定点在苹果中存在较高的PR家族基因表达，称为PR-10家族。这些基因在苹果中被广泛表达，与免疫系统的反应有关。因此，对于过敏人群而言，了解苹果中PR-10家族基因的表达水平是非常重要的。这项研究为开发新的低过敏性苹果品种提供了理论基础。
real-time PCR analysis and the commonly used are housekeeping genes that are proved to be non-regulated (Nicot et al., 2005; Garg et al., 2010).

The objective of the study was the analysis of expression levels changes of the Mal d 1.03A allergen of apple fruit during the ripening in the varieties of Golden and Spartan.

MATERIAL AND METHODS

Plant material

Apple pulps of varieties Golden and Spartan were used in the study. Apple fruits were collected during the ripening (table 1) from the trees that grow under the condition of fruit garden in the locality of Rišňovec, Slovak Republic. Collected samples were stored in -20°C until the processing.

Table 1 Codes and dates of the sampling of analysed apple pulps of varieties Golden and Spartan.

<table>
<thead>
<tr>
<th>code of sample</th>
<th>date of collection</th>
</tr>
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<tbody>
<tr>
<td>X1</td>
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</tr>
<tr>
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</tr>
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<td>X5</td>
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<td>20.8.2016</td>
</tr>
<tr>
<td>X38</td>
<td>13.9.2016</td>
</tr>
</tbody>
</table>

Legend: X – Golden; Z – Spartan

Extraction of RNA and preparation of cDNA

Apple pulps were firstly homogenized using the liquid nitrogen. Total RNA was extracted following the manufacturers recommendations of commercial analytical kit GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Reverse transcription was performed by Thermo Scientific™ RevertAid TM First Strand cDNA Kit Text from 1μg of RNA (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using the random primers and following the manufacturers recommendations.

Semiquantitative real-time PCR analysis and data processing

Amplification of Mal d 1.03A transcripts were performed by Maxima SYBR Green qPCR Master Mix (2x)(Thermo Fisher Scientific, Waltham, Massachusetts, USA) using the 600 nmol × dm⁻³ of both of the primers and 1 μl of the 1:100 diluted cDNA. Primers used for the amplification of Mal d 1.03A originate in the study of Pagliarani et al., (2013) and primers for the amplification of actin were designed on the base of nucleotide sequence stored in NCBI under the accession number D7002474 with the following sequences: forward 5’CTATGTCCCTGGTTAGGTCAGAGC3’ and reverse 5’GCCACAACTTGTGGTTTACG3’. Primers were designed by Primer3web version 4.0.0 (http://primer3.ut.ee/).

The following amplification profile was used: 95 °C, 10 minutes, 50 x (95 °C for 10seconds; 62 °C for 20 seconds; 72 °C for 20 seconds) and final 72 °C 5 minutes. Amplified products specificity was checked by 2% agarose electrophoretic separation using the FastRuler™ Ultra Low Range DNA Ladder ready-to-use (Thermo Scientific). The analysis of the amount of amplified transcripts were performed in the 5μl of amplified products by the interpolation of density of pixels to those of the amount of 1000 ng × μl⁻¹ using the software GeneTools 4.01.04 (SynGene).

RESULTS AND DISCUSSION

The different analysis connected to the plant genome variability and to the plant gene expression are in the focus of researchers actually. The genome mapping using different DNA based markers is used for the purpose of characterization of natural variability of plant food sources (Balážová et al., 2014; Vivodik et al., 2015). The real-time PCR based technique are widely applied to gene expression analysis, pathogen identification, environmentally specific stress related answer of plants or plant allergen analysis (Kántor et al., 2014; Kačianiová et al., 2012; Žiarovská et al., 2013; Račná et al., 2014).

In central and northern Europe as well as in North America asignificant proportion of patients who suffer from birch pollenallergy develop intolerance to certain kinds of fruits andvegetables(Ballmer-Weber, 2015). In apples the major allergen that is responsible for birch pollen-related food allergies is the 17.5kDa protein Mal d 1 (Vancic-Krebitz et al., 1995). Real-time PCR based analysis of food allergen per se are well established for almost all of the main of them. Today, routine protocol exist for the detection of allergens in different foodstuff, but the knowledge about their expression dynamics, epigenetic background or genomics interactions directly in the plants are very limited (Žiarovská and Zeleňáková, 2016).Here, the changes of the expression levels of Mal d 1.03A allergen transcripts were evaluated during the ripening of two apple varieties – Golden and Spartan.

Amplification of Mal d 1.03A allergen resulted in the Ct values that ranged from 23.03 up to the 27.25. The difference among the starting of the ripening and physiological maturity of apple pulps was about 4 in the Ct values. The specificity of amplified product was confirmed electrophoretically where a product of the expected length of 86 bp for actin and 96 bp for the Mal d 1.03A was obtained (figure 1).

Using the reverse transcription for the detection of the transcripts is actually well established and choosing the quantitative or semiquantitative approach can be done. One of the questions that are behind is necessity of the knowledge of very precisely measuring of number of RNA molecules or the basic information about the variation in RNA levels (Marone et al., 2001). This strategy provide a possibility of the detection the very low quantities of expressed genes through their mRNA transcripts (Tajvidin et al., 2014). Different procedures are used in the semiquantitative amplification of products ranged from fluorescent labeling of amplicons up to the visualization on agarose and acrylamide gels and other gel densitometry methods (Heid et al., 1996; Valasek et al., 2005).

Both, semiquantitative and quantitative approach are methodologically dependent on the selection of appropriate housekeeping genes that are used as an internal controls in the analysis. Transcriptional profiles of different housekeeping genes were analysed for the Malus domestica, Borkh tissues - actin, protein disulfide isomerase, ubiquitin-conjugatin enzyme E2, glyceraldehyde 3-phosphate dehydrogenase, histone 1, nucleossome assembly 1 protein, 18S ribosomal RNA, ribosomal protein S19 or Rubisco (Gadiou and Kundu, 2012; Storch et al., 2015). Different plant organs, fruit developmental stages or ripe fruits kept at room temperature and under long term cold storage, were subjected to analysis of this housekeeping genes stable expression as well as different types of treatments such as exogenous ethylene or controlled atmosphere conditions (Storch et al., 2015).

Figure 1 A – Electrophoretic separation of amplified actin and Mal d 1.03A sequences of apple varieties Golden (X40) and Spartan (Z40) – checking of the amplification specificity.

Here, the semiquantitative strategy was used for the analysis of the variations in the transcripts levels of Mal s 1.03A allergen in the pulps of two apple varieties – Golden and Spartan when using actin as an internal control. The analysis of density profiles of amplified actin and Mal d 1.03A transcripts in the agarose gel was performed against the defined amount of 1000 ng × μl⁻¹. The levels of amplified levels of actin were balanced for all the samples and has varied from the amount of amplified product in ng 4206 up to the 4228. In the comparing analysis, the levels of amplified transcripts of Mal d 1.03A were more balanced in the first stages of ripening in the variety Spartan. A high increasing of the amount of transcripts were obtained in the first stages of ripening in the variety Golden (figure 2; table 2). Continual increasing of Mal d 1.03A transcripts was observed the middle ripening stage for both varieties. At the end of the ripening a different situation was observed in analysed apple varieties. The expression of Mal d 1.03A allergen was balanced for the variety Golden but a high increase was observed at the end of the ripening for the variety Spartan.
Figure 2 Determination of the amplified transcripts of Mal d 1.03A allergen and actin in the samples

The very similar data was proved by us for the Golden variety - expression of Mal d 1.03A allergen was increasing continuously, too. The quantification of Mal d 1 allergen during the storage was performed by Kiewnig et al. (2013). Increasing of this allergen expression has depend strongly on the Apple variety and resulted in heterogeneous levels - the highest content of Mal d 1 was obtained in cultivars Golden Delicious and Gala and the lowest one for Elise and Pinova. This results correspond to the previous studies where a strong variety dependence is reported for Mal d 1 content in peel or pulp of apples (Matthes and Schmitz-Eiberger, 2009; Kiewning and Schmitz-Eiberger, 2014).

Expression of Mal d allergens in apples is reported as to be affected not only by growing, but by storage conditions, too (Matthes and Schmitz-Eiberger, 2009; Sancho et al., 2006, Schnitz-Eiberger and Matthes, 2011). They are reported as labile proteins that can be degraded very easy by proteolytic enzymes (Jense-Jarolis et al., 1999). The proteomic approach of analysis is limited here by the fact, that Mal d allergens possible to be extracted in the active form only if reactions with phenolic compounds present in apple are inhibited (Szamos et al., 2011).

Table 2 Quantified amount of amplified transcripts of Mal d 1.03A allergen in the apple varieties Golden and Spartan during the ripening

<table>
<thead>
<tr>
<th>Sample</th>
<th>AAT/ng</th>
<th>Photographic documentation</th>
<th>Sample</th>
<th>AAT/ng</th>
<th>Photographic documentation</th>
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<td><img src="golden.png" alt="Golden Apple" /></td>
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<td>2123</td>
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<td>X5</td>
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<td>Z5</td>
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</table>
Actually only a some data are available about the expression pattern of Mal d 1 allergen in peel and pulp of the apples. Marzban et al. (2005) reported the variety Golden Delicious as one of the varieties that has one of the highest potential to express allergenic proteins. Actually, different strategies are used in the quantitation of allergens in fruits/apples. Szamos et al. (2011) has used the chromatographic quantification of Mal d 1 and Mal d 2 allergens from five samples of Golden Delicious during the ripening time. The content of Mal d 1 was slowly increasing during the ripening.

CONCLUSION

The analysis of transcript level of Mal d 1.03A allergen was performed in the study using the reverse transcription approach. The changes of the transcription levels were compared in two apple cultivars, Golden and Spartan, during the ripening. Golden was proved as to possess the high increasing of the amount of Mal d 1.03A transcripts in the first stages of ripening and then a continuously increasing of transcripts in the pulp. In Spartan, Mal d 1.03A transcripts were expressed in a balanced manner up to the middle phase of ripening and at the end of ripening, the content quickly raised.

Acknowledgments: This research was supported by European Community under project no 2622020180: Building Research Centre “AgroBioTech” and by The Danube strategy project DS-2016-0051 Genomics, transcriptomics, digestomics and a mouse allergy of sensitization to allergenic lipid transfer proteins.

REFERENCES


Biochemical and Biophysical Research Communications, 396, 283-288. DOI: http://dx.doi.org/10.1016/j.bbrc.2010.04.079


Legend: X – Golden, Z – Spartan; AAT – amount of amplified transcripts

X40 3209
Z40 3627

Table 1: validation of internal control genes for quantitative gene expression studies in chickpea (Cicer arietinum L.).

Fritsch, R., Bohle, B., Vollmann, U., Wiedermann, U., Jahn-Schmidt, B., Krebitz, M., Breiteneder, H., Kraft, D., Eben, C.1998. Bet v 1, the major birch pollen allergen, is only expressed in the major birch pollen allergen, cross-react at the level of allergen specific T helper cells. Journal of Allergy and Clinical Immunology, 102, 679-686.


