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Effect of Postharvest Storage on the Expression of the Apple Allergen Mal d 1

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Consumption of fresh apples can cause allergy in susceptible individuals. A competitive enzyme-linked immunosorbent assay (ELISA) has been developed to determine Mal d 1 levels in apple pulp using a monoclonal antibody (BIP-1). The ELISA was able to rank ten cultivars according to their Mal d 1 content (between 3.8 and 72.5 μg/g pulp). For the first time, it has been demonstrated that growing conditions and postharvest storage, using three different treatments over a 5 month period in 2 consecutive years, increase Mal d 1 expression at a translational and transcriptional level (3.5- and 8.5-fold under controlled atmosphere storage). Expression of three major Mal d 1 isoforms was observed by real-time polymerase chain reaction over the 5 month storage period, and Mal d 1.02 was the most highly expressed isoform. In conclusion, Mal d 1 gene expression was significantly increased during modified atmosphere storage. Individuals suffering from birch pollen−apple allergy syndrome might experience fewer problems consuming freshly picked apples.

KEYWORDS: Apple; allergens; Bet v 1; cultivar; Mal d 1; Mal d 1 expression; PR 10; storage

INTRODUCTION

It is estimated that between 5 and 8% of children of less than 3 years of age and approximately 2% of the adult population experience IgE-mediated food allergy (*1*). Allergies to a range of fruits such as apple, pear, apricot, cherry, and plum; vegetables such as celeriac (celery tuber), potato, and carrot; and nuts such as hazelnut and walnut are associated with prior sensitization to pollen, especially from birch (*2*, *3*). This is due to immunological cross-reactivity between Bet v 1, the major allergen in birch pollen, and its homologues present in apple (Mal d 1) and other foods (*4*−*6*). Patients allergic to Mal d 1 generally develop mild symptoms (oral allergy syndrome), restricted to the lips, tongue, and throat (*7*), only after ingestion of fresh fruit. This can be explained by the lability of this allergen to cooking (*8*), oxidation (*9*), and proteolytic digestion (*10*).

The biological role of Mal d 1 is still unknown, but it may involve binding and transport of plant steroids (*11*, *12*), and a role in intracellular signalling has been suggested because of its ability to bind a novel apple protein, MdAP (*13*). Mal d 1 belongs to the pathogenesis-related protein 10 family (PR-10) (*14*) thought to be expressed in response to fungal or bacterial infection and stress (*15*−*17*). Consequently, abiotic and biotic stress on the apple tree and fruits might affect the levels of this allergen. In addition, it has been demonstrated that storage at low temperature (2−4 °C) under uncontrolled oxygen conditions quantitatively affects levels of Mal d 1 in apples (*18*) and has an effect on the skin reactivity to apples in Mal d 1-reactive patients (*19*). Similarly, the allergen level varies between cultivars as demonstrated quantitatively by enzyme-linked immunosorbent assay (ELISA) and semiquantitatively by immunoblotting (*20*, *21*) matching the in vivo reactivity to apples indicated by SPT and open challengers in patients allergic to apple (*19*, *22*). Variability in the allergenic potency of apple cultivars might be due to the different expression levels of any of the 18 Mal d 1 isoforms clustered into four groups (Mal d 1.01−Mal d 1.04) identified to date (*13*, *23*, *24*). A mixture of isoforms is present in the fruit (skin and pulp) at different levels but with a similar pattern of expression in unrelated cultivars

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(*17*, *21*, *23*, *24*). In addition, different IgE binding capacities of the Mal d 1 isoforms have been reported, being higher with Mal d 1.01 than with Mal d 1.02 (*23*). At present, there are no reports on the evolution of Mal d 1 isoforms throughout time at different types of storage conditions.

In this paper, we describe the expression of Mal d 1, at both transcriptional and translational levels, as a function of apple cultivar and postharvest storage. Such knowledge is needed to support the development of knowledge-based strategies to reduce allergen loads in fresh products through optimization of postharvest handling of fruits.

MATERIALS AND METHODS

Apple Fruits. Apple fruits were provided by Norfolk Fruit Growers (Hoveton, Norfolk, United Kingdom) for 2 consecutive years. In 2001 (year 1), apples from cvs. Cox's Orange Pippin (orchard 3) and Jonagored were harvested on the 22nd of September and the 11th of October, respectively. Immediately after harvest, fruits were treated with fungicide (Ridomil MBC, FRAG-UK, York) at half label strength according to the manufacturer's instructions. Different storage conditions were applied with samples stored under ambient conditions (AMB, 20 °C), cold (2 °C) with no controlled atmosphere (CS), or controlled atmosphere (CA) conditions (<0.5% CO_2 and 1.25% O_2 at 3.8 °C) for up to 5 months. Six apple fruits per sample from a group of six trees were randomly selected for analysis.

In 2002 (year 2), selected apple fruits, cv. Cox's Orange Pippin from two orchards (orchards 3 and B), were picked from a group of five trees, and for two consecutive picks, six fruits were selected from each tree. Two picking dates were chosen as follows: September 10 (pick 1), optimal for long storage, and September 17 (pick 2), optimal for short storage. Fruits were treated with fungicide and stored under CA for up to 5 months as described for year 1. For comparison, apple fruits (cv. Jonagored) were harvested (October 24) at Orford (United Kingdom) and stored under CA as for cv. Cox.

Fruits from five cultivars grown in The Netherlands and five cultivars grown in the Adriatic seaside were harvested at a stage of ripeness used routinely by growers and stored for 3 months at 4 °C. Protein extracts were prepared according to Zuidmeer et al. (*20*) and Marzban et al. (*21*). These 10 cultivars were chosen to validate the ELISA as described below.

Apple Extracts. Apple pulp (50 g) was frozen in liquid nitrogen and homogenized in 23 mM Na_2PO_4 buffer (200 mL, pH 7.0) containing 0.8 mM ethylenediamine tetraacetic acid disodium salt, 10 mM sodium diethyldithiocarbamate, 4% (w/v) polyvinylpolypyrrolidone (PVPP), 0.6 mM benzamide, 0.5 mM phenylmethanesulfonyl fluoride, and a protease inhibitor cocktail (Roche, Germany) for 2 min at 1 °C in a Waring blendor (Waring Commercial, CT). The extract was centrifuged at 10000*g* for 30 min at 1 °C, and the resulting supernatant was filtered through a Millex-HA 0.45 μm syringe filter (Millipore, Millex-HV, Bedford). Apple extracts were stored in aliquots at −40 °C.

Protein Determination. The protein concentration was determined using the bicinchoninic acid assay (Sigma Diagnostics Co., St. Louis, MO) following the manufacturer's instructions with bovine serum albumin (BSA) as the standard protein (*22*). Apple extracts were dialyzed (2 kDa cutoff dispo-biodialyzer, Sigma) against distilled water prior to analysis.

ELISA. Natural Mal d 1 from apple was purified as described by Zuidmeer et al. (*20*) including PVPP in the purification protocol. Monoclonal anti-Bet v 1 antibody (BIP-1) was produced as described by Weiss et al. (*26*). Recombinant Mal d 1 (Mal d 1.01) was purchased from Biomay AG (Vienna, Austria). Polystyrene microtitre plates (Nunc-Immuno plate, Nalge Nunc International, Denmark) were coated with 220 μL of rMal d 1 (2 μg/mL) in coating buffer (5.3 g/L $NaHCO_3$ and 4.0 g/L Na_2CO_3, pH 9.6) overnight at 1 °C. After they were washed five times with PBST (0.05% v/v Tween-20 in phosphate-buffered saline), plates were blocked with 1% (w/v) BSA for 2 h at room temperature and washed as before. Samples (100 μL diluted in PBST) were incubated with 100 μL of monoclonal anti-Bet v 1 antibody diluted 1:1000 (v/v) in PBST for 3 h at 1 °C. After they were washed five times, plates were incubated with 200 μL of goat anti-mouse IgG-labeled with horseradish peroxidase (Sigma) diluted 1:2000 (v/v) in PBST containing 1% (w/v) BSA overnight at 1 °C. Following a final washing step, 200 μL of horseradish peroxidase substrate (Biovet, Canada) was added and the color development was stopped by adding 50 μL of 2 M H_2SO_4. The optical density was determined at 450 nm wavelength using a Dynatech MR5000 plate reader (Dynatech Laboratories, Billingshurst, United Kingdom). Amounts of Mal d 1 in apple extracts were quantitatively determined based on the standard curve using native Mal d 1, by GraphPad Prism software (GraphPad Software Inc., CA). All determinations were run in triplicate, and Mal d 1 concentrations were expressed as μg Mal d 1/g pulp wet weight.

To assess a possible matrix effect on the Mal d 1 ELISA assay, extracts were diluted up to 20-fold in the buffer described for apple extract protocol. To assess the reproducibility of this protocol, triplicate apple extracts of cvs. Jonagored and Cox (orchard 3) were prepared and Mal d 1 levels were determined by ELISA. To study extract stability, one extract was prepared, aliquoted, and stored at −20 °C for up to 10 days. As part of the ELISA validation, 10 apple cultivars were selected as described in the apple fruits section.

Total RNA Extraction. To study the expression of Mal d 1 at a transcriptional level, the peel and pulp of apple fruits (cvs. Cox 3 and Jonagored) harvested in year 1 and stored under CA conditions for up to 5 months were frozen in liquid nitrogen separately, and total RNA was extracted according to the method of Chang et al. (*27*) but modified. Frozen apple material (5 g) was homogenized to fine powder in a mortar under liquid nitrogen. Then, the powder was extracted with 15 mL of extraction buffer containing 2% (v/v) β-mercaptoethanol for 10 min at 65 °C and then for another 10 min at room temperature. Extraction with equal volumes of chloroform:isoamylalcohol was carried out three times. RNA precipitation was carried out as described (*27*).

The yield and the quality (ratio A260/A280) of the RNA were determined spectrophotometrically (Perkin-Elmer, Boston, MA) and by gel electrophoresis using ethidium bromide to stain as described by Marzban et al. (*21*).

Real-Time Polymerase Chain Reaction (PCR). Quantitative expression of three Mal d 1 isoforms was measured by means of SYBR1Green I-based real-time PCR. cDNA synthesis and real-time PCR experiments were performed as described previously (*13*).

Statistical Analysis. Analysis of variance (ANOVA) models were employed to examine the relationships between the Mal d 1 levels and the various factor explanatory variables. Initially, models were created that contained all of the explanatory variables and all of their two-way interactions. Backward elimination was then employed to reduce the model to one containing only terms that significantly affect Mal d 1. When a two-way interaction term is present, this means that the effect of one of the variables in the interaction depends upon the value of the other variable in the interaction. Standard regression diagnostics indicated that these models were appropriate for the data, i.e., no need to use nonparametric models. Apple cultivars were ranked according to their Mal d 1 content by calculating the tertiles. Data were ranked from 1 to n and then grouped into three sets with a 33% cut-off point. The Wilcoxon test was chosen to assess median differences between Italian and The Netherlands non-normally distributed populations. Pearson's product−moment correlation was used to test for a linear relationship between two continuous variables. For all such testing, significance was accepted at the standard level of $p = 0.05$. All analysis was performed using the R software package (http://www.R-project.org/).

RESULTS

Validation of Mal d 1 ELISA. An indirect competitive ELISA was developed to determine Mal d 1 content in apple pulp using recombinant Mal d 1 as the ELISA solid phase, natural Mal d 1 as a calibrant, and a mouse monoclonal antibody (BIP 1) directed to Bet v 1, the major birch pollen allergen. A typical calibration curve is represented in **Figure 1a** and shows that the ELISA had a working range from 0.5 to 5 μg/mL and

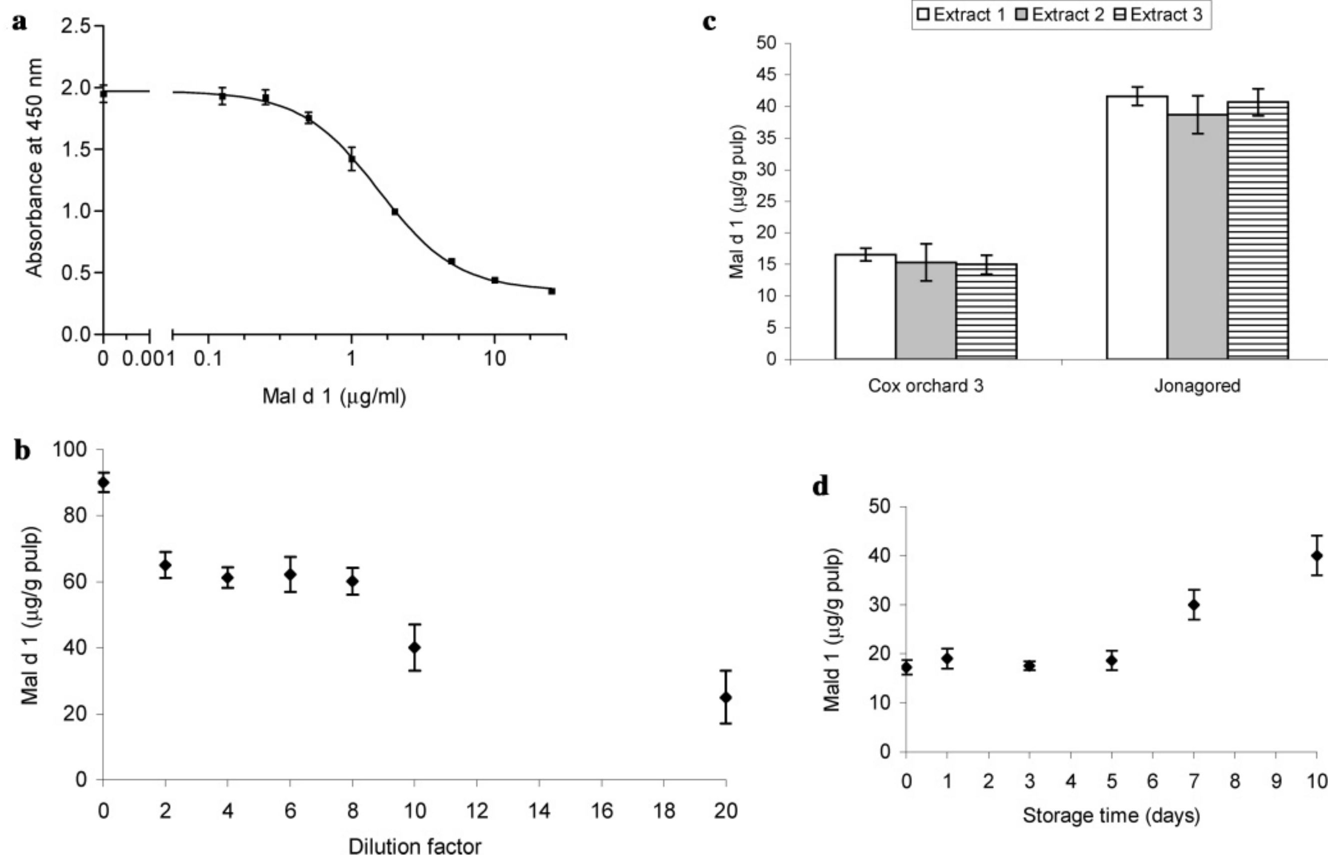

Figure 1. Standard curve of mouse anti-Bet v 1 monoclonal antibody in indirect competitive ELISA assay (**a**) and study of the matrix effect (**b**), reproducibility (**c**), and stability (**d**) of the apple extract preparation as determined by ELISA.

a IC_{50} of 0.79 μg/mL. The matrix effects at different dilutions of apple extract measured in ELISA were investigated for several apple cultivars under different storage conditions and times. When extracts were diluted up to 20-fold, a matrix effect was observed at a very high or very low dilutions as shown in this example (**Figure 1b**). However, a linear dose−response was observed for Mal d 1 between 2- and 8-fold dilutions of all of the extracts prepared. Consequently, all extracts were diluted in this working range for analysis. No significant differences were observed in Mal d 1 levels when triplicate apple pulp extracts were prepared for two different cultivars and assayed by ELISA (**Figure 1c**). No variation in the allergen levels for up to 5 days was observed after storing the extract at −20 °C although longer-term storage did result in an apparent increase in Mal d 1 levels (**Figure 1d**). This may be because changes in immunoreactivity occurred rather than changes in amount. Consequently, extracts were prepared and stored for only 3 days before analysis. This ELISA was shown to discriminate cultivars with low and high Mal d 1 levels (*20*) in a collection of 10 cultivars: five harvested from the Adriatic seaside (I) and five from The Netherlands (NL). Mal d 1 levels ranged from 3.8 to 72.5 μg/g pulp (**Table 1**). The median values for the I and the NL cultivars differed; they were 16.14 and 57.33 μg/g pulp, respectively.

Table 1. Mal d 1 Concentration (μg/g Pulp) in 10 Apple Cultivars, Five from Italy (I) and Five from The Netherlands (NL), Determined by Indirect Competitive ELISA Using a Monoclonal anti-Bet v 1 Antibody[a]

cultivar	Mal d 1 (μg/g pulp)	SD	country of origin	ranking
D3	3.8	0.1	NL	1
G 362	8.8	0.5	I	1
G 185	14.1	0.5	I	1
Mela campanina	16.1	0.5	I	1
Schoone	26.1	1.0	NL	2
Ambrosia	27.5	0.3	I	2
DL35 Bio	28.8	1.6	I	2
Fuji	50.8	0.9	NL	3
Septer	63.9	0.9	NL	3
87031–029	72.5	1.2	NL	3

[a] The cultivars were ranked according to their Mal d 1 concentration by calculating the tertiles in three groups: high (3), medium (2), and low (1).

Effect of Storage Conditions on Expression of Mal d 1 Translation. The effect of environmental conditions (during growth or storage) and genetic (cultivar-to-cultivar) factors on the expression of Mal d 1 at a translational level in the pulp of fruits was studied by ELISA. In year 1, fruits were stored up to 5 months in either cold or CA conditions and only up to 3 months under AMB conditions due to the initial onset of senescence (**Table 2**). An increase in Mal d 1 levels was observed during storage time for all of the cultivars and conditions. This increase was 2.5-fold (AMB), 4-fold (CS), and 3.5-fold (CA) for cv. Cox orchard 3 and 3.5-fold (AMB) and 4.5-fold (CS and CA) for cv. Jonagored (**Table 2**). This pattern was confirmed by ANOVA analysis (**Table 3**, **year 1**) showing that the interactions between storage time vs cultivar and between storage time vs storage type did not affect Mal d 1 levels. However, the two-way interactions storage type vs cultivar significantly affected Mal d 1 levels. Cv. Jonagored showed similar levels of Mal d 1 for all of the storage conditions, whereas cv. Cox Mal d 1 levels were, on average, higher for AMB and lowest for CS (**Figure 2a−c**). When comparing Mal d 1 levels in both cultivars for each storage condition, it was observed that the allergen levels in cv. Cox were greater under AMB conditions, similar under CA conditions, and lesser under

Table 2. Mal d 1 Concentration (μg/g Pulp) in Year 1 in Apple Pulp Determined by ELISA[a]

		Mal d 1 (μg/g tissue)									
		storage time (months)									
cultivar	storage conditions	1	SD	2	SD	3	SD	4	SD	5	SD
Cox orchard 3	AMB	31.1	0.8	58.3	2.2	71.9	3.5	NA		NA	
	CS	8.0	0.7	16.8	0.9	17.3	0.6	23.9	1.5	30.9	1.4
	CA	19.0	1.2	33.3	0.5	27.7	1.8	38.1	0.5	64.2	0.8
Jonagored	AMB	17.6	2.0	48.1	3.6	61.9	3.9	NA		NA	
	CS	12.1	0.7	32.3	2.2	36.0	1.2	42.7	3.4	54.2	2.2
	CA	22.8	1.9	18.5	1.7	34.2	2.8	32.7	0.4	102.9	4.8

[a] Apple fruits were stored under AMB, CS or CA conditions for up to 5 months; NA, not available.

Table 3. ANOVA of Mal d 1 Levels (μg/g Pulp) Measured in Apple Fruit Harvested in Year 1 and Year 2[a]

	Df	Sum Sq	*F* value	Pr(>*F*)
	year 1			
apple cultivar	1	0.13	2.40	0.1409
storage type	2	2.46	21.93	0.0000
storage time	4	5.03	22.41	0.0000
storage type vs apple cultivar	2	0.97	8.68	0.0028
residuals	16	0.90		
	year 2			
apple cultivar	2	10.95	118.06	0.0000
storage time	4	60.87	328.25	0.0000
storage time vs apple cultivar	8	7.53	20.31	0.0000
residuals	10	0.46		

[a] Functions analyzed for year 1 were storage time and type and apple cultivar, and for year 2, functions analyzed were orchard and storage time. Degrees of freedom (Df) indicate the number of quantities that must be estimated to define the effect of the variable. The sum of squared error (Sum Sq) is a measure of how much of the variance in the response is explained by the variable. The *F* value formally relates the Sum Sq for a variable to the total amount of variation of the response (incorporating the Df information). The *p* value [Pr(>*F*)] is the probability of observing the experimental *F* value under the assumption that the variable has no effect on the response. If this *p* value is less than 0.05, this assumption is rejected; therefore, the variable is significantly related to the response.

CS conditions. The highest increase of Mal d 1 levels was observed under CA conditions at time 5 (**Figure 2a−c**). Pearson's product−moment correlation was calculated to determine any linear relationship between Mal d 1 content and total extractable protein over the 5 month storage period. No significant correlation was found at $p < 0.05$ for cv. Jonagored ($r = 0.646$; p value $= 0.117$) and cv. Cox 3 ($r = 0.191$; p value $= 0.650$).

Apples (cvs. Cox orchard 3 and B and Jonagored) harvested in year 2 and stored under CA conditions were analyzed for Mal d 1 levels in fruit pulp by ELISA (**Table 4**). An overall increase in Mal d 1 levels was observed during storage time for all of the cultivars and orchards, as seen for year 1 apples. For cv. Cox orchard 3 and B, this increase was between 7- and 8.5-fold but was lower (3.5-fold) for cv. Jonagored (**Table 4**). The sharpest increase of Mal d 1 levels was observed after 5 months of CA storage (**Figure 2d**). Mal d 1 levels were higher in apples (cv. Cox) originating from orchard B as compared to orchard 3 (**Figure 2d**). Unlike year 1, ANOVA analysis (**Table 3, year 2**) showed a significant effect of the storage time vs cultivar on Mal d 1 levels. The results seem to indicate a different profile between cv. Jonagored and cv. Cox. In addition, the effect of harvesting time on the translation of Mal d 1 was studied on apples harvested on two consecutive picks in year 2

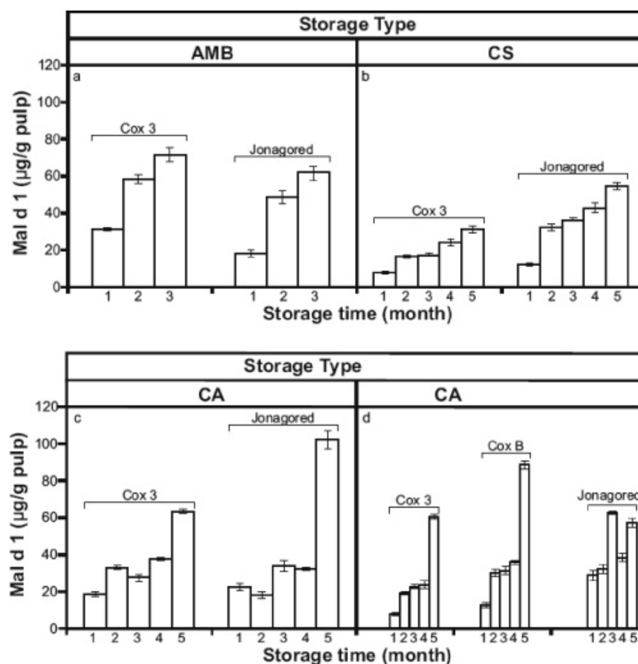

Figure 2. Effect of storage time (month 0, 1, 2, 3, 4, and 5) and cultivar (Cox orchard 3, and Jonagored) on Mal d 1 levels (μg/g pulp) in apples stored under AMB (**a**), CS (**b**), and CA (**c**), year 1 harvest. Effect of storage time and cultivar (Jonagored, Cox orchard 3, and B) on Mal d 1 levels in apples harvested in year 2 and stored under CA (**d**).

Table 4. Mal d 1 Concentration (μg/g Pulp) in Year 2 in Apple Pulp Determined by ELISA[a]

		Mal d 1 (μg/g pulp)			
		time of picking			
cultivar	storage time (months) under CA conditions	1	SD	2	SD
Cox orchard 3	0	NA		NA	
	1	7.1	0.7	8.1	0.8
	2	16.5	0.6	21.5	0.2
	3	20.6	0.6	23.3	0.8
	4	22.4	1.7	25.2	1.5
	5	61.2	1.3	60.2	1.9
Cox orchard B	0	NA		NA	
	1	12.9	0.9	12.1	1.7
	2	29.2	2.3	30.3	2.0
	3	31.0	2.2	31.0	1.2
	4	32.7	0.4	38.6	0.6
	5	92.2	1.9	85.5	2.4
Jonagored	0	17.2	1.5		
	1	28.8	2.5		
	2	32.3	2.2		
	3	63.0	0.8		
	4	38.2	2.1		
	5	57.6	2.2		

[a] Fruits from cv. Cox 3 and B were harvested on September 10 (pick 1) and on September 17 (pick 2), and cv. Jonagored was harvested on October 24. Apple fruits were stored under CA conditions for up to 5 months; NA, not available.

(**Table 4**). ANOVA analysis (**Table 3, year 2**) showed no significant differences for pick time and for any of its interaction with the other parameters.

The interaction between the two different harvest years and storage time (up to 5 months under CA conditions) on Mal d 1 levels was determined in cvs. Cox orchard 3 and Jonagored. Storage time had a significant effect on Mal d 1 levels in apples from both cultivars ($p = 0.0000$) whereas the effect of harvest year was cultivar-dependent ($p = 0.0138$). Thus, Mal d 1 levels in apples harvested in year 1 were higher than in year 2 for cv.

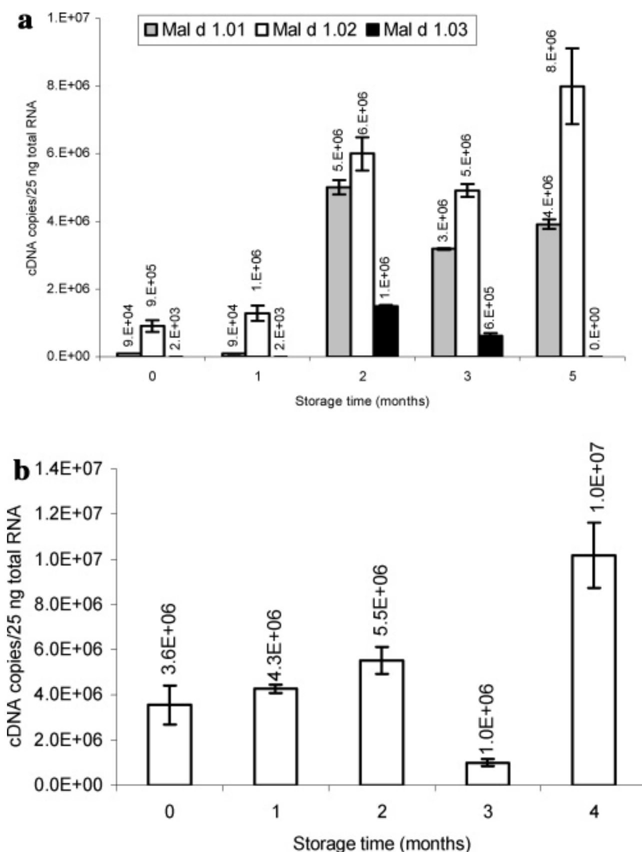

Figure 3. Quantification of Mal d 1 isoform transcripts in apple pulp (Mal d 1.01, Mal d 1.02, and Mal d 1.03) (**a**) and peel (Mal d 1.02) (**b**) (cv. Jonagored) by Real-time-Q PCR. Results are expressed as cDNA copies per 25 ng total RNA. Values represent the mean and SD of two measurements.

Cox 3; the opposite effect was observed for cv. Jonagored. The observed difference in cv. Jonagored data over storage time for the 2 years (**Figure 2**) could not be statistically investigated since restricting data to just the Jonagored apples is not enough to fit a model to test the interaction between year and storage time. Therefore, we cannot assess whether the difference is a significant effect.

Effect of Storage Conditions on Expression of Mal d 1 Transcription. Complimentary studies were undertaken using peel and pulp from fruits of cv. Jonagored harvested in year 2 and storage for up to 5 months under CA conditions, to study the expression of Mal d 1 at a transcriptional level. No mRNA could be extracted from apple pulp for month 4 or apple peel for month 5; hence, no analysis could be performed on these samples. The expression profile of the Mal d 1 isoforms Mal d 1.01 (accession no. AJ417551), Mal d 1.02 (accession no. AF020542), and Mal d 1.03 (accession no. AY186248) in apple pulp was investigated by real-time PCR using isoform-specific primers. The three Mal d 1 isoforms were constitutively expressed over the storage period (**Figure 3a**), Mal d 1.02 being the most abundant, Mal d 1.01 showing intermediate levels of expression, and Mal d 1.03 having the lowest levels of expression (**Figure 3a**). This ranking of isoform expression is in agreement with data published on several cultivars (*13*, *17*, *21*). An increase of the Mal d 1 transcripts was observed during storage, the highest values being obtained after 2 and 5 months of storage. In contrast, Mal d 1.02 is the most abundant isoform in apple peel (*17*) but the effect of its expression in peel during storage has never been reported. Real-time PCR analysis showed that this isoform was also continually expressed over the storage period in peel showing an overall increase during storage expression being highest on month 4 (**Figure 3b**).

DISCUSSION

A competitive ELISA was developed using a monoclonal antibody, BIP-1, directed to Bet v 1, the major birch pollen allergen. BIP-1 binds to at least nine isoforms of Bet v 1 (*26*), which is thought to recognize discontinuous (i.e., conformational) epitopes on the molecule (*28*). BIP-1 also cross-reacts with Mal d 1 in immunoblots (*28*). This assay was shown to be effective at determining Mal d 1 levels in apple pulp. Among the 10 apple cultivars studied, Mal d 1 levels varied between 3.8 and 72.5 μg/g pulp.

Apple ripening is associated with the onset of ethylene biosynthesis, which influences the subsequent softening and storage capability of the fruit. Thus, postharvest treatments such as storage are commonly used to slow down the softening process and to delay the onset of senescence by reducing the respiration rate of the fruit and the enzymatic activity, increasing the storage life of apples, and maintaining fruit availability for as long as economically desirable. Previous studies have shown that apple maturity affects Mal d 1 expression at a translational level in some cultivars and very weakly in others (*29*). Our analysis indicated that cv. Cox is a variety in which Mal d 1 levels are unaffected by harvesting time. Mal d 1 content increases in all cultivars and orchards during storage under three different conditions (AMB, CS, or CA) used in this study over a 5 month period, and the type of storage affected Mal d 1 in a cultivar-specific manner. The effect of storage under CA on apple allergenicity has been studied by Bolhaar et al. (*19*). They observed a moderate reduction (15%) in allergenicity as compared to apple stored without CA, which was only significant when all cultivars were analyzed together. This indicates the complexity of assessing apple allergenicity, as factors such as individuals variation and prick-to-prick factors have to be taken into consideration as well as allergen levels. These prick-to-prick factors might be the pricking position and the depth of the needle into the apple and subsequently into the patient's underarm skin, the amount of apple allergen taken up by the needle and transferred into the skin, differences between individual apples fruits from the same cultivar batch, variation in skin reactivity over the individual patient's arm, and variation of skin reactivity among individual patients.

Our data suggest that plant genetic factors may play an important role on apple allergenicity. However, environmental effects, such as growing site, may well be part of the complex regulation as indicated by greater Mal d 1 levels for cv. Cox orchard B as compared to those of orchard 3. The lack of correlation between Mal d 1 detected by ELISA and total apple protein seems to indicate specific alterations in Mal d 1 gene expression. Real-time PCR analysis confirmed this showing that the changes in Mal d 1 levels resulted from an up-regulation in gene expression through increased transcription.

Studies on the levels and pattern expression of Mal d 1 isoform at different storage conditions over a period are sparse. Beuning et al. (*17*) reported no changes of Mal d 1-related mRNA in fruits (cv. Cox's Orange Pippin) but an increase for other cultivars (including Golden Delicious) stored at 20 °C for 2 weeks, in contrast to the conditions used in our study, which are relevant to commercial practices. Our results demonstrated the expression of the isoforms Mal d 1.01; Ma l d 1.02, the most abundant in pulp (*17*); and Mal d 1.03, in freshly picked apples. With storage, temporal changes in expression of each of the isoforms were apparent. There was a sharp

increase in mRNA levels after 2 months of storage followed by a sharp decrease after 3 months of storage although these levels increased again at month 5. This cyclical pattern of expression is also mirrored at the translational level although repression at the mRNA level coincided with higher levels of the allergen being present (and visa versa). This would suggest the presence of a feedback mechanism, which could involve the protein itself whereby expression is maintained at levels that are nontoxic but sufficient in order for it to carry out its biological role (*30*). The same mechanism was observed for Ma l d 1.02 in peel. Variations on the expression of any of the Mal d 1 isoforms may account for the variability of allergenic potency of apple cultivars, which suggests that genetic factors have a major role in controlling the Mal d 1 allergenicity in mature apples. In addition, Mal d 1 expression appears to be up-regulated upon biotic stress as shown by Puehringer et al. (*16*) in leaves; however, there are no studies on fruits. Therefore, different tissues might respond differently to the stimuli and Mal d 1 genes may also be under control of different promoters. The control of gene expression for Mal d 1, which belongs to the pathogenesis-related (PR) family 10, appears to be different to the nonspecific lipid transfer protein Mal d 3, belonging to the PR 14 (*14*), since stored apples contained the lowest levels of Mal d 3 (*31*). Several approaches such as selection of low-allergenic cultivars (*19*), side-directed mutagenesis (*32*), and gene silencing (*33*) have been developed to reduce Ma l d 1 content and hence allergenicity of apples. Future studies into the way in which Mal d 1 gene expression is controlled may allow the development of new approaches to reduce allergen levels, through selection of cultivars where Mal d 1 expression is not up-regulated during storage.

Although a threshold dose has not been determined, our data suggest that individuals with Bet v 1-related fruit allergies might minimize their symptoms by selecting apple varieties with low allergen content and by eating freshly picked apples, which have significantly lower levels of allergens as compared to stored fruits.

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