönci magyarkajszi’ and ‘Preventa’. The antioxidant capacity and total phenolic content of ‘Preventa’ exceeded the values measured in ‘Gönci magyarkajszi’ fruits. ‘Preventa’ had higher total phenolic contents than any other cultivars previously tested.

‘Preventa’ fruit skin showed the highest values at all methods. ‘Gönci magyarkajszi’ fruit skin tissues showed 50-70 % lower values than those of ‘Preventa’ fruit.

The ABTS radical-scavenging activity (TEAC values) showed that fruit flesh tissues of both apricot genotypes were similar in their antioxidant capacity. The ratio of FRAP values in

the unripe mezocarpium of ‘Gönci magyarkajszi’ and ‘Preventa’ fruits was very similar to their corresponding total phenolic content values. The differences in the FRAP values of fruit flesh of the two genotypes decreased in the later ripening stages, and increased again in the fully ripe ‘Preventa’ fruits reaching a 15-times higher value. On the whole, the antioxidant capacity values of ‘Preventa’ fruit flesh exceeded the values of ‘Gönci magyarkajszi’, especially in unripe (1st ripening stage) and fully ripe (5th ripening stage) fruits.

4.2. Characterisation of the ripening process of sour cherry fruits

The ripening process of ‘VN-1’ and ‘Pipacs 1’ sour cherry fruits was characterized through the change of color, size, weight and soluble solid contents.

4.2.1. Color change of fruits through ripening

At the early ripening stages of ‘Pipacs 1’ fruits, skin tissues started to accumulate anthocyanin pigments over the green colour, while fruit flesh was still green because of the chlorophyll content. The colour change of unripe ‘Pipacs 1’ fruit showed different tendency in both skin and flesh: the a^* and H^o values of fruits at the third and fourth ripening stages showed a more intensive change on the surface of the fruit than in fruit flesh, because anthocyanins occured first in fruit skin. Observing ‘VN-1’, similarly, first fruit skin turns to red, but the process began already at the secong ripening stage, which was showed by the a^* and H^o values in case of base colour and flesh colour. In contrast, the a^* and H^o values of ‘VN-1’ at later ripening stages are very similar in case of fruit skin and flesh. The L^* values of ripe fruit flesh showed a difference between ‘VN-1’ and ‘Pipacs 1’ with ‘VN-1’ showing lower values. Its explanation is that ‘VN-1’ is a *Morello* type sour cherry with dark red fruit flesh color, whereas the fruit flesh of the *Amarella* type ‘Pipacs 1’ is nearly colourless (Papp et al., 2010).

Colour parameters were different in the fruit skin tissues of the two sour cherry genotypes. ‘VN-1’ fruit skin showed a stronger decrease in hue angle value than ‘Pipacs 1’. It shows that the formatin of red colour in fruit skin of ‘VN-1’ is more intensive and shows higher values at the early ripening stages than ‘Pipacs 1’.

4.2.2. Other parameters showing the development of fruits through the different ripening stages

The two sour cherry genotypes used in the experiments showed different ripening dynamics. The soluble solid contents of ‘VN-1’ and ‘Pipacs 1’ fruits increased according to a different tendency throughout ripening.

4.3. RNA-isolation from apricot and sour cherry fruits

The RNA isolation is a crucial step of sample preparation for real-time PCR experiments. Numerous protocols have been developed in the last few decades for RNA isolation from plant samples containing huge amounts of carbohydrates and phenols. We attempted to extract RNA from apricot and sour cherry fruit flesh and skin tissues with RNeasy Plant Kit (QIAGEN), with an aceton-based protocol of Asif et al. (2006), with the CTAB-based method described by Jaakola et al. (2001) and with the hot borate method developed by Wan and Wilkins (1994). Comparing all protocols, hot borate was the most effective, which was later used for all samples.

4.4. Identification of genes in the flavonoid biosynthesis of apricot and sour cherry

The first step of the experiments was the determination of partial or full gene sequences encoding the key enzymes in flavonoid biosynthesis. We identified the partial gene sequences of the following genes from the next apricot cultivars (‘Gönci magyarkajszi’, ‘Preventa’, ‘Ceglédi arany’, ‘Harcot’, ‘Aurora’ and hybrid 18/61): *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *F3’H*, *F3’5’H*, *DFR*, *ANS*, *ANR*, *LAR* és *UFGT*. We determined a partial gene sequence from the GAPDH gene of apricot, which was used as a reference gene. We identified the following partial gene sequences of sour cherry (‘VN-1’ and ‘Pipacs 1’): *C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *F3’5’H*, *ANR*, *LAR* és *UFGT*. We also identified a partial sequence of the *GAPDH* and *MYB* genes.

We have carried out sequence identification based on homology searches using the NCBI BLASTn search platform. The homology-based search revealed that our sequences showed high similarity with sequences from other species. Based on the E=0 or near zero values, we supposed that the identified sequences indeed form a part of the target genes.

In case of apricot *4CL*, *F3’5’H*, *LAR* and *GAPDH* genes (used as reference genes), our sequences are the first known sequences in the *Prunus* genus. Except *PAL* gene (Pina and Errea, 2008), all other examined genes were first sequenced in our study in apricot. Sour

cherry *4CL*, *F3'5'H*, *LAR*, *GAPDH* and *MYB* gene sequences are again new within the *Prunus* genus, and the remaining sequences were first determined in sour cherry.

4.5. Optimization of the real-time PCR experiments

To ensure reliability for the real-time PCR assays, numerous conditions (annealing temperature of the primers, primer concentration, cDNA quantity, $MgCl_2$ concentration) were optimized. We designed target gene and assay specific primers for real-time PCR experiments with melting temperatures ranging from 65 to 66 ºC. For all real-time PCR experiments, 60 ºC was used as annealing temperature.

4.6. Dilution matrix

We determined the optimal cDNA quantity, primer and $MgCl_2$ concentrations using dilution matrices. When applying 50 ng cDNA, PCR products were obtained after an adequate cycle number and with good amplifiction efficiency, hence the quantity of cDNA was not increased. The efficiency of real-time PCR experiments increased with higher primer concentrations and melting analyses confirmed the exclusive amplification of the specific PCR products, hence primer concentration was increased from the recommended value of 0,3 µM to 0,6 µM. The $MgCl_2$ concentration of the PCR solutions was 3 mM. The elevation of the $MgCl_2$ concentration resulted reactions with slightly decreased efficiency, and consequently all real-time PCR experiments were carried out at the concentration of 3 mM.

4.7. Choosing the optimal reference gene

Real-time PCR is an efficient technique for gene expression analyses, but its reliability depends on the stable expression profile of the reference genes used for data normalisation. Our samples measured included two different species, apricot and sour cherry, five different ripening stages and two different genotypes and two different fruit tissues for each species. In consequence, finding a reference gene with stable (practically unchanged) expression in all those different samples was a challenging task.

Tong et al. (2009) tested eleven different reference genes in peach fruits. *TEF-II*, *UBQ10*, *TUB*, *GAPDH*, *ACT* and *RP-II* genes showed stable expression through ripening. Marty et al. (2005) examined carotenoid biosynthesis in apricot, and chose 26S ribisomal RNA gene as reference. González-Agüero et al. (2009) used *DAP* (*dehydro dipicolinate reductase*) as reference gene in order to follow the formation of aromatic components in ripening apricot.

Our first real-rime PCR experiments were carried out to test the expression stability of the *ACT*, *GAPDH*, *RP-II*, *TEF-II*, *UBQ10* and *18S rRNA* reference genes. *RP-II* (RNA polymerase II) was found to have the most stable expression in all samples. Hence, we chose using *RP-II* as reference gene for the real-time PCR examination of all samples. Because we applied one reference gene for all target experiments, we had the opportunity to evaluate the nascent datas all together with a data pooling technique. Since preparation of all samples and conditions of each reaction were identical, data of the real-time PCR analysis could be pooled for a collective analysis and comparison.

During the expreiments the way to make cDNA – applying oligo$(dT)_{18}$ or random hexamer primer– was innefective to the relative gene expression values, which coincided with the results of Hansen et al. (2010).

4.8. Examination of the influence of residual genomic DNA

The residual genomic DNA present in RNA and hence in cDNA solutions can also be used as template during the qPCR experiments, which may influence the results. So elmination of the genomic DNA from the examined samples is recommended. Several processes were developed for this purpose in the last few years. According to our experiences, the cDNA isolated from agarose gels was not suitable for further PCR-based experiments. An often used technique is the treatment of RNA solutions with DN-ase I enzyme, followed by the irreversible inatcivation of the enzyme with high temperature. In our study we were not able to prepare cDNA solution suitable to PCR from DN-ase I treated RNA samples. In consequence, a method had to be built up, which will not have negative effects on RNA solutions and keeps away genomic DNA.

PCR and real-time PCR techniques were used to check that the cDNA solutions are free from residual genomic DNA. For the conventional PCR experiments, we designed primers anneal to exon regions with two introns between the targeted exons. So applying genomic and cDNA templates, amplicons will have different sizes. PCR carried out on the cDNA solutions exclusively amplified the fragments with size without intron. There was no case to detect any fragment of size different from the expected, or only in such a small quantity which did not influence further experiments, because could not compete for reaction components.

We have also worked out a new, real-time PCR based experiment to detect genomic DNA contamination. We used the sequence-specific primers designed for *LAR* and *ANR* genes. The *LAR* gene in the F1+R1 primer combination has to give successful amplification both on genomic and cDNA templates, because those are designed from exon regions. Since

between the exons there is an intron region, the amplicon containing the intron can be identified with two methods. One of them is gel electrophoresis, the other is melting analysis.

The melting temperature of the amplicon containing an intron will be higher than the shorter amplicon, which does not contain an intron. The q*LAR*-F2 primer was designed from two subsequent exon regions, hence the primer is not capable to PCR amplify genomic DNA. A similar method was applied by Wong and Medrano (2005). If q*LAR*-F1+R1 and q*LAR*-F2+R1 show different expression or the reaction with the q*LAR*-F1+R1 primer pair provides a longer amplicon due to the additional size of the intron, the cDNA solution surely contain genomic DNA contamination. Experiments with q*LAR*-F1+R1 and q*LAR*-F2+R1 primer pairs showed identical results, hence cDNA solutions did not contain any genomic DNA or contained a quantity just under the detection level. Primer pairs designed for the *ANR* gene showed similar arrangements and gave identical results.

4.9. Expression analysis of the apricot flavonoid genes

We detected a high transcript abundance at the first and second ripening stages of apricot, which decreased during the subsequent ripening stages. Fruits of 'Preventa' were characterized by higher relative transcript quantities for several genes (*PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS*, *ANR* és *LAR*) as compared with the fruits of 'Gönci magyarkajszi', especially in fruit skin. In 'Preventa', mRNA molecules are intensively synthetised already at the first ripening stage, which are necessary for the synthesis of enzymes included in the flavonoid biosynthesis, so we can suppose that already unripe fruit contains a huge amount of key enzymes of the flavonoid biosynthesis.

A similar expression profile was shown by Dardick et al. (2010) in yellow fleshed peach. The *4CL*, *DFR* and *ANS* genes showed continuously decreasing expression, while the expression of *PAL*, *C4H*, *CHS*, *CHI* and *F3H* genes decreased only after a short increasing tendency. A decreasing tendency in expression was observed throughout ripening in case of several fruit crops for more flavonoid biosynthetic genes, for example for *PAL* in pear (Steyn et al., 2004); *CHS*, *CHI*, *F3H*, *DFR*, *FLS*, *LAR*, *ANR* and *UFGT* in green skin apple (Takos et al., 2006).

A significant difference between the two genotypes that the fruit flesh and especially fruit skin of 'Preventa' has a many times higher transcriptome quantity compared to that of 'Gönci magyarkajszi' at the first ripening stages. In the flesh and skin of 'Preventa' fruits, the genes putatively encoding PAL and CHS enzymes – the first enzymes of phenylpropanoid and flavonoid biosynthesis – had several times higher relative expression values compared to

‘Gönci magyarkajszi’. The fruit flesh of ‘Preventa’ showed higher *C4H* gene expression and flavonoid content than the fruit flesh of ‘Gönci magyarkajszi’. Saud et al. (2009) showed similar tendencies in strawberry.

‘Preventa’ showed several times higher antioxidant capacity values than ‘Gönci magyarkajszi’ (Hegedűs et al., 2010). After the identification of different polyphenolic compounds, we can suppose that catechin accumulating in ‘Preventa’ fruit flesh in higher quantity than in ‘Gönci magyarkajszi’ can be responsible for the difference in antioxidant capacity of fruits of the two genotypes. Catechin belongs to the group of flavan-3-ols. The following enzymes are required for catechin biosynthesis: PAL, C4H, 4CL, CHS, CHI, F3H, DFR and LAR. The expression of these genes in the fruit flesh of the two genotypes indicated that ‘Preventa’ had in average four times higher relative transcriptome quantity for all genes at the first ripening stage. This transcriptome abundancy seems responsible for the higher antioxidant capacity and bigger catechin contents. Catechin content of ‘Preventa’ fruit skin and flesh increased throughout the ripening, then showed a stable quantity, whereas it decreased in ‘Gönci magyarkajszi’. We suppose that catechin in ‘Gönci magyarkajszi’ fruits is utilized for the formation of condensed tannins (or other compounds). Also in ‘Cripps Red’ apple cultivar with red fruit skin higher *ANR* and *LAR* expression was associated with the accumlation of condensed tannins (Takos et al., 2006).

The mesocarp of ‘Gönci magyarkajszi’ contains in average two or three times more catechin than that of the ‘Preventa’. Because both catechin and epicatechin belong to the group of flavan-3-ols, the enzymes responsible for their production are more or less identical, with the exception that instead of LAR, the enzymes ANS and ANR take part in the biosynthesis. ‘Preventa’ had higher relative expression values for *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *DFR*, *ANS* and *ANR* than ‘Gönci magyarkajszi’. So the higher epicatechin content of ‘Gönci magyarkajszi’ fruit is supposedly originated from the fact, that in ‘Preventa’ condensed tannins are formed mainly from epicatechin. Degradation or interchanges of flavan-3-ols to other molecules can be also a reason of the difference between the two genotypes.

The red overcolour in fruit skin is induced by anthocyanin accumlation. The ANS and UFGT enzymes catalyze the last steps of the anthocyanin biosynthesis pathway. The 3-OH-anthocyanidins produced by ANS, can turn into anthocyanins or colorless flavan-3-ols in a reaction catalyzed by the ANR enzyme. On the sun exposed surface of the fruit ‘Gönci magyarkajszi’ accumulated anthocyanin pigments from the fourth ripening stage. In contrast, the skin of ‘Preventa’ fruit did not contain anthocyanins. The skin of unripe ‘Preventa’ fruits

showed a higher relative transcriptome quantity for *ANS*, *ANR*, *LAR* and *UFGT* genes, than fruit skin of ‘Gönci magyarkajszi’. The pigmentation of ‘Gönci magyarkajszi’ fruit skin can be probably explained by the relatively higher transcriptome ratio of the UFGT– although it had a lower quantity than ‘Preventa’ – to the *ANS*, *ANR* and *LAR* genes, which was much smaller in ‘Preventa’ fruit skin. Consequently, ‘Gönci magyarkajszi’ displayed relatively higher *UFGT* expression – compared with the expression values of other genes – than ‘Preventa’, which may have a role in anthocyanin pigmentation.

The *ANR* and *LAR* expression was higher in ‘Preventa’ fruit skin than in ‘Gönci magyarkajszi’. During the accumlation of anthocyanin pigments, the transcriptome level of CT biosynthesis genes was lower (Takos et al., 2006) than that of other flavonoid biosynthesis genes. It indicates that the CT and anthocyanin biosynthesis in apricot skin tissue, similarly to apple, are under different regulation.

Nevertheless we can suppose that the identified *UFGT* gene homologue in ‘Preventa’ fruit flesh does not produce the enzyme responsible for the glycosylation of the 3-OH anthocyanidins, so they are used for flavan-3-ol biosynthesis by ANR. The UFGT enzyme also has a crucial role in the formation of anthocyanin pigments in other fruits. The UFGT produces anthocyanins (stable and coloured molecules), the glycosylated anthocyanidins. The UFGT enzyme was not detected in the skin of white grapes and the expression of other genes was also lower compared with red grape cultivars (Boss et al., 1996).

Some MYB transcription factors are able to tissue specifically activate or inhibit the flavonoid biosynthesis in plant. Lin-Wang et al. (2010) demonstrated that MYB10 transcription factor accumulated in the red flesh of apple while white fruit flesh had only minor MYB10 transcriptome quantities. Real-time PCR experiments indicated low transcriptome quantity in both tissue types of apricot throughout ripening. The *MYB10* transcriptome quantity increased in fruit skin then showed a decreasing tendency until the fifth ripening stage. Since neither ‘Gönci magyarkajszi’ nor ‘Preventa’ fruit flesh contained anthocyanin pigments, low *MYB10* transcriptome quantity correlated with the observations of Lin-Wang et al. (2010). *MYB* showed higher trancriptome quantity during ripening than *MYB10*. Fruit skin of ‘Gönci magyarkajszi’ showed higher *MYB* transcriptum abundance in the second, third and fourth ripening stages, while fruit flesh in all ripening stages had higher transcriptum quantity than ‘Preventa’ skin and flesh. According to these – comparing the two genotypes – there is no correlation between the transcriptum quantity of the flavonoid biosynthesis enzymes and that of the *MYB* in fruit flesh or skin. Although ‘Gönci magyarkajszi’ had higher relative *MYB* expression level in skin than ‘Preventa’, it does not

explain the differences in the anthocyanin pigmentation of skin, because the difference is small.

4.10. Expression analysis of the sour cherry flavonoid genes

Sour cherries – in contrast with apricot – had the highest transcriptum quantity usually at the fourth and fifth ripening stages. The growing tendency was closely exponential. Throughout the ripening and paralell with the anthocyanin accumulation an increase in the expression was shown in peach with red fruit skin (‘Akatsuki’ and ‘Flavortop’) for the genes *CHS*, *CHI*, *F3H*, *DFR*, *ANS* and *UFGT* (Tsuda et al., 2004), in apples with red skin for *CHS*, *CHI*, *F3H*, *DFR*, *FLS*, *LAR*, *ANR* and *UFGT* (Takos et al., 2006), in red grapes for *F3H*, *F3’H*, *F3’5’H*, *FLS*, *DFR*, *ANS* and *UFGT* (Castellarin et al., 2007a,b), in blueberry for *PAL*, *CHS*, *F3H*, *DFR* and *ANS* (Jaakola et al., 2002) and in *Fragaria* × *ananassa* for *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *DFR*, *ANS* (Saud et al., 2009).

The transcript abundance of *PAL*, *C4H*, *CHS*, *CHI*, *F3’H*, *DFR* and *ANS* in the fruit skin of ‘VN-1’ sour cherry (characterized by high anthocyanin content) exceeded the respective quantities in the skin of ‘Pipacs 1’ (containing less anthocyanins). Transcription factors MYB and MYB10 are likely to take part in the transcriptional regulation of some sour cherry flavonoid biosynthesis genes. The abundance of transcripts in sour cherry fruits increased throughout the ripening process, which was closely correlated with the evolution of anthocyanic pigmentation. Comparing all results, the highest relative gene expression values were detected in the fruit skin of ‘Preventa’ and ‘VN-1’.

The results of real-time PCR experiments carried out with ‘VN-1’ fruit samples doesn’t seem to be reliable comparing to the other data. We suppose that the fruits of ‘VN-1’ contained one or more components, which were not removed during the RNA isolation procedures and hence inhibited the cDNA synthesis or PCR.

Papp et al. (2010) characterized the antioxidant capacities of ‘VN-1’ and ‘Pipacs 1’ fruits. They could detect high antioxidant capacity value and vitamin C content in ‘Pipacs 1’, and high anthocyanin content in ‘VN-1’. The huge anthocyanin content and gene expression values of ‘VN-1’ fruit skin are well correlated. The lower anthocyanin content of ‘Pipacs 1’ fruits is associated with lower expression levels of anthocyanin biosynthesis genes. In previuos experiments (Papp et al., 2010) we examined the gene expression of *PAL*, *CHS*, *DFR* and *ANS* with non-quantitative PCR. All of the four genes were expressed both in fruit skin and flesh tissues, hence we supposed that the lower anthocyanin pigmentation is

attributable to the lack or decreased levels of expression of other genes (e.g. *UFGT*) or regulating elements (e.g. transcription factors).

In order to clarify this point, 'VN-1' and 'Pipacs 1' fruits were examined with real-time PCR technique at five different ripening stages. 'Pipacs 1' fruit skin showed lower *PAL*, *C4H*, *CHS*, *CHI*, *DFR*, *F3'H* and *ANS* transcriptum content than 'VN-1', which correlated with the lower anthocyanin content of 'Pipacs 1'. In case of peach, *CHS* and *DFR* genes semed to be crucial in anthocyanin accumlation, because in cultivars with white skin color *CHS* and *DFR* expression was not detectable (Tsude et al., 2004). White strawberry (*Fragaria chiloensis*) without anthocyanin pigmentation showed lower expressions for several genes (*PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *ANS* and *UFGT*) in contrast with *F. ananassa* red fruits (Saud és mts., 2009). In case of conventional and blood orange, *PAL*, *CHS*, *DFR* and *ANS* genes are supposed to play a role in the formation of the difference of anthocyanin pigmentation (Licciardello et al., 2008).

There is visible difference in the expression *MYB10* transcription factor. 'VN-1' shows a several times higher *MYB10* expression at all ripening stages, than the fruit skin of 'Pipacs 1'. It results into a higher expression of other genes (*PAL*, *C4H*, *CHS*, *CHI*, *DFR*, *F3'H* and *ANS*) as well. A similar tendency was described by Lin-Wang et al. (2010): two sweet cherry cultivars with different anthocyanin contents showed different expression levels of *CHS*, *ANS* and *MYB10*. The fruit of 'Stella' with dark red fruit flesh showed higher *CHS*, *ANS* and *MYB10* transcriptum quantities than the 'Rainier' cultivar with yellow fruit colour. The regulating role of MYB transcription factors in anthocyanin biosynthesis is also confirmed in some other fruit crops (Cultrone et al, 2010; Espley et al., 2007).

'Pipacs 1' fruit skin showed a higher *F3H* expression, than 'VN-1'. Based on this fact we can suppose that while flavonoid biosynthesis in 'VN-1' fruits is channeled to the formation of anthocyanins, colourless flavonoids are formed in fruits of 'Pipacs 1', which contribute to the formation of the antioxidant capacity of the fruit. Genistein was detected in 'Pipacs 1' sour cherry at the Department of Applied Chemistry, Corvinus University of Budapest (Abrankó et al., 2011b).

4.11 Identification of some flavonoid components of apricot

A hyphenated HPLC-ESI-(Q)TOF MS technique was used to identify the main flavonoid components in apricot and determine their relative quantities. Quercetin-hexoside, rutin, naringenin-hexoside (first time detected in apricot) as well as catechin and epicatechin were identified in higher quantities in apricot fruits. We concluded that the outstanding

antioxidant capacity of ‘Preventa’ is, at least partly, due to the high catechin contents in fruit flesh, which is a consequence of the elevated expression of genes involved in the flavanol biosynthesis.

The most innovative parts of my PhD work are the following achievements: we were the first to identify the most important putative flavonoid biosynthesis genes in apricot and sour cherry and characterize their expression throughout the ripening process of fruits. We have also identified some differently expressed genes that might be responsible for the high antioxidant capacity.

5. NEW SCIENTIFIC RESULTS

1. We designed PCR primers based on homolog gene sequences encoding the key enzymes of the flavonoid biosynthesis, than we characterised their applicability in apricot and sour cherry fruits.
2. We identified a partial DNA sequence of 19 apricot and 10 sour cherry flavonoid biosynthesis genes.
3. Using the sequences we designed primers suitable to real-time PCR experiments.
4. We worked out an effective protocol for real-time PCR analysis (adequate RNA isolation, evaluation of the influence of genomic DNA contamination, choosing optimal reference gene, optimization of reaction conditions) of apricot and sour cherry fruits.
5. Our results confirmed that apricot skin has higher antioxidant capacity and total phenolic content than fruit flesh in both genotypes. Fruit skin and flesh of 'Preventa' had higher antioxidant capacity than those of the 'Gönci magyarkajszi'; the highest values were measured in the skin of 'Preventa' fruit. In general, fruits of 'Preventa' showed increasing, while those of 'Gönci magyarkajszi' exerted decreasing antioxidant capacities throughout the ripening process.
6. Our analysis showed differences in the expression pattern of flavonoid biosynthesis genes of apricot and sour cherry throughout the ripening: in apricot, the unripe (small, green) fruit had the highest expression level, while in sour cherry it increased until the fully ripe stage.
7. Our results confirmed that the formation of the outstanding antioxidant capacity of 'Preventa' fruit is contributed by numerous genes (*PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS*, *ANR* és *LAR*), which show markedly higher expression at the first ripening stage than in 'Gönci magyarkajszi'. The MYB and MYB10 transcription factors putatively play a role in the transcriptomic regulation of the anthocyanin biosynthesis.
8. Using an HPLC-ESI-(Q)TOF MS hyphenated technique we concluded that the outstanding antioxidant capacity of 'Preventa' is, at least partly, due to the high

catechin contents in fruit flesh, which is a consequence of the elevated expression of genes involved in the flavanol biosynthesis.

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