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Maturity and Storage Influence on the Apple (*Malus domestica*) Allergen Mal d 3, a Nonspecific Lipid Transfer Protein

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Consumption of apples can provoke severe allergic reactions, in susceptible individuals, due to the presence of the allergen Mal d 3, a nonspecific lipid transfer protein, found largely in the fruit skin. Levels of Mal d 3 were determined in peel as a function of apple cultivar, position of the fruit growing on the tree, apple maturity, and postharvest storage by ELISA. As the apples mature, Mal d 3 levels increased, although the rate was dependent on cultivar and tree position. During storage, levels of Mal d 3 decreased in all cultivars (cvs. Cox, Jonagored, and Gala), the rate of overall decrease being greatest under controlled atmosphere conditions. There was no correlation between Mal d 3 levels and total apple peel protein, indicating specific alterations in Mal d 3 expression. Thus pre- and postharvest treatments (i.e., storage) can modify the allergen load in apple peel, the highest levels being found in overly mature and freshly harvested fruits.

KEYWORDS: Apple; allergens; nonspecific lipid transfer protein; Mal d 3; cultivar; storage; maturity; nsLTP; Mal d 3 expression

INTRODUCTION

Consumption of fruits and vegetables forms part of a healthy diet, although they pose a risk to certain sectors of the population of triggering allergic reactions (*1*). In Mediterranean countries, patients allergic to Rosaceae fruits (such as apple, peach, apricot, plum, and cherry), but not sensitized to birch pollen, react to a nonspecific lipid transfer protein (nsLTP) present in these fruits (*2*, *3*), presenting mild to severe (including life-threatening) symptoms (*4*).

nsLTPs are ubiquitous proteins widely distributed throughout the plant kingdom (*5*) and classified as plant panallergens (*3*). They have been identified in apple, where it is referred to as Mal d 3 (*3*), and in other fruits of the Rosaceae family such as peach (*6*), apricot (*7*), plum (*8*), and cherry (*9*). Clinical cross-reactivity between nsLTPs from botanically unrelated plant-derived foods has also been reported (*10*). These proteins have been implicated in plant defense from pathogens and environmental stress (*5*, *11*) belonging to family 14 of the pathogenesis-related proteins (*12*). They are also thought to participate in the transport of suberin monomers during cutin synthesis (*13*). These biological roles are consistent with their accumulation in the outer epidermal layers of plants (*5*), which is in agreement with the stronger allergenicity of peels with respect to pulps in Rosaceae fruits in individuals sensitized to nsLTPs (*14*).

Apple fruit quality is greatly influenced by both the maturity at the time of picking and postharvest processing (*15*, *16*). Time and temperature, during storage, have a great impact on firmness, in particular, and on the postharvest shelf life of the fruit in general (*16*). Preliminary data on the occurrence of the Bet v 1 superfamily member Mal d 1, the major allergen found in apple flesh (*17*), and its in vitro and in vivo IgE binding potency show that this allergen is influenced by the cultivar, the degree of maturity, and storage conditions of the fruits (*18*, *19*). In contrast, the allergenic potential of mango fruits, another climacteric fruit, remains unchanged during ripening for all of the strains studied (*20*). However, the effect of those factors on the levels and allergenicity of the apple nsLTP (Mal d 3) has not been determined. In this paper, we describe the occurrence of the allergen Mal d 3 as a function of apple cultivar, maturity, and postharvest storage with a view to reducing allergen loads in food by optimizing postharvest handling (storage, etc.) of fruits.

MATERIALS AND METHODS

Apple Fruits. Apple fruits were provided by Norfolk Fruit Growers (Hoveton, Norfolk, U.K.) for two consecutive years. In 2001 (year 1), cv. Cox's Orange Pippin apples from three orchards were harvested

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on Sept 22 (orchard 3) and Sept 25 (orchard 49) and on Oct 2 (orchard 9). Cvs. (Royal) Gala and Jonagored apples were harvested on Oct 5 and 11, respectively. Immediately after harvest, fruits were either treated with fungicide (Ridomil MBC, FRAG-UK, York, U.K.) at half label strength according to the manufacturer's instructions or left untreated. Different storage conditions were applied with samples stored under ambient conditions (AMB), cold (2 °C) with no controlled atmosphere (CS), or controlled atmosphere conditions (CA) (<0.5% CO_2 and 1.25% O_2 at 3.8 °C) for up to 5 months. Six apple fruits per sample 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 each of the four consecutive pickings six fruits were selected from each tree. Picking dates were as follows: pick 1, Sept 6 (too early for storage); pick 2, Sept 10 (optimal for long storage); pick 3, Sept 17 (optimal for short storage); and pick 4, Sept 25 (near eating ripe). Fruits were also picked from the upper and lower positions of the tree and the sunny and shady sides. Fruits were treated with fungicide, and those from pick 2 were stored under CA for up to 5 months as described for year 1. For comparison, apple fruits (cv. Jonagored) were harvested (Oct 24) at Orford (U.K.) and stored under controlled atmosphere as for cv. Cox.

Apple Extracts. Apples were peeled (3 mm) using an automatic apple peeler (Lakeland Limited), and the peel was frozen in liquid nitrogen. Frozen peel (30 g) was homogenized in 23 mM Na_2PO_4 buffer (50 mL, pH 7.0) containing 0.8 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 10 mM sodium diethyldithiocarbamate, and 4% (w/v) polyvinylpolypyrrolidone (PVPP), for 2 min in a Waring blendor (Waring Commercial, Hartford, CT). After 20 min of stirring at room temperature, the extract was centrifuged at 17000*g* for 3 min, and the resulting supernatant was filtered through a Millex-HA 0.45 μm syringe filter (Millipore, Millex-HV, Bedford, MA). Apple extracts were stored in aliquots at −40 °C. nsLTP from apple (Mal d 3) (cv. Golden Delicious) was purified according to the method of Sancho et al. (*21*).

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

SDS-PAGE and Western Blotting. Apple peel extracts (cvs. Jonagored and Cox, orchard 3; 80 μg of protein) and purified Mal d 3 (0.4 μg of protein) were separated by SDS-PAGE under reducing conditions using a 10% Bis-Tris gel in a NuPAGE system (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. Proteins were visualized by SilverXpress silver staining (Invitrogen).

After electrophoresis, proteins were transferred onto a 0.2 μm nitrocellulose membrane (Sartorius, Gottingen, Germany) using the trans-blot SD semidry electrophoretic transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA). Blots were blocked with PBST [0.05% v/v Tween-20 in phosphate-buffered saline (PBS)] containing 5% (w/v) skimmed milk powder for 1 h at room temperature. After washing with PBST, blots were incubated with polyclonal rabbit antibody serum raised against Mal d 3 (Dr. Laurian Zuidmeer, Sanquin, The Netherlands) diluted 1:1000 (v/v) in PBST, for 1 h at room temperature. After washing, specific IgG binding was detected with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma) diluted 1:1000 (v/v) in PBST for 1 h at room temperature. Blots were washed five times with PBST and stained with BCIP/NBT buffered substrate tablets (Sigma).

Enzyme-Linked Immunosorbent Assay (ELISA). Polystyrene microtiter plates (Nunc-Immuno plate, Nalge Nunc International, Roskilde, Denmark) were coated with 200 μL of Mal d 3 at 0.2 μg/mL in PBS overnight at 1 °C. After washing with PBST (0.05% v/v Tween-20 in PBS), samples (100 μL diluted in PBST) were incubated with 100 μL of polyclonal rabbit antibody serum raised against Mal d 3 diluted 1:20000 (v/v) in PBST for 2.5 h at 20 °C. After five washings, plates were incubated with 200 μL of goat anti-rabbit IgG labeled with horseradish peroxidase (Sigma) diluted 1:1000 (v/v) in PBST overnight at 1 °C. Following a final washing step, 200 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Biovet) 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, U.K.). Amounts of LTP in apple extracts were quantitatively determined on the basis of the standard curve using GraphPad Prism software (GraphPad Software, Inc.). All determinations were run in triplicate, and Mal d 3 concentrations were expressed as micrograms of Mal d 3 per gram of peel wet weight.

To assess a possible matrix effect on the Mal d 3 ELISA assay, extracts were diluted up to 50 times in the buffer described for apple extract protocol. To assess the reproducibility of this protocol, triplicate apple extracts of cvs. Gala, Jonagored, and Cox (orchard 49) were prepared, and Mal d 3 levels were determined by ELISA. To study extract stability, an extract was prepared, aliquoted, and stored at −20 °C for up to 15 days.

Statistical Analysis. Analysis of variance (ANOVA) models were employed to examine the relationships between Mal d 3 levels and the various factor-explanatory variables. Standard regression diagnostics indicated that these models were appropriate for the data; that is, there was no need to use nonparametric models. 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

Apple Extract Characterization. Mal d 3 is located predominantly in the epidermis of the apple fruit (*17*), showing a distribution pattern different from that of other apple allergens. In addition, Fernandez-Rivas and Cuevas (*14*) observed that apple peel has a higher clinically relevant allergenicity than pulp. Consequently, only apple peel was used for allergen determination. SDS-PAGE analysis of protein extracts of apple peel from two cultivars (cvs. Jonagored and Cox, orchard 3) showed a similar protein pattern for both cultivars (**Figure 1a**). A major dominant polypeptide of $M_r \sim 9000$ was observed with a complex mixture of less abundant polypeptides running with M_r 18000−70000. The major polypeptide runs with a slightly larger M_r than that of purified apple nsLTP (**Figure 1a**, track 3). Thus, its identity was confirmed by immunoblotting using a rabbit polyclonal anti-Mal d 3 antibody, which recognized only the M_r 9000 polypeptide in the apple extract, and the purified Mal d 3. In addition to confirming the identity of the M_r 9000 polypeptide as Mal d 3, the data also show the antibody recognized only Mal d 3 in the apple extracts, demonstrating its specificity (**Figure 1b**).

This antibody was then used to develop an indirect competitive ELISA using purified Mal d 3 as both the ELISA solid phase and a calibrant. A typical calibration curve is represented in **Figure 2a** and shows the ELISA had a working range from 0.03 to 2.5 μg/mL and a IC_{50} of 0.29 μg/mL. When extracts were diluted up to 40-fold, a linear dose−response was observed for Mal d 3, indicating the lack of matrix effect other than at very high or very low dilutions (**Figure 2b**). Subsequently, all extracts were diluted between 10- and 30-fold. No significant differences were observed in Mal d 3 levels when triplicate extracts were prepared and assayed by ELISA (**Figure 2c**). No variation in the allergen levels for up to 10 days was observed after the extract had been stored at −20 °C (**Figure 2d**).

Between 0.2 and 1.5 mg of Mal d 3/apple could be detected in the apples used in this study, as compared to 0.3−10 mg of Mal d 1/apple in the same apples (results not shown).

Effect of Apple Maturity on Mal d 3 Levels. The concentration of Mal d 3 in the fruit peel at harvesting time in year 1 was determined by ELISA and found to be greater in cv. Cox from orchard 3 (70.2 μg/g of peel), orchard 9 (58.8

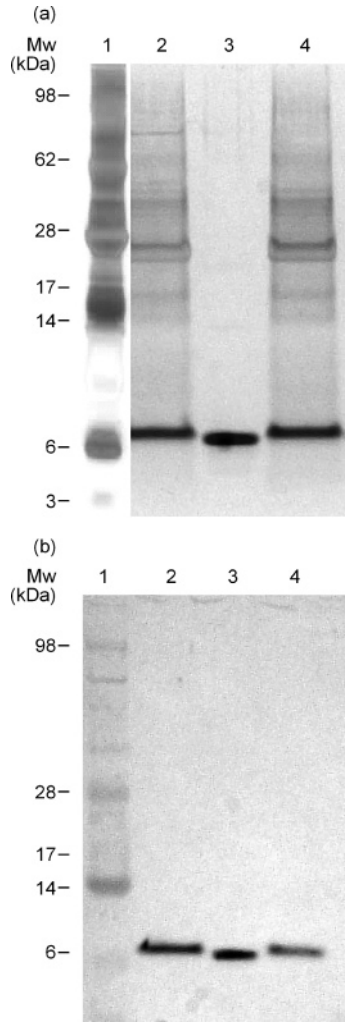

Figure 1. SDS-PAGE (**a**) and immunoblot analysis with a rabbit polyclonal anti-Mal d 3 antibody (**b**) of apple extracts from cv. Jonagored (lane 2), Cox (orchard 3, lane 4), and purified Mal d 3 (lane 3). Molecular weight markers (lane 1) were insulin (B chain) (M_r 3000), aprotinin (M_r 6000), lysozyme (M_r 14000), myoglobulin (M_r 17000), carbonic anhydrase (M_r 28000), BSA (M_r 62000), and phosphorylase (M_r 98000).

μg/g of peel), and orchard 49 (58.5 μg/g of peel) compared to either cv. Jonagored or cv. Gala (~31 μg/g of peel) as determined by ELISA (**Table 1**). Similarly, cv. Cox, orchard B, apples harvested at their mature time (picking times 2 and 3) in year 2 had higher Mal 3 concentration (~77 μg/g of peel) compared to fruits from cv. Cox, orchard 3 (~48.5 μg/g of peel), and cv. Jonagored (18.5 μg/g of peel) (**Table 2**).

The effect of position on the tree of the fruit (cv. Cox, orchards B and 3) (sunny versus shady side and upper versus lower part) and its maturity (picking date) in year 2 on Mal d 3 levels was investigated by ANOVA. This analysis showed that the two-way interactions picking time versus orchard and picking time versus apple position and apple height (lower/upper) were the main effect and significantly affected Mal d 3 levels (**Table 3**). Allergen levels increased as apples matured, being orchard dependent. This increase was sharper for cv. Cox between picks 3 and 4 (2-fold) at orchard 3 compared to orchard B (1.2-fold) (**Figure 3a**). In the same way, Mal d 3 levels were increased 2-fold in apples harvested from the shady site of the tree compared to the sunny site (1.4-fold) between picks 3 and 4 (**Figure 3b**). On average, apples picked from the lower part of the tree had ≈12 μg of Mal d 3/g of peel more than apples from the upper part of the tree (**Table 2**).

Effect of Storage on Mal d 3 Levels. All Mal d 3 analyses for apple fruits (cvs. Cox, orchards 3, 9, and 49; Gala; Jonagored) harvested in year 1 and stored for up to 5 months in either ambient, cold, or controlled-atmosphere conditions (either treated or untreated with fungicide) were evaluated by ANOVA (**Table 4**). This analysis showed that the two-way interactions storage time versus variety and storage time versus storage type significantly affected Mal d 3 levels. Cv. Cox apples from orchards 3, 9, and 49 were treated as separate varieties for statistical purposes. Fungicide treatment and the interaction between variety and storage type did not affect Mal d 3 levels. An initial increase in Mal d 3 levels was observed during the first month of storage for all of the cultivars and conditions: AMB (**Figure 4a**), CS (**Figure 4b**), and CA (**Figure 4c**). Subsequently, the Mal d 3 concentration decreased over the storage period, although the rate of decrease was cultivar-dependent, with a steeper decrease during the first 2 months for cv. Cox compared to cvs. Gala and Jonagored. Mal d 3 concentration reached a similar level for all varieties at the end of the storage time. There was also a strong environmental effect as indicated by the different profiles observed between orchards 3, 9, and 49 for cv. Cox (**Figure 4a−c**). On average, Mal d 3 levels in cv. Cox, orchard 9, were greater than those in all of the other varieties (including the other two cv. Cox orchards).

Apples stored under CA showed a steadier but also a greater overall decrease (~3-fold) of Mal d 3 levels than fruits stored under CS or AMB conditions (~2-fold) (**Figure 4c**). Data collection on apples stored in ambient condition was not possible after 3 months due to initial onset of senescence. Apples harvested in year 2 and stored under CA conditions showed an overall decrease in Mal d 3 levels for all of the cultivars and confirmed the different profiles of cvs. Cox and Jonagored (**Figure 4d**) observed in year 1. This decrease was greater for cv. Cox, orchard B (from 77.5 to 22 μg/g of peel) compared to orchard 3 apples (from 48.5 to 13 μg/g of peel) and cv. Jonagored (from 20 to 7 μg/g of peel).

Pearson's product−moment correlation was calculated to determine any linear relationship between Mal d 3 content and total extractable protein over the 5 month storage period. No significant correlation was found at $p < 0.05$ for cv. Jonagored ($r = 0.1308$; p value $= 0.6853$) and cv. Cox 3 ($r = -0.3044$; p value $= 0.2193$).

The effect of harvest year and storage time (up to 5 months under CA conditions) on Mal d 3 levels was determined in cvs. Cox, orchard 3, and Jonagored. For cv. Jonagored, storage time but not year had a weakly significant effect ($p = 0.0947$), whereas for cv. Cox, orchard 3, both factors were significant ($p = 0.0214$ for storage time and $p = 0.0022$ for year).

DISCUSSION

Mal d 3 levels increased as apple fruits reached their physiological maturity, initially being slow followed by a sharp increase at the end of maturity (pick 4) when apples are suitable for immediate consumption but are too ripe for storage. This sudden rise might coincide with increased respiration activity (climacteric) of the fruit and the onset of ethylene biosynthesis, which initiates ripening, influencing the subsequent softening and storage capability of the fruit in a cultivar-dependent manner (*15*). Thus, cv. Gala produces very little ethylene during maturity and storage, resulting in a slower rate of softening (*23*) as compared to cv. Cox (*24*). Interestingly, our results showed a lower Mal d 3 concentration for cv. Gala compared to cv. Cox. Although ethylene control of Mal d 3 gene expression has not been described, there have been several reports on other plants'

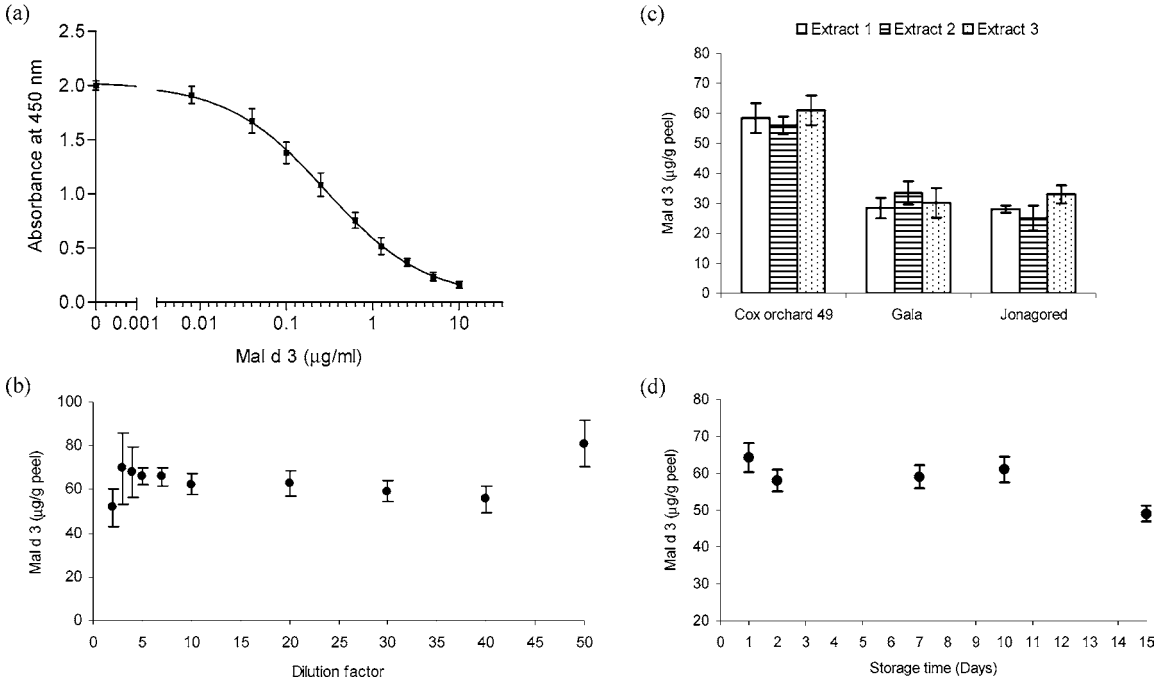

Figure 2. Standard curve of anti-Mal d 1 polyclonal antibody in indirect competitive ELISA assay (**a**) and study of the matrix effect (**b**), reproducibility (**c**), and stability (**d**) of the apple extract preparation as determined by ELISA.

Table 1. Mal d 3 Concentrations in Year 1 in Apple Peel Determined by ELISA[a]

cultivar	storage conditions	fungicide	Mal d 3 (µg/g of peel) after storage for 0 months	SD	1 month	SD	2 months	SD	3 months	SD	4 months	SD	5 months	SD
Cox, orchard 3	AMB	undrench	70.2	5.4	83.0	1.9	55.1	5.6	23.8	1.9	na		na	
		drench	70.2	5.4	99.7	4.6	43.6	5.0	23.8	2.6	na		na	
	CS	undrench	70.2	5.4	80.3	7.2	40.2	6.8	40.0	3.6	48.6	6.1	18.5	2.0
		drench	70.2	5.4	81.1	4.4	33.4	4.4	35.6	4.9	45.1	8.7	17.8	1.3
	CA	undrench	70.2	5.4	83.1	6.8	38.7	5.7	59.8	6.4	41.6	4.1	16.5	9.1
		drench	70.2	5.4	70.0	6.3	54.7	9.0	60.1	3.4	55.0	3.9	24.3	5.8
Cox, orchard 9	AMB	undrench	58.8	0.3	121.1	8.1	54.0	7.8	33.9	1.3	na		na	
		drench	58.8	0.3	100.6	0.9	74.9	6.8	55.7	4.4	na		na	
	CS	undrench	58.8	0.3	100.4	5.0	76.3	7.0	48.5	1.4	59.7	6.4	33.6	0.9
		drench	58.8	0.3	92.7	6.8	61.9	7.0	46.5	9.3	51.1	7.2	45.6	7.6
	CA	undrench	58.8	0.3	58.2	1.3	48.5	8.5	68.6	3.2	72.3	9.1	41.5	6.3
		drench	58.8	0.3	65.9	1.9	65.6	9.7	70.1	5.2	78.0	5.0	29.8	2.0
Cox, orchard 49	AMB	undrench	58.5	4.5	94.6	3.2	9.1	1.1	27.4	1.8	na		na	
		drench	58.5	4.5	83.4	4.3	6.6	0.1	26.1	1.9	na		na	
	CS	undrench	58.5	4.5	103.0	1.4	10.2	1.5	25.8	4.7	42.3	9.6	30.3	4.4
		drench	58.5	4.5	81.6	8.7	7.4	0.1	43.1	2.2	53.2	2.1	30.0	3.3
	CA	undrench	58.5	4.5	80.8	0.2	74.6	7.0	33.0	3.8	35.8	3.1	5.7	0.7
		drench	58.5	4.5	64.3	2.9	67.9	6.4	37.3	6.3	25.6	2.7	6.6	4.9
Jonagored	AMB	undrench	30.4	2.1	64.2	3.3	45.4	8.4	38.7	4.2	na		na	
		drench	30.4	2.1	52.5	7.8	37.9	2.8	58.9	5.3	na		na	
	CS	undrench	30.4	2.1	47.2	3.4	61.3	3.6	61.2	1.3	18.6	1.2	32.1	3.6
		drench	30.4	2.1	42.2	4.4	63.0	6.5	63.3	3.7	21.1	0.8	30.7	4.5
	CA	undrench	30.4	2.1	64.1	1.2	58.4	2.1	48.7	2.5	11.5	1.1	15.6	0.6
		drench	30.4	2.1	54.4	0.2	69.0	4.7	28.0	1.1	11.3	1.2	15.1	1.6
Gala	AMB	undrench	31.3	0.4	36.1	5.8	37.2	6.5	15.0	0.3	na		na	
		drench	31.3	0.4	34.8	2.9	22.2	0.8	28.4	0.4	na		na	
	CS	undrench	31.3	0.4	59.0	0.1	56.4	6.3	28.5	1.6	16.2	3.1	24.4	3.8
		drench	31.3	0.4	40.4	2.9	42.3	6.3	33.2	8.7	15.9	1.2	22.0	0.2
	CA	undrench	31.3	0.4	49.7	1.3	64.1	1.7	32.6	2.2	33.9	2.6	13.5	0.7
		drench	31.3	0.4	48.7	0.5	67.1	2.3	23.6	2.6	26.0	3.6	15.0	0.9

[a] Immediately after harvest, fruits were either treated with fungicide (drench) or left untreated (undrench). Apple fruits were stored under ambient conditions (AMB), cold with no controlled atmosphere (CS), or controlled atmosphere conditions (CA) for up to 5 months. na, not available.

nsLTPs responding to this regulator. Thus, two of the three nsLTP genes in pepper (*Capsicum*) were up-regulated by ethylene treatment (*25*). No changes were observed in any of the LTP mRNAs from barley leaves following ethylene treatment, although no studies on barley storage tissue have been reported (*26*).

The extracellular cuticle mainly allows the plant to reduce water loss by increasing cuticle thickness and provides protection

Table 2. Mal d 3 Concentration in Year 2 in Apple Peel Determined by ELISA[a]

cultivar	tree position	storage time (months) under CA conditions	Mal d 3 (μg/g of peel) pick 1	SD	pick 2	SD	pick 3	SD	pick 4	SD
Cox, orchard 3	lower/sunny	0	36.0	1.9	37.5	7.7	57.7	4.0	130.2	5.6
	upper/sunny	0	43.4	4.4	29.3	5.2	62.0	5.6	93.5	5.8
	lower/shady	0	37.4	7.1	52.6	7.9	49.5	6.0	152.5	7.0
	upper/shady	0	35.8	8.6	51.5	3.5	45.8	9.2	128.2	9.1
	pool	1			40.8	9.7				
		2			31.6	5.5				
		3			39.4	6.0				
		4			22.8	4.5				
		5			12.7	2.8				
Cox, orchard B	lower/sunny	0	na		81.3	4.3	101.4	5.7	102.7	3.6
	upper/sunny	0	na		66.6	3.1	76.2	7.3	78.2	0.6
	lower/shady	0	na		63.9	5.0	87.5	2.5	115.7	5.4
	upper/shady	0	na		62.8	6.3	75.3	8.0	107.3	3.8
	pool	1			52.6	6.0				
		2			47.5	1.9				
		3			49.8	7.0				
		4			30.4	3.0				
		5			21.8	5.4				
Jonagored	pool	0			18.5	0.5				
		1			36.9	2.7				
		2			37.6	5.2				
		3			25.9	2.6				
		4			28.0	1.2				
		5			7.3	1.0				

[a] Fruits were picked at four consecutive times: pick 1, Sept 6, (too early for storage); pick 2, Sept 10 (optimal for long storage); pick 3, Sept 17 (optimal for short storage); and pick 4, Sept 25 (near eating ripe). Fruits were also picked from the upper and lower positions of the tree and the sunny and shady sides. Apple fruits were treated with fungicide, and those from the second picking stored under CA conditions for up to 5 months. na, not available.

Table 3. ANOVA of Mal d 3 Levels (Micrograms per Gram of Peel) Measured in Apple Fruit Harvested in Year 2[a]

	Df	sum Sq	*F* value	Pr (>*F*)
orchard	1	599.8	6.54	0.0228
time of picking	2	15203.0	82.87	1.739e-08
tree position 1: sunny vs shady	1	297.3	3.24	0.0933
tree position 2: upper vs lower	1	903.4	9.85	0.0072
orchard vs picking time	2	4191.6	22.85	3.900e-05
picking time vs tree position 1	2	1376.8	7.50	0.0061
residuals	14	1284.1		

[a] Functions analyzed were the following: orchard, time of picking, tree position (sunny vs shady sides and branch height upper vs lower). Degrees of freedom (Df) value indicates the number of quantities that must be estimated to define the effect of the variable. Sum of squared error (sum Sq) is a measure of how much of the variance in the response is explained by the variable. *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 <0.05, this assumption is rejected; therefore, the variable is significantly related to the response.

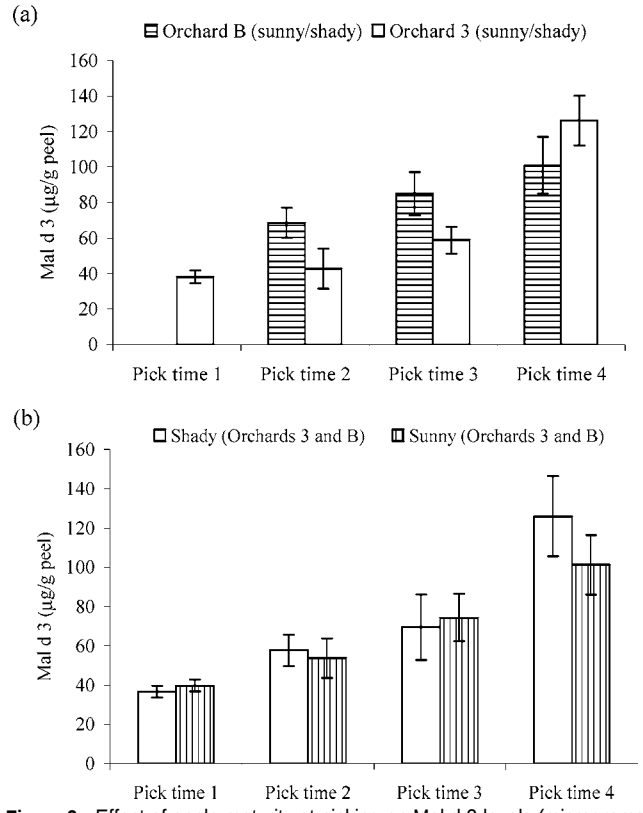

Figure 3. Effect of apple maturity at picking on Mal d 3 levels (micrograms per gram of peel) showing environmental effects of growing site (**a**) and tree position (**b**). Apple fruits were harvested in year 2 and Mal d 3 levels determined by ELISA. Pick 1, too early for storage; picks 2 and 3, optimal for long storage; pick 4, too late for storage and near eating ripe.

against infection. When reaching the climacteric, free fatty acids, one of the main components of the wax layer, are no longer utilized for the formation of this layer in apple fruit, leading to their accumulation in the cuticle (*27*). Although the role of Mal d 3 on cuticle formation has not been established, Douliez et al. (*13*) have shown that a nsLTP from barley is capable of binding two molecules of ω-hydroxypalmitic acid, one member of a family of cutin monomers. If Mal d 3 is involved in cutin formation, it might explain its accumulation at the end of maturity and its localization in the epidermal tissues of apple fruits.

Cutin biosynthesis is generally stimulated by pathogen infection, which might induce pathogenesis-related proteins and particularly nsLTPs (*5*, *12*). Thus, Molina and Garcia-Olmedo (*26*) described a 9-fold increase of mRNA levels of three barley nsLTPs 12 h after fungal pathogen infection. The effect of

Table 4. ANOVA of Mal d 3 Levels (Micrograms per Gram of Peel) Measured in Apple Fruit Harvested in Year 1[a]

	Df	sum Sq	*F* value	Pr (>*F*)
storage time	4	29407.8	34.24	0.0000
apple cultivar	4	12246.5	14.26	0.0000
storage type	2	391.7	0.91	0.4054
storage time vs apple cultivar	16	10840.9	3.16	0.0003
storage time vs storage type	6	4128.9	3.21	0.0068
residuals	89	19108.9		

[a] Functions analyzed were the following: storage time and type, fungicide treatment, and apple cultivar (abbreviations as for **Table 3**).

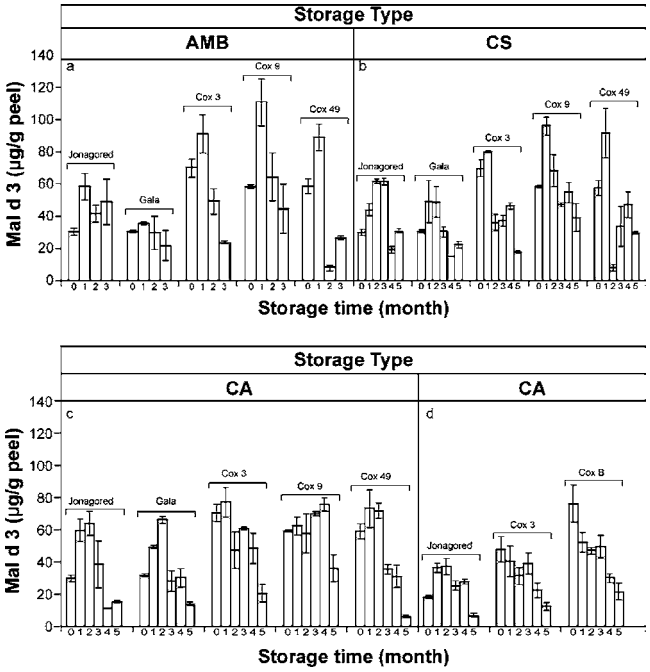

Figure 4. Effect of storage time (0, 1, 2, 3, 4, and 5 months) and cultivar (Jonagored, Gala, Cox, orchards 3, 9, and 49) on Mal d 3 levels (micrograms per gram of peel) in apples stored under ambient (AMB) conditions (**a**), stored cold with no controlled atmosphere (CS) (**b**), and stored under controlled atmosphere (CA) (**c**), year 1 harvest. Effect of storage time and cultivar (Jonagored, Cox, orchards 3 and B) on Mal d 3 levels in year 2 (**d**). Apples were stored under controlled atmosphere.

pathogen on expression of apple nsLTP (Mal d 3), a member of family 14 of pathogenesis-related proteins, has yet to be demonstrated, but our results showed that fungicide treatment did not affect the levels of Mal d 3.

In addition, nsLTP expression due to abiotic stress has been described. Certain nsLTP genes are induced by drought in tomato (*28*) and pepper (*25*) but not in barley (*26*); by salinity in tomato (*29*), barley (*26*), and pepper (*25*); and by wounding in pepper (*25*) but not in barley (*26*). No response to cold stress has been observed in barley nsLTP (*26*). According to these studies, it is likely that Mal d 3 is expressed by abiotic stresses, explaining the variation and different profile observed between orchards.

Apples are harvested commercially just as they begin to ripen and then stored for several months to maintain availability for as long as economically desirable. Storage under CA has become an important means of slowing the softening process controlled by ethylene biosynthesis and delaying the onset of senescence by reducing the respiration rate of the fruit, the rate of ethylene production, and the enzymatic activity, increasing the storage life (shelf life) of apples (*16*). Our results demonstrate that the three storage conditions used in this study are efficient post-harvest treatments to reduce Mal d 3 content in all of the cultivars, especially for apples stored under CA, which showed an overall steadier and greater decrease. In contrast, Hsieh et al. (*19*) observed that another major apple allergen, the Bet v 1 superfamily member Mal d 1, located in the flesh, increased during storage at 4 °C in the three apple cultivars studied. Our data suggest differences in the control of gene expression for Mal d 1 and Mal d 3, the latter belonging to the pathogenesis-related (PR) 14 family, which responds to different stimuli to the PR10 family to which Bet v 1 and its homologues Mal d 1 belong (*12*). In apple fruit, two genes encoding mature nsLTP proteins have been identified and named Mal d 3.01 and Mal d 3.02 (*30*). Mal d 3.01 corresponds to the protein described by Pastorello et al. (*2*), but Mal d 3.02 has not yet been identified. The sequence variation between these two genes suggests their expression is independent (*30*) and might be subjected to different stimuli as seen for the three nsLTP genes from pepper (*Capsicum*) (*25*). Our results indicate that the expression of nsLTPs in apple fruits may be mediated by a complex mixture of genetic and environmental stimuli. Ethylene, a major ripening-hormone regulator in apple, may have a role as the signal to activate nsLTP gene expression, although this has yet to be demonstrated. Although the levels of Mal d 3 remaining in mature fruits at the end of storage are significantly lower than those in fresh fruits, it is unlikely that they are low enough to avoid triggering an allergic reaction in sensitized individuals. However, a greater understanding of how the expression of Mal d 3 is altered may allow the development of knowledge-based strategies for achieving the reductions necessary for effective low-allergen cultivars in the future.

ABBREVIATIONS USED

nsLTP, nonspecific lipid transfer protein; CS, cold storage; AMB, ambient storage; CA, controlled atmosphere; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

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