

Environmental factors affecting the expression of apple (*Malus × domestica* L. Borkh) allergen-encoding genes

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SUMMARY

Apples can cause allergic reactions in the worldwide population because of the presence of four classes of allergens (Mal d 1, Mal d 2, Mal d 3, and Mal d 4), and their cross-reactivity with sensitising allergens in other species. Knowledge of the factors that affect the allergenic potential of apples would provide important information to apple growers and consumers for the adoption of agronomic practices to decrease the allergenic potential, and for the consumption of fruits with reduced levels of allergens. Expression studies on apple genes that encode allergens were performed by means of real-time PCR. Samples were collected from fruit in different trials set up to assess the effects of shading, elevation, storage, and water stress on the expression of apple allergen genes. Shading, elevation, and storage significantly affected the transcription of all genes encoding allergens, whereas water stress had only a slight influence on the expression of the *Mal d 4* family of genes. The implications of these results for growers and consumers are critically discussed.

Apple (*Malus × domestica* L. Borkh) is an important fruit crop, worldwide, and its consumption is highly recommended for a healthy diet because of its efficacy in reducing the risk of stroke, heart disease, and lung cancer (Knekt *et al.*, 1996; 2000; Le Marchand *et al.*, 2000). Unfortunately, apple also represents an important cause of allergic reactions (Fernandez-Rivas *et al.*, 2006), in both children and adults, due to the presence of four main classes of allergens (namely Mal d 1, Mal d 2, Mal d 3, and Mal d 4) with different clinical relevance, depending on the geographical area. Allergic reactions caused by Mal d 1, the major apple allergen, belong to class-II allergies (Breiteneder, 2004), which mainly affect northern and central European populations and are often associated with birch pollinosis due to cross-reactivity with Bet v 1 (Fritsch *et al.*, 1998). Both are 17 – 18 kDa allergens and belong to group-10 of the pathogenesis-related proteins (PR-10), which are expressed in response to biotic and abiotic stresses, and may be involved in the binding and transport of plant steroids (Markovic-Housley *et al.*, 2002) and cytokinins (Zubini *et al.*, 2009). The accumulation of Mal d 1 in apple fruit was shown to change according to genotype, ripening stage, and after storage (Zuidmeer *et al.*, 2006; Sancho *et al.*, 2006a). Genetic mapping studies have confirmed the existence of at least 18 genes encoding different isoforms of Mal d 1, with different levels of expression (Botton *et al.*, 2008; Gao *et al.*, 2005a; 2008; Puehringer *et al.*, 2003).

Little is known about the occurrence of Mal d 2-related allergy. Hsieh *et al.* (1995) previously identified an apple TLP (thaumatin-like protein) as a major allergen, and, recently, a 23.2 kDa TLP deduced from a

full-length cDNA sequence, named Mal d 2, was characterised as an antifungal protein (Krebitz *et al.*, 2003). This allergen was one of the most prominent proteins in ripe apple fruit, and was particularly resistant to protease degradation and heat treatment because of having eight disulphide bonds formed by 16 conserved cysteine residues (Oh *et al.*, 2000). TLPs belong to the PR-5 family of pathogenesis-related proteins and are considered to be a new class of pan-allergens in foods and pollen (Breiteneder and Ebner, 2000). Recent genetic mapping studies confirmed the existence of multiple copies of Mal d 2-encoding genes in the apple genome (Gao *et al.*, 2005b), whereas no information is available concerning gene expression levels and protein accumulation in different varieties, and/or after different pre- and post-harvest treatments. Gene expression data are available for peach, and show that such genes are specifically induced by ethylene, wounding, and during fruitlet abscission (Ruperti *et al.*, 2002), as well as during fruit ripening (Botton *et al.*, 2009). An extensive genomic characterisation of these peach TLP genes was also published recently (Chen *et al.*, 2008).

Mal d 3 is a non-specific lipid transfer protein (nsLTP) causing class-I food allergies (Pastorello *et al.*, 1999) mainly in the Mediterranean area (Ballmer-Weber, 2002). Plant nsLTPs form large multigene families that encode 9 kDa proteins with eight conserved cysteines, forming four disulphide bonds, and are included in the PR-14 family. According to Kader (1996), nsLTPs may have multiple biological functions in plants, due to the presence of isoforms showing moderate levels of amino acid sequence identity and different pattern of gene expression, as reported in peach by Botton *et al.* (2002; 2009). Lipid transfer proteins are considered pan-allergens, as they belong to a family of structurally

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TABLE I
Sequences of the PCR primers used to quantify allergen-related transcripts in apple fruit tissue

Gene	Forward	Reverse	Size (bp) [†]	T _m (°C) [†]
<i>Mal d 1.01</i>	5'-AAGCTGAAATCCTTGAAGGAA-3'	5'-GTGCTCTTCCTTGATTTCATG-3'	275	79.0
<i>Mal d 1.02</i>	5'-ACACCTCTGAGATTCACCAC-3'	5'-CAACTTGGTYTCGTAAGAGAC-3'	287	79.0
<i>Mal d 1.03</i>	5'-ACCTCCATCCCCCTG-3'	5'-TCTTCTCAATTGTCTCAGAGAT-3'	265	79.0
<i>Mal d 1.04</i>	5'-CATCGAAGGCGATGGAGGT-3'	5'-CCTTAGCAYGGTAGTGGCTA-3'	241	77.5
<i>Mal d 2.01</i>	5'-GTGTGCCCGGCTCCACTT-3'	5'-TTCGAATCACCAAACGCAAG-3'	86	79.0
<i>Mal d 2.02</i>	5'-CCCGGCTGAGTTACAAGTGA-3'	5'-TACTTCGGCTCACCGAAAAGC-3'	84	79.0
<i>Mal d 2.03</i>	5'-TGGCAGCAAATGAAGAAGTG-3'	5'-ATGTGCACCTGCGAAGAAGA-3'	92	74.5
<i>Mal d 3.01</i>	5'-GTGACCAGCAGCCTTGCG-3'	5'-TTCAGGCAGTTGCAAGCAGT-3'	140	80.0
<i>Mal d 3.02</i>	5'-AACATGTGGCCAGGTGAGATC-3'	5'-TGATTCCATTGCAGCAAGC-3'	92	79.0
<i>Mal d 4.01</i>	5'-GCTCTGGTGGCGTAACTGTG-3'	5'-CCTGGAGTCAAAGGCTCCTC-3'	76	74.5
<i>Mal d 4.02</i>	5'-CTCCGACCGGGTTGTATCTT-3'	5'-GCCCTTCTTTCCTCGAATCA-3'	83	74.5
<i>Mal d 4.03</i>	5'-GTCTCAGAGCGCCTCTTCC-3'	5'-GGTTACCCTGGATCACCAT-3'	138	74.5
<i>Mal d UBI*</i>	5'-CATCCCCCAGACCAGCAGA-3'	5'-ACCACGGAGACGCAACACCAA-3'	121	80.0

*The primers used to amplify the reference gene (*ubiquitin*) are also listed.

[†]For each pair of primers, the size of the PCR product (in bp) and its melting temperature are reported.

highly-conserved proteins, which are also present in vegetable species not belonging to the family Rosaceae, (Asero *et al.*, 2000). Two genes encoding mature nsLTPs are present in apple (Gao *et al.*, 2005c). Recently, Sancho *et al.* (2006) demonstrated that various pre- and post-harvest treatments could modify the levels of LTP-related allergens in apple peel. Changes in the accumulation of nsLTP transcripts in different varieties were recently demonstrated in apple and peach (Botton *et al.*, 2006; Sancho *et al.*, 2008).

Mal d 4-related allergies are found predominantly in the Mediterranean area of southern Europe, as reported for Mal d 3 (van Ree *et al.*, 1995). These allergens belong to the profilin family of proteins, whose members are also involved in allergic reactions to the fruits of other species (Westphal *et al.*, 2004), and also have strong cross-reactivity to birch pollen profilin, Bet v 2 (van Ree *et al.*, 1995). The main biological functions of profilins have been related to cell elongation, the maintenance of cell shape, flowering, seedling development, and pollen-tube growth (McKenna *et al.*, 2004).

In the present paper, the effects of shading, elevation, water stress, and fruit storage on the levels of expression of known apple allergen-encoding genes were assessed using real-time PCR. The experimental findings, and potential practical implications for common horticultural practices, are discussed, pointing out the main factors that affect the allergenic potential of apple fruit.

MATERIALS AND METHODS

Plant material

This research was carried out on apples harvested from 7-year-old trees of the apple cultivar 'Golden Delicious' on M9 rootstock, grown in different experimental orchards of the Istituto Agrario San Michele all'Adige (Trento, Italy) over two growing seasons (2005 and 2006). The picking dates of the fruits were decided according to a Streif index of approx 0.08 ± 0.009 , assessed on control fruit in the same orchard for each trial, which is suitable for long-term storage of 'Golden Delicious' apples grown in the Trentino area. The Streif index was calculated as [firmness / (soluble solids \times starch index)] according to De Long *et al.* (1999).

All trees underwent exactly the same agronomic practices, following the standard integrated pest management (IPM) allowed in European apple orchards. For each trial, a cube of cortex (1 cm³) and a square of

epidermis (1 cm²) was excised from fifty fruit, frozen immediately in liquid nitrogen, and stored at -80°C.

Fruits of the 'shaded' trial were harvested in the experimental orchard at Revò (Val di Non, Trento, Italy; 800 m a.s.l.) from 20 homogeneous trees divided into two groups. Ten trees were shaded from May (at full bloom) until harvest, by means of plastic nets that decreased the light intensity by approx. 30%, and ten trees were kept as controls under normal daylight. Apples in the 'elevation and storage' trial were harvested from 40 homogeneous trees at Denno (Val di Non, Trento, Italy; 340 m a.s.l.) and at Romeno (Val di Non, Trento, Italy; 900 m a.s.l.), for the low and the high elevation trials, respectively. Samples from ten trees at each location were collected at harvest, and after 5-months storage at 1.2°C in a standard controlled atmosphere (95% relative humidity, 2.5 - 3.0% CO₂, 1.5% O₂). The 'water stress' trial was carried out at the Maso Maiano orchard (Val di Non, Trento, Italy; 650 m a.s.l.) on 20 homogeneous apple trees split into two groups. Ten trees were completely deprived of artificial irrigation from June until harvest, whereas the ten remaining trees were kept as a control with normal irrigation. The soil water potential was measured throughout the growing season and ranged from -2.2 MPa to -1.8 MPa for the water-stressed trees, and from -0.5 MPa to -0.3 MPa for the control trees.

RNA extraction, cDNA synthesis, and real-time PCR

Total RNA isolation, cDNA synthesis, and real-time

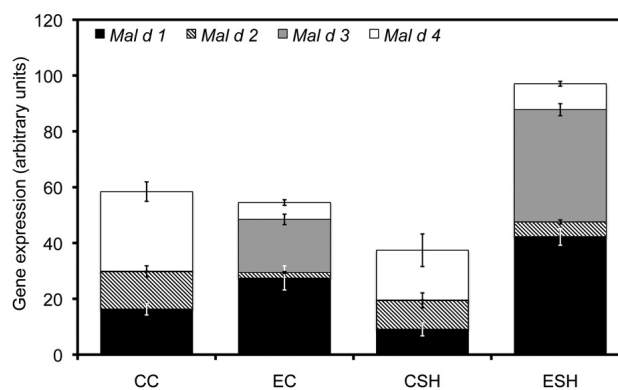


FIG. 1

Expression profiles of the *Mal d 1* (black), *Mal d 2* (cross-hatch), *Mal d 3* (grey), and *Mal d 4* (white) genes in the cortex and epidermis of 'Golden Delicious' apples grown under normal light conditions (CC and EC), or shading (CSH and ESH). Bars indicate \pm standard errors (LSD test is reported in Table II).

TABLE II

Effect of shading on the expression of apple allergen-encoding genes

Gene (family) name	CC [†]	EC [†]	CSH [†]	ESH [†]
<i>Mal d 1</i>	ab*	c	a	d
<i>Mal d 2</i>	c	a	c	ab
<i>Mal d 3</i>	a	b	a	c
<i>Mal d 4</i>	d	a	c	b

*Different lower-case letters are used when significant differences between gene expression values in different tissues were found, as assessed by LSD test at $P < 0.05$.

[†]CC = control cortex; EC = control epidermis; CSH = shaded fruit cortex; ESH = shaded fruit epidermis.

PCR experiments were carried as reported previously (Ruperti *et al.*, 2001; Botton *et al.*, 2008). The primers used are reported in Table I. Mean normalised expression data were reported by aggregating the expression values related to all the isoforms of each class of allergen, to provide an overall estimate of the allergenic potential.

Statistical analysis

Statistical analyses were performed using the CoStat Version 6.311 software package (CoHort Software, Monterey, CA, USA). Means were compared with LSD tests at a significance level of $P = 0.05$.

RESULTS

The reduction in light intensity (i.e., shading) was shown to have a significant effect on the overall transcription of apple allergen-encoding genes, with a differential trend in the cortex and the epidermis (Figure 1; Table II). The *Mal d 1* genes were expressed at higher levels in the skin than in the flesh, and a statistically significant interaction was observed between shading and the tissue samples. Expression of this gene family was down-regulated by 44% in the cortex, and up-regulated by 53% in the epidermis, following partial (30%) shading. For the *Mal d 2* gene class, no significant change in the rate of transcript accumulation was noted, but a constantly higher level of accumulation was measured in the fruit cortex; as high as seven-fold that in control fruit. The *Mal d 3* genes were expressed mainly in the epidermis, with 100–1,000-fold higher levels of

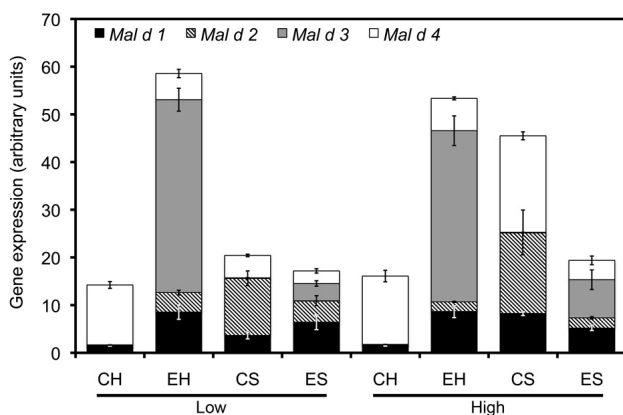


FIG. 2

Expression profiles of the *Mal d 1* (black), *Mal d 2* (cross-hatch), *Mal d 3* (grey), and *Mal d 4* (white) genes in the cortex and epidermis of 'Golden Delicious' apples grown at low (Low) or high (High) elevation, at harvest (CH and EH) or stored for 5 months in a controlled atmosphere at low temperature (CS and ES). Bars indicate \pm standard errors (LSD test is reported in Table III).

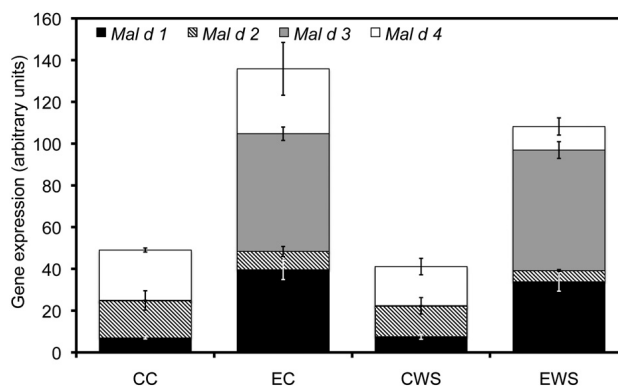


FIG. 3

Expression profiles of the *Mal d 1* (black), *Mal d 2* (cross-hatch), *Mal d 3* (grey), and *Mal d 4* (white) genes in the cortex and epidermis of 'Golden Delicious' apples grown with normal irrigation (CC and EC), or under water stress conditions (CWS and EWS). Bars indicate \pm standard errors (LSD test is reported in Table IV).

accumulation compared to the cortex. The effect of shading was again significant and dependent on the tissue being sampled. Up-regulation (by 111%) was again observed in the epidermis; whereas, in the cortex, there was a slight down-regulation, although non-significant. In the case of the *Mal d 4* class of genes, generally lower levels of transcripts were measured in the epidermis, two- to five-fold lower than in the cortex. Shading had a significant effect on overall transcription rate for this class of allergen-coding genes, with a decrease of 37% in the cortex and an increase of 53% in the epidermis. As far as the total amounts of allergen-related transcripts were concerned, shading caused down-regulation by 36%, and up-regulation by 78%, in the cortex and epidermis, respectively.

A significant effect of orchard elevation on the transcription of allergen-encoding genes was observed (Figure 2; Table III). As far as *Mal d 1* transcripts were concerned, a significant effect was observed in the cortex of stored fruit, with an increase of 126% in samples harvested at the higher location. *Mal d 2* gene expression was also significantly affected by elevation, using the same samples as for the *Mal d 1* measurements, in this case showing a 42% up-regulation. In contrast, the overall level of *Mal d 3* transcripts was not significantly affected. For the *Mal d 4* gene class, a four-fold increase was again observed in the cortex of fruit harvested at higher elevations and stored for 5 months.

As far as the effect of fruit storage was concerned, major changes in expression levels were observed for most of the allergen-coding genes analysed. *Mal d 1* gene expression was stimulated by storage, as described above, whereas transcription of the *Mal d 2* genes was significantly enhanced in the cortex at both elevations. Extremely strong down-regulation, by 91% and 78%, was observed for *Mal d 3* gene expression in the skins of apple fruit harvested at low and at high elevations, respectively. Storage also strongly affected the accumulation of *Mal d 4* transcripts, with a down-regulating effect in all samples, except in the cortex of the higher elevation fruit, where a 41% up-regulation was observed. Concerning the overall levels of *Mal d 4* gene transcripts, no significant differences were observed at harvest between samples collected at low or high elevations. Different transcription behaviours were observed in fruit after storage according to orchard

TABLE III
Effect of orchard elevation and 5-months of fruit storage on the expression of apple allergen-encoding genes

Gene (family)	Low elevation				High elevation			
	CH [†]	EH [†]	CS [†]	ES [†]	CH [†]	EH [†]	CS [†]	ES [†]
<i>Mal d 1</i>	a*	c	ab	bc	a	c	c	bc
<i>Mal d 2</i>	a	b	c	b	a	b	d	b
<i>Mal d 3</i>	a	d	a	b	a	d	a	bc
<i>Mal d 4</i>	c	ab	ab	a	c	ab	d	a

*Different lower-case letters are used when significant differences between gene expression values in different tissues were found, as assessed by LSD test at $P < 0.05$.

[†]CH = cortex at harvest; EH = epidermis at harvest; CS = cortex after storage; ES = epidermis after storage.

elevation. Apples from lower locations showed an overall increase in allergen gene transcripts of 43% and a decrease of 70% in the cortex and epidermis, respectively; whereas fruit from high elevations showed an up-regulation of 183% and a down-regulation of 64% in the same two tissues, respectively.

Water shortage had a dramatic effect on fruit size, and on the general condition of the apple trees. Final fruit diameter was reduced by approx. 10 mm, and vegetative growth of the trees was noticeably reduced, even in the following year (data not shown). In spite of these dramatic effects on reproductive and vegetative growth, water stress exerted only limited effects on the levels of expression of almost all apple allergen-related genes (Figure 3; Table IV). In fact, only the *Mal d 4* gene class was significantly affected by water shortage, with an overall decrease in transcription as high as 64%.

DISCUSSION

In this paper, we describe the importance of orchard elevation, fruit storage, tree shading, and water stress in determining the allergenic potential of apple fruit, with possible implications for common horticultural practices and post-harvest fruit management. Gene sequences encoding all known isoforms of the major apple allergens were searched-for in public databases, and specific primers were designed to quantify the relative amounts of their transcripts by means of real-time PCR. Previous studies were carried out mainly at the allergenic protein level, and often reported clear discrepancies in the quantification of allergenic proteins (Sancho *et al.*, 2006b; Borges *et al.*, 2006).

Concerning the effect of tree shading, the rates of transcription of all four classes of allergen-encoding genes were affected by this factor, often with a clear and significant interaction with the tissue (cortex or epidermis) being studied. Although fruit skin is more directly exposed to sunlight, tree shading also affected the levels of transcription of allergen-related genes in the cortex, but usually to a lesser extent than in the epidermis, and with an opposite trend (i.e., up-regulation in cortex vs. down-regulation in the epidermis, or *vice*

versa). Since fruit can be peeled before consumption and processing, the actual reduction in allergenic potential (–36%) in the cortex following partial light deprivation deserves further investigation at the protein level, as well as by allergological tests *in vivo*. Indeed, tree shading may represent an important cultural practice to reduce the allergenicity of peeled apple fruit.

Apple cultivation at different altitudes is widespread in temperate climates, and the final organoleptic properties, as well as the chemical properties of fruit at harvest may vary significantly according to this environmental parameter. These properties are generally improved when fruits are produced at higher elevations. Orchard elevation is also important for the storage ability of this commodity, which is a major factor in determining the quality of apple fruit, and contributes to their final market value. Considering harvest and post-storage separately, fruit from the lower sites showed major reductions (–70%) in overall allergen-related gene expression in their skin after 5-months in storage, whereas fruit grown at higher altitudes had a significant increase (+183%) in the levels of the same gene transcripts in their cortex, after storage. Consequently, the allergenicity of apples should be evaluated carefully throughout storage, taking into account the elevation at which the fruits were originally grown and harvested, as well as their storage conditions. However, allergen gene expression profiles need to be coupled to clinical, allergological trials (i.e., by skin prick tests and/or oral challenge) before making recommendations to sensitised consumers.

Although drought has often been reported to stimulate the expression of many PR protein genes (Ouvrard *et al.*, 1996; Romo *et al.*, 2001; Jang *et al.*, 2004), and despite its dramatic effects on fruit size and the vegetative growth of trees, water stress did not significantly affect the rates of transcription of the apple allergen genes considered here, except for the *Mal d 4* class.

In conclusion, once suitable hypo-allergenic cultural practices have been validated by *in vivo* allergological trials, apple growers should be informed by Extension Service personnel to pursue such practices to reduce the allergenic potential of their apples, or at least to label their products for allergic consumers.

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TABLE IV

Effect of water stress on the expression of apple allergen-encoding genes

Gene (family)	CC [†]	EC [†]	CWS [†]	EWS [†]
<i>Mal d 1</i>	a*	b	a	b
<i>Mal d 2</i>	b	a	b	a
<i>Mal d 3</i>	a	b	a	b
<i>Mal d 4</i>	ab	c	ab	a

*Different lower-case letters are used when significant differences between gene expression values in different tissues were found, as assessed by LSD test at $P < 0.05$.

[†]CC = control cortex; EC = control epidermis; CWS = water-stressed cortex; EWS = water-stressed epidermis.

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