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COMPARATIVE ANALYSIS OF Mal d 1.03A EXPRESSED TRANSCRIPTS IN PULP OF APPLE CULTIVARS GOLDEN AND SPARTAN

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ABSTRACT

Apples are one of the oldest, most desirable and most consumed fruit in the world and in our agroclimatic conditions apples are the fruit that are also in the winter mostly consumed fresh. As apples are available all the year, they are a very important source of secondary plant metabolites. On the other hand, apples do not provide only benefits and belong to the fruit that cause an allergic reaction in a sensitized the population. Mal d 1 protein, one of the apple allergens, belongs to the PR proteins and is encoded by multiple isoforms in the genome of *Malus domestica* Borkh. The aim of the study was to compare the level of expression of Mal d 1.03A isoform in the pulps of apple varieties Golden and Spartan during the ripening. Expression analysis of Mal d 1.03A allergen were performed in pulps from a total of six ripening stages ranged from the early up to the physiological maturity at approximately monthly intervals. Assessment of the level of amplification of transcripts of Mal d 1.03A allergen was performed by software comparing them to a well-defined value of 1000 ng × μl^{-1}. 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. In 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.

Keywords: Mal d 1, allergen expression, apple pulp, Golden, Spartan

INTRODUCTION

Apples are very popular among the consumers in large quantities not only for their taste and presence of healthy metabolites, but apples are a very substantial part in many traditional receipts in many national cuisines. Unfortunatelly, during the last years the presence of allergens became a very important question for many of consumers and for many foodstuffs, on the other hand apple has been demonstrated to be an important source of secondary metabolites able to promote human health. For this reason the integrated information about the different allergenic potential together with the amount of biological active metabolites and phytonutrient is crutial to provide a background that must guide the consumer to choose the preferable one.Diversification of food resources available for consumers is in concordance with the the aim of personalized nutrition, much more when the nutrigenomic relationships became an important part of the nutrition research.

Allergens identification has become an integral part of the characteristics of many foodstuffs. The research in this area is important not only from the scientific point of view, but also from the view of impact's to the health as the increasing number of people suffering from allergies. Allergens of individual plant food sources are very well described and structural details are known as well as the interaction with the immune system of patients. But at the level of regulation and expression of the genes themselves in plants, our knowledge is very limited for the known allergenic proteins. One of these is the Mal d 1 allergen of apple fruits. A variety of allergens from different fruits were identified based on experimental immunology and molecular biology, i.e., by sequencing, leading to gene and protein identification. Among the different fruit allergens, the pathogenesis-related (PR) proteins, classified into 17 families based on sequence, diverse structure, function and biological activity **(van Loon *et al.*, 2006)**, and are produced in response to different biotic and abiotic stresses **(Maghuli *et al.*, 2009)**.

Mal d 1 allergen belong to PR-10 proteins and is a Bet v 1-homologue and large cross-sensitization was previously demonstrated between pollen allergen Bet v 1 and apple Mal d 1 **(Fritsch *et al.*, 1998)**. Mal d 1 is coded by a large gene family of 18 members **(Gao *et al.*, 2005a, 2005b, 2005c)**. Different hypothesis exist for the allergenic potential of Mal d allergens in apples. **Son *et al.* (1999)** reported allergenic differences between apple cultivars due to the different expression levels of Mal d 1 without the connection to the presence of its different isoformes. **Gao *et al.* (2008)** reported that differences in allergenic potential is associated with the allelic composition of two specific allergens - Mal d 1.04 and Mal d 1 1.06 A.

Apple allergens are responsible of a fast hypersensitive reaction mediated by immunoglobulin E (IgE). Mal d 1- allergen is supposed to be accumulated at cytosolic level in apple fruits. It is the major apple allergen causing type I allergy in tree-pollen sensitized patients. It was reported to be a variety dependent in its expression, and mainly present in commercial apples **(Botton *et al.*, 2008; Pagliarani *et al.*, 2013)**. According to the allergen surface reacting with the immune system, apple allergies can be distinguished into the following: Class-I, identified as gastro-intestinal and systemic symptoms, because the allergen is not degraded by gastric digestion **(Pastorello and Rivolta, 2004)** and Class-II, identified as oral allergy syndrome, in which the pollen proteins are acting as primary sensitizing agents and the fruit ingestion immediately provokes mild symptoms in the mouth **(Eriksson *et al.*, 1982)** and in the skin in contact with apple during consumption **(Fernandez-Rivas *et al.*, 2006)**.

Apple cultivars have different allergenicity levels, as reported in various clinical studies. Unluckily, the majority of commercial varieties shows high allergenic potential but a few displays a very promising hypoallergenicity, such as Santana, Topaz, Elise, Braeburn **(Bolhaar *et al.*, 2005; Kootstra *et al.*, 2007)**, Pink Lady, Gloster, McIntosh, Modi **(Vlieg-Boerstra *et al.*, 2011, 2013)** and Durello di Forli **(Ricci *et al.*, 2010)**. In spite of the very good knowledge about fruit allergen protein characteristics or their immunological interaction in senzitized patients **(Rona *et al.*, 2007)**, the knowledge about their expression characteristics in plants per se is very limited.

Currently, the methods based on the reverse transcription are considered to be the most sensitive, precise and reproducible techniques to detect specific mRNAs and were employed in a wide range of applications **(Nicot et al., 2005)**. The crucial step for the accurate quantification of analysed transcripts is the identification of stable reference genes to normalize the target´s levels **(Philips et al., 2009)**. Different housekeeping genes were reported as internal controls in

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 growth under the condition of fruit garden in the locality of Rišňovce, 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.

code of sample	date of collection
X1	18.4. 2016
Z1	18.4. 2016
X5	4.5.2016
Z5	4.5.2016
X16	17.6.2016
Z16	17.6.2016
X32	20.8.2016
Z32	20.8.2016
X38	13.9.2016
Z38	13.9.2016
X40	21.9.2016
Z40	21.9.2016

Legend:X – Golden; Z - Spartan

Extraction of RNA and preparation of cDNA

Apple pulps were firstly homogenized using the liquid nitrogen. Total RNAwas 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^{-1} of both of the primers and 1 µl of the 1:100 dilluted 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 DT002474 with the following seguences: forward 5´CTATGTTCCCTGGTATTGCAGACC3´ and reverse 5´GCCACAACCTTGTTTTTCATGC3´. 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 specifity was checked by 2% agarose electrophoreotic 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; Vivodík *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; Kačániová *et al.*, 2012; Žiarovská *et al.*, 2013; Ražná *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 isresponsible for birch pollen-related food allergies is the 17.5kDa protein Mal d 1 **(Vanek-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 specifity of amplified product was confirmed electrophoreotically 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 neccesity of the knowledge of very pecisely 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 **(Tajadiny *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 diferent housekeeping genes were analysed for the *Malus domestica*, Borkh tissues - actin, protein disulphide 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)**.

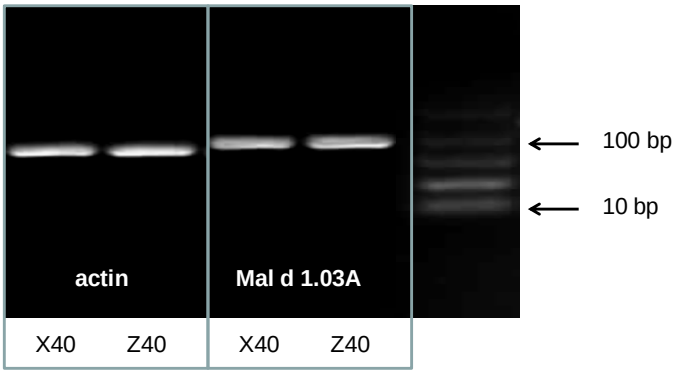


Figure 1 A – Electrophoreotic separation of amplified actin and Mal d 1.03A sequences of apple varieties Golden (X40) and Spartan (Z40) – checking of the amplification specifity.

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 × μ^{-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.

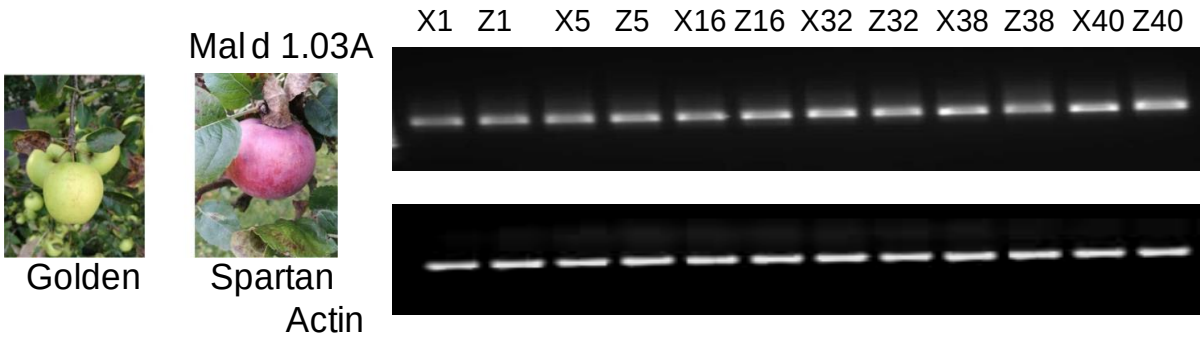
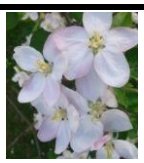
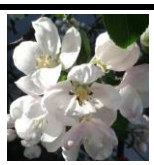
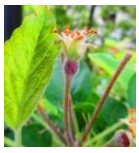


Figure 2 Determination of the amplified transcripts of Mal d 1.03A allergen and actin in the samples

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)**. Increasinf 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 andSchmitz-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, Schmitz-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, thatMal d allergenis 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 ripenig

Sample	AAT/ng	Photographic documentation	Sample	AAT/ng	Photographic documentation
X1	1826		Z1	2123	
X5	2556		Z5	2887	
X16	2620		Z16	2998	
X32	3052		Z32	2942	
X38	3238		Z38	2661	

X40	3209		Z40	3627	

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

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.

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