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Effect of long-term storage on the change in the expression of selected *Mal d 1* gene isoforms in the apple cultivar Opal®

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Abstract: Apples are available all year round since they can be stored for long time. However, the expression of the major apple allergen Mal d 1 can increase in the fruit during storage and incease so the risk of allergies in sensitive consumers. Therefore, we studied the change in the expression of the *Mal d 1* gene during storage in the modern cultivar UEB 32642 (known under the brand name Opal®) and the cultivar Gala Brookfield (cv. Gala) as a reference. The cultivars were stored under two different conditions, ultra-low oxygen (ULO) and standard cold conditions. The gene expression was evaluated both in fresh fruits and in fruits stored for four, six, and nine months. Nine isoforms of the gene *Mal d 1* with the highest published expression were quantified using real-time PCR. The most expressed isoforms *Mal d 1.01*, *Mal d 1.02* and *Mal d 1.06A* were found in the fresh and also in the stored fruits. The expression of the *Mal d 1.03G* and *Mal d 1.06D* isoforms was higher in the stored fruits. Our study confirmed that (*i*) Opal® had a lower overall expression of the *Mal d 1* gene than cv. Gala, both in the fresh and stored fruits; (*ii*) standard cold storage is superior in preserving lower *Mal d 1* levels in Opal®apples compared to the ULO conditions; and (*iii*) less expressed isoforms may be responsible for the general increase in the *Mal d 1* gene expression during storage.

Keywords: apple allergy; cold storage; controlled atmosphere; *Malus* × *domestica* Borkh.; relative quantification

An important part of a healthy diet is fruit that is a source of many vitamins, minerals, fibre, and other healthy substances. The apple (*Malus* × *domestica* Borkh.) is one of the most cultivated fruit crops worldwide. Its advantages are good storability providing for year-round availability, as well as the content of health-promoting substances in a completely natural form. However, eating apples can cause serious health problems, such as allergic reactions, to a certain group of people (e.g., Hassan & Venkatesh 2015). Therefore, allergenicity is an important feature in determining the quality of the fruit. So far, four allergen groups have been identified in the apple: Mal d 1, Mal d 2, Mal d 3, and Mal d 4 (Savazzini et al. 2015). Since the Mal d 1 allergens belong to PR-10 proteins or Bet v 1-homologues, their presence is of particular importance in Central, Northern, and Eastern Europe, as well as in North America, i.e., areas populated with birches, where cross-sensitisation between the birch pollen allergen Bet v 1 and the apple allergen Mal d 1 can occur (Fritsch et al. 1998). Clinical observations of patients allergic to birch pollen demonstrated that up to 70% of these people confirmed allergic symptoms after

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eating apples (Ebner et al. 1995). A comparison of the *Bet v 1* allergen and the *Mal d 1* allergen sequences revealed that, at the nucleic acid level, the sequence identity was about 56%, and, at the amino acid level, the sequence identity was about 65% (Vanek-Krebitz et al. 1995). Allergies caused by the Mal d 1 allergen are usually mild, featuring the so-called oral allergy syndrome (OAS) (Bohle et al. 2006).

Currently, 31 isoforms of the *Mal d 1* gene have been described. Their expressions can differ substantially despite their only small difference at the sequence level (Pagliarani et al. 2013). The level of expression of the *Mal d 1* gene isoforms varies between apple cultivars and is also affected by the locality, growing conditions, diseases, fruit ripeness, and storage conditions (Sancho et al. 2006; Botton et al. 2008). Different studies have shown that the content of allergens in the fruit and the ability to elicit an allergic reaction varied significantly between apple cultivars. Some cultivars exhibit a high potential to cause an allergic reaction while others have been declared hypoallergenic. Santana is considered to be a hypoallergenic cultivar, the low-allergenic cultivars include Elise, Topaz and Braeburn (Bolhaar et al. 2005; Vlieg-Boerstra et al. 2011). Most commercial cultivars are classified as high-allergenic (Savazzini et al. 2015), for example, Golden Delicious, Gala or Jonagored (Bolhaar et al. 2005). People with a Mal d 1 allergy, i.e., with mild manifestations, can typically consume these hypoallergenic cultivars without adverse effects. The regular, gradually increasing consumption of low-allergenic apples can lead to a tolerance to highly allergenic cultivars (Kopac et al. 2012; Bergmann et al. 2020). Nothegger et al. (2020) reported that the consumption of low-allergenic apple cultivars can also represent a suitable immunotherapy for hay fever caused by the allergic reaction to birch pollen. Therefore, it is very important for consumers to know the level of the *Mal d 1* expression and the presence of allergens in apple fruit. This especially applies to novel commercially successful cultivars, e.g., Opal®.

The aim of this study was to describe effect of the type of storage and its length on the change in the *Mal d 1* gene expression. For the first time, not only the three main isoforms of the Mal d 1 allergen were studied, but also its minor isoforms in two commercially successful apple cultivars, Opal® and Gala. We also evaluated the contribution of these nine isoforms to the total *Mal d 1* gene expression in both cultivars during their long-term storage.

MATERIAL AND METHODS

Plant material and storage condition. The apple cultivar UEB 32642 (also known under the brand name Opal® used herein) and the well-known cultivar Gala Brookfield (abbreviated as cv. Gala) were used for comparison. The cv. Gala fruits were harvested at the experimental plantings of Research and Breeding Institute of Pomology Holovousy while the Opal® fruits were obtained from a grower in Ostroměř. Fruits were harvested at their optimal harvest maturity. Analyses were conducted using freshly harvested fruits and those stored under two different conditions: (*i*) controlled in an ultra-low oxygen atmosphere (ULO; 2% O_2, 1% CO_2, temperature 1.5–2 °C, humidity 99%) and (*ii*) cold storage (no controlled atmosphere, temperature approx. 2 °C). The samples stored under both conditions were analysed immediately after harvest and after four, six and nine months of storage.

RNA extraction. Apple peel was used for the RNA extraction. Each sample was prepared as a mixture of peels from three fruits of the given cultivar. The total RNA was isolated from the samples using a Ribospin™ Plant kit (GeneAll®, Korea) and the potential genomic DNA contamination was removed via the use of a DNA-free kit (Ambion by Life Technologies, USA) following the manufacturer's instructions. The total RNA (1 µg) was reverse transcribed using an M-MLV Reverse Transcriptase (ThermoFisher Scientific, USA) and hexanucleotide random primers (Roche, Switzerland).

Relative quantification of gene expression. The gene expression of the selected *Mal d 1* isoforms (*Mal d 1.01*, *Mal d 1.02*, *Mal d 1.03D*, *Mal d 1.03G*, *Mal d 1.06A*, *Mal d 1.06B*, *Mal d 1.06D*, *Mal d 1.07* and *Mal d 1.13A*) was determined using primers described by Pagliarani et al. (2013) except for the *Mal d 1.06A* isoform for which new primers were designed (Mal d 1.06AF: 5' -CATCATGGGTGTCCTCACATACG-3'; Mal d 1.06AR: 5'-GAGCAATCTTCGGAATGAGAT-3'). *Actin* was used as a reference gene to which the relative gene expression levels were normalised:

Actin F: 5'-TGACAGAATGAGCAAGGAAATTACT-3'
Actin R: 5'-TACTCAGCTTTGGCAATCCACATC-3'.

Primers for *Actin* and for the *Mal d 1.06A* isoform were designed using Geneious Prime software (https://www.geneious.com). Sequences obtained from the GenBank database (https://www.ncbi.nlm.nih.gov/nuccore) were used for the primer design (XM008342515 for *Mal d 1.06A* and XM008347230 for Actin).

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For the polymerase chain reaction (PCR), a qPCR 2× Blue Master Mix (Top-Bio, Czech Republic) supplemented with EvaGreen (Biotium, USA) was used and the reactions were run using a Rotor-Gene Q (Qiagen, Netherlands) cycler with the following temperature profile: 95 °C/5 min; 50 cycles (95 °C/20 s; 55 °C/20 s; 72 °C/10 s). Each sample was analysed in a technical triplicate. The relative gene expression of the selected isoforms was determined by applying the ΔΔCT method (Livak & Schmittgen 2001).

The specificity of the amplification reaction was confirmed by melting analysis (Figure S3 in the Electronic Supplementary Material (ESM)) that was performed post-PCR (temperature from 76 °C to 88 °C; ramp 0.2 °C 2 s), and by sequencing using a BigDye™ Terminator v3.1 Cycle Sequencing Kit, BigDye XTerminator™ Purification Kit, with an Applied Biosystems 3500 Genetic Analyser (all Thermo Fisher Scientific) following the manufacturer's instructions.

Statistical analysis. The statistical analysis was performed using Student's *t*-test with a significance level of $P < 0.05$. The equality of variances was evaluated using *F*-test.

RESULTS

***Mal d 1* gene expression in fresh fruits.** The isoforms *Mal d 1.02*, *Mal d 1.01*, *Mal d 1.06A* were those most expressed in the fresh fruit of both cultivars (Figure 1A). These three isoforms in the fresh Opal® accounted for 83% of the total relative gene expression of the *Mal d 1* gene. In contrast, the same three most expressed isoforms represented almost 96% of the total *Mal d 1* gene expression in the fresh cv. Gala (Figure 1B). These three main isoforms in cv. Gala exhibited a higher expression than in Opal®. On the other hand, the minor isoforms *Mal d 1.06B*, *Mal d 1.07* and *Mal d 1.13A* were higher expressed in Opal® (Figure 1A). After summing all of the isoforms, the overall relative expression of the *Mal d 1* gene is 45 % lower in Opal® compared to cv. Gala (Figure S1 in the ESM).

***Mal d 1* gene expression in fruits stored under controlled atmosphere ULO.** The total relative expression of the *Mal d 1* gene in the Opal® increased during the first 6 months of storage under the ULO condition, but the gene expression decreased after

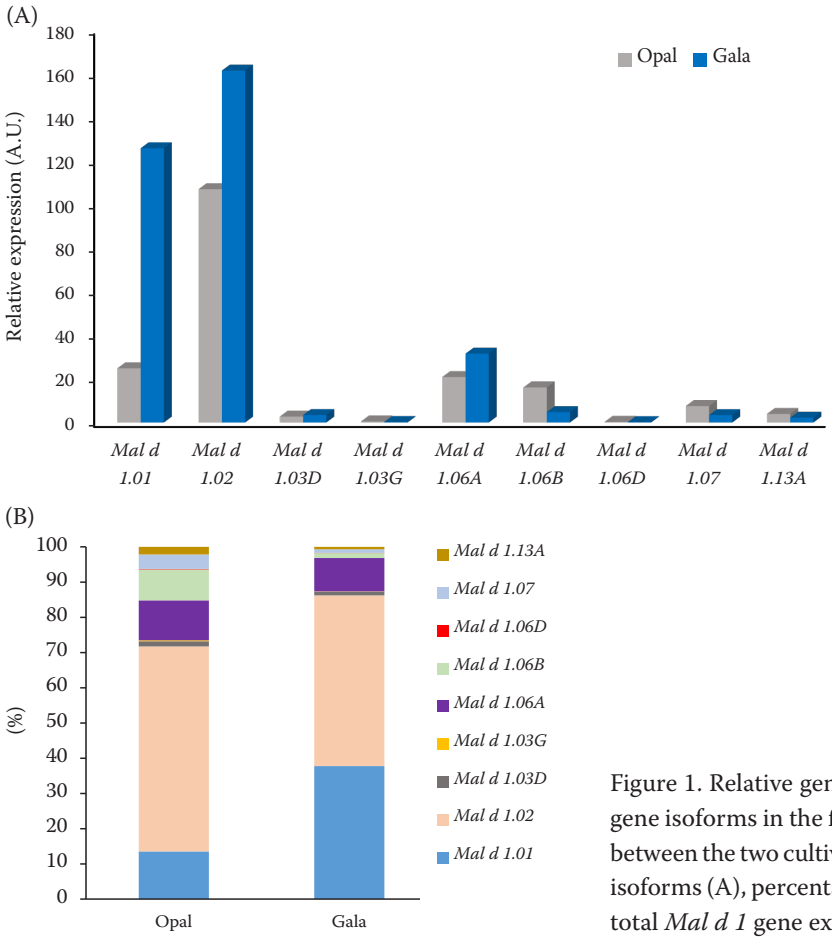

Figure 1. Relative gene expression for all the studied *Mal d 1* gene isoforms in the fresh Opal® and cv. Gala fruit: difference between the two cultivars in the gene expression of the selected isoforms (A), percentage representation of each isoform in the total *Mal d 1* gene expression (B)

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9 months. Significant changes in the gene expression were observed for all the main isoforms (*Mal d 1.01*, *Mal d 1.02* and *Mal d 1.06A*) as well as for two minor isoforms *Mal d 1.03G* and *Mal d 1.06D* (Figure 2A; Table S1 in ESM). Similar to the fresh fruits, the *Mal d 1.02* isoform in Opal® remained the most expressed even after storage under the ULO conditions, followed by the *Mal d 1.01* and *Mal d 1.06A* isoforms.

The total relative expression of the *Mal d 1* gene gradually increased during storage in the cv. Gala fruits stored under the ULO conditions. The *Mal d 1.01* isoform was the most expressed, followed by the *Mal d 1.02* isoform (Figure 2A). Under these conditions, the expression of the *Mal d 1.01* isoform increased significantly (6.3×) in comparison with the fresh fruits (Figure S2 and Table S1 in the ESM). A slight increase in the *Mal d 1.06A* isoform was observed after 9 months of storage (Figure 2A). However, the percentage representation of the total expression of this isoform tended to decrease (Figure 2B). The expression of the minor isoforms *Mal d 1.03G* and *Mal d 1.06D* in cv. Gala also increased significantly during storage under the ULO conditions (Figure 2A, Table S1 in the ESM) as can be also seen in their percentage representation (Figure 2B).

***Mal d 1* gene expression in fruits stored under standard cold conditions.** The *Mal d 1.02* isoform remained the most expressed in the Opal® fruits stored in the cold storage which was followed by the *Mal d 1.01* isoform, although its expression did not change much during storage (Figure 3A). For the other isoforms in Opal®, no distinct increase in the expression occurred during the entire length of storage except for the *Mal d 1.06D* isoform for which a significant increase in expression was observed (Figure 3A; Table S1 in the ESM). This is mainly evident from the percentage representation of the individual isoforms in the overall expression (Figure 3B). Storage of Opal® fruits under standard cold conditions resulted in a generally lower gene expression of the *Mal d 1* isoforms compared to the ULO (significant for nearly all combinations isoforms vs. storage; Table S1 in the ESM).

The total relative expression of the *Mal d 1* gene in the cv. Gala fruits gradually increased during cold storage. The *Mal d 1.02* isoform was the most expressed (Figure 3A) followed by the *Mal d 1.01* isoform. However, the percentage representation of this isoform in the total expression decreased in the first six months while increasing strikingly after nine

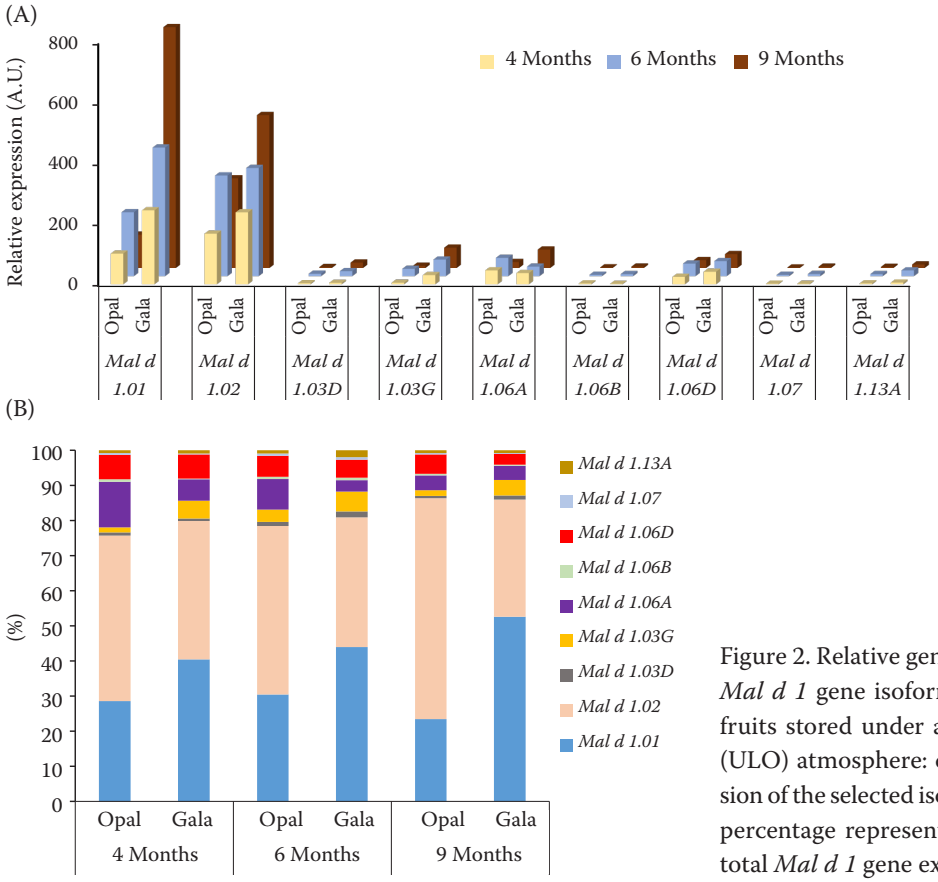

Figure 2. Relative gene expression of all the studied *Mal d 1* gene isoforms in the Opal® and cv. Gala fruits stored under a controlled ultra-low oxygen (ULO) atmosphere: difference in the gene expression of the selected isoforms of the two cultivars (A), percentage representation of each isoform in the total *Mal d 1* gene expression (B)

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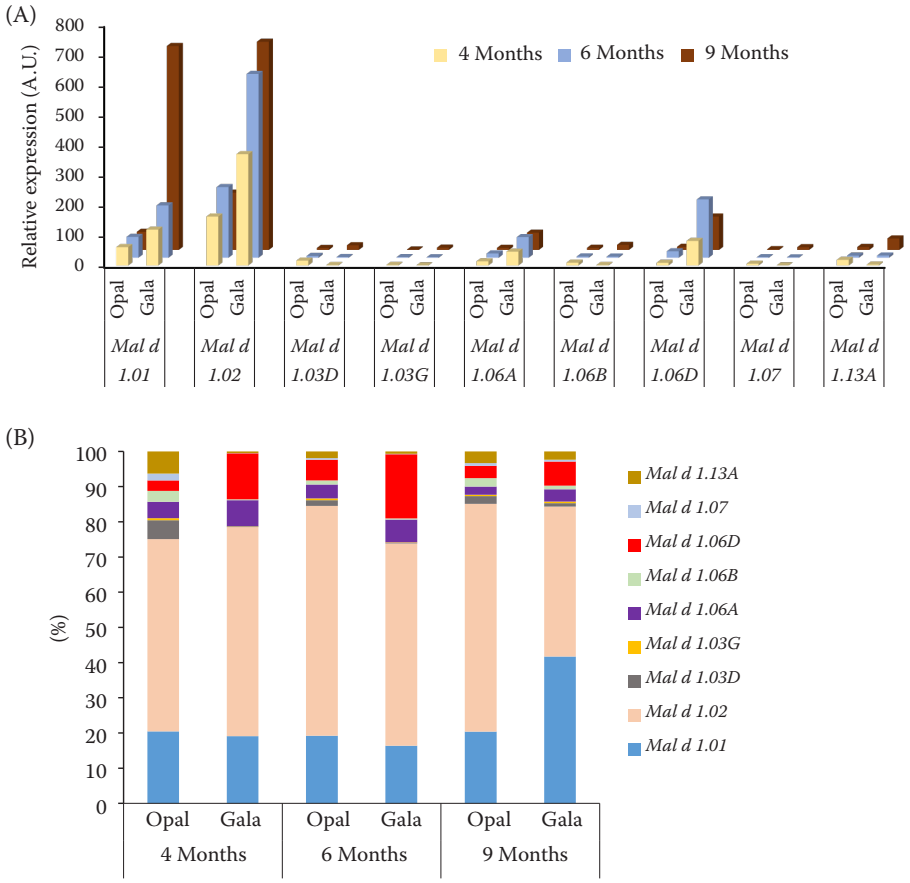

Figure 3. Relative gene expression for all the studied *Mal d 1* gene isoforms in the Opal® and cv. Gala fruit stored under the standard cold conditions: difference between the two cultivars in the gene expression of the selected isoforms (A), percentage representation of each isoform in the total *Mal d 1* gene expression (B)

months of storage (Figure 3B). While the expression of the *Mal d 1.06A* isoform in the cv. Gala increased slightly (Figure 3a) in cold storage, the percentage representation of the total expression of this isoform had a tendency to decrease (Figure 3B) similar to storage in the ULO conditions. The fruit of cv. Gala stored in cold storage exhibited a significant increase in the expression of the *Mal d 1.06D* isoform that reached a higher relative expression value than the *Mal d 1.01* isoform after six months of storage (Figure 3A; Table S1 in the ESM). In general, the gene expression of the *Mal d 1* isoforms in Opal® was distinctively lower in comparison to cv. Gala.

DISCUSSION

This work aimed at comparing the gene expression of the most expressed isoforms of the *Mal d 1* gene in the Opal® and cv. Gala apple cultivars stored under two different storage conditions. Opal® is a modern Czech apple cultivar while cv. Gala was chosen as a reference since it is well-recognised and grown worldwide. In addition, the fruits of both cultivars can be stored for a long time till the end of spring. Opal® apples have a yellow skin colour and excellent taste. This cultivar also bears resistance to scab and tolerates powdery mildew quite well (Tupy et al. 2005). Thus, it could be a suitable substitute for the sensitive and more agrotechnical demanding cultivars such as the cv. Golden Delicious.

Isoforms representing more than 0.5 % of the total *Mal d 1* gene expression were selected for the analyses (Žďárská et al. 2021). Furthermore, the selected isoforms had to have their relative gene expression measured using real-time PCR present at detectable levels. The isoforms *Mal d 1.01*, *Mal d 1.02*, *Mal d 1.03D*, *Mal d 1.03G*, *Mal d 1.06A*, *Mal d 1.06B*, *Mal d 1.06D*, *Mal d 1.07*, and *Mal d 1.13A* met these criteria. The melting analysis (Figure S3 in the ESM) and sequencing (data not shown) confirmed the identity of all the selected *Mal d 1* isoforms. Isolation from the peel was preferred based on the finding of Pagliarani et al. (2013) in which, for most isoforms of the *Mal d 1* gene, the expression was higher in the peel than in the pulp.

We observed differences between the relative gene expression of the individual isoforms in the fresh and stored fruits, as well as between the two storage

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methods. We also found significant differences in the expression of the individual isoforms between the two studied cultivars (also see the supplementary data, Figures S1, S2 and Table S1 in the ESM). Previous works concluded that the content of the allergen Mal d 1 increased during storage (Bolhaar et al. 2005; Sancho et al. 2006; Kiewning et al. 2013; Siekierzynska et al. 2021). Our results correspond with this finding since the overall *Mal d 1* gene expression increased in the stored fruits. The relative gene expression in Opal® was lower than in cv. Gala both in the fresh fruits and during storage. Similar to the cv. Gala, the relative gene expression of the gene *Mal d 1* in Opal® increased during storage, but, unlike in the cv. Gala, the increase in Opal® followed an inverse U-curve, reaching the highest values after 6 months of storage. Significant differences were also observed between cold storage and storage under the ULO conditions. While the relative gene expression of the gene *Mal d 1* in the cv. Gala increased in the cold storage more than under the ULO conditions, the opposite was true for Opal®. This indicated that the changes in the gene expression during storage under different conditions are apparently variety dependent. Mal d 1 belongs to the group of PR (pathogenesis related) proteins which are activated in plants in response to various types of stress (Fernandes et al. 2013). Therefore, it is possible that the change in the allergen expression is related not only to the storage of apples *per se*, but also to the potential diseases in storage, cold effect on the stored fruit, and exposure to other types of stress. Stress, in general, along with the maturing of fruits during storage, can affect an increase in the levels of certain isoforms of the Mal d 1 allergen and, thus, an increase in allergenicity of the stored fruits. Opal® is known to tolerate storage very well even without the use of special conditions and to retain good fruit characteristics for a long time. Based on our results, the best trade-off between the potential allergenicity and storage conditions in Opal® apples could be achieved either by marketing fresh fruits (55 % of the *Mal d 1* gene expression compared to cv. Gala) or under cold storage conditions resulting in the same or lower *Mal d 1* levels in comparison with the fresh Gala fruit. The cultivars Gala together with Golden Delicious are considered cultivars with the highest allergenicity (Bolhaar et al. 2005; Kiewning et al. 2013), with the allergenic potential likely growing upon storage.

Regarding the individual isoforms, the highest gene expression in fresh fruits was noticed in the *Mal d 1.02* isoform, followed by the *Mal d 1.01* and *Mal d 1.06A* isoforms in both studied cultivars. These together accounted for 83% and 96% of the total expression of the *Mal d 1* gene allergen in Opal® and cv. Gala, respectively. These isoforms are generally considered to be the most highly expressed (Botton et al. 2008; Pagliarani et al. 2013; Siekierzynska et al. 2021). The *Mal d 1.01* and *Mal d 1.02* isoforms remained the most expressed even during storage, whereby the *Mal d 1.01* isoform expression increased even more under the ULO conditions than in the cold storage. In the cv. Gala, it even reached a higher value of the relative gene expression than the *Mal d 1.02* isoform. In both cultivars, the expression of the *Mal d 1.06D* isoform significantly increased during storage (Figure S2 and Table S1 in the ESM). This was especially apparent in the cv. Gala under cold storage conditions, where this isoform reached the highest expression values after *Mal d 1.01* and *Mal d 1.02,* and it even exhibited a higher expression than the *Mal d 1.01* isoform after 6 months of storage. Thus, it is possible that the *Mal d 1.06D* isoform may play an important role in an increase in the allergenicity of apples during storage. Under the ULO conditions, the expression of the *Mal d 1.03G* isoform increased indicating that this isoform can also take a part in the overall allergen contents during storage (Figure S2 in the ESM).

CONCLUSION

The effect of various storage conditions on the *Mal d 1* gene expression was studied in the Opal® and Gala cultivars. Although the *Mal d 1.01* and *Mal d 1.02* isoforms remained the most expressed in both the fresh and stored fruits, other isoforms, such as *Mal d 1.06D* and *Mal d 1.03G*, cannot be neglected in studies of hypoallergenic apples. An increase in the relative gene expression during storage was also observed in these less expressed isoforms which could also affect the change in allergenicity of apple cultivars. Opal® had generally a lower gene expression of the main *Mal d 1* isoforms under all the studied conditions, rendering it less allergenic than the cv. Gala. Use of standard storage conditions for Opal® apples led to only a minimal increase in the overall *Mal d 1* gene expression and, from this perspective, these conditions should be preferred to the ULO storage.

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