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RESEARCH ARTICLE

# Transcript expression levels of apple allergens in Granny Smith and Golden Delicious fruits across European retail stores

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## Abstract

The complex interplay between genetics and environmental factors shapes the allergenic potential of apples. All 4 described allergens of apples vary in their protein content, but the variability at the transcriptomic level is not well known. In this study, the natural variability of the transcriptomic levels of apple genes for allergen isoforms was analysed for fruits from retail chains in various European countries. Real-time quantitative polymerase chain reaction (RT-qPCR) was utilised to quantify the expression of PR-10 (Bet v 1 family), thaumatin-like proteins, nsLTP1 and profilins. The expression levels exhibited variability among apples originating from different countries, to varying degrees. Only minor variation was observed in Golden Delicious, with a change of 0.11-fold to 6.82-fold relative to the calibrator that was the fruit from the Slovak Republic. Among the *Mal d 1* isoforms, *Mal d 1.02* showed the highest fold change and the greatest variability. However, in Granny Smith, the *Mal d 4.01* exhibited substantial up-regulation, reaching approximately 280-fold. Furthermore, the fold changes demonstrated greater variability compared to those observed in Golden Delicious. Insights into the molecular variability and regulation of *Mal d 1* isoforms can inform both public health guidance and precision agriculture strategies. As food allergies continue to rise globally, region-specific allergen profiling and cultivar selection may contribute to safer, more transparent and more inclusive food systems.

**Keywords:** allergens; Golden Delicious; Granny Smith; *Mal d*; PR-10

## Introduction

Allergic reactions to fruits such as apples frequently occur as part of Pollen–Food Allergy Syndrome (PFAS), in which sensitisation to airborne pollen allergens results in IgE-mediated responses to structurally related food proteins. In central and northern Europe, sensitisation to apple is mainly due to cross-reactivity between the major birch pollen allergen *Bet v 1* and its homologous apple allergen *Mal d 1*, whereas in Mediterranean regions, where birch pollen exposure is low, sensitisation is more commonly associated with peach (1, 2).

To date, 4 major classes of allergens have been identified in apples- *Mal d 1*, *Mal d 2*, *Mal d 3* and *Mal d 4* (3). *Mal d 1*, *Mal d 2* and *Mal d 3* correspond to PR proteins (PR-10, PR-5 and PR-14 respectively), while *Mal d 4* belongs to profilins (4). These allergens differ in tissue localisation, with Mal d 1 and Mal d 2 present in both peel and pulp, whereas *Mal d 3* is predominantly peel-specific (5).

*Mal d 1* is a stress-responsive protein expressed in apple trees in response to biotic and abiotic stresses and plays a role in the binding and transport of plant steroids. Its levels in apples are influenced by several factors, including apple cultivar, storage time and storage conditions. Some studies suggest that organically grown apples, which are often exposed to more environmental challenges, may contain higher levels of *Mal d 1*, an allergen induced by environmental stressors (6). However, findings on the effect of cultivation systems remain inconsistent. For instance, researchers found that apples produced organically generally had lower *Mal d 1* levels than apples produced using integrated systems (7).

Storage conditions also have a notable impact. *Mal d 1* accumulation during storage is promoted by low temperatures under controlled atmosphere conditions, leading to significantly higher levels compared to regular air storage (8). The 'Golden Delicious' cultivar reached peak *Mal d 1* levels after 8 weeks of storage, followed by a decline during extended storage (7).

Furthermore, environmental factors such as shading and elevation strongly influence the transcription of allergen-encoding genes, while water stress has only a minor effect. Shading may serve as a useful method to reduce allergen levels in apple cortex tissue (9).

Using enzyme-linked immunosorbent assay (ELISA), a broad range and high inter-apple variability of *Mal d 1* levels were detected among the 39 cultivars, with values ranging from 0.84 to 33.17 µg/g fresh weight. The occurrence of Mal d 1 isoforms appears to be not cultivar-specific and the major isoforms generally show a comparable expression pattern (5). To date, over 100 *Mal d 1* allergen isoforms have been identified in apples. These are grouped into 12 subgroups (*Mal d 1.01–Mal d 1.12*) based on their amino acid sequences (10). The variation in *Mal d 1* content is due to different

levels of expression of different isoforms, with the most abundant isoforms belonging to the *Mal d 1.01* and *Mal d 1.02* subfamilies (11). In a previous study, which also included the Granny Smith cultivar, the *Mal d 1.02* was the predominant isoform expressed in ripe apples, showing the highest expression levels by a wide margin. In comparison, *Mal d 1.01* was expressed at levels nearly ten times lower, while *Mal d 1.03* showed even less expression, making it the least abundant among the three (5). The expression levels were significantly higher, ranging from 10– 10000-fold, compared to all other *Mal d 1* genes. The total amount of RNA for these genes exceeded the combined amount of all other genes in *Mal d 1* (12). These isoforms, *Mal d 1.01* and *Mal d 1.02*, were associated with hyperallergenic characteristics because they were highly expressed in the hyperallergenic varieties and less frequently transcribed in hypoallergenic varieties (13).

Therefore, the aim of this study was to evaluate the natural variability of transcription levels of major apple allergen genes (*Mal d 1*–*Mal d 4*) in fruits of commonly consumed apple cultivars obtained from European retail chains, with the hypothesis that allergen gene expression shows significant isoform- and region-dependent variability.

## Materials and Methods

### Biological material

The biological material used in this study consisted of fruits from two apple cultivars: Golden Delicious and Granny Smith (*Malus domestica* (Suckow) Borkh). A total of 13 samples (Table 1) were collected from retail chains in different European countries. Each country was represented by one biological replicate per cultivar. Since the study did not involve field sampling or access to restricted plant material, no collection permits or institutional approvals were necessary. As the apples were not grown, harvested or stored under uniform conditions, variations in growth and storage environments were observed.

**Table 1.** Characteristics of biological materials used in the study

| Sample ID | Cultivar | Country |
|---|---|---|
| MD1z | Golden Delicious | Austria |
| MD3z | Golden Delicious | |
| MD4z | Granny Smith | |
| MD5z | Golden Delicious | Slovakia |
| MD6z | Granny Smith | |
| MD7z | Golden Delicious | Serbia |
| MD8z | Granny Smith | |
| MD9z | Golden Delicious | Croatia |
| MD10z | Granny Smith | |
| MD11z | Golden Delicious | Hungary |
| - | - | |
| MD13z | Golden Delicious | Italy |
| - | - | |
| MD15z | Golden Delicious | Slovenia |

### RNA isolation

RNA was isolated using the Ribospin™ Seed/Fruit Kit (GeneAll) according to the manufacturer's protocol. Samples (0.6 g) consisting of skin and pulp were homogenised using a pestle and mortar with the addition of sea sand. The RNA concentration and purity were assessed using a NanoPhotometer™ (IMPLEN). The RNA concentrations ranged from 21.2 to 111 ng/µL. The A260/280 ratios ranged from 1.71 to 2.15, indicating generally good purity with minimal protein contamination. Despite slight variation in purity metrics, all samples were suitable for downstream cDNA synthesis and qPCR following standard normalization procedures. All samples (MD1-15z) were standardised to 140 ng for consistency in subsequent analyses. The template amount of 140 ng was selected on the basis that it represented the highest RNA concentration that had been reliably obtained from the applied extraction procedure, thus providing the maximal input that was permissible for the reaction. The subsequent dilution factors (Table 2) were applied in accordance with the results of the preceding optimisation process. RNA was then reverse transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR.

**Table 2.** Reverse transcription quantitative polymerase chain reaction (RT-qPCR) condition characteristics for primers used in the study

| | Isoform | Annealing temperature (°C) | Melt temperature (°C) | Primer concentration (nmol.dm-3) | Dilution factor |
|---|---|---|---|---|---|
| | *Mal d 1.01* | 63 | 83,5 | 900 | 10X |
| | *Mal d 1.02* | 64 | 84 | 600 | 10X |
| | *Mal d 1.03A* | 62 | 81 | 600 | 10X |
| | *Mal d 1.03C* | 66 | 80,5 | 600 | 10X |
| | *Mal d 1.03D* | 63 | 82,5 | 600 | 10X |
| | *Mal d 1.03E* | 64 | 82,5 | 600 | 10X |
| | *Mal d 1.03F* | 63 | 79,5 | 600 | 10X |
| | *Mal d 1.03G* | 63 | 81 | 600 | 10X |
| **Pathogenesis-related protein, PR-10, Bet v 1 Family member** | *Mal d 1.06A* | 63 | 8 | 600 | 10X |
| | *Mal d 1.06B* | 64 | 81 | 600 | 10X |
| | *Mal d 1.06C* | 60 | 80 | 600 | 10X |
| | *Mal d 1.07* | 63 | 81 | 600 | 10X |
| | *Mal d 1.08* | 63 | 81,5 | 600 | 10X |
| | *Mal d 1.11A* | 60 | 70,5 | 600 | 10X |
| | *Mal d 1.11B* | 62 | 81 | 800 | 10X |
| | *Mal d 1.13A* | 64 | 78 | 600 | 10X |
| | *Mal d 1.13B* | 60 | 81,5 | 600 | 10X |
| | *Mal d 1.13D* | 64 | 78 | 600 | 10X |
| | *Mal d 2.01* | 64 | 83 | 300 | 50X |
| **Thaumatin-like protein** | *Mal d 2.02* | 64 | 82,5 | 300 | 100X |
| | *Mal d 2.03* | 64 | 79 | 300 | 10X |
| **Non-specific lipid transfer protein type 1 (nsLTP1)** | *Mal d 3.01* | 64 | 86 | 300 | 100X |
| | *Mal d 3.02* | 64 | 82,5 | 300 | 100X |
| | *Mal d 4.01* | 64 | 80 | 300 | 50X |
| **Profilin** | *Mal d 4.02* | 64 | 80,5 | 300 | 100X |
| | *Mal d 4.03* | 64 | 82 | 300 | 100X |

### Expression levels analysis

RT-qPCR was performed to quantify gene expression targeting pathogenesis-related proteins (PR-10, Bet v 1 family), thaumatin-like proteins, non-specific lipid transfer proteins (nsLTP1) and profilins. The experiment was performed using EliZyme™ Green MIX (Elisabeth Pharmacon). The RT-qPCR premix was prepared for each reaction to ensure consistency across 3 technical replicates. The reaction mixture had a final volume of 10 µL and consisted of EliZyme™ Green MIX (Elisabeth Pharmacon), diluted cDNA template, forward and reverse primers (Table 2). Primers designed were specific to the isoforms of the *M. domestica* genes listed in Table 2 (11).

Actin was selected as the housekeeping gene, with specific primers designed for its amplification: forward (F) - CTATGTTCCCTGGTATTGCAGACC and reverse (R) - GCCACAACCTTGTTTTTCATGC. Actin was used as the reference gene for $2^{-\Delta\Delta Ct}$ normalization and its Ct values were monitored across all samples to ensure suitability as a reference gene. Technical replicates were used to ensure consistency and reliability of results. The majority of samples exhibited comparable actin Ct values, indicating generally stable expression. Serial dilution was then performed to generate a standard curve with concentrations at 1x (undiluted), 10x, 100x and 1000x. The RT-qPCR reactions were performed using a standard time-temperature protocol to ensure optimal amplification and specificity. The protocol began with an initial denaturation step at 95 °C for 2 min to fully denature the DNA and activate the polymerase. This was followed by 45 cycles of denaturation at 95 °C for 5 sec, annealing at a temperature of 60 °C to 66 °C (depending on the primer used) for 30 s and extension at 72 °C for 30 sec to allow efficient extension of the primers. Following the amplification cycles, a melting curve analysis was performed to assess the specificity of the amplified products. The melting step involved a gradual increase in temperature from 70 °C to 95 °C, allowing the detection of specific amplicons and the identification of any non-specific products or primer dimers. The efficiency of the primer pairs ranged from 90.1 % to 109.6 %, falling within the acceptable range for RT-qPCR. Melt curve analysis confirmed the amplification of single, specific amplicons for all primer sets.

### Data analysis

Relative expression levels of analysed apple allergen isoforms were calculated using an efficiency-corrected ΔΔCt method (14). The data were presented as fold change relative to samples from the Slovak Republic, which served as the calibrator. Actin was used as the reference gene and for each gene isoform, relative expression values were derived from 3 technical replicates.

Statistical analyses were performed in R using the RStudio environment. Differences in gene expression among countries were evaluated using one-way analysis of variance (ANOVA). When significant effects were detected, Tukey's honestly significant difference (HSD) test was applied for post-hoc pairwise comparisons. Results are presented as mean ± standard deviation (SD). Statistical significance was accepted at $p<0.01$. Graphical visualizations were generated using R.

## Results

Here, apple fruits from different European countries were analysed for differences in the expression levels of individual isoforms of genes of apple allergens. Samples from Slovakia were used as a calibrator, with their expression values normalised to 1. Amplification of *Mal d 1.08* was not successful in Granny Smith and *Mal d 2.02* could not be amplified in either cultivar. Fold change values for each isoform were visualized using map-based representations generated in Microsoft Excel, enabling comparative analysis of spatial patterns for both 'Golden Delicious' and 'Granny Smith'. The corresponding graphs are provided in the Supplementary Fig. 1 and 2.

### Variability of transcription levels of apple allergens in Granny Smith

Expression levels of *Mal d 1* isoforms in 'Granny Smith' apples exhibited pronounced regional variability. Among *Mal d 1* isoforms, *Mal d 1.02* emerged as the most biologically relevant and variable isoform (Fig. 1). The highest expression was observed in a sample from Croatia (5.58 ± 0.23-fold). In contrast, *Mal d 1.01* showed minimal variation, with expression levels close to the calibrator across all regions.

Most *Mal d 1.03* isoforms (e.g., *1.03A*, *1.03C* and *1.03D*) displayed consistently low expression below the calibrator level. The Croatian sample showed particularly low expression, with *Mal d 1.03A* reaching 0.0025-fold relative to Slovakia ($p<0.01$). Similarly, *Mal d 1.06B* and *Mal d 1.06C* were expressed at low levels across all regions, whereas *Mal d 1.06A* exhibited moderate upregulation, with the highest expression observed in the Croatian sample (3.57 ± 0.43-fold, $p<0.01$). *Mal d 1.07* showed the highest expression in Slovakia and the lowest in Croatia (0.11 ± 0.04-fold, $p<0.01$). Tukey's test confirmed statistically significant differences between Slovakia and samples from other countries ($p<0.01$).

For *Mal d 1.11* isoforms, the highest expression levels were observed in the Croatian sample, with *Mal d 1.11B* reaching 8.00 ± 2.24-fold ($p<0.01$) and *Mal d 1.11A* reaching 5.05 ± 0.87-fold ($p<0.01$) relative to the calibrator. These expression levels were significantly higher compared to those in other samples. In contrast, *Mal d 1.13* isoforms (*1.13A*, *1.13B* and *1.13D*) exhibited comparatively low variability, with the highest expression consistently observed in Slovakia and the lowest in Austria, with significant differences between samples from Slovakia and Austria.

*Mal d 1* isoforms in Granny Smith apples showed marked differences in fold change values (Fig. 2). Higher expression and greater variability were observed for *Mal d 1.02*, *Mal d 1.06A*, *Mal d 1.11A* and *Mal d 1.11B*, while most remaining isoforms exhibited low and stable expression levels. Occasional outliers indicate isoform-specific upregulation in individual samples.

Regarding *Mal d 2* isoforms (Fig. 3), *Mal d 2.01* showed the highest expression in the Austrian sample (14.83 ± 2.50-fold, $p<0.01$). In contrast, *Mal d 2.03* showed a divergent trend, with decreased expression in Austria (0.29 ± 0.07-fold) and Serbia (0.23 ± 0.16-fold) compared to Slovakia, although differences for *Mal d 2.03* were not statistically significant.

Expression levels of *Mal d 3.01* in Granny Smith were generally upregulated relative to the calibrator (Fig. 4). *Mal d 3.01* showed the highest expression in samples from Serbia, reaching approximately a 20-fold increase ($p<0.01$). A similar expression pattern was observed for *Mal d 3.02*, with elevated levels across all regions and the highest levels recorded in Serbia (5.49 ± 0.70-fold, $p<0.01$), followed by Austria (2.27 ± 0.08-fold, $p<0.1$).

Expression levels of *Mal d 4* isoforms in Granny Smith varied among regions (Fig. 5). One-way ANOVA results for all three isoforms, indicate strong statistical significance, suggesting at least one region

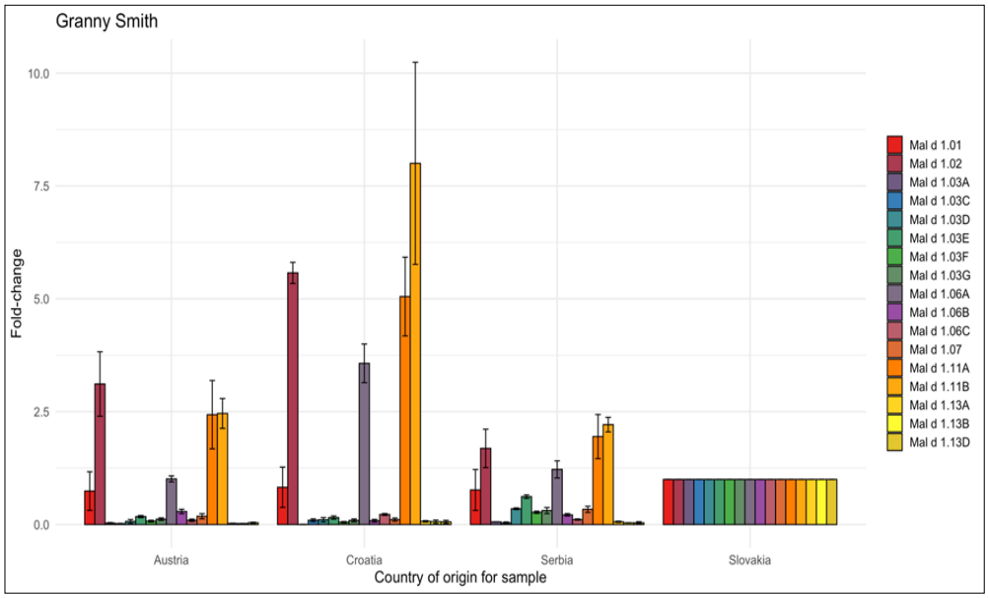


**Fig. 1.** Detected mRNA transcript levels of *Mal d 1* isoforms (*Mal d 1.01–Mal d 1.13D*) in Granny Smith variety apple fruits from different retail chains of European countries. Gene expression was normalised to the reference gene and calculated relative to the calibrator sample (Slovakia), which was set to 1. Error bars represent the standard deviation of biological replicates. Observed fold-change values ranged from ~0.05 to ~10.2, depending on the isoform and country of origin.

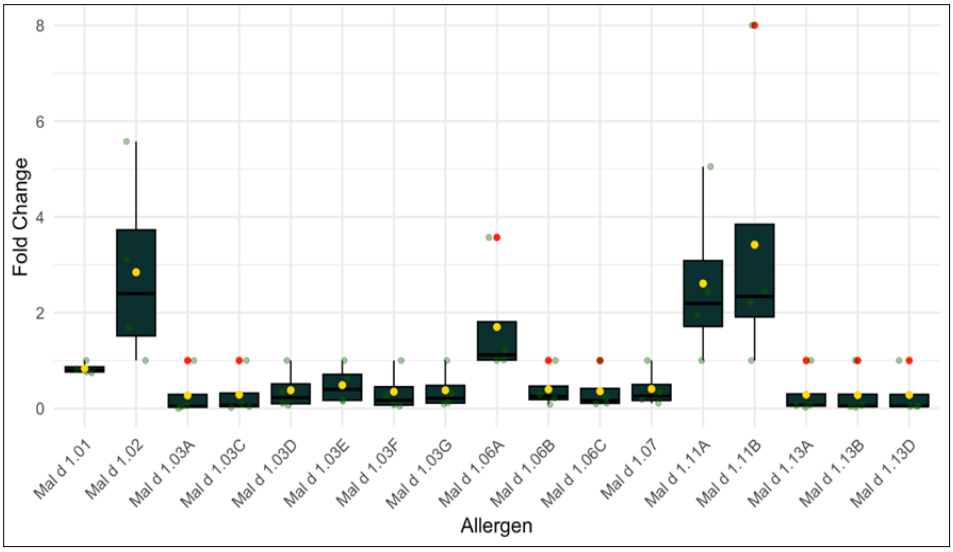


**Fig. 2.** Distribution of fold change by allergen (*Mal d 1*) in Granny Smith. The boxplot illustrates the fold change in expression levels across multiple apple allergen isoforms, belonging to *the Mal d 1* family. Observed fold-change values ranged approximately from 0.1 to 8.0 across isoforms. The x-axis represents individual allergen isoforms, while the y-axis shows the magnitude of fold change. Each dot reflects an individual data point (sample- green dots), with boxplots summarising the distribution (mean- yellow dot and outlier- red dot).

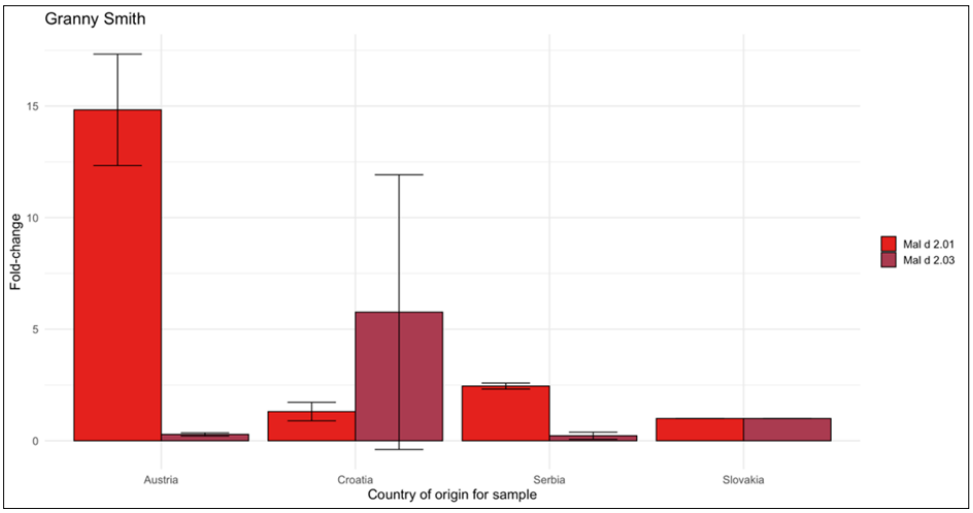


**Fig. 3.** Detected mRNA transcript levels of *Mal d 2* isoforms (*Mal d 2.01* and *Mal d 2.03*) in Granny Smith variety apple fruits from different retail chains of European countries. Gene expression levels were normalised to the reference gene and calculated relative to the calibrator sample (Slovakia), which was set to 1. Observed fold-change values ranged from ~0.1 to ~17.5, depending on the isoform and country of origin.

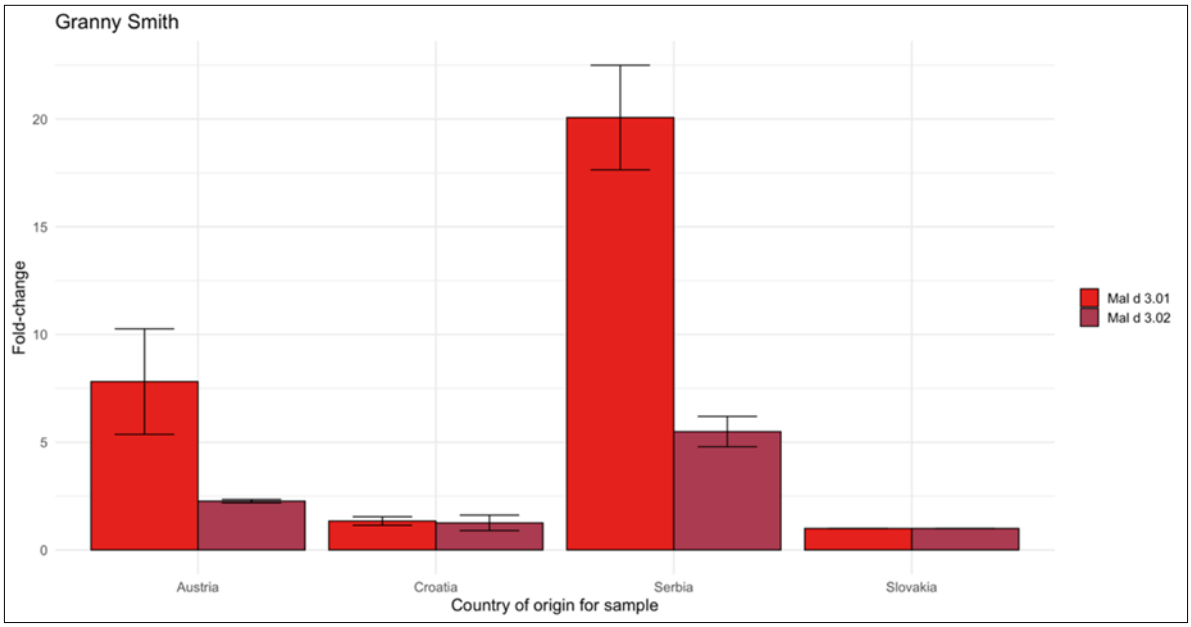


**Fig. 4.** Detected mRNA transcript levels of *Mal d 3* isoforms (*Mal d 3.01* and *Mal d 3.02*) in Granny Smith variety apple fruits from different retail chains of European countries. Gene expression levels were normalised to the reference gene and calculated relative to the calibrator sample (Slovakia), which was set to 1. Observed fold-change values ranged from ~1.0 to ~22.5, depending on the isoform and country of origin.

had significantly different expression compared to others. Notably, the Croatian sample showed the highest expression levels of *Mal d 4.01*, with a significant 280-fold increase compared to the calibrator, representing the highest fold change observed among all analysed isoforms in Granny Smith apples, highlighting a particularly strong upregulation. Serbia and Austria also showed elevated expression (74.29 ± 23.76-fold and 83.03 ± 13.34-fold respectively), although these were not significantly different from that observed in Slovakia. For *Mal d 4.02* and *Mal d 4.03*, the highest expression levels were detected in Serbia and Austria, with significantly higher expression compared to Slovakia ($p$<0.01).

Out of *Mal d 2*, *Mal d 3* and *Mal d 4*, *Mal d 4.01* stood out as the most variable isoform within this group, showing a broad range of fold change values across samples. Remarkably, *Mal d 4.01* exhibited an extreme outlier, with an approximate 280-fold increase in expression in the sample from Croatia. This suggests a highly inducible response under specific conditions (Fig. 6). These findings highlight the complex expression patterns of *Mal d* isoforms in 'Granny Smith' apples across different regions, highlighting the importance of considering regional variations in allergen expression.

## Variability of transcription levels of apple allergens in Golden Delicious

Expression levels of allergen isoforms in Golden Delicious apples differed among regions. Among *Mal d 1* isoforms (Fig. 7.), *Mal d 1.02* exhibited the highest expression in the sample from Germany (27.66 ± 5.77-fold, $p$<0.01). All other samples showed higher expression levels above the calibrator, with samples from Germany, Slovenia and Italy generally exhibiting higher fold changes than the calibrator. *Mal d 1* showed lower variability with the highest expression

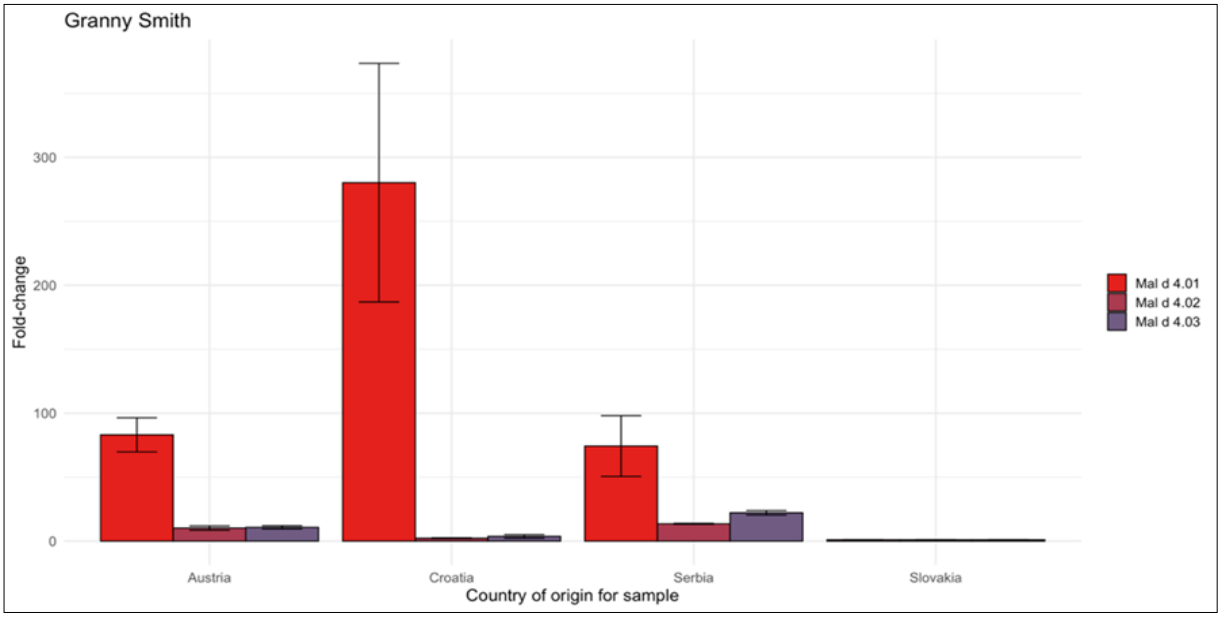


**Fig. 5.** Detected mRNA transcript levels of *Mal d 4* isoforms (*Mal d 4.01, Mal d 4.02* and *Mal d 4.03*) in Granny Smith variety apple fruits from different retail chains of European countries. Gene expression levels were normalised to the reference gene and calculated relative to the calibrator sample (Slovakia), which was set to 1. Observed fold-change values ranged from ~1.0 to ~360, depending on the isoform and country of origin.

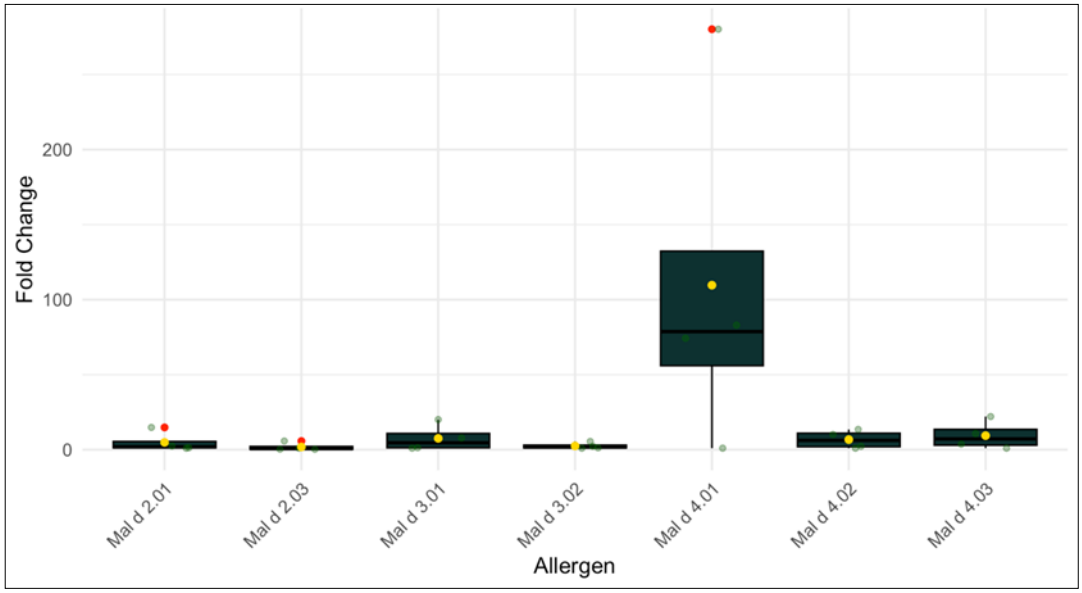


**Fig. 6.** Distribution of fold change by allergen (*Mal d 2, Mal d 3* and *Mal d 4*) in Granny Smith. The boxplot illustrates the fold change in expression levels across multiple apple allergen isoforms, belonging to the *Mal d 2, Mal d 3* and *Mal d 4* families. Observed fold-change values ranged approximately from ~0.5 to ~260 across allergen families. The x-axis represents individual allergen isoforms, while the y-axis shows the magnitude of fold change. Each dot reflects an individual data point (sample- green dots), with boxplots summarising the distribution (mean- yellow dot and outlier- red dot).

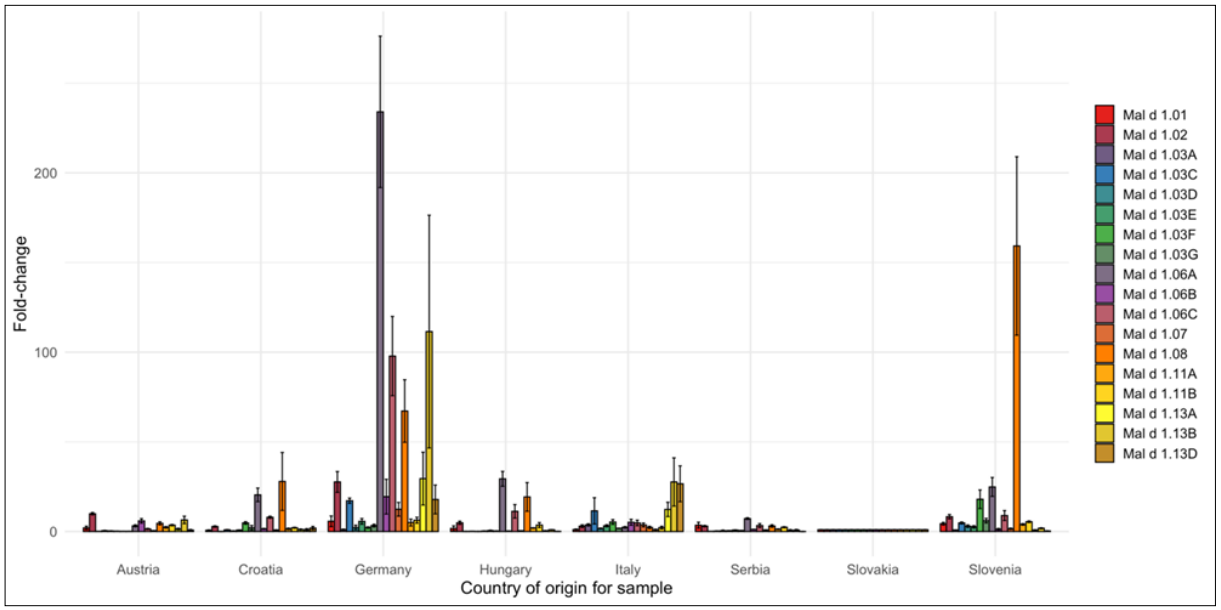


**Fig. 7.** Detected mRNA transcript levels of *Mal d 1* isoforms in Golden Delicious variety apple fruits from different retail chains of European countries. Gene expression levels were normalised to the reference gene and calculated relative to the calibrator sample (Slovakia), which was set to 1. Observed fold-change values ranged from ~0.1 to ~260, depending on the isoform and country of origin.

detected in the sample from Germany (5.71 ± 2.97) and Slovenia (4.36 ± 0.74).

*Mal d 1.03A* showed the greatest difference between Slovakia and Italy (3.71 ± 0.61, $p<0.01$), while samples from Croatia, Hungary, Austria and Serbia exhibited significantly lower expression compared to Slovakia. *Mal d 1.03C* showed the highest expression in samples from Germany (17.19 ± 1.46, $p<0.01$) and Italy (11.52 ± 7.33, $p<0.01$). The results for *Mal d 1.03D* showed that the highest fold change (2.99 ± 0.68-fold, $p<0.01$) compared to the calibrator was observed in the sample from Slovenia. The sample from Slovenia also showed the highest expressions for *Mal d 1.03F* (18.00 ± 5.18-fold, $p<0.01$) and *Mal d 1.03G* (6.07 ± 1.27-fold, $p<0.01$).

*Mal d 1.06A* exhibited extreme upregulation in samples from Germany (234.02 ± 42.22-fold, $p<0.01$). *Mal d 1.06B* and *Mal d 1.06C* were also significantly upregulated in German samples (19.45 ± 9.70-fold and 97.84 ± 22.09-fold respectively, $p<0.01$), whereas the lowest expression was detected in Slovakia (*Mal d 1.06A* and *Mal d 1.06C*) and Hungary (*Mal d 1.06B*). Differences between Slovakia and Hungary were not statistically significant.

For *Mal d 1.07*, the highest expression was observed in the sample from Germany (12.42 ± 3.81-fold, $p<0.01$), whereas most other samples showed expression below the calibrator, with the lowest level detected in Hungary (0.45 ± 0.03-fold, statistically not significant). In contrast, *Mal d 1.08* exhibited strong region-specific upregulation, with the highest expression in the sample from Slovenia (159.26 ± 49.74-fold, $p<0.01$) and the lowest in Slovakia.

For both *Mal d 1.11A* and *Mal d 1.11B*, the sample from Germany exhibited the highest fold changes with values of 5.02 ± 1.91-fold ($p<0.01$) and 6.33 ± 1.64-fold ($p<0.01$) respectively. The lowest expression levels were consistently found in the sample from

Slovakia (calibrator). The expression levels of *Mal d 1.13* varied significantly between countries, with the highest levels observed in Germany (29.50 ± 14.73, $p<0.001$ for *Mal d 1.13A*; 111.49 ± 64.89, $p<0.001$ for *Mal d 1.13B* and 17.89 ± 8.03, $p<0.001$ for *Mal d 1.13D*). In contrast, Serbia, Hungary and Slovenia exhibited markedly lower expression levels, with Serbia showing downregulated expression for *Mal d 1.13D* (0.03 ± 0.01, $p<0.001$).

Overall, while most *Mal d 1* allergen isoforms showed relatively stable expression, *Mal d 1.06A* (>220-fold) and *Mal d 1.08* (>150-fold) displayed extreme fold-change outliers, indicating strong isoform-specific inducibility and sample-dependent regulation (Fig. 8)

The most significant upregulation of the *Mal d 2.01* (Fig. 9) in Golden Delicious was observed in the sample from Italy (32.34 ± 12.65, $p<0.1$), followed by Germany (19.10 ± 8.82, $p<0.01$). In contrast, samples from Serbia, Hungary and Slovenia showed marked downregulation, with the lowest expression detected in Serbia (0.02 ± 0.01-fold). The *Mal d 2.03* gene expression values varied, with the Slovenian sample exhibiting extreme upregulation (117.14 ± 59.62-fold, $p<0.01$ compared to the calibrator). Additionally, an upregulation was observed in samples from Italy (28.24 ± 14.76), Croatia (21.95 ± 10.07) and Hungary (11.45 ± 8.51), indicating substantial region-dependent variation in *Mal d 2.03* transcript levels.

*Mal d 3.01* (Fig. 10) was highest in the sample from Slovenia (7.96 ± 0.94-fold, $p<0.01$), followed by Hungary (4.58 ± 0.44-fold, $p<0.01$) and Croatia (3.48 ± 0.62-fold, $p<0.01$), while the lowest expression was detected in Italy (0.46 ± 0.22-fold, $p<0.01$). Similarly, *Mal d 3.02* showed upregulation in the sample from Hungary (14.38 ± 0.48-fold, $p<0.01$), Slovenia (12.77 ± 1.28-fold, $p<0.01$) and Croatia (10.55 ± 2.56-fold, $p<0.01$). Downregulation was observed only in the sample from Italy (1.44 ± 0.16, statistically not significant).

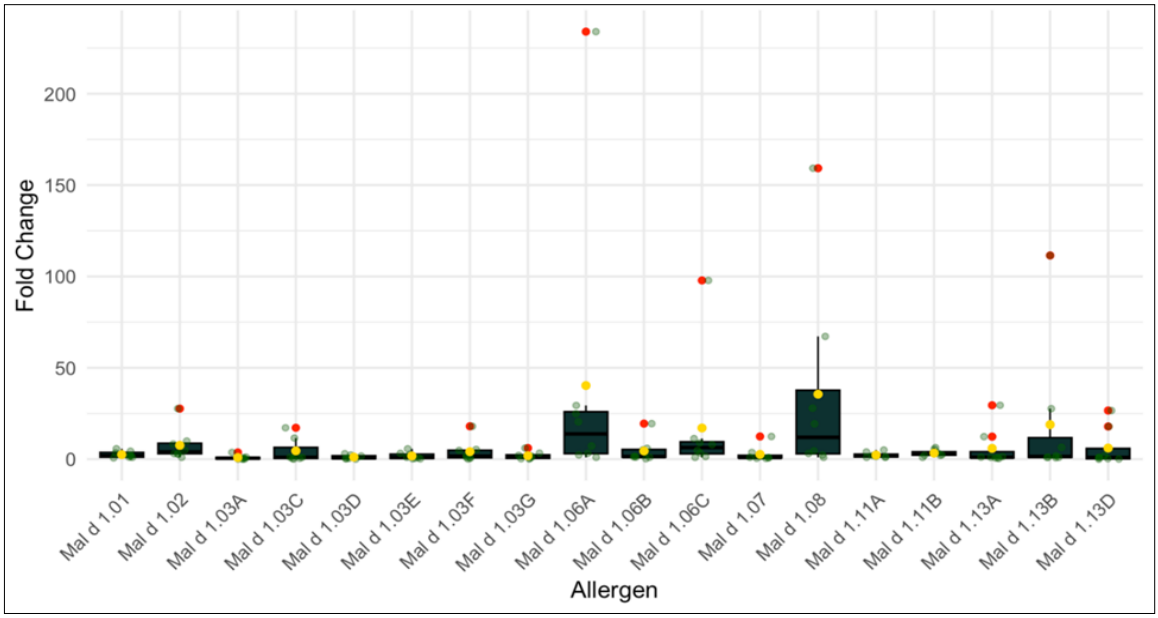


**Fig. 8.** The boxplot illustrates the fold change in expression levels across multiple apple allergen isoforms, belonging to the *Mal d 1* family. Observed fold-change values ranged approximately from ~0.1 to ~230 across isoforms. The x-axis represents individual allergen isoforms, while the y-axis shows the magnitude of fold change. Each dot reflects an individual data point (sample- green dots), with boxplots summarising the distribution (mean- yellow dot and outlier- red dot).

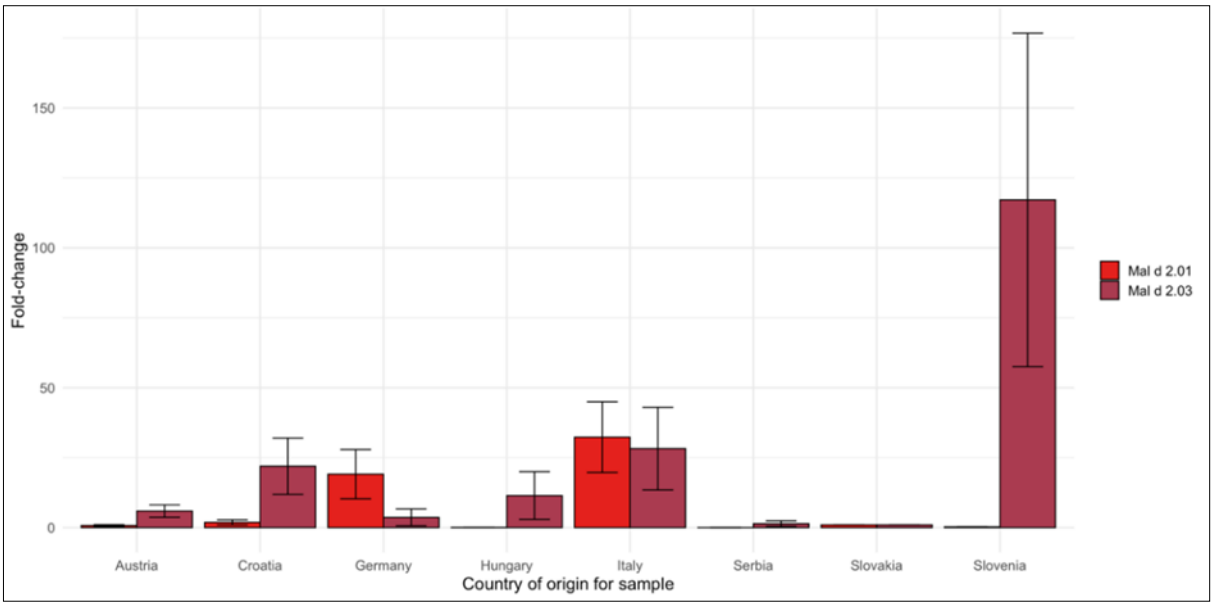


**Fig. 9.** Detected mRNA transcript levels of *Mal d 2* isoforms (*Mal d 2.01* and *Mal d 2.03*) in Golden Delicious variety apple fruits from different retail chains of European countries. Gene expression levels were normalised to the reference gene and calculated relative to the calibrator sample (Slovakia), which was set to 1. Observed fold-change values ranged from ~0.5 to ~175, depending on the isoform and country of origin.

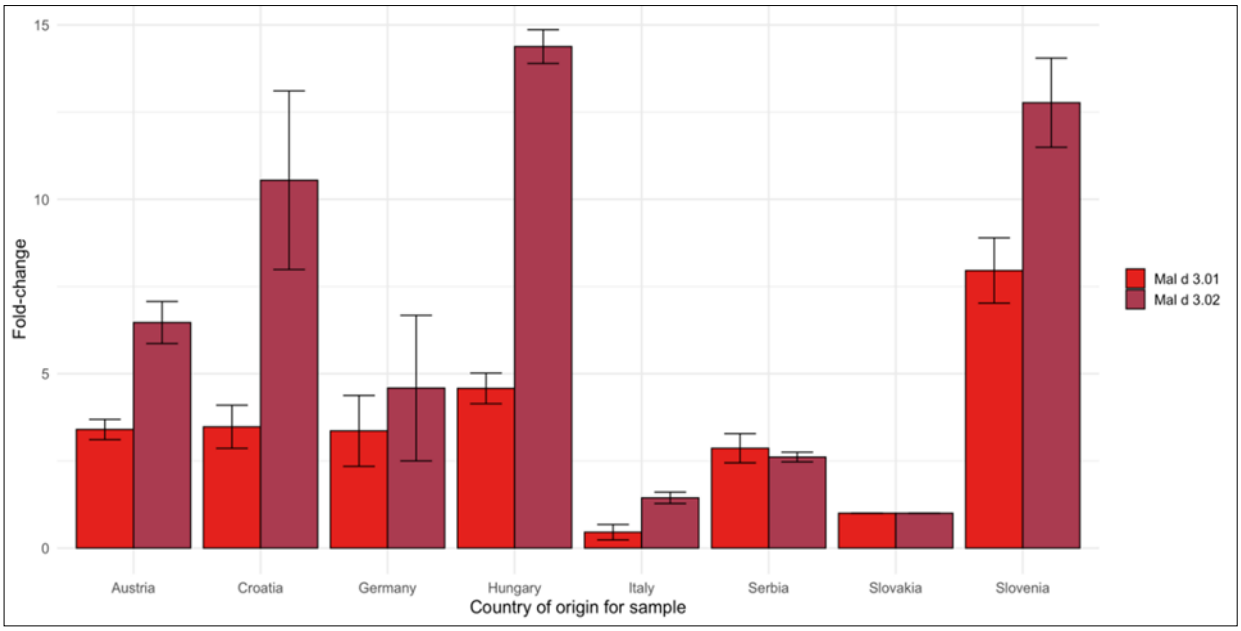


**Fig. 10.** Detected mRNA transcript levels of *Mal d 3* isoforms (*Mal d 3.01* and *Mal d 3.02*) in Golden Delicious variety apple fruits from different retail chains of European countries. Gene expression levels were normalised to the reference gene and calculated relative to the calibrator sample (Slovakia), which was set to 1. Observed fold-change values ranged from ~0.5 to ~15.0, depending on the isoform and country of origin.

For *Mal d 4* isoforms (Fig. 11), *Mal d 4.01*, showed statistically significant upregulation in samples from Hungary (6.81 ± 1.49-fold, $p<0.01$) and Slovenia (5.97 ± 1.07-fold, $p<0.01$), while the lowest expression was detected in Italy (0.11 ± 0.01-fold, $p<0.01$). It was observed that *Mal d 4.02* and *Mal d 4.03* exhibited less variation than *Mal d 4.01*. Upregulation of *Mal d 4.02* was observed in samples from Austria (2.06 ± 0.15-fold, $p<0.01$), while samples from Serbia (0.71 ± 0.08-fold, $p<0.01$) and Italy (0.90 ± 0.08-fold, $p<0.01$) were downregulated compared to Slovakia. For *Mal d 4.03*, no statistically significant differences were detected among regions, with fold change values ranging from 0.54 to 1.58.

Isoform *Mal d 2.03* shows the highest variability with a large interquartile range and an extreme outlier of almost a 120-fold change. This indicates that this isoform is highly variable between samples and can be significantly upregulated under certain conditions. Isoform *Mal d 2.01* also shows moderate variability, with a few outliers reaching ~30-fold change, although the median is relatively lower. This suggests that some samples show increased expression. The *Mal d 4* isoforms are low or unresponsive with low fold change values clustered below 5 (Fig. 12).

## Discussion

The present study provides a comprehensive transcriptional analysis of four clinically relevant *Mal d* allergens (*Mal d 1, 2, 3* and *4*) in 2 apple cultivars, Golden Delicious and Granny Smith, sourced from eight European countries. The expression profiles demonstrate genotype- and environment-dependent modulation of allergenic proteins, thus providing substantial support for the hypothesis that both intrinsic genetic makeup and extrinsic environmental conditions jointly shape the allergenicity landscape of apples. Importantly, the

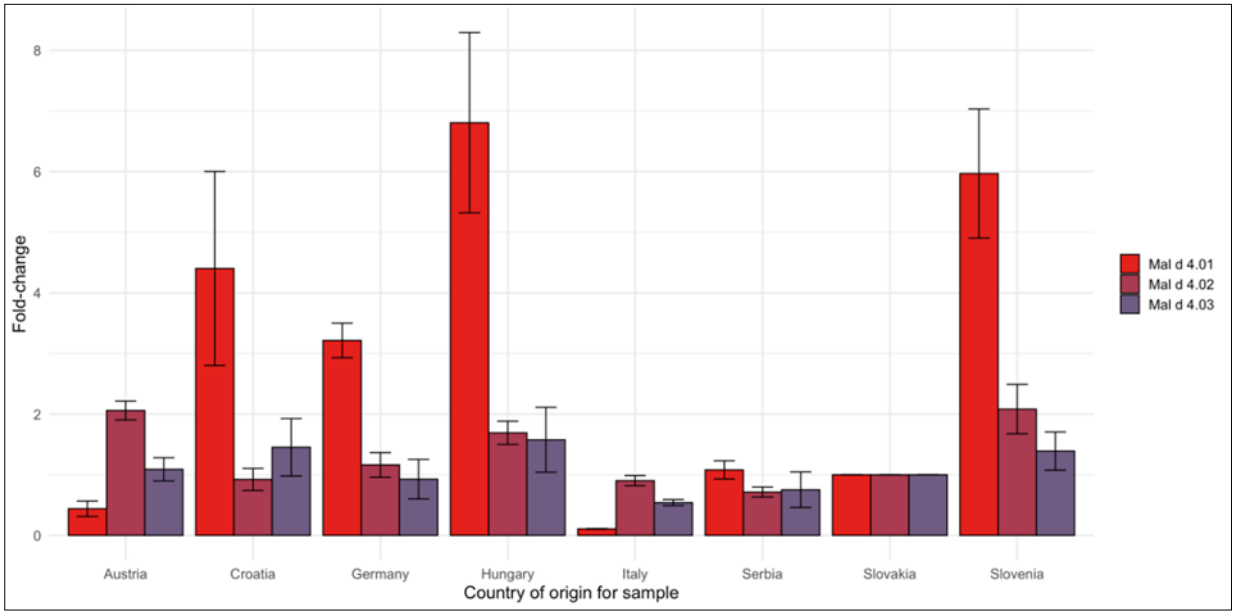


**Fig. 11.** Detected mRNA transcript levels of *Mal d 4* isoforms (*Mal d 4.01, Mal d 4.02* and *Mal d 4.03*) in Golden Delicious variety apple fruits from different retail chains of European countries. Gene expression levels were normalised to the reference gene and calculated relative to the calibrator sample (Slovakia), which was set to 1. Observed fold-change values ranged from ~0.1 to ~8.5, depending on the isoform and country of origin.

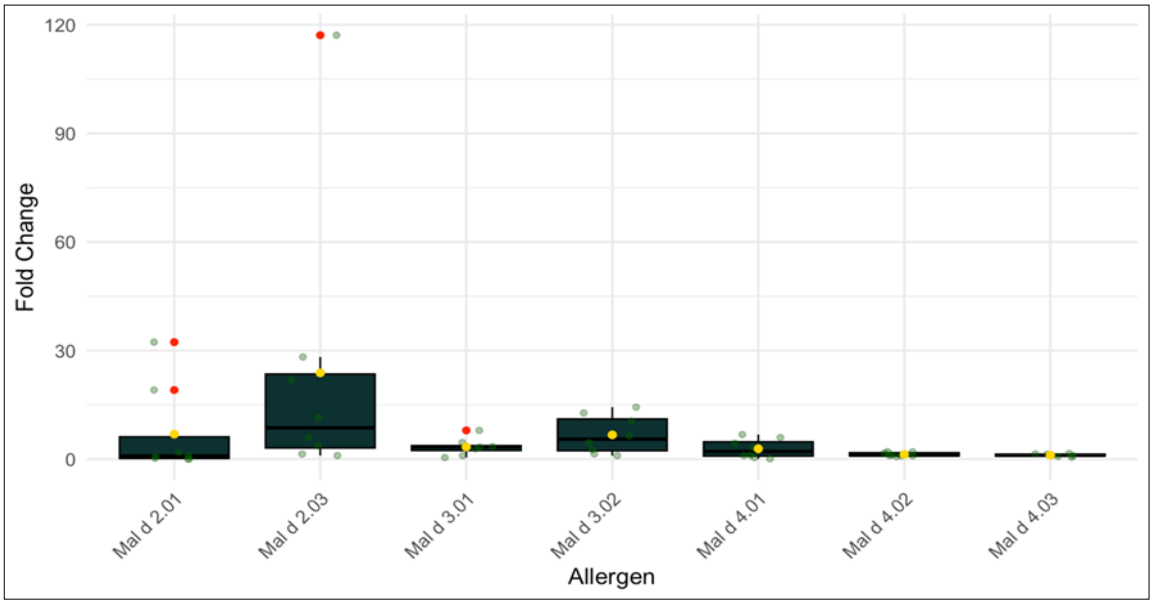


**Fig. 12.** The boxplot illustrates the fold change in expression levels across multiple apple allergen isoforms, belonging to the *Mal d 2, Mal d 3* and *Mal d 4* families. Observed fold-change values ranged approximately from ~0.5 to ~120 across allergen families. The x-axis represents individual allergen isoforms, while the y-axis shows the magnitude of fold change. Each dot reflects an individual data point (sample- green dots), with boxplots summarising the distribution (mean- yellow dot and outlier- red dot).

observed variability reflects differences at the transcript level and does not necessarily imply proportional changes in allergenic protein abundance, highlighting the need for cautious interpretation of gene expression data in a clinical context. These findings have substantial implications for both personalised allergen risk assessment and targeted hypoallergenic breeding programs.

Both apple varieties analysed in this study are reported in the literature as having high levels of *Mal d 1* protein content (15, 16). The transcription patterns of *Mal d 1* isoforms presented here reveal a new layer of complexity in allergen regulation. While previous studies have recorded differential expression across tissues and genotypes (5, 13), our data confirm the predominance of *Mal d 1.02* among the *Mal d 1.01–1.03* subgroup, corroborating findings of a previous study (17). Although not the most highly expressed isoform overall, *Mal d 1.02* remains the most abundant within this subgroup. Such variability suggests that allergen gene expression is highly plastic and responsive to environmental cues, including climatic conditions, pathogen pressure and post-harvest handling. Furthermore, our data provide direct molecular evidence that environmental and post-harvest factors profoundly modulate allergen gene expression.

The *Mal d 1.03* gene expression was reported previously as tissue-specific with higher values in peel than in pulp of all analysed varieties (18). The extreme upregulation observed for *Mal d 1.06A* likely reflects the substantial transcriptional plasticity of this allergen, which is likely influenced by environmental and physiological factors such as abiotic or biotic stress exposure, post-harvest storage conditions and differences in fruit maturity stage. However, it should be noted that increased transcript levels may not directly translate into increased protein accumulation. These findings underscore that total allergen expression may not reliably predict immunogenicity, pointing to the critical importance of isoform-specific and environmentally influenced expression patterns.

As supported by recent proteomic and immunoassay data, even lowly expressed isoforms, such as *Mal d 1.03* and *Mal d 1.06*, can have a disproportionate impact on the overall allergenic potential of apples (19). Their enrichment in hyperallergenic cultivars and absence from hypoallergenic ones suggests that the relative proportions of individual isoforms may be more relevant than their absolute quantities in determining allergenicity (9, 13). Moreover, the observed intra-cultivar variability indicates that even within the same cultivar, there may be distinct allergen profiles depending on extrinsic factors- an aspect often overlooked in food safety risk assessments. Some varieties, such as Elise, maintained relatively stable *Mal d 1* expression throughout the storage period and may be better tolerated by allergic patients (20). In addition, three independent studies have shown that older apple varieties tend to be less allergenic than modern varieties, suggesting a potential loss of hypoallergenic traits through breeding for other agronomic traits (13).

Levels of allergens such as *Mal d 1* are influenced not only by genotype and region, but also by post-harvest factors such as storage duration and conditions. This is evidenced by the fact that the levels of *Mal d 1* in Golden Delicious apples after storage protein levels were up to nine times greater than those in Pinova apples. This clearly demonstrates how handling and storage practices have the capacity to significantly impact allergen concentrations (20).

The only apple protein demonstrated to retain allergenic properties after technological processing is *Mal d 3*, a protein known for its exceptional stability. As a major allergen, especially in patients with non-pollen-related fruit allergies, it is particularly prevalent in Mediterranean regions (1). Patients frequently exhibit cross-reactivity between apple *Mal d 3* and peach *Pru p 3* allergens (21). It is well established that the concentration of *Mal d 3* in the apple pulp is extremely low (22). Immunoblotting analyses demonstrated that 68 % of tested patients (13 out of 19) exhibited IgE reactivity to *Mal d 3*, confirming its clinical relevance (23). In contrast to the more extensively researched *Mal d 1*, fewer studies have addressed *Mal d 3*. A previous study showed that cultivation method significantly influenced *Mal d 3.01* gene expression only in the skin, not in the flesh, of modern apple cultivars (1). Both organic and intensive farming methods led to increased expression in the peel. Additionally, previous studies also reported that pathogen infection led to the downregulation of *Mal d 3*, a pathogenesis-related (PR) protein (24).

Regarding cultivar differences, it was reported that Granny Smith, Golden Delicious and Fuji cultivars contain higher *Mal d 3* protein levels in the fruit cortex (17, 12 and 9 µg/g, respectively) compared to other cultivars, where levels range between 1 and 3 µg/g. Moreover, nsLTP proteins such as *Mal d 3* are predominantly localised in the peel, where their concentration can exceed that of the flesh by 10- to 30-fold, particularly in cultivars like Granny Smith (25).

From a practical standpoint, our results emphasise the importance of considering geographic origin when assessing food allergenicity. Variability in allergen expression may influence the clinical severity of reactions in LTP-sensitised individuals and should be considered when developing guidelines for safe consumption and food labelling.

As indicated by the literature, *Mal d 2* and *Mal d 4* are less clinically important than *Mal d 1* and *Mal d 3*. However, they are also associated with hyperreactivity to apples (1). The *Mal d 2* allergen is one of the major protein components of ripe apples (26). However, molecular studies on the apple allergen *Mal d 2* are still limited. In a previous study, traditional (older) cultivars showed significantly higher transcript levels of *Mal d 2.01* compared to modern cultivars (1). Furthermore, the farming practices that were employed had a significant impact on the expression of *Mal d 2.01*: organic farming was associated with lower transcript levels in the flesh. The results of this study indicate that both genotype and farming practices play a role in regulating *Mal d 2.01* expression. The present study confirms this finding, as apples from different countries exhibited varying expression levels. On a genotype basis, the results obtained demonstrate variability at the cultivar level. For example, *Mal d 2.01* was found to be upregulated in Granny Smith, whereas the same isoform in Golden Delicious was slightly downregulated relative to the calibrator.

As demonstrated by a previous study, the *Mal d 4* exhibited minimal variability in expression across various varieties. A relatively constant gene expression pattern was detected for *Mal d 4* (9). This stability suggests a more limited contribution of *Mal d 4* to overall allergen variability compared to *Mal d 1* and *Mal d 3*.

Taken together, these results show considerable variability in the expression of these isoforms even within a single cultivar as they were grown, harvested and stored in different countries and under different conditions. Beyond genetic background, our findings confirm that geographical origin, agricultural practices and post-harvest storage conditions significantly influence allergen gene expression - supporting a multifactorial control model for isoform-level variability in apple allergens.

Limitations of the present study include the relatively small number of biological replicates per country and the lack of detailed environmental metadata, such as storage duration, temperature and agricultural inputs. These factors may contribute to the observed variability and should be addressed in future studies using controlled growth conditions combined with parallel proteomic and immunological analyses to link transcriptomic variability with clinically relevant allergen levels.

This work highlights the complex interplay between genetics and environmental factors in shaping the allergenic potential of apples. The observed high expression levels of *Mal d* isoforms in certain cultivars and regions emphasise the clear need for region-specific allergen profiling. The identification of less allergenic or hypoallergenic cultivars could be of great value for dietary recommendations, especially for individuals with apple allergy. In conclusion, our study underscores the intricate and multifactorial nature of allergen expression in apples.

## Conclusion

In the present study, only minor variation was observed in Golden Delicious, with fold changes ranging from 0.11 to 6.82-relative to the calibrator. However, in Granny Smith, the *Mal d 4.01* exhibited substantial up-regulation, reaching approximately 280-fold. Furthermore, the fold changes demonstrated greater variability compared to those observed in Golden Delicious. Clarifying all the aspects and specificities of *Mal d 1* expression will help in the strategy of a personalized approach in allergy management in the future.

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## Authors' contributions

LK carried out the software analyses, contributed to the investigation, participated in the writing of the original draft and prepared the visualisations. AJ carried out the software analyses, contributed to the investigation and participated in the writing of the original draft. LU contributed to the validation, formal analysis and investigation. LZ contributed to the validation. JŽ conceived and designed the study, provided resources, performed data curation, contributed to validation and participated in the writing of the original draft, review and editing, as well as supervision. All authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest:** Authors do not have any conflict of interests to declare.

**Ethical issues:** None

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