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Genetic and Environmental Factors Affecting Allergen-Related Gene Expression in Apple Fruit (*Malus domestica* L. Borkh)

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Freshly consumed apples can cause allergic reactions because of the presence of four classes of allergens, namely, Mal d 1, Mal d 2, Mal d 3, and Mal d 4, and their cross-reactivity with sensitizing allergens of other species. Knowledge of environmental and endogenous factors affecting the allergenic potential of apples would provide important information to apple breeders, growers, and consumers for the selection of hypoallergenic genotypes, the adoption of agronomical practices decreasing the allergenic potential, and the consumption of fruits with reduced amount of allergens. In the present research, expression studies were performed by means of real-time PCR for all the known allergen-encoding genes in apple. Fruit samples were collected from 15 apple varieties and from fruits of three different trials, set up to assess the effect of shadowing, elevation, storage, and water stress on the expression of allergen genes. Principal components analysis (PCA) was performed for the classification of varieties according to gene expression values, pointing out that the cultivars Fuji and Brina were two good hypoallergenic candidates. Shadowing, elevation, and storage significantly affected the transcription of the allergen-encoding genes, whereas water stress slightly influenced the expression of only two genes, in spite of the dramatic effect on both fruit size and vegetative growth of the trees. In particular, shadowing may represent an important cultural practice aimed at reducing apple cortex allergenicity. Moreover, elevation and storage may be combined to reduce the allergenic potential of apple fruits. The possible implications of the results for breeders, growers, and consumers are discussed critically.

KEYWORDS: Apple allergens; Mal d 1; Mal d 2; Mal d 3; Mal d 4; gene expression; apple cultivars; shadowing; elevation; storage; water stress

INTRODUCTION

Apples (*Malus domestica* L. Borkh) are an important worldwide commodity, and their consumption is highly recommended for a healthy diet because of their efficacy in reducing the risk of stroke, heart disease, and lung cancer (*1–3*). Unfortunately, apples can also represent an important cause of allergic reactions (*4*), both in children and adults, due to the existence of four main classes of allergens, with different clinical relevance according to the geographical area, namely, Mal d 1, Mal d 2, Mal d 3, and Mal d 4. Allergic reactions caused by Mal d 1, the major apple allergen, belong to class II food allergies (*5*), mainly affect the northern and central European population and are often associated with birch pollinosis due to cross-reactivity with Bet v 1 (*6*). Both are 17−18 kDa allergens belonging to the group 10 of pathogenesis-related proteins (PR-10), expressed in response to biotic and abiotic stresses, and likely to be involved in binding and transport of plant steroids (*7–12*). Mal d 1 accumulation in the fruit was shown to change according to the genotype, the ripening stage, and storage (*13–15*). Genetic mapping studies pointed out the existence of at least 18 genes encoding Mal d 1 isoforms (*16*), which can be subdivided into four subgroups: *Mal d 1.01*, *1.02*, *1.03*, and *1.04* (*17*).

Concerning Mal d 2-related allergy occurrence, little is known. Hsieh et al. (*14*) previously identified an apple TLP (Thaumatin-like protein) as a major allergen, and recently, a 23.2 kDa protein deduced from a full-length cDNA encoding a TLP was characterized as an antifungal protein and named Mal d 2 (*18*). This allergen is one of the most represented proteins in ripe apple fruit and is particularly resistant to protease and heat treatments because of its eight disulfide bonds formed by 16 conserved cysteines (*19, 20*). TLPs belong to the PR-5 family

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Table 1. Characteristics of 15 Apple Varieties Assessed in This Study

acronym	variety	group	origin	susceptibilities[a]	resistances[a]	commercial harvest
BN	Brina	autumn/winter variety	Italy		S, M, A	second−third week of September
BR	Braeburn "Hilwell"	winter variety	New Zealand	BP, BW, FL	S	first week of October
DB	DCA-BO-78-406-031	summer variety	Italy			third week of August, first week of September
DE	Delorina	autumn variety	Germany	M	S	second−third week of September
F9	FG-96-CIV	autumn/winter variety	Italy			third week of September, first week of October
FJ	Fuji "Kiku 8"	winter variety	Japan			first week of October
GD	Golden Delicious	autumn variety	U.S.	R		second week of September
GO	Golden Orange	winter variety	Italy	M	S, A	first week of October
GS	Granny Smith	winter variety	Australia	PD, S, M		second week of October
HK	Hapke Delicious	autumn variety	Canada	S		first week of September
I3	INRA-33-18	summer variety	France			third week of August, first week of September
MD	Morgenduft Dallago	winter variety	Italy			first−second week of October
RE	Resista	autumn/winter variety	Czech Republic		S	second−third week of September
RJ	Rajca	summer variety	Czech Republic	BP	S	third week of August, first week of September
VE	Vesna	autumn variety	Czech Republic		S	first−second week of September

[a] A: aphids; BP: bitter pit; BW: browning; FL: firmness loss during storage; M: mildew; PD: preharvest fruit drop; R: rust; and S: scab.

of pathogenesis-related proteins and are considered as a new class of panallergens in food and pollen (*7, 20*). Recent genetic mapping studies pointed out the existence of multiple copies of Mal d 2-encoding genes in the apple genome (*21*), namely, *Mal d 2.01*, *2.02*, and *2.03*, whereas no information is available about gene expression and protein accumulation in different varieties and/or upon different pre- and postharvest treatments. Expression data are available for peach, pointing out that such genes are specifically induced by ethylene, wounding, and abscission (*22*).

Mal d 3 is a nonspecific lipid transfer protein causing class I food allergies (*23*), prevalently in the Mediterranean area (*24*). Plant nsLTPs form large multigene families encoding 9 kDa proteins, with eight conserved cysteines forming four disulfide bonds, and are included in the PR-14 family. According to Kader (*25*), nsLTPs may have multiple biological functions in plants, also due to the presence of isoforms showing moderate levels of amino acid sequence identity and different gene expression patterns, as reported in peach by Botton et al. (*26*). Lipid transfer proteins are considered to be panallergens since they belong to a family of structurally highly conserved proteins also present in non-Rosaceae vegetable food (*27*). In apple, two genes encoding mature nsLTPs are present and are named *Mal d 3.01* and *3.02* (*28*). Recently, Sancho et al. (*29*) demonstrated that pre- and postharvest treatments can modify the LTP-related allergen load in apple peel. Changes in LTP accumulation in different varieties were recently demonstrated in apple and peach (*30, 31*).

Regarding Mal d 4-related allergy, a prevalence similar to that of Mal d 3 was frequently reported (*32*), being predominantly found in the Mediterranean area. These allergens belong to the profilin protein family and are also involved in allergic reactions to fruits of other species (*33–36*), with a strong cross-reactivity to the birch pollen profilin Bet v 2 (*32*). Profilins are small cytosolic proteins of 12−15 kDa, present in all eukaryotic cells. The main biological functions of profilins have been related to cell elongation, cell shape maintenance, flowering, seedling development, and pollen tube growth (*37–39*). Therefore, profilins play essential roles during plant development (*40*). In apple, the three gene-encoding profilin isoforms were mapped and named *Mal d 4.01*, *4.02*, and *4.03* (*21*). No information is available concerning gene expression and protein accumulation in different apple varieties and after pre- and postharvest treatments.

On the basis of the available information, the patterns of accumulation of Mal d 1 and Mal d 3 may vary according to the genotype and pre- and postharvest conditions. Previous research focused mainly on the protein levels of two allergens (*13, 14, 29, 30, 41*), and inconclusive information was supplied concerning the overall gene expression patterns and factors affecting the allergenic potential of apples. In the present paper, a comprehensive transcriptional profiling was performed for all the known apple allergen-encoding genes in different varieties characterized by diverse origins, resistance traits, and ripening times. The effect of shadowing, elevation, water stress, and storage was assessed in Golden Delicious apples by real-time PCR. Experimental findings and potential practical implications are discussed, emphasizing the main factors affecting apple's allergenic potential.

MATERIALS AND METHODS

Plant Material. The research was carried out on apples harvested for two subsequent growing seasons (2005 and 2006). However, since the results of the two seasons did not differ significantly, only data concerning 2005 are reported. The fruits of the 15 apple varieties reported in **Table 1** were all collected in Maso Part (Val d'Adige, Trento, Italy, 210 m a.s.l.). Fifty fruits were picked from five homogeneous trees of each variety at comparable ripening stages assessed through the Streif index suitable for commercial harvest. The Streif index was calculated as [firmness/(soluble solids × starch index)] (*42*). A cube of cortex of equal dimensions was excised from each fruit, immediately frozen in liquid nitrogen, and stored at −80 °C. The fruits of the shadowing trials were harvested in an experimental orchard at Revò (Val di Non, Trento, Italy, 800 m a.s.l.) from 20 homogeneous trees (cv. Golden Delicious/M9) divided into two groups. Ten of them were shadowed (Sh) from May (at full bloom) until harvest, by means of plastic nets decreasing the light radiation by ∼30%, and 10 were kept as a control under normal light (NL). The apples of the elevation (E) and storage (S) trials were harvested from 40 homogeneous trees (cv. Golden Delicious/M9) in a private orchard at Denno (Val di Non, Trento, Italy, 340 m a.s.l.) and an experimental orchard at Romeno (Val di Non, Trento, Italy, 900 m a.s.l.) for the low (LE) and high (HE) elevation trials, respectively. Samples were collected both at harvest from 10 trees for each location and after a 5 month storage at 1.2 °C in a standard controlled atmosphere (95% relative humidity, 2.5−3% CO_2, 1.5% O_2) from the same number of fruits. The water stress (WS) trials were carried out in the Maso Maiano experimental orchard (Val di Non, Trento, Italy, 650 m a.s.l.) on 20 homogeneous apple trees (cv. Golden Delicious/M9) split into two groups. Ten of them were completely deprived of the artificial irrigation from June until harvest (WS), whereas the remaining trees were kept as a control with the normal irrigation (NI). The groundwater potential was measured throughout the growing season and spanned from −2.2 to −1.8 MPa in the WS and −0.5 to −0.3 MPa in the NI.

The picking dates of the fruits were decided according to a Streif index, assessed on the control fruits in the same orchard for each trial,

Table 2. Sequences of Family- and Gene-Specific Primers Used To Quantify Allergen-Related Transcripts in Apple Fruit Tissues[a]

gene	forward	reverse	size (bp)	T_m (°C)
Mal d 1.01	5′-AAGCTGAAATCCTTGAAGGAA-3′	5′-GTGCTCTTCCTTGATTTCAATG-3′	275	79.0
Mal d 1.02	5′-ACACCTCTGAGATTCCACCAC-3′	5′-CAACTTGGTYTCGTAAGAGAC-3′	287	79.0
Mal d 1.03	5′-ACCTCCGTCATCCCCCCTG-3′	5′-TCTTCTCAATTGTCTCAGAGAT-3′	265	79.0
Mal d 1.04	5′-CATCGAAGGCGATGGAGGT-3′	5′-CCTTAGCAYGGTAGTGGCTA-3′	241	77.5
Mal d 2.01	5′-GTGTGCCCGGCTCCACTT-3′	5′-TTCGAATCACCAAACGCAAG-3′	86	79.0
Mal d 2.02	5′-CCCGGCTGAGTTACAAGTGA-3′	5′-TACTTCGGCTCACCGAAAGC-3′	84	79.0
Mal d 2.03	5′-TGGCAGCAAATTGAAGAAGTG-3′	5′-ATGTGCACCTGCGAAGAAGA-3′	92	74.5
Mal d 3.01	5′-GTGACCAGCAGCCTTGCG-3′	5′-TTCAGGCAGTTGCAAGCAGT-3′	140	80.0
Mal d 3.02	5′-AACATGTGGCCAGGTGAGATC-3′	5′-TGATTCCATTGCAGCAAGC-3′	92	79.0
Mal d 4.01	5′-GCTCTGGTGGCGTAACTGTG-3′	5′-CCTGGAGTCAAAGGCTCCTC-3′	76	74.5
Mal d 4.02	5′-CTCCGACCGGGTTGTATCTT-3′	5′-GCCCTTCTTTCCTCGAATCA-3′	83	74.5
Mal d 4.03	5′-GTCTCAGAGCGCCTCTTTCC-3′	5′-GGTTCACCCTGGATCACCAT-3′	138	74.5
MdUBI	5′-CATCCCCCCAGACCAGCAGA-3′	5′-ACCACGGAGACGCAACACCAA-3′	121	80.0

[a] Oligos used to amplify the reference gene are also listed. For each couple of primers, the size of the PCR product and its melting temperature are reported.

around 0.08 ± 0.009, which is suitable for long storage of Golden Delicious fruits grown in the Trentino area (*42*). This apple cultivar was chosen for its broad diffusion in the worldwide market and its consumption both fresh and transformed, especially in children's nutrition.

All the trees underwent exactly the same agronomical and fertilization practices, following the standard integrated pest management (IPM) allowed in European apple orchards. For each trial, a cube of cortex and a square of epidermis were excised from 50 fruits, immediately frozen in liquid nitrogen, and stored at −80 °C.

RNA Extraction and cDNA Synthesis. Total RNA was extracted following the method of Ruperti et al. (*43*), starting from 4 g of cortex and 1 g of epidermis. Minor adaptations to the protocol were brought by adding 50 μL (cortex) and 250 μL (epidermis) of a calcium hydroxide suspension just before the first centrifugation step to facilitate the precipitation of contaminating pectic sugars. cDNA was synthesized from 2 μg of DNA-free total RNA in a final volume of 25 μL containing 200 units of MMLV reverse transcriptase (Promega, Madison, WI), 1X MMLV buffer, 25 units of RNasin (RNase inhibitor, Amersham Biosciences, Piscataway, NJ), 1 μg of random hexamers (Invitrogen, Carlsbad, CA), and 2 mM dNTPs. The reaction was carried out for 1 h at 37 °C in a Gene Amp PCR System 9700 termocycler (Applied Biosystems, Foster City, CA).

Real-Time PCR. Real-time PCR relative quantification was performed in a total volume of 10 μL using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with 3 pmol of each primer and 2 μL of a 1:10 dilution of cDNA. The cluster-specific primers for *Mal d 1* genes were those used by Puehringer et al. (*17*), whereas the gene-specific primers for the remaining genes (**Table 2**) were designed with Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the instructions reported in the SYBR Green PCR Master Mix protocol (Applied Biosystems). The specificity of amplification was assessed by subsequent subcloning and sequencing of the PCR products obtained under the same conditions adopted in the real-time experiments. The reaction mixture was amplified in a 7500 real-time PCR system (Applied Biosystems) under the following conditions: initial step at 50 °C for 2 min, activation step at 95 °C for 10 min, 45 cycles including 15 s of denaturation at 95 °C, and 30 s of annealing/extension at 60 °C. After every PCR cycle, a data acquisition step was introduced to record the fluorescent signals at the optimum temperature, previously determined by melting point analysis of every specific amplification product (**Table 2**). Data were acquired, elaborated, and exported with the software SDS Sequence Detection System v1.2 (Applied Biosystems), whereas all the final calculations were carried out with the automated Excel spreadsheet Q-Gene designed by Simon (*44*), using the modifications of the delta Ct method suggested by Pfaffl (*45*). Gene expression values were normalized to the ubiquitin gene (DQ438989, **Table 2**) and reported as arbitrary units (A.U.) of mean normalized expression (*45*), using eq 2 of Q-Gene. The correct size of the amplification products was checked by running each reaction in a 1.5% agarose gel stained with ethidium bromide and viewed under UV light. All PCR experiments were carried out in three technical replicates using RNA from two independent extractions.

Statistical Analysis. Statistical analyses were performed using the CoStat version 6.311 software package (CoHort Software, Monterey, CA). Means were compared with LSD tests at a 0.05 significance level. Apple varieties were ranked according to the mean normalized expression values by calculating the tertiles. Data were ranked from 1 to 15 and then grouped into three sets with a 33% cutoff point.

The classification and ordination analyses were performed according to the unweighted pair group method arithmetic mean (UPGMA) clustering algorithm (*46*), and the centroids of all apple varieties were constructed from the symmetrical genetic distance matrix determined using the Euclidean coefficient. Interval measurement data were standardized according to the following linear transformation: $y' = (y_i - y_m)/SD$ (i.e., the mean value of each variable was subtracted to each individual value and the difference was divided by the standard deviation). Standardized quantitative values were subjected to PCA to gain information on the variables most effective in discriminating and grouping varieties on the basis of their allergenic potentials. Common component coefficients, eigenvalues, and relative and cumulative proportions of the total variance explained by single allergen-related transcript amounts were calculated. The first two components having maximum variance were then selected for the ordination analysis. Eigenvectors from the matrix of correlation among variables were extracted and used for the projection of centroids into a bidimensional plot. All calculations and analyses were conducted using the appropriate routines of the software NTSYS v2.1x (Exeter software, Setauket, NY) (*47*).

RESULTS

Gene Expression in Different Apple Varieties. The 15 apple varieties considered in the present research showed significantly different expression profiles for all the genes analyzed, except for *Mal d 1.04*, whose transcript accumulation in the fruit cortex was almost undetectable and for which no significant difference was observed among the epidermis samples of the different varieties (data not shown).

Concerning *Mal d 1* genes, the minimum level of expression was observed for the *Mal d 1.01* cluster, for which three statistically different classes of transcript accumulation were assessed. *Mal d 1.02* transcripts were the most abundant in all the varieties, whereas the *Mal d 1.03* cluster showed intermediate levels of expression with respect to the previous two *Mal d 1* classes. Considering the *Mal d 1* expression as a whole, the highest values were encountered in INRA-33-18, Resista, DCA-BO-78-406-031, Golden Delicious, and Rajca varieties, whereas Granny Smith, Golden Orange, Hapke Delicious, and Vesna displayed the lowest amounts (**Figure 1A** and **Table 3**).

As far as *Mal d 2* genes, the highest expression was assessed for *Mal d 2.01* and the lowest for *Mal d 2.03*. Concerning the former, the expression level was quite constant in all varieties, the maximum range of difference being as low as 1 log factor. Regarding *Mal d 2.03*, a quite

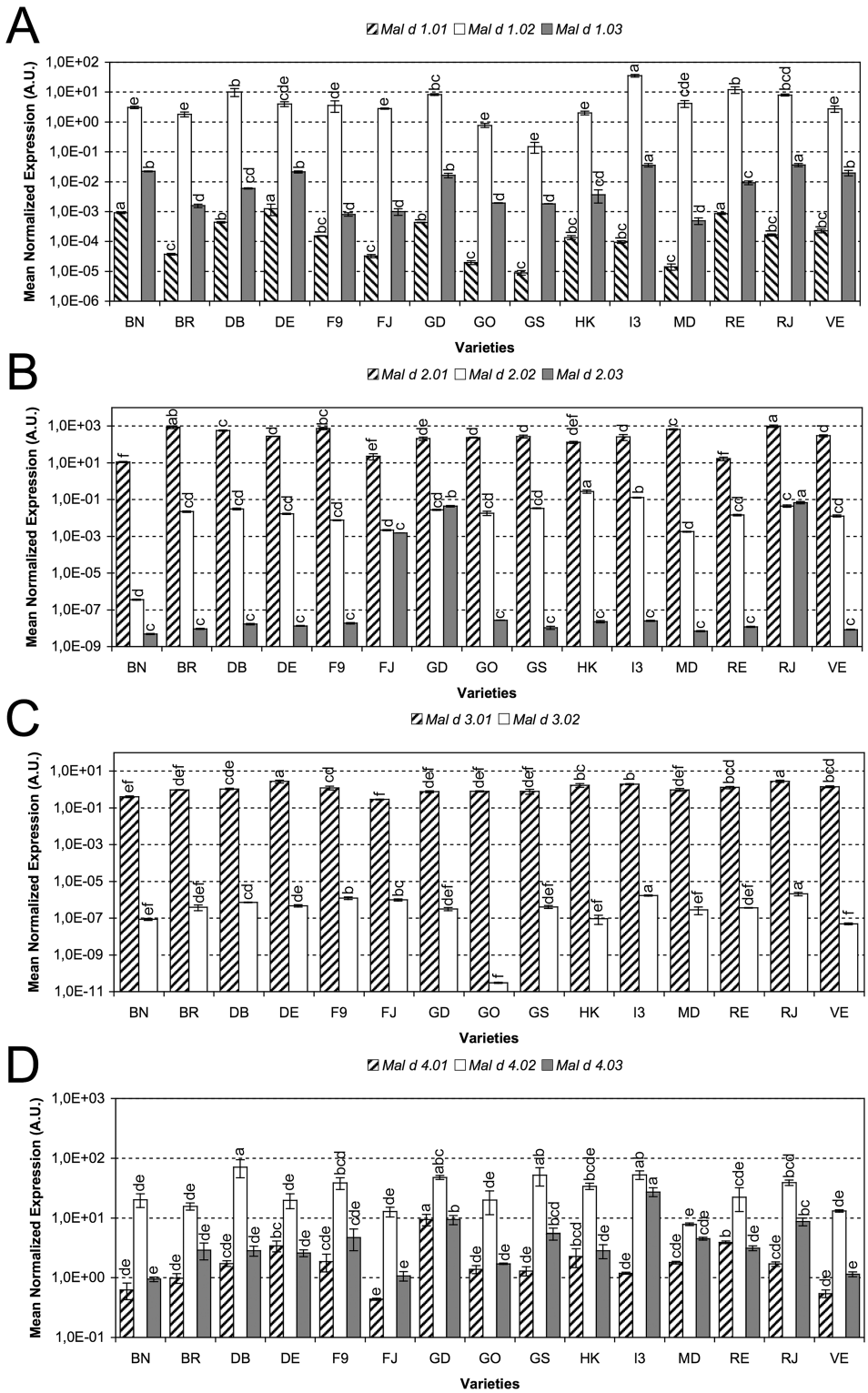

Figure 1. Expression profiles of *Mal d 1* (**A**), *Mal d 2* (**B**), *Mal d 3* (**C**), and *Mal d 4* (**D**) in the cortex of 15 apple cultivars. Transcript accumulation is reported as mean normalized expression and expressed in A.U.. The values in the charts resulted from three technical replicates and by using two independently extracted RNA samples. The letters on the bars show the nonsignificant ranges according to the LSD test ($P \leq 0.05$). The apple cultivars are Brina (BN), Braeburn (BR), DCA-BO7840-6031 (DB), Delorina (DE), FG 96 CIV (F9), Fuji (FJ), Golden Delicious (GD), Golden Orange (GO), Granny Smith (GS), Hapke Delicious VT (HK), INRA-33-18 (I3), Morgenduft Dallago (MD), Resista (RE), Rajca (RJ), and Vesna (VE).

interesting pattern was found, being expressed at very low levels in all samples except for Rajca, Golden Delicious, and to a lower extent also in the Fuji cortex. Interestingly, only in the former two varieties the *Mal d 2.03* transcript amount was higher than that of *Mal d 2.02*. In general, the varieties with the lowest levels of *Mal d 2* expression were Brina, Resista, Fuji, Hapke Delicious, and Golden Delicious, whereas Rajca, Braeburn, FG-96-CIV, Morgenduft Dallago, and DCA-BO-78-406-031 showed the highest amount of transcripts (**Figure 1B** and **Table 3**).

The level of expression of the *Mal d 3.01* gene was quite stable, within a 1 log range. Concerning *Mal d 3.02*, the Golden

Table 3. Ranking of Apple Varieties by Class of Allergen-Encoding Gene Transcript Amount (*Mal d 1*, *Mal d 2*, *Mal d 3*, and *Mal d 4*) and Total[a]

Mal d 1	*Mal d 2*	*Mal d 3*	*Mal d 4*	total	rank
I3	RJ	RJ	I3	I3	I
RE	BR	DE	DB	RJ	I
DB	F9	I3	GD	DB	I
GD	MD	HK	GS	F9	I
RJ	DB	VE	RJ	DE	I
MD	VE	RE	F9	GD	II
DE	DE	F9	HK	BR	II
F9	GS	DB	RE	MD	II
BN	I3	MD	DE	GS	II
FJ	GO	BR	GO	HK	II
VE	GD	GO	BN	RE	III
HK	HK	GS	BR	VE	III
BR	FJ	GD	VE	GO	III
GO	RE	BN	FJ	BN	III
GS	BN	FJ	MD	FJ	III

[a] Varieties were ranked according to gene expression levels by calculating the tertiles in three groups: high (I), medium (II), and low (III).

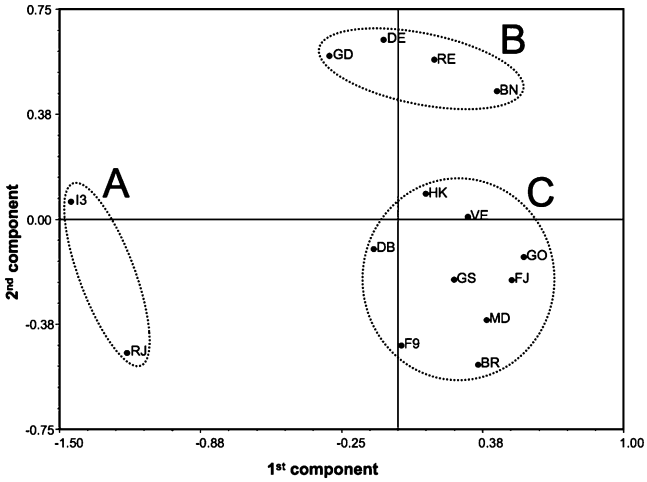

Figure 2. Centroids obtained by the PCA of the 15 apple varieties, using Euclidean coefficients based on the whole allergen-related gene expression data. Three subgroups of varieties were identified (**A**−**C**) according to the first two components.

Orange sample showed a very lower amount of specific transcripts as compared to the other varieties. Considering the whole *Mal d 3* expression, Rajca was also in this case the variety with the highest level of transcripts along with Delorina, INRA-33-18, Hapke Delicious, and Vesna, whereas Fuji, Brina, Golden Delicious, Granny Smith, and Golden Orange showed minimum levels of *Mal d 3* specific mRNAs (**Figure 1C** and **Table 3**).

Three genes of the *Mal d 4* class were analyzed, with *Mal d 4.02* having the highest expression level and the other two genes showing similar patterns. On the whole, INRA-33-18, DCA-BO-78-406-031, Golden Delicious, Granny Smith, and Rajca varieties showed the highest expression of *Mal d 4* genes, whereas Morgenduft Dallago, Fuji, Vesna, Braeburn, and Brina had the lowest ones (**Figure 1D** and **Table 3**). Considering the total allergenic potential, INRA-33-18, Rajca, DCA-BO-78-406-031, FG-96-CIV, and Delorina ranked into the highest level of expression, whereas Fuji, Brina, Golden Orange, Vesna, and Resista were in the lowest one (**Table 3**).

PCA allowed the definition of centroids of all varieties. Thirteen of the 15 varieties were clustered into two main subgroups of nine and four varieties each, as can be seen from the scatter diagram plotted according to the first two components (**Figure 2**). Commercially important varieties such as Granny Smith and Fuji were closely clustered and clearly discriminated

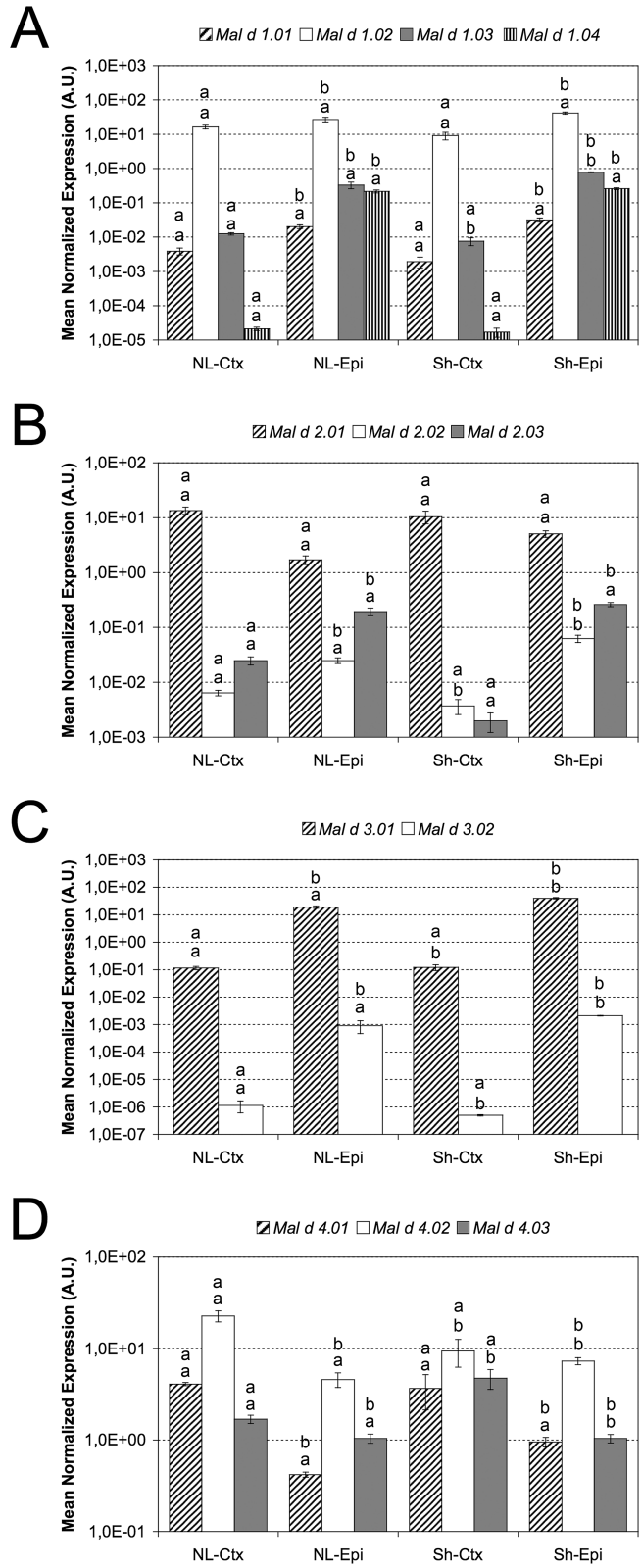

Figure 3. Expression profiles of *Mal d 1* (**A**), *Mal d 2* (**B**), *Mal d 3* (**C**), and *Mal d 4* (**D**) in cortex (Ctx) and epidermis (Epi) of Golden Delicious apples grown under normal light conditions (NL) or shadowing (Sh). The values in the charts resulted from three technical replicates and by using two independently extracted RNA samples. The letters on the bars show the nonsignificant ranges according to the LSD test ($P \leq 0.05$) performed separately for the two factors considered. The letters closer to the bars concern the shadowing effect, whereas the upper letters describe the comparison of the means for the two tissues (for descriptive statistics and *P* values, see **Table 4**).

Table 4. Statistics Summarizing the Effects of Shadowing (Sh), Tissue (T, Cortex and Epidermis), and Related Interaction (Sh × T) on Transcript Accumulation of Apple Allergen-Related Genes[a]

gene	Sh	T	Sh × T
Mal d 1.01	ns ($P = 0.1031$)	*** ($P = 0.0000$)	* ($P = 0.0317$)
Mal d 1.02	ns ($P = 0.2777$)	*** ($P = 0.0001$)	** ($P = 0.0072$)
Mal d 1.03	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0003$)
Mal d 1.04	ns ($P = 0.1285$)	*** ($P = 0.0000$)	ns ($P = 0.1284$)
Mal d 2.01	ns ($P = 1.0000$)	ns ($P = 1.0000$)	ns ($P = 1.0000$)
Mal d 2.02	** ($P = 0.0083$)	*** ($P = 0.0001$)	** ($P = 0.0038$)
Mal d 2.03	ns ($P = 0.3015$)	*** ($P = 0.0000$)	ns ($P = 0.0526$)
Mal d 3.01	*** ($P = 0.0001$)	*** ($P = 0.0000$)	*** ($P = 0.0001$)
Mal d 3.02	* ($P = 0.0303$)	*** ($P = 0.0002$)	* ($P = 0.0302$)
Mal d 4.01	ns ($P = 0.9488$)	** ($P = 0.0032$)	ns ($P = 0.5465$)
Mal d 4.02	* ($P = 0.0497$)	** ($P = 0.0022$)	** ($P = 0.0083$)
Mal d 4.03	* ($P = 0.0322$)	** ($P = 0.0061$)	* ($P = 0.0324$)

[a] P values of ANOVA statistical tests ($P < 0.05$) are also provided (ns: nonsignificant).

from Golden Delicious. The two remaining varieties (i.e., INRA-33-18 and Rajca) were clustered apart resulting to be highly differentiated for quantitative values associated with the first three components.

The first four components with eigenvalues >1 were able to explain more than 77% of the total quantitative variation found for expression patterns of allergen-related genes. In particular, the first component, which explains 25.35% of the total variation, was positively associated with *Mal d 1.01* and negatively associated with *Mal d 1.02*, *Mal d 2.03*, and *Mal d 4.03*, respectively. This means that most varieties with high values of component 1 revealed low expression levels of *Mal d 1.02*, *Mal d 2.03*, and *Mal d 4.03* genes. The second component, which explains 21.56% of the total variation, was positively associated with *Mal d 1.02*, *Mal d 1.03*, and *Mal d 4.01* and negatively associated with *Mal d 2.01*. Varieties of the two main subgroups with antagonist values of component 2 are therefore characterized by differential expression levels of the genes *Mal d 1.02*, *Mal d 1.03*, *Mal d 4.01*, and *Mal d 2.01*. The third component (not shown in the graph), which explains 16.65% of the total variation, was positively associated with *Mal d 2.01* and negatively associated with *Mal d 1.02* and *Mal d 4.03*. In particular, Rajca and INRA-33-18 proved to be highly differentiated for expression levels of the *Mal d 2.01*, *Mal d 1.02*, and *Mal d 4.03* genes, *Mal d 2.01* being highly expressed in the former and *Mal d 1.02* and *Mal d 4.03* in the latter.

Effect of Shadowing. Shadowing had a significant effect on the transcription of six genes out of 12 analyzed (**Figure 3** and **Table 4**). Considering *Mal d 1* genes (**Figure 3A**), only for *Mal d 1.03*, a statistically significant difference was observed. A statistical interaction was also assessed between shadowing and tissue factors, the gene being down- and up-regulated in the cortex and epidermis, respectively. An interacting effect was also observed for *Mal d 1.01* and *1.02*, whereas no interaction was pointed out for *Mal d 1.04*. The fruit skin constantly showed higher amounts of specific transcripts than the cortex, ranging from 1.7- to 4.7-fold in the control fruits and shadowed ones, respectively.

Considering the *Mal d 2* class (**Figure 3B**), only *Mal d 2.02* expression was significantly affected by the light deprivation, in a tissue-dependent manner, the transcription being inhibited in the cortex and stimulated in the epidermis. No major interaction was observed between shadowing and tissue for the other *Mal d 2* genes. Taking into account the overall *Mal d 2* transcript amount, a higher accumulation was assessed in the cortex, as high as 7-fold in the control fruits, especially for *Mal d 2.01* that was the most expressed of this class.

The *Mal d 3* genes (**Figure 3C**) were mainly expressed at the skin level, with 2−3 log difference with respect to the cortex. The effect of shadowing was faint but significant, and for both *Mal d 3.01* and *3.02* was dependent on the tissue. A clear up-regulation was observed again in the epidermis, whereas in the cortex there was an opposite effect. In the case of *Mal d 4* (**Figure 3D**), a generally lower amount of transcripts was measured in the epidermis, from 2- to 5-fold less expressed than in the cortex. Shadowing had no significant effect on *Mal d 4.01* transcription but enhanced gene expression of *Mal d 4.02* and *Mal d 4.03* in the skin and cortex, respectively, with a proved interaction with the tissue factor.

Effect of Elevation and Storage. A significant effect of elevation on the transcription of *Mal d 1.04*, *2.02*, *2.03*, *4.01*, *4.02*, and *4.03* genes was pointed out (**Table 5**). Specifically, *Mal d 1.04* expression was reduced in samples grown at a higher elevation (HE), with a more visible effect on the epidermis after a 5 month storage (**Figure 4A**). A decrease of *Mal d 2.02* transcripts was observed only in the epidermis, whereas concerning *Mal d 2.03*, two opposite trends were pointed out, the gene being up- and down-regulated in the cortex and epidermis, respectively (**Figure 4B**). As far as the Mal d 4 class is concerned, three different patterns of expression were observed as a response to elevation. *Mal d 4.01* transcription was stimulated mainly in the cortex, whereas *Mal d 4.02* was up-regulated in both tissues. *Mal d 4.03* showed a quite peculiar behavior, its expression being inhibited and stimulated by altitude in both tissues at harvest and after storage, respectively (**Figure 4D**). Concerning the other genes, the differences were in the nonsignificant range (**Table 5**).

Table 5. Statistics Summarizing the Effects of Elevation (E), Storage (S), Tissue (T, Cortex and Epidermis), and Related Interactions (E × S, E × T, S × T, and E × S × T) on Transcript Accumulation of Apple Allergen-Related Genes[a]

gene	E	S	T	E × S	E × T	S × T	E × S × T
Mal d 1.01	ns ($P = 0.0644$)	*** ($P = 0.0000$)	ns ($P = 0.7496$)	ns ($P = 0.2093$)	* ($P = 0.0328$)	*** ($P = 0.0000$)	ns ($P = 0.3072$)
Mal d 1.02	ns ($P = 0.4207$)	ns ($P = 0.4071$)	ns ($P = 0.1084$)	ns ($P = 0.2507$)	ns ($P = 0.2163$)	ns ($P = 0.1048$)	ns ($P = 0.4781$)
Mal d 1.03	ns ($P = 0.9650$)	*** ($P = 0.0002$)	** ($P = 0.0067$)	ns ($P = 0.6745$)	ns ($P = 0.2229$)	*** ($P = 0.0005$)	ns ($P = 0.4493$)
Mal d 1.04	*** ($P = 0.0004$)	*** ($P = 0.0008$)	*** ($P = 0.0000$)	ns ($P = 0.6412$)	*** ($P = 0.0004$)	*** ($P = 0.0008$)	ns ($P = 0.6410$)
Mal d 2.01	ns ($P = 0.8196$)	*** ($P = 0.0000$)	** ($P = 0.0043$)	ns ($P = 0.3789$)	ns ($P = 0.0849$)	*** ($P = 0.0000$)	ns ($P = 0.3065$)
Mal d 2.02	*** ($P = 0.0000$)	*** ($P = 0.0000$)	ns ($P = 0.9380$)	ns ($P = 0.4747$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	ns ($P = 0.3945$)
Mal d 2.03	*** ($P = 0.0001$)	ns ($P = 0.0503$)	*** ($P = 0.0000$)	ns ($P = 0.9312$)	*** ($P = 0.0000$)	ns ($P = 0.2866$)	ns ($P = 0.7174$)
Mal d 3.01	ns ($P = 0.9445$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	ns ($P = 0.0634$)	ns ($P = 0.9789$)	*** ($P = 0.0000$)	ns ($P = 0.0604$)
Mal d 3.02	* ($P = 0.0115$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0004$)	* ($P = 0.0115$)	*** ($P = 0.0000$)	*** ($P = 0.0004$)
Mal d 4.01	*** ($P = 0.0001$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0006$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0002$)
Mal d 4.02	*** ($P = 0.0000$)	ns ($P = 0.1120$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)
Mal d 4.03	*** ($P = 0.0009$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	*** ($P = 0.0001$)

[a] P values of ANOVA statistical tests ($P < 0.05$) are also provided (ns: nonsignificant).

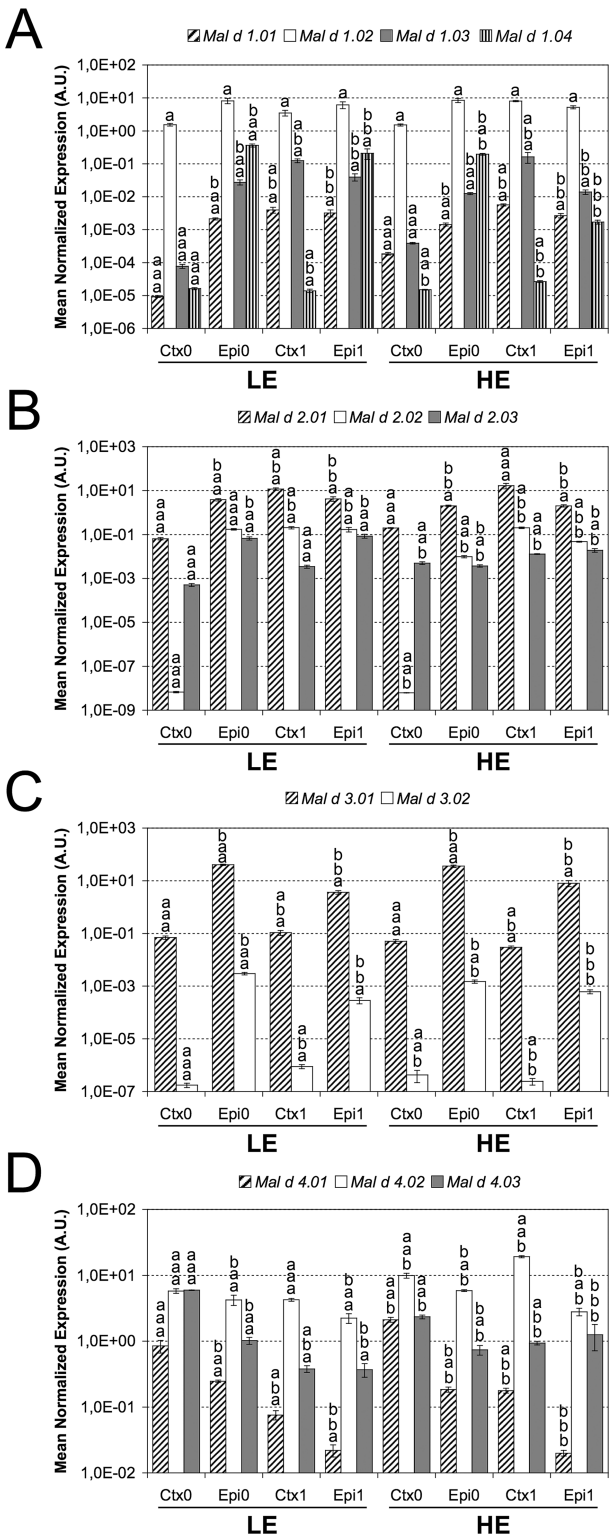

Figure 4. Expression profiles of *Mal d 1* (**A**), *Mal d 2* (**B**), *Mal d 3* (**C**), and *Mal d 4* (**D**) in cortex (Ctx) and epidermis (Epi) of Golden Delicious apples grown at a low (LE) or high (HE) elevation and harvested (0) or stored for 5 months in a controlled atmosphere and low temperature. The values in the charts resulted from three technical replicates and by using two independently extracted RNA samples. The letters on the bars show the nonsignificant ranges according to the LSD test ($P \leq 0.05$) performed separately for the three factors considered. The letters closer to the bars concern the elevation effect, and those in the middle are related to storage, whereas the upper letters describe the comparison of the means for the two tissues (for descriptive statistics and *P* values, see **Table 5**).

Table 6. Statistics Summarizing the Effects of Water Stress (WS), Tissue (T, Cortex and Epidermis), and Related Interaction (WS × T) on Transcript Accumulation of Apple Allergen-Related Genes[a]

gene	WS	T	WS × T
Mal d 1.01	ns ($P = 0.1259$)	** ($P = 0.0055$)	ns ($P = 0.1420$)
Mal d 1.02	ns ($P = 0.4204$)	*** ($P = 0.0000$)	ns ($P = 0.3430$)
Mal d 1.03	ns ($P = 0.1314$)	*** ($P = 0.0000$)	ns ($P = 0.1258$)
Mal d 1.04	** ($P = 0.0021$)	** ($P = 0.0021$)	** ($P = 0.0021$)
Mal d 2.01	ns ($P = 0.3501$)	* ($P = 0.0193$)	ns ($P = 0.9896$)
Mal d 2.02	ns ($P = 0.1138$)	*** ($P = 0.0000$)	ns ($P = 0.1064$)
Mal d 2.03	ns ($P = 0.1388$)	* ($P = 0.0179$)	** ($P = 0.0020$)
Mal d 3.01	ns ($P = 0.9155$)	*** ($P = 0.0000$)	ns ($P = 0.9298$)
Mal d 3.02	ns ($P = 0.7737$)	*** ($P = 0.0004$)	ns ($P = 0.7740$)
Mal d 4.01	* ($P = 0.0295$)	* ($P = 0.0287$)	ns ($P = 0.2321$)
Mal d 4.02	ns ($P = 0.0808$)	ns ($P = 0.7248$)	ns ($P = 0.3277$)
Mal d 4.03	ns ($P = 0.3650$)	ns ($P = 0.1677$)	ns ($P = 0.3780$)

[a] *P* values of ANOVA statistical tests ($P < 0.05$) are also provided (ns: nonsignificant).

Storage had a significant effect on the transcription of nine genes out of 12 (**Table 5**). A positive effect was revealed on *Mal d 1.01*, *1.03*, and *1.04* expression, mainly in the cortex samples (**Figure 4A**). The same trend was observed also for *Mal d 2.01* and *2.02* (**Figure 4B**), whereas the two *Mal d 3* genes were clearly down-regulated, mainly in the epidermis (**Figure 4C**). *Mal d 4* expression patterns were similar to the previous ones, but with a stronger effect of the storage also in the cortex, as high as 1 log in the case of *Mal d 4.01* (**Figure 4D**).

The expression levels in cortex and epidermis were significantly different for *Mal d 1.03*, *1.04*, *2.01*, *2.03*, *3.01*, *3.02*, *4.01*, *4.02*, and *4.03*. Concerning *Mal d 1.03* and *2.01*, a higher expression in the epidermis was pointed out at harvest, whereas an opposite trend was evidenced after storage. For *Mal d 1.04*, *2.03*, *3.01*, and *3.02*, a higher amount of transcripts was detected in the skin of all samples. As far as *Mal d 4* genes, higher expression levels were always found in the cortex, more markedly for *Mal d 4.01* and *4.02* at HE (**Figure 4**). Several interactions also occurred between and among factors, particularly concerning *Mal d 3.02* and *Mal d 4* (**Table 5**).

Effect of Water Stress. The water shortage had a dramatic effect on the dimensions of the fruits and on the general conditions of the apple trees, even in the following year. The fruit size was reduced by ~10 mm with respect to the control, and the vegetative growth of the trees in the following season was noticeably reduced if compared to that of the trees with normal irrigation (data not shown). Therefore, the trees were actually stressed by water deprivation. However, water stress was shown to have a small effect on the expression of apple allergen-related genes (**Table 6**). Only two genes out of 12 showed a modified expression as a response to water deficiency. *Mal d 1.04* was strongly up-regulated (2 log) by drought, reaching values of *Mal d 1.03* in the epidermis (**Figure 5A**), and *Mal d 4.01* was stimulated mainly in the skin (**Figure 5D**).

Concerning the tissue, the levels of expression paralleled those previously pointed out, although with few differences in the statistical meaning. Significant interactions between water stress and tissue factors were assessed for *Mal d 1.04* and *2.03*, being up- and down-regulated in cortex and skin, respectively (**Table 6**).

DISCUSSION

In the present research, the actual importance of several external (elevation, light, water, and storage) and internal (genotype and tissue) factors in the determination of the

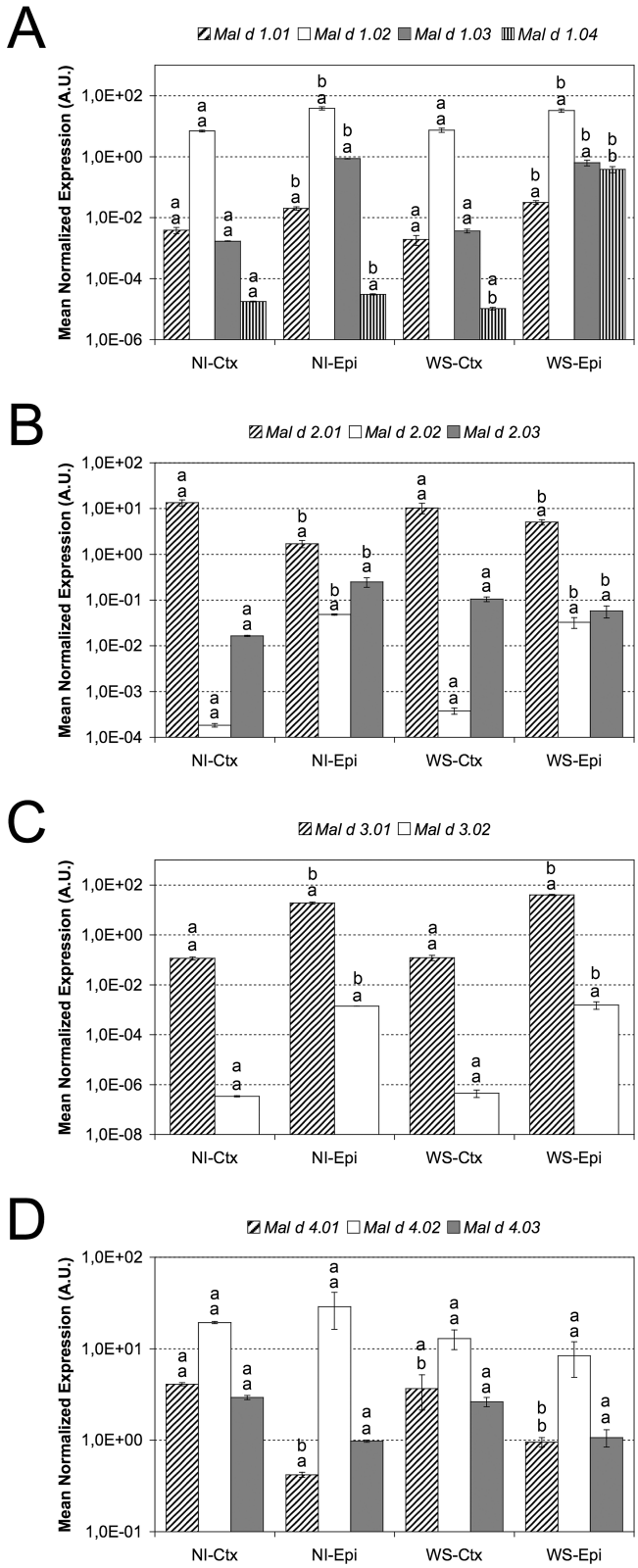

Figure 5. Expression profiles of *Mal d 1* (**A**), *Mal d 2* (**B**), *Mal d 3* (**C**), and *Mal d 4* (**D**) in cortex (Ctx) and epidermis (Epi) of Golden Delicious apples grown with a normal irrigation (NI) or upon water stress conditions (WS). The values in the charts resulted from three technical replicates and by using two independently extracted RNA samples. The letters on the bars show the nonsignificant ranges according to the LSD test ($P \leq 0.05$) performed separately for the two factors considered. The letters closer to the bars concern the WS effect, whereas the upper letters describe the comparison of the means for the two tissues (for descriptive statistics and *P* values, see **Table 6**).

allergenic potential of apple fruit is described, with implications in common agronomical practices and postharvest management, as well as in breeding programs for the selection of hypoallergenic apple genotypes. The results provide the widest available report of apple allergen-related gene expression patterns along with their inter- and intralocus variation as function of genotype, environmental conditions, and postharvest management, supplying direct evidence of the actual effects of such factors on the transcription of apple allergen-encoding genes. All the genes encoding isoforms of apple allergens were searched in public databases, and specific primers were designed to quantify the relative amount of transcripts by means of real-time PCR. Previous studies were carried out mainly at the protein level, often reporting clear discrepancies in the quantification of allergenic proteins (*29, 41*). Moreover, a lack of exhaustive information about the gene transcription of the different classes of allergens exists. This information, taking into account the difficulty in assessing the protein level for each isoform, appears to be quite relevant since a strong correlation between the transcript accumulation and the protein level has been demonstrated (*15*).

The gene expression profiles in the 15 apple varieties herein considered varied significantly, giving rise to a ranking of individual and clusters of varieties characterized by distinct allergenic potentials. According to the ranks assigned by statistical analyses, the varieties with the highest allergenic potential were those harvested earlier, whereas those with the lowest amount of allergen-related transcripts were the late-ripening ones (**Tables 1** and **3** and **Figure 2**). The differences between the maximum and the minimum transcript amount spanned from 6-fold in the case of *Mal d 4* to 200-fold in the case of *Mal d 1* (**Figure 1**). This may lead to a difference in terms of the final allergenic protein content, as pointed out for Mal d 3 and Mal d 1 by Borges et al. (*41*) and Sancho et al. (*15, 29, 30*), respectively. Moreover, three varieties (Fuji, Golden Delicious, and Granny Smith) out of the 15 included in the present study were previously shown not to differ significantly from the point of view of Mal d 3 allergenic protein content in the cortex (*41*), fully agreeing with gene expression data. On the other hand, more recently, Sancho et al. (*30*) showed that the same varieties span a 2-fold range in terms of Mal d 3 content, from the minimum value of Fuji up to the maximum of Granny Smith. It is worth noting that a similar behavior also was found for the gene expression values herein described, although without statistical significance. Among the different varieties, the minimum variability in terms of expression was pointed out for *Mal d 4* genes which encode profilin isoforms. Since profilins cover essential cellular functions (*48*), the cellular level of such important proteins and the related gene transcription rate have to be almost constant, and indeed, a quite constant gene expression pattern was detected for *Mal d 4*, as reported in **Figure 1**.

As far as practical implications are concerned, all available information in addition to a further exploitation of the natural variability should be used in breeding programs addressed to the selection of hypoallergenic apple varieties, for which Fuji and Brina may be two good candidates. For this purpose, PCA may be adopted as a standard method for classifying apple varieties according to their allergenic potential. The same approach could also be adopted using the allergenic protein content as well as in vivo allergological tests.

Concerning the effect of shadowing, the transcription rate of six genes was affected, often with a clear and significant interaction with the tissue factor. Although the fruit skin is more

directly exposed to light, shadowing affected the transcription of allergen-related genes also in the cortex, but usually to a lesser extent than in the fruit epidermis and with an opposite trend. Since fruits can be peeled before consumption and processing, the actual reduction of allergenic potential in the cortex following a partial light deprivation deserves further investigation at the protein level as well as by allergological in vivo tests. Indeed, shadowing may represent an important cultural practice aimed to reduce apple cortex allergenicity.

Apple cultivation is widespread at different altitudes in temperate climate areas, and the final organoleptic as well as chemical properties may vary significantly according to this environmental parameter, being generally improved when fruits are produced at higher elevations. This factor is also important for the storage of this commodity, which is a major feature determining the quality of apple fruits and contributing to the final price. The fruits grown at low altitude (LE) showed an overall decreased (~20%) gene expression with respect to those of higher elevation (HE), when the global values of all the four allergen classes are considered. Considering the situation at harvest and after storage separately, the LE fruits showed a 50% reduction of global gene expression after storage for 5 months, whereas the HE fruits did not change significantly throughout storage. Consequently, the allergenicity of apples after storage should be evaluated allergen by allergen separately, as well as considering the elevation at which fruits were harvested.

During the last few years, strong climate changes were observed in Europe, mainly in the Mediterranean area (*49*). Water shortages are becoming a serious problem in many countries where apple cultivation represents an important economic resource; thus, we assessed the effect of water stress on the accumulation of allergen-encoding gene transcripts in apples. Despite the dramatic effect on fruit size and the vegetative growth of the trees, water deprivation did not significantly affect the transcription rates of genes considered here, except for *Mal d 1.04* and *4.01*, although drought was often reported to stimulate the expression of many PR genes (*50–52*). As a concluding remark, apple growers should be addressed by extension services to follow suitable agricultural practices for the reduction of the allergenic potential of apples, and breeders should take into account the putative hypoallergenic traits pointed out in this and in previous research to release multiple hypoallergenic varieties.

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