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Apple (*Malus domestica* L. Borkh.) Allergen Mal d 1: Effect of Cultivar, Cultivation System, and Storage Conditions

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It has been reported on the basis of skin prick tests and gene expression studies that apple cultivars differ in their allergenic potential. Only a few studies have tried to measure the amount of the major apple allergen Mal d 1 so far. Mal d 1 belongs to the pathogenesis-related proteins, a family of proteins that are induced by pathogens and environmental stress. Due to cross-reactivity between Bet v 1 and proteins present in several plant-derived foods, birch pollen allergic patients develop food allergies, most frequently to apples. Mal d 1 content was quantified in different apple cultivars, cultivated at the research stations Klein-Altendorf and Bavendorf, dependent on cultivation method and storage conditions by sandwich-ELISA. Apple cultivars differ considerably in their Mal d 1 content. A high variability in Mal d 1 content was determined within one cultivar and between the two locations for the same apple cultivar. In most cases organically cultivated fruit showed lower Mal d 1 content in comparison to fruit from integrated production. At harvest the detected concentration of Mal d 1 was low, but during storage the Mal d 1 content increased significantly.

KEYWORDS: Apple allergy; birch pollen; Bet v 1; food allergy; Mal d 1; cross-reactions; pathogenesis-related protein; PR-10; *Malus domestica*

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INTRODUCTION

The prevelance of allergies is increasing throughout the world, which can also be seen in the rising number of patients showing reactions to birch pollen related food allergens. Today, up to 90% of birch pollen allergic patients may have developed intolerances to fruits and vegetables (*1*). Up to 2% of the central European and North American population suffer from apple allergies (*2*). Apple fruit (*Malus domestica* L. Borkh.) represents one of the most popular fruits consumed during the whole year. Therefore, apples are an important source of secondary plant metabolites. Current evidence supports a role of secondary plant metabolites in the prevention of cardiovascular diseases, cancers, and osteoporosis (*3*). Consumption of fresh apples can provoke several allergic reactions due to the presence of the apple allergen Mal d 1. Allergic symptoms are often limited to the oral allergy syndrome, because the allergen is labile and sensitive to pepsin digestion and heat; therefore, it does not survive most processing steps (*4*, *5*). Parallel appearance of birch and food allergies can be explained by cross-reactive IgE. The major birch pollen allergen, Bet v 1, and the apple allergen Mal d 1 share allergenic epitopes leading to these IgE cross-reactivities (*6*, *7*). Especially during the birch pollen season, an increase in clinical reactions to apples occurs (*8*). Apple allergy in southern Europe is characterized by reactions against Mal d 3, a nonspecific lipid transfer protein. It leads to more severe symptoms due to its high heat and pepsin resistance (*9*). Mal d 1 has been identified as a 17−18 kDa protein (*6*, *10*), equally present in the pulp and peel of apple fruit (*11*). Research done by Son and Lee (*12*) showed that allergenic differences between apple cultivars are mainly related to the expression levels of Mal d 1 and not to the presence of different isoformes, whereas Gao et al. (*13*) stated that differences in allergenicity are associated with the allelic composition of two specific genes (Mal d 1.04 and Mal d 1 1.06 A). Both Bet v 1 and Mal d 1 belong to the family 10 of pathogenesis-related proteins (PR-10), which are induced by pathogens, wounding, or certain environmental stress (*14*). PR-10 are developmentally regulated in different plant tissues and are involved in resistance to several pathogens (*15*). The biological role of Mal d 1 is unknown; it may be involved in the binding and transport of plant steroids (*16*).

Patients allergic to apples show great interest in consuming apple fruit, so it is important to evaluate the allergenicity of different apple cultivars and the impact of agronomical practices (cultivation and postharvest treatment). Fruit with low allergenic potential might be tolerated by patients with mild apple allergy. Gilissen et al. (*17*) went one step further and showed a reduction of Mal d 1 gene expression by silencing the genes for Mal d 1 by RNA interference.

The allergenic composition of most apple cultivars has not yet been fully characterized. Data on the IgE binding potency of Mal d 1 in vitro and in vivo and gene expression studies showed that this allergen is influenced by cultivar, degree of maturity, and storage conditions of the fruit (*4*, *7*, *18*). However, the amount of Mal d 1 depending on these factors has rarely been determined so far. The aim of the study was to quantify the Mal d 1 content of the fruit depending on cultivation method (organic and integrated production), cultivar, and different periods of postharvest storage.

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MATERIALS AND METHODS

Plant Material and Harvest. Fruits of different apple cultivars were cultivated at the Centre of Competence in Klein-Altendorf, Germany (Bonn University), and Bavendorf, Germany (Hohenheim University). The orchard in Klein-Altendorf is characterized by a growing period of 170 days, an average yearly temperature of 9.2 °C, a rainfall of 1534 nm, and 596 sunshine hours. Bavendorf Centre of Competence had an average temperature of 8.1 °C, 1678 h of sunshine, and 861 nm rainfall, and the growing period lasted 185 days. Orchic luvisol ('Parabraunerde') is the prevailing soil type at both orchards. The literature shows that variability between individual apple fruits is high, so that sampling has to be standardized (*4*). We used the Streif index to warrant that apple fruits were picked at optimal harvest date, so that every cultivar had the same stage of maturity. The index was calculated as fruit firmness/[soluble solid content × starch degradation value] (*19*). Starch degradation was used as the main factor. In this study fruit were picked from definite positions of the tree with comparable irradiation conditions, and picking was conducted by the same person at each location to minimize variability. Harvest lasted from the beginning of September ('Gala', 'Rubens') to the end of October ('Braeburn', 'Fuji Kiku'). Due to climatic differences between the two orchards, harvest was performed about 1 week later at the Centre of Competence at Bavendorf. Subsequent to harvest, fruits were washed and protein was extracted. Integrated plant protection was carried out according to the German integrated production guideline QS-Gap 9. Organic cultivation was performed according to EU directive 2092/91 (Klein-Altendorf) and "Bioland" guidelines (Bavendorf), respectively. For storage experiments fruits of cultivars 'Jonagold' and 'Golden Delicious' were stored in a cold chamber at 2 °C for 4, 8, and 12 weeks.

Preparation of Extract. Extraction of the protein was carried out according to the method of Björksten et al. (*20*). Peel and pulp were homogenized with potassium phosphate buffer (10 mM K_2HPO_4, 10 mM KH_2PO_4, pH 7) containing sodium dietyldithiocarbamate trihydrate (10 mM), ethylenediaminetetraacetic acid (2 mM), and polyvinylpolypyrrolidone (2%) in a relation of 1:1.5 w/v using a Retsch (Retsch, Haan, Germany) grinder. The extracts were prepared from the same ratio of peel and pulp for each cultivar. The core was removed before extraction. After incubation in a flask on a shaker for 4 h at room temperature, the homogenates were centrifuged at 4 °C for 15 min at 5000*g*. The supernatants were collected and subsequently frozen in aliquots at −80 °C. For each variety extracts were prepared 5-fold.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). To warrant stability of the extraction procedure, five apple fruits of the cultivar 'Greenstar' were extracted twice. The extracts were subjected to SDS-PAGE according to the method described by Laemmli (*21*). A 4−18% acrylamide stacking gel was used. Proteins were separated at 20 mA for 1.5 h under reducing conditions. Protein was stained with Coomassie Brilliant Blue to confirm the bands. As molecular weight marker Page Ruler Protein Ladder (Fermentas International Inc., Burlington, ON, Canada) was used.

Protein Determination. Total protein concentration was determined with the Roti-Nanoquant protein assay kit (Carl Roth, Germany), which is a colorimetric method based on the Bradford assay. The reaction mixture was composed of 720 μL of Roti-Nanoquant and 180 μL of sample. Bovine serum albumin (BSA) was used as standard protein.

Antibodies. The monoclonal antibody specific for Mal d 1 and the polyclonal rabbit serum with specificity for Bet v 1 were provided by Paul-Ehrlich-Institut (PEI, Division of Allergology, Langen, Germany). All other antibodies are commercially available.

Sandwich-ELISA. Quantification of the Mal d 1 content of every sample was determined in triplicate by sandwich enzyme-linked immunosorbent assay (ELISA). A sandwich-ELISA was developed for measurement of Mal d 1. Microtiter plates (Nunc-Immuno Plate Maxi Sorp surface, Nalge Nunc International, Denmark) were coated with 1:500 sheep anti-mouse Ig AP 302 (Chemicon, Germany) diluted in PBS buffer (phoshate-buffered saline, pH 7) at room temperature for 1 h. After every incubation step, four washes with PBS-T (v/v 0.05% Tween 20 in PBS) were conducted. A monoclonal antibody specific for Mal d 1 diluted in PBS-T (1:10) was incubated at 4 °C overnight. All further dilutions were performed with PBS-T buffer. In the next step the wells were incubated with dilutions of the apple extracts (1:4 to 1:512 in dual steps) in triplicate, and recombinant Mal d 1 was used as reference (from 2000 to 2.4 ng/mL). After incubation for 1 h at room temperature, a polyclonal rabbit serum with specificity for Bet v 1 was incubated (1:5000). Detection was performed with goat anti-rabbit IgG (Sigma, A0545, Germany) labeled with a peroxidase (1:5000), which was incubated at room temperature for 1 h. Finally the plates were incubated for 10 min with the substrate 3,3′,5,5′-tetramethylbenzinidine (citrate buffer, pH 3.95). To stop the reaction, 50 μL of 25% sulfuric acid was added. Photometric detection was performed at 450 nm in a microplate reader (Labsystems Multiscan RC). Recombinant Mal d 1 (Biomay, Austria) was used as standard, and total Mal d 1 content was calculated by a four-parametric calibration curve. Values were expressed as nanograms of Mal d 1 per milliliter and converted to micrograms per gram of fresh weight (FW).

Statistical Analysis. The experimental data were analyzed using the statistic program SPSS 14.0 for Windows (Munich, Germany). Statistical analysis was performed by one-way analysis of variance (ANOVA), with a significance level of $\alpha = 0.05$. Comparisons of mean values were performed by the Tukey test.

RESULTS

Validation of Extraction and Mal d 1 ELISA. Findings from SDS-PAGE of 10 extracts made of 5 apple fruits are presented in **Figure 1**. A band corresponding to Mal d 1 was visualized in all extracts after staining with Coomassie Brilliant Blue at 18 kDa. The obtained results revealed that protein extraction is consistent and reproducible.

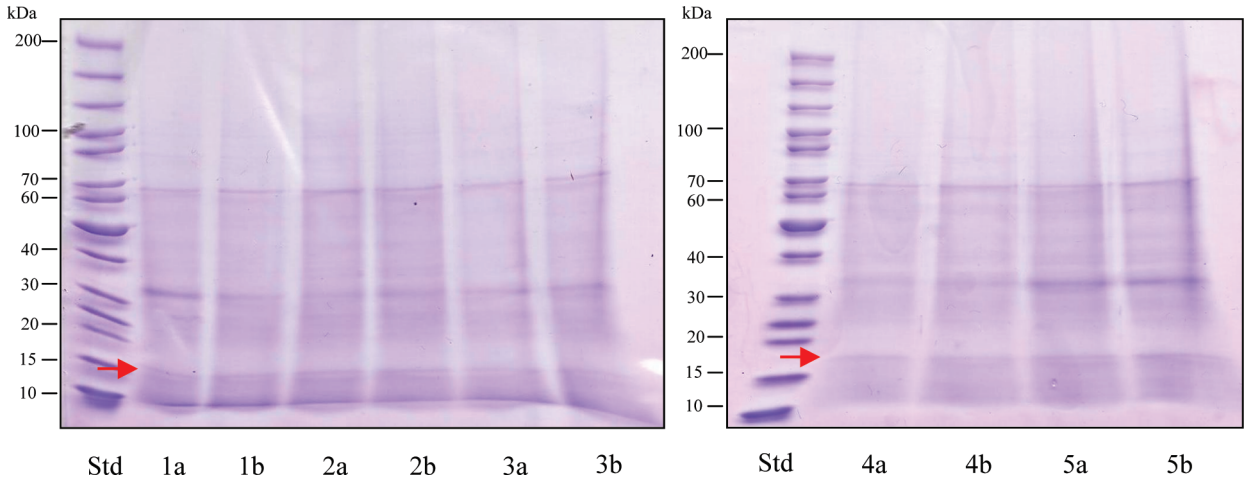

Figure 1. Findings from sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Five fruits of the cultivar 'Greenstar' (1−5) were investigated; two extracts were prepared each (a, b). "Std" indicates molecular weight marker. The 18 kDa band, corresponding to Mal d 1, is marked with an arrow.

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Table 1. Mal d 1 Content, Total Soluble Protein, Percentage of Mal d 1/Total Soluble Protein, and Streif Index of Selected Apple Cultivars Cultivated in Klein-Altendorf[a]

cultivar	Mal d 1 (μg/g of FW)	total soluble protein (μg/g of FW)	% Mal d 1/total soluble protein	Streif index
Jonagold	1.3 c	271.4	0.5	0.11
Kanzi	1.6 c	319.8	0.5	0.15
Greenstar	2.4 c	247.8	0.9	0.15
Pinova	3.4 c	266.8	1.3	0.21
Topaz	4.7 c	312.0	1.5	0.1
GD	6.2 c	372.8	1.7	0.1
Braeburn	6.4 bc	230.4	2.8	0.2
Diwa	6.5 bc	162.4	4.0	0.27
Fuji Kiku	8.9 abc	265.8	3.3	0.1
Cameo	9.1 abc	292.8	3.1	0.2
Rubens	14.2 ab	184.0	7.7	0.14
Gala	14.6 a	338.6	4.3	0.19

[a] Values followed by different letters are significantly different at the 5% significance level. Cultivars are ranked according to their Mal d 1 content (mean, $n = 5$).

Table 2. Mal d 1 Content, Total Soluble Protein, Percentage of Mal d 1/Total Soluble Protein, and Streif Index of Selected Apple Cultivars Cultivated in Bavendorf[a]

cultivar	Mal d 1 (μg/g of FW)	total soluble protein (μg/g of FW)	% Mal d 1/total soluble protein	Streif index
Braeburn	2.3 b	236.8	1.0	0.22
Topaz	5.5 b	336.1	1.6	0.13
Fuji Kiku	6.2 b	315.8	2.0	0.09
Greenstar	7.2 b	218.2	3.3	0.11
GD	7.6 b	248.6	3.0	0.1
Pinova	8.0 ab	415.7	1.9	0.09
Cameo	8.6 ab	343.5	2.5	0.13
Jonagold	8.7 ab	126.9	6.9	0.08
Kanzi	9.4 ab	260.2	3.6	0.16
Diwa	11.7 ab	180.8	6.5	0.26
Rubens	20.1 a	325.0	6.2	0.19

[a] Values followed by different letters are significantly different at the 5% significance level. Cultivars are ranked according to their Mal d 1 content (mean, $n = 5$).

To assess reproducibility of the sandwich-ELISA, a Mal d 1 standard solution and two apple extracts were assayed fivefold. The interassay coefficient of variation was always under 20%, with increasing values with higher dilutions, the intra-assay coefficient of variation was under 5%, so that reproducibility was confirmed.

Cultivar Ranking and Influence of Orchard on Mal d 1 Content. Harvest of every cultivar was performed at the optimal stage of maturity according to the Streif index. No differences in these values were detected between the locations (**Tables 1** and **2**). The apple cultivars differed significantly in their Mal d 1 contents (**Tables 1** and **2**). In fruit cultivated at the center of competence in Klein-Altendorf, lowest amounts of Mal d 1 were found in 'Jonagold', 'Kanzi', 'Greenstar', 'Pinova', 'Topaz', and 'Golden Delicious' fruits. The significantly highest amounts in comparison to cultivars with low Mal d 1 levels were determined in cultivars 'Rubens' and 'Gala'. The lowest concentration in fruit from Bavendorf occurred in cultivars 'Braeburn', 'Topaz', 'Fuji Kiku', 'Greenstar', and 'Golden Delicious'. The highest Mal d 1 content was determined in fruit of 'Rubens'. We analyzed a high variability in Mal d 1 content between the two locations. In most cases higher amounts were found in fruit cultivated at Bavendorf. This was not the case for cultivars 'Topaz', 'Golden Delicious', and 'Cameo', which showed similar Mal d 1 concentrations.

Table 3. Mal d 1 Content of 'Jonagold' and 'Topaz' Fruits Cultivated at Klein-Altendorf (KAD) and Bavendorf (Bav) under Organic and Integrated Production Systems[a]

cultivar	orchard	Mal d 1 content (μg/g of FW): organic production	Mal d 1 content (μg/g of FW): integrated production	ANOVA
Jonagold	KAD	2.0 a	1.3 b	$p = 0.004$
	Bav	1.3 b	8.7 a	$p = 0.003$
Topaz	KAD	2.7 b	4.7 a	$p = 0.035$
	Bav	2.0 b	5.5 a	$p = 0.018$

[a] Values followed by different letters within an orchard and cultivar are significantly different. p values of ANOVA statistical tests ($p < 0.05$) are also provided (mean, $n = 5$).

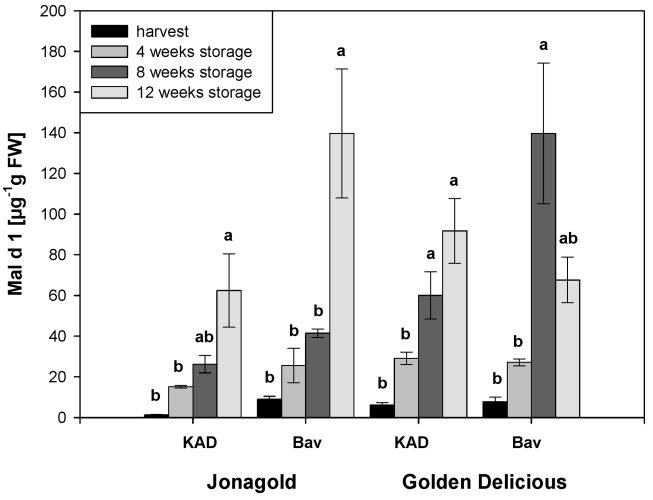

Figure 2. Mal d 1 content (μg/g of FW) during cold storage in 'Jonagold' and 'Golden Delicious' fruits cultivated at Klein-Altendorf (KAD) and Bavendorf (Bav). Significant differences were calculated for each cultivar and orchard at a significance level of 5% (mean, $n = 5$).

Higher Mal d 1 amounts for fruit from Klein-Altendorf were determined in cultivars 'Braeburn' and 'Fuji Kiku'. Differences between the locations were significant for the cultivars 'Jonagold' (ANOVA, $p = 0.02$), 'Greenstar' (ANOVA, $p = 0.00$), and 'Kanzi' (ANOVA, $p = 0.007$).

The calculated percentage of Mal d 1/total soluble protein was between 0.5 and 6.9%. This ranking of the cultivars showed similarities to the ranking based on total Mal d 1 content (**Tables 1** and **2**).

Organic versus Integrated Production. In most cultivars fruit from integrated production showed significantly higher Mal d 1 concentrations in comparison to those cultivated according to organic production guidelines. This was not observed in 'Jonagold' fruit cultivated at Klein-Altendorf (**Table 3**), which showed significantly higher Mal d 1 content in organically cultivated fruit.

Storage Experiments. Fruits of cultivars 'Jonagold' and 'Golden Delicious' ranked as cultivars with low ('Jonagold') and high allergenicity ('Golden Delicious') (*12*) were stored for 4, 8, and 12 weeks in a cold chamber at 2 °C. In both apple cultivars Mal d 1 protein increased significantly during storage. 'Jonagold' fruit of both orchards showed a permanent accumulation of Mal d 1, so significantly highest amounts were analyzed after 12 weeks of cold storage (**Figure 2**). In 'Golden Delicious' fruit from Klein-Altendorf significantly higher allergen amounts were found after 8 weeks in comparison to storage for 4 weeks, and a further increase of Mal d 1 during the following storage time was also

seen. 'Golden Delicious' fruit cultivated in Bavendorf showed the highest Mal d 1 values after 8 weeks, with lower concentrations after 12 weeks (**Figure 2**).

Percentage Mal d 1/total soluble protein increased during the storage period up to 15%, but in the cultivar 'Golden Delicious' highest percentages were calculated after 8 weeks of storage and declined if fruits were further stored (data not shown).

DISCUSSION

Among the cultivars the detected Mal d 1 content varies between 0.5 and 15 μg/g of FW. Other studies analyzed Mal d 1 levels between 3.8 and 72.5 μg/g of FW in the pulp (*22*), 0.84−33.17 μg/g of FW (*11*), 0.84−12.18 μg/g of FW (*4*), and 5.95−455 μg/g of FW (*23*). Our results are at the lower end of this range. Stability of the extraction procedure and reproducibility of the sandwich-ELISA were shown by pretests. Nevertheless, we had a high variability in Mal d 1 content within one cultivar. The high variability in Mal d 1 content could also be seen in differences shown by the same cultivar cultivated at different orchards. In a study by Asero et al. (*4*) 'Jonagold' fruit showed an interapple variability up to 20-fold and an intra-apple variability up to 5-fold. 'Golden Delicious' fruit showed a lower variability. They stated that the calculated Mal d 1 content is not valid for each individual fruit. A hypoallergenic cultivar can have concentrations in a single fruit that are as high as that of a cultivar with high allergenicity. A cultivar screening in two following years indicated that the results of 2004 did not correspond to those determined in 2003. The differences in Mal d 1 amount between fruits with low and high allergenicity were less pronounced in 2004. Differences between two orchards were also described by Sancho et al. (*22*) for the cultivar 'Cox Orange'. In our study sampling was standardized as far as possible with the same standards at every location, as described under Materials and Methods. From that, the importance of environmental factors for Mal d 1 regulation became obvious. Besides, plant genetic factors have a major impact on allergenicity of mature fruit (*22*). However, regulation of Mal d 1 expression is influenced by several biotic and abiotic factors; therefore, it is difficult to obtain reliable data on a ranking of different apple cultivars.

Data on the influence of cultivation method on Mal d 1 concentration are scarce. Most studies did not find any differences in Mal d 1 content between the different cultivation practices. Klockenbring et al. (*24*) did not detect any significant differences in the IgE-binding potency between organically and integratedly produced fruit by western Blot. Prick-to-prick tests obtained higher allergenicity of organic cultivated 'Boskoop' fruit in comparison to integrated produced ones. All other cultivars showed no differences. Marzban et al. (*11*) quantified Mal d 1 in a high number of cultivars. Some of the selected cultivars were cultured either conventionally or under organic farming conditions. According to cluster analysis they found no significant differences in allergen content between different production systems. Marzban et al. (*11*) stored cultivars for 3 months at 4 °C before extraction, so any influences of this treatment have to be taken into consideration when the different modes of production are compared. Mal d 1 belongs to the pathogenesis-related proteins, so it can be concluded that stress factors leading to protein synthesis are different in both cultivation systems. Mal d 1 synthesis in organic fruit may be induced by biotic stress factors such as fungi, viruses, and bacteria. Integratedly produced fruit might not be affected by biotic factors due to pesticide treatment, but this treatment itself might cause a response of the fruit in the form of PR-10 accumulation including Mal d 1. In this study for the first time significant differences were detected; in most cases the integratedly produced fruit showed higher contents of allergenic proteins. It seems that pesticide treatment led to stronger responses than any biotic factors as shown in this study. Which mechanism led to the activation of Mal d 1 synthesis and the metabolic pathways has yet to be investigated in detail. Response to any stress factors might be cultivar dependent, so that the influence of any agronomical practices and environmental conditions should be investigated in more detail.

Further factors affecting PR synthesis seem to be processes that occur during senescence and storage. Mal d 1 content increased in this study during storage in a cold chamber throughout 12 weeks. This is consistent with other studies in which higher Mal d 1 contents were determined over a 5 month storage period under ambient storage conditions (20 °C), cold storage, and storage under modified atmosphere (*22*). It should be pointed out that in 'Golden Delicious' fruit the highest Mal d 1 concentrations were analyzed after 8 weeks of storage, so response to cold stress seems to be cultivar dependent. Bolhaar et al. (*25*) detected a reduction of allergenicity by 15% if the fruit was stored under controlled atmosphere in comparison to the cold-stored fruit by skin prick tests. Real-time PCR analysis run by Sancho et al. (*22*) showed that changes in Mal d 1 levels resulted from up-regulation in gene expression through increased transcription.

Ripening of the fruit is associated with an upsurge in the rate of respiration and ethylene production. Hsieh et al. (*5*) observed an increase in Mal d 1 content during ripening and tried to evaluate if this could be caused by ethylene. They did not find any correlation between Mal d 1 content and the expression of one of the enzymes of ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid oxidase, so they suggest that this allergen is not affected by senescence processes that occur during storage, but by factors that are related to resistance to several diseases. This finding was corroborated by Pühringer et al. (*15*), who did not find any correlation between the precursor of ethylene, ethephon, and the activity of the Ypr10*a promotor in young apple leaves, which leads to inducibility of its gene product Mal d 1, whereas Mal d 1 expression was up-regulated in response to biotic stress (*15*). Nevertheless, protein metabolism is influenced by ethylene as shown in several studies (*26*, *27*), and gene expression of a protein sharing 90% sequence homology with Mal d 1 was up-regulated in response to ethylene (*28*). The increase in percent Mal d 1/total soluble protein during storage shows that Mal d 1 gene expression is more stimulated by cold stress than gene expression of other proteins.

Assessment of the allergenicity of apple cultivars has been conducted on the basis of in vitro studies (gene expression, ELISA, and immunoblotting) and in vivo studies (skin prick tests and oral food challenges). We used a sandwich-ELISA to quantify Mal d 1 content in apple fruit. Both in vivo and in vitro techniques have typical problems. In a patient-dependent assay factors such as individual variation within the patients and technical factors of prick-to-prick testing affect the results. Reproducibility is complicated through smaller sample numbers and fluctuation in allergic responses during the year (*25*). Comparison of different in vitro assays by Zuidmeer et al. (*23*) showed that analysis of allergenicity is highly dependent on reliable sample preparation and the assay itself. Some assays cannot distinguish between the native form of Mal d 1 and any aggregated form that might exist in extracts. This may lead to underestimation of Mal d 1 content. Assessment of the allergenicity of apple cultivars is complex, and only parallel analysis with in vitro and in vivo assays may obtain reliable results. Results obtained from different assays are not always consistent. Therefore, the cultivar 'Fuji' was classified as highly allergenic according to Mal d 1 content (*11*), as moderate by skin prick testing (*25*), and as

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hypoallergenic according to gene expression values (*18*). However, in our study 'Fuji' fruit from Bavendorf showed higher allergenicity in comparison to fruit cultivated in Klein-Altendorf.

Up-regulation of Mal d 1 gene expression seems to be influenced by a wide range of biotic and abiotic factors. The effect of cultivar, growing site, cultivation-related factors, and environmental factors has not been fully characterized and requires further studies. High variability between fruit cultivated at different locations showed that the impact of environmental conditions and pesticide treatment need to be investigated in detail. Studies in which all environmental conditions can be standardized, for example, in a climate chamber, and just the influence of one factor can be varied should give valid results on the impact of single environmental conditions on Mal d 1 content. Further studies with long-term series of Mal d 1 determination in a wide range of cultivars need to be carried out to clarify which cultivar constantly expresses low amounts of this allergen. This knowledge would allow breeders and growers to select hypoallergenic cultivars and agronomical practices to provide fruit with decreased allergenic potential, which might be tolerated by patients with apple allergy.

ABBREVIATIONS USED

PR-10, pathogenesis-related 10 proteins; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BSA, bovine serum albumin; ELISA, sandwich enzyme-linked immunosorbent assay.

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