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Isoform-specific expression patterns of the *MdPR10* gene in apple cultivars with Golden Delicious pedigree

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Abstract
The Mal d 1 protein, a member of the PR-10 protein family encoded by the *MdPR10* gene in *Malus domestica* Borkh., responds to biotic and abiotic stress stimuli. It may accumulate in plant tissues and is known to trigger allergic reactions in humans. Empirical observations show that the same individual may exhibit varying allergic responses depending on the apple cultivar consumed. The underlying cause is still not fully understood, although several hypotheses are being considered, and it is likely to be multifactorial.
In this study, the expression of eight *MdPR10* isoforms (*1.01, 1.02, 1.03, 1.06, 1.07, 1.08, 1.11*, and *1.13*) was measured across twelve *M. domestica* cultivars, all sharing Golden Delicious as a maternal pedigree. Isoforms *1.01* and *1.02* were consistently highly expressed with low variability, while isoforms *1.03, 1.06, 1.07*, and *1.08* showed high expression variability among cultivars. Freyberg exhibited the closest expression profile to Golden Delicious, followed by Sirius and Shalimar; in contrast, Mutsu, Spencer, and Delbarestivale showed the greatest divergence.

Keywords: *Malus domestica* Borkh., variability, pathogen-related protein.

Introduction

Malus domestica Borkh. (family Rosaceae) belongs to the genus *Malus*, which includes 8 to 78 species due to frequent hybridization [1]. Today, China is the leading global producer of apples with more than half of the worldwide production of more than 49-million tons [2]. Apple cultivation is most common in temperate regions, with over 10,000 known cultivars developed for various purposes [3]. One of the most commercially important cultivars is Golden Delicious (GD), an old variety developed in the 19th century in the USA [4]. Its entire genome has been sequenced, making GD a suitable reference cultivar for molecular analyses. From an allergenic perspective, it is considered hyperallergenic due to its high content of the Mal d 1 protein in the fruit [5].

The major apple allergen, Mal d 1 (17.5 kDa), belongs to the PR-10 protein family. Expression of PR-10 proteins is triggered by various stress factors and is part of the plant's defense response [6, 7]. The transcription level of Mal d 1 depends on the cultivar, as well as biotic and abiotic factors, storage conditions, and duration [8]. Cultivar selection significantly influences the chemical composition of apples, as breeding has targeted different uses. In recent years, the chemical composition of apple cultivars has been recognized as one of the key factors influencing their allergenic potential. Higher polyphenol content has been associated with reduced allergenicity [9]. These compounds are typically more abundant in red-skinned and traditional cultivars compared to green or modern bred varieties.

Due to their bioactivity, PR proteins can trigger immune responses in sensitized individuals upon contact with mucosal surfaces. Identifying these proteins is clinically important, especially for hypersensitive patients [10]. Not only major allergens but also their isoforms and structurally similar IgE-binding proteins are relevant, as they may induce cross-reactive immune responses. Isoforms of PR proteins share 69.8 – 99.4% sequence identity, yet their 3D structures differ only slightly [8]. Despite this, some isoforms show marked differences in allergenicity. For example, the most common birch pollen isoform, Bet v 1.0101 (35% of all Bet v 1), induces a strong Th2-mediated IgE response [11, 12]. In contrast, Bet v 1.0102 (10%) elicits minimal IgE production, despite 95.6% sequence similarity [13].

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Clinical observations suggest that different cultivars may vary in allergenicity, an aspect not yet well understood but important for future breeding strategies. As mentioned above, it is already known that Mal d 1 protein content widely varies among apple cultivars [14]. This assumption forms the basis of the hypothesis that the natural expression dynamics of *MdPR10* gene isoforms in apple cultivars are also highly variable and cultivar-dependent, even among cultivars that share the same maternal parent and may relate to level of allergenicity.

Material and Method

Biological material

The biological material consisted of fruits from 12 cultivars of *Malus domestica* Borkh. (listed in Table 1), all harvested on the same day at the physiological maturity stage and a cultivar of Golden delicious as a reference variety. Immediately after collection, the fruits were processed and frozen at −20 °C until total mRNA extraction. A selection criterion for the cultivars was a shared maternal parentage with the cultivar Golden Delicious (GD).

Table 1. List of varieties of biological material with shared maternal parentage of Golden delicious.

Variety	Parentage
Delor	Golden delicious x Lord Lambourne
Freyberg	Golden delicious x Cox's Orange Pippin
Heliodor	Golden delicious x Topaz
Mutsu	Golden delicious x Indo
Orion	Golden delicious x Otava
Rezistent Opal	Golden delicious x Topaz
Sirius	Golden delicious x Topaz
Angold	Golden delicious x Antonovka
Shalimar	Golden delicious x Topaz
Spencer	Golden delicious x McIntosh
Delbarestivale	Golden delicious x Stark Jonagrimes
Jantár	Golden delicious x Jonathan

RNA Isolation and cDNA Synthesis

Total mRNA was isolated using the Ribospin™ Seed/Fruit RNA isolation kit (GeneAll®), following a modified version of the original protocol. During the initial lysis step, the lysis buffer was supplemented with 10 µL of dithiothreitol (DTT) and 10 µL of polyvinylpyrrolidone (PVP), with a corresponding reduction of 20 µL in the volume of lysis buffer to maintain the total volume. RNA concentration and purity were determined spectrophotometrically using a NanoPhotometer® P-360 (Implen) at an absorbance ratio of A260/280, with purity values of 2.0 ± 0.1.

Total mRNA (150 ng) was reverse transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase® (Thermo Fisher Scientific), following the original protocol's instructions. Gene-specific primers targeting isoforms *MdPR10*: *1.01, 1.02, 1.03, 1.06, 1.07, 1.08, 1.11,* and *1.13* were used, as designed by Pagliarani et al. [15] (Table 2). The metabolic gene *MdActin* was used as the reference gene.

Table 2. List of used primers designed by Pagliarani et al. [15].

Gene	Forward primer	Reverse primer
Mdactin	CTATGTTCCCTGGTATTGCAGACC	GCCACAACCTTGTTTTTCATGC
MdPR10 1.01	GATTGAAGGAGATGCTTTGACA	GTAATGACTGATGCTCTTGATGG
MdPR10 1.02	GATTGAAGGAGATGCTTTGACA	TTGGTGTGGTAGTGGCTGATA
MdPR10 1.03	ATCTGAGTTCACCTCCGTCATT	ACTGCTTGTGGTGGAATCTTT
MdPR10 1.06	CTATAGCTATAGCTTGATTGAAGGG	TTCCAACCTTAACATGTTCTTCT
MdPR10 1.07	CAACTTTGTGTACCAGTACAGTGTC	TAGTGGCTGATGCTCTTGATAAC
MdPR10 1.08	TCTTCGGTGAAGGTAGCACAA	ACCCTTAGTGTGGTAGTGGCAT
MdPR10 1.11	GGAGGATGCATCTGTCATTTG	CCATGAGATAGGCTTCCAAAACT
MdPR10 1.13	GTGTTGGAACCATCAAGAAGATTAG	ACATCTCCTTCAATCAAACTGTAAT

Quantitative Real-Time PCR

Each qPCR reaction contained 5 µL of DyNAmo Flash® SYBR Green Master Mix (Thermo Fisher Scientific), 600–900 nmol·dm^{-3} of each primer, 0.5 – 1 µL of 10-fold diluted cDNA, ROX reference dye, and PCR water. The thermal cycling protocol was as follows: initial denaturation at 95 °C for 7 minutes; followed by 45 cycles of 95 °C for 30 seconds, 60 – 66 °C for 15 seconds, and 72 °C for 15 seconds, with fluorescence signal measured after each extension step. To assess product specificity, a melt curve analysis was performed from 60 °C to 90 °C with temperature increments of 0.1 °C per step.

Statistical analysis

The relative expression levels were calculated using the ddCt method adjusted for PCR efficiency according to Pfaffl [16]. Fold changes for all cultivars were calculated relative to the reference cultivar Golden Delicious, the maternal parent. Calculations and graph generation were performed using Microsoft Excel. The function "*VAR.P*" was used for the variability in fold-changes among cultivars. The similarity/difference of isoforms activities relative to GD was calculated by Euclidean Distance.

Results and Discussion

Based on the hypothesis that a high degree of natural variability exists in the expression of *MdPR10* genes among *Malus domestica* Borkh. cultivars (*unpublished*, Urbanová et al.), this study quantified the expression variability of selected *MdPR10* isoforms in cultivars sharing the same maternal parent. The cultivar Golden Delicious was chosen as a reference cultivar due to its frequent use in breeding programs and abundance of information at a genetic level [17, 18].

Gene expression levels of eight *MdPR10* isoforms (*1.01*, *1.02*, *1.03*, *1.06*, *1.07*, *1.08*, *1.11*, and *1.13*) were analyzed using real-time qPCR. All measurements were performed in triplicate, including no-template controls, and gene specificity was verified via melt curve analysis. The reaction efficiencies, melting temperatures of the PCR products, and the sequences of primers used are summarized in Table 3.

Table 3. Reaction efficiencies and melting temperatures for all eight *MdPR10* isoforms.

Gene	PCR effectivity [%]	PCR effectivity (R^2)	Product melting temperature [°C]
Mdactin	94.5	0.9973	79.5
MdPR10 1.01	91.4	0.9982	83.5
MdPR10 1.02	91.4	0.9982	84
MdPR10 1.03	101.6	0.9987	82
MdPR10 1.06	100.0	0.999	81
MdPR10 1.07	99.0	0.9936	81
MdPR10 1.08	103.8	0.997	81.5
MdPR10 1.11	99.8	0.9985	80
MdPR10 1.13	96.7	0.9974	79

A possible answer to the proposed hypothesis is that the level of natural variability in the expression of *MdPR10* gene isoforms among *M. domestica* cultivars is indeed high, despite all studied cultivars sharing the same maternal parent. Almost all measured differences were significantly different from the reference cultivar, except for seven cases. Five cultivars (Delor, Orion, Angold, Delbarestivale, and Jantár) showed no significant differences in expression of isoform *1.02* and were very similar to the maternal variety. Similarly, the remaining cultivars (except Sirius) showed only low levels of statistical significance. In the case of Sirius, the fold change in isoforms *1.01* and *1.08* was not statistically significant, but for the other isoforms, expression levels varied markedly – ranging from a 7-fold decrease (isoform *1.02*) to a 70-fold increase (isoform *1.03*). The activity of isoform *1.08* was consistently downregulated compared to the reference cultivar, ranging from a 3.35-fold decrease in Spencer to a 239.93-fold decrease in R. Opal, except for not significant Sirius. In contrast, the expression of isoforms *1.03*, *1.06*, *1.07*, and *1.11* was consistently upregulated relative to GD. Among the analyzed cultivars, Freyberg exhibited the most similar gene expression profile to GD, followed by Sirius and Shalimar. In contrast, Mutsu showed the greatest divergence from GD profile, followed by Spencer and Delbarestivale.

All cultivars analyzed showed lower expression of isoform *1.01* compared to GD. The highest variability in fold change – both in comparison to the GD and among the material collection – was observed for isoform *1.03*, followed by *1.08* and *1.07*. In contrast, isoform *1.02*, followed by *1.01*, exhibited the most

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stable expression levels among cultivars when compared to GD. This claim is also supported by proteomic data from Gao et al. [19], which identified these two isoforms as highly conserved at the proteomic level.

These findings open a new hypothesis: that *MdPR10* isoform *1.02* gene activity in apple fruit flesh is quite conserved, particularly in cultivars that share one of their ancestors. Further, isoform *1.01* also showed low variability. Given that isoforms *1.01* and *1.02* are the most abundant *MdPR10* isoforms, expressed at levels 10- to 10,000-fold higher than other isoforms [15] and they are the most transcribed isoforms [20], this observation is of particular interest. Because of their abundancy, these two isoforms are also the most studied [21, 22]. The expression of the other isoforms studied was highly variable, suggesting either a lack of inheritance from the maternal lineage, or that their expression variability may be linked to the inheritance of other genes involved in their biological pathway. However, it is very important to note that three cultivars – Heliodor, Opal, and Sirius – which share both parents, exhibited identical activity in isoform *1.06*, which may suggest a link to heredity. In the other isoforms, their activity differed significantly.

It has been hypothesized that differences in patient immune responses to various apple cultivars are related to the Mal d 1.06 isotype A [19]. However, as shown by Savazzini et al. [22], patients' age also plays an important role. Their findings indicate that pediatric patients were more sensitive to isoforms 1.07 and 1.03 than to 1.06A, which was the dominant isoform in adult patients. As mentioned above, the variability in the activity of all three isoforms was relatively high, with isoforms *1.03* and *1.07* showing particularly high variability among studied cultivars. Mal d 1 content and its protein activity in apples is also influenced by fruit color, primarily due to differences in polyphenol content, as well as by breeding objectives – particularly the emphasis on improving disease resistance. Kschonsek et al. [9] synthesized findings from Schmitz-Eiberger & Matthes [23] and Nothegger et al. [24], concluding that older cultivars of *M. domestica* are generally better tolerated by allergic patients. This may be related to the selective breeding of newer cultivars for improved disease resistance and reduced post-harvest browning. Older varieties may not only exhibit reduced synthesis of Mal d 1 proteins but also contain lower levels of other potentially allergenic or reactive compounds, such as chlorogenic acid, caffeic acid, or epicatechin.

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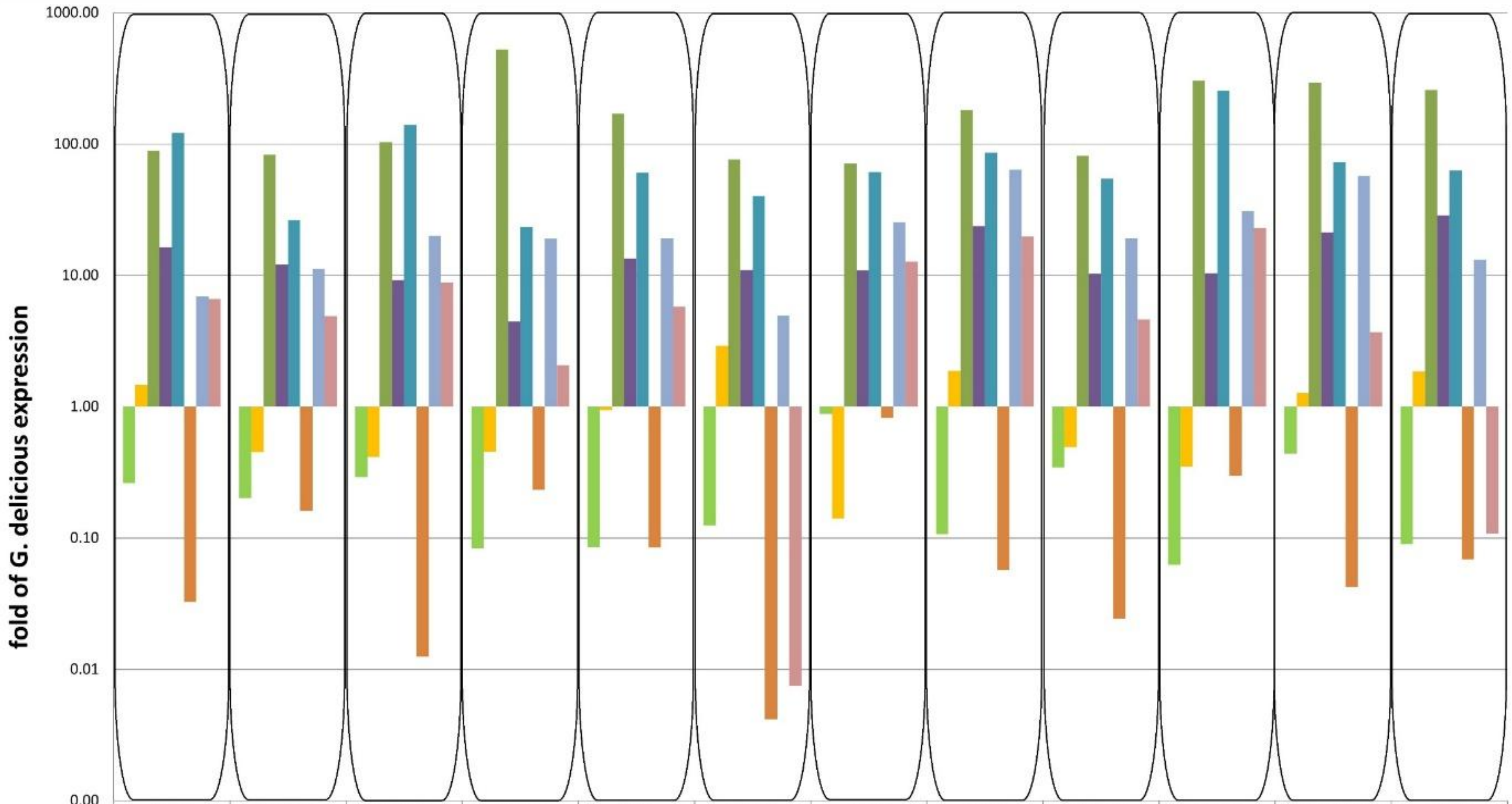
Figure 1 Expression activity of eight *MdPR10* gene isoforms in twelve *Malus domestica* Borkh. cultivars compared to the reference variety Golden Delicious, the maternal parent of all studied cultivars.



	Delor	Freyberg	Heliodor	Mutsu	Orion	R. Opal	Sirius	Angold	Shalimar	Spencer	Delbarestivale	Jantár
Mal d 1.01	0.26	0.20	0.29	0.08	0.09	0.12	0.88	0.11	0.35	0.06	0.44	0.09
Mal d 1.02	1.46	0.45	0.42	0.45	0.94	2.91	0.14	1.87	0.49	0.35	1.27	1.85
Mal d 1.03	89.29	82.59	103.27	525.45	170.52	76.44	70.91	181.50	81.33	303.91	294.21	257.73
Mald 1.06	16.40	12.10	9.18	4.44	13.44	10.93	10.88	23.66	10.31	10.39	21.25	28.70
Mal d 1.07	121.58	26.35	140.05	23.37	60.58	40.13	61.01	86.06	54.45	254.35	72.92	62.98
Mal d 1.08	0.03	0.16	0.01	0.23	0.09	0.00	0.82	0.06	0.02	0.30	0.04	0.07
Mal d 1.11	6.90	11.21	20.10	19.03	19.17	4.96	25.40	63.83	19.15	30.79	57.04	13.17
Mal d 1.13	6.63	4.90	8.80	2.07	5.77	0.01	12.75	19.72	4.63	22.89	3.69	0.11

Conclusions

There is substantial natural variability in the activity of *MdPR10* gene isoforms among apple cultivars sharing the same maternal lineage. The least variability was observed in the most abundant isoforms, *1.01* and *1.02*, whereas isoforms *1.03*, *1.06*, and *1.07* showed higher variability, which is consistent with previous protein-level studies by other authors. Isoform *1.08* also exhibited very high variability, with consistently reduced activity across all studied cultivars compared to the maternal parent cultivar (Golden Delicious), but it was not identified as likely to be responsible for variability in allergic response in previous studies.

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