

## Cloning and expression of the major allergen genes in apple fruit

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(Accepted 1 September 2009)

### SUMMARY

Apple, the most common fruit in European and North American diets, can cause allergic reactions in susceptible individuals, and genes for four families of apple allergens have been identified to date: *Mal d 1*, *Mal d 2*, *Mal d 3*, and *Mal d 4*. Our remit was to evaluate the effects of genotype, tissue, and stage of fruit development on the expression of these allergen genes and, hence, on the potential allergenicity of apple fruit. Transcript levels were investigated in fruit of the apple cultivars, ‘Gala’ and ‘Florina’, using quantitative Real-Time PCR. The resulting patterns of allergen gene expression differed, with *Mal d 1* and *Mal d 2* being the most highly expressed in the skin and flesh of ripe fruit, respectively. Overall, ‘Florina’ fruit showed higher levels of expression than ‘Gala’ fruit for all allergens tested.

High fruit consumption is reported to be beneficial to human health (Boyer and Liu, 2004). However, like other *Rosaceae* fruit species, apples can cause severe allergic reactions. The four major gene families of apple allergens reported to date are included in the official list of the World Health Organisation’s allergen nomenclature (<http://www.allergen.org>): *Mal d 1*, *Mal d 2*, *Mal d 3* and *Mal d 4*.

Two distinct patterns of apple allergy have been described across Europe. In Northern and Central areas, apple allergy is mainly due to an IgE-mediated cross-reaction between the major birch-pollen allergen, Bet v 1 and the highly homologous apple allergen, *Mal d 1* (Son *et al.*, 1999). This class of food allergy is frequent in patients sensitised to birch pollen and provokes mild local symptoms. In contrast, allergies to apple in Mediterranean areas are frequently found in patients without pollen sensitisation (Fernandez-Rivas *et al.*, 2006). This form of allergy can provoke more severe symptoms, and *Mal d 3* is the allergen mainly involved (Diaz-Perales *et al.*, 2002).

*Mal d 1* is a multigene family containing 18 different loci mainly clustered on the homeologous linkage groups (LG) 13 and 16. Four different sub-families (I – IV) have been described in relation to the presence and length of the intron. Comparisons of *Mal d 1* gene coding sequences have revealed different levels of identity: 71 – 83% between sub-families; 86 – 98% within a sub-family; and 98 – 100% between alleles of a single gene (Gao *et al.*, 2005a). *Mal d 1* genes code for a 17 – 18 kDa protein consisting of 158 – 159 amino acids (aa), classified as a pathogenesis-related (PR) protein 10 (van Loon *et al.*, 2006). High levels of expression of *Mal d 1* have been found in ripe apple fruit and in mature leaves (Pühringer *et al.*, 2003).

While *Mal d 2* was the second apple allergen to be identified (Krebitz *et al.*, 2003), its relevance to allergenicity is still unclear. It is an apoplastic, 31 kDa protein containing 246 aa encoded by 1,119 – 1,121

nucleotides (nt) organised in two exons (61 nt and 680 nt) and one intron (378 – 380 nt). Gao *et al.* (2005b) assumed that the intron size was locus-specific, and named the two loci *Mal d 2.01A* and *Mal d 2.01B*, respectively, and both were mapped on LG 9. The N-terminus of the mature protein is about 50% identical to the superfamily of thaumatin-like proteins (TLPs), also known as PR-5 proteins, with antifungal activity (Kebitz *et al.*, 2003). TLPs contain 16 conserved cysteine residues that form eight disulphide bonds which are essential for the overall folding of the proteins, and possibly for their anti-fungal and allergenic potential (van Loon *et al.*, 2006).

*Mal d 3* is a non-specific lipid transfer protein (nsLTP) belonging to the PR-14 protein family. It is characterised by having resistance to pepsin hydrolysis and thermal denaturation (van Loon *et al.*, 2006). Gao *et al.* (2005c) described two *Mal d 3* genomic sequences called *Mal d 3.01* and *Mal d 3.02* that were mapped on LG 12 and LG 4, respectively. Both genes contain a single exon of 348 nt, of which the first 72 nt code for a putative signal peptide (Kader, 1996). The two *Mal d 3* consensus sequences share 89% identity in their coding region, although the similarity is very low in the upstream region. LTPs are found mainly in aerial plant organs such as leaves, seeds, flowers, and fruit, with expression levels being low (or even nil) in roots. LTPs accumulate preferentially in exposed surfaces such as fruit skin (Borges *et al.*, 2006). It is known that the *Mal d 3.01* gene is expressed in apple fruit (Diaz-Perales *et al.*, 2002).

*Mal d 4* is a small (12 – 15 kDa) cytosolic protein belonging to the profilin protein family. Profilins are found in all eukaryotic cells, and their allergenic potency has frequently been reported (Asero *et al.*, 2003). Three distinct profilin sequences (*Mal d 4.01*, *Mal d 4.02*, and *Mal d 4.03*) have been reported in apple, with 75 – 80% identity in both their coding and amino acid sequences (Gao *et al.*, 2005b). All apple profilins have a coding sequence of 396 nt and two introns of different size in conserved positions. *Mal d 4.01* was mapped on LG 9, *Mal d 4.02* on LG 2, and *Mal*

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*d 4.03* on LG 8 (Gao *et al.*, 2005b).

It is known that allergenicity in apple is influenced both by genotype and by many 'external' factors such as storage conditions and fruit processing (Bolhaar *et al.*, 2005; Botton *et al.*, 2008). At present, the only therapy for apple allergy is the avoidance of apples and related fruits, even if this deprives the sufferer of an important source of vitamins, minerals, and fibre in his/her diet. Our aim, in the present study, was to evaluate the effects of different factors on the expression of several genes for apple allergens. We cloned the *Mal d 1.02*, *2*, *3.01*, *3.02*, *4.01*, *4.02* and *4.03* genes and determined their levels of expression by quantitative Real-Time PCR (qRT-PCR) as a function of genotype, fruit tissue, and stage of development.

## MATERIALS AND METHODS

### Plant material

Apple fruit were collected at the Cadriano Experimental Station, Bologna University, Northern Italy, from 'Gala' and 'Florina' trees at different stages of fruit development: after June drop (T0), in July (T1), and in August (T2) for both cultivars, and in September (T3) for 'Florina' alone. Skin and flesh samples were frozen separately in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction.

### Total RNA isolation and cDNA synthesis

Total RNA was extracted from 6–8 g of frozen tissue using a phenol/SDS method (Paris *et al.*, 2009) and adding 300  $\mu\text{l}$  0.5 M  $\text{Ca}(\text{OH})_2$  before the first centrifugation to facilitate the precipitation of polymeric sugars (Dal Cin *et al.*, 2005). The extraction was repeated twice for each sample. Total RNA was quantified using a Nanodrop<sup>TM</sup> ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 40  $\mu\text{g}$  were treated with 10 Units DNaseI (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) at  $37^{\circ}\text{C}$  for 20 min. First-strand cDNA was synthesised according to Paris *et al.* (2009).

### Primer design

Allergen-specific primers (Table I) were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3/input.htm>) based on the consensus sequences

retrieved from public databases. For *Mal d 1*, a primer pair able to amplify predominantly those sequences belonging to sub-family I (*Mal d 1.01* and *Mal d 1.02*) was designed because these are known to be highly expressed (Puehringer *et al.*, 2003). Only one primer pair was designed for *Mal d 2* because the differences between the gene isoforms *Mal d 2.01A* and *Mal d 2.01B* were due only to intron length. For *Mal d 3*, two specific primer pairs were designed for the gene isoforms *Mal d 3.01* and *Mal d 3.02*. For *Mal d 4.01*, *4.02*, and *4.03* specific primers reported by Gao *et al.* (2005b) were used.

### Gene cloning and sequencing

A total of 50 ng cDNA was used for each polymerase chain reaction (PCR). PCR amplification was performed using Bio-x-ACT<sup>TM</sup> DNA polymerase (Bioline, London, UK) as follows:  $94^{\circ}\text{C}$  for 2 min, then 35 cycles of 2 min at  $94^{\circ}\text{C}$ , 45 s at the optimised annealing temperature, and 2 min at  $72^{\circ}\text{C}$ , followed by a final extension of 20 min at  $72^{\circ}\text{C}$ . The PCR products of *Mal d 1*, *2*, *3.01*, and *3.02* were then ligated into the pGEM<sup>®</sup>-T Easy Vector (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. To obtain the sequences of *Mal d 4.01*, *4.02*, and *4.03*, we screened a 'Florina' BAC library (Vinatzer *et al.*, 1998) according to Cova (2008), using isoform-specific primers. The pGEM<sup>®</sup>-T Easy Vector DNA was purified as described by Sambrook *et al.* (1989) and BAC plasmid DNA was purified using alkaline lysis/PEG treatment (User's Manual for DNA sequencing reactions; Applied Biosystem, Foster City, CA, USA). The sequencing reactions were performed by MWG-Biotech AG (Ebersberg, Germany) and the sequences were analysed using BLAST ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) and CLUSTAL W software (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

### Gene expression analysis

End-point PCR was performed in a 17.5  $\mu\text{l}$  reaction containing 1  $\mu\text{l}$  of 1:3 diluted cDNA, 0.1  $\mu\text{M}$  gene-specific primers (Table I), 1.5 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  dNTPs, 0.5 Unit DNA Polymerase (Fisher Molecular Biology, Hampton, NH, USA), and 1X reaction buffer. The reaction included an initial 3 min denaturation step at  $94^{\circ}\text{C}$ , followed by 40 PCR cycles of 45 s at the optimised annealing temperature, 2 min at  $72^{\circ}\text{C}$ , and 30 s at  $94^{\circ}\text{C}$ ,

TABLE I  
Sequences of primers used for apple allergen gene cloning and end-point PCR

Allergen gene	Primer Code	Sequence (5' – 3')	Product size (bp)	Ta ( $^{\circ}\text{C}$ )
<i>Mal d 1</i>	M1For	TCCACCACCAAGATTGTTCA	420	60
	M1Rev	TGGCCCTTGAGGTAGCTCTC		
<i>Mal d 2</i>	M2nFor	AAAGCTAGCCGATCAGTGGA	478	60
	M2nRev	GAGGGCACTGCTTCTCAAAG		
<i>Mal d 3.01</i>	M301For2	GTGGCCAAGTGACCAGCAG	249	60
	M3Rev2	TGGTGGAGGTGCTGATCTTG		
<i>Mal d 3.02</i>	M302For2	TGGCCAGGTGAGCTCCAA	247	60
	M3Rev2	TGGTGGAGGTGCTGATCTTG		
<i>Mal d 4.01</i>	Mald4-For*	TGTCGTGGCAGGCGTACGTC	2,017	60
	Mald4.01-Rev*	TTATAGGCCTTGATCAATCAGGTAGTCT		
<i>Mal d 4.02</i>	Mald4-For*	see above	826	62
	Mald4.02-Rev*	TTAGAGACCCTGCTCAATGAGATAATCC		
<i>Mal d 4.03</i>	Mald4For*	see above	1,032	62
	Mald4.03-Rev*	TTAGAGACCCTGCTCGATAAG		

Ta = annealing temperature.

\*Primers reported by Gao *et al.* (2005b).

with a final extension of 10 min at 72°C. The amplicons were visualised on an Image Station 440 CF (Kodak, Rochester, N., USA) after electrophoresis in 1.5% (w/v) agarose gels.

All qRT-PCR experiments were carried out according to Paris *et al.* (2009) using an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA) and the Platinum SYBR Green master mix (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA). Gene-specific primer pairs suitable for qRT-PCR experiments (Table II) were designed for each allergen based on cloned sequences, using Primer Express software Version 1.5. In the complex *Mal d 1* gene family, *Mal d 1.01* and *Mal d 1.02* (from sub-family I) are reported to be the most highly expressed gene isoforms in ripe fruit (Puehringer *et al.*, 2003). The abundance of the *Mal d 1.02* protein in fruit has also been confirmed at the proteomic level (Helsper *et al.*, 2002). Given these findings, the expression profile of the *Mal d 1* gene family was investigated using a primer pair able to amplify, preferentially, the *Mal d 1.02* isoform. In effect, aligning all *Mal d 1* gene sequences enabled the *Mal d 1.02* primers even to amplify *Mal d 1.04* sequences, although the absence of *Mal d 1.04* ESTs in public databases from fruit suggests that this isoform is probably not expressed in fruit. Marzban *et al.* (2005) also report no detectable levels of *Mal d 1.04* gene expression in fruit.

The melting curve of the qRT-PCR products confirmed that a single gene was specifically amplified. Gene quantification was performed using a standard dilution curve for each gene studied (Larionov *et al.*, 2005). Gene expression values were normalised to the *actin* gene and reported as arbitrary units (A.U.) of mean normalised expression. All samples were run in triplicate per experiment, and the analyses were repeated twice starting from two independent extractions of total RNA. The average values of gene expression in the two biological replicates, and standard errors, were then calculated.

## RESULTS AND DISCUSSION

### Gene cloning and sequencing

Partial sequences of the apple allergen genes *Mal d 1*, 2, 3.01, and 3.02 were obtained from cloned cDNAs of 'Gala' and 'Florina' apples and deposited in GenBank

under accession numbers (ID) FM887022 – 29. For *Mal d 1*, the *Mal d 1.02* gene isoform was found in both cultivars, as expected, after Puehringer *et al.* (2003) reported *Mal d 1.02* was the most abundantly expressed member of this gene family in apple fruit and leaves. BLAST sequence analysis showed 100% identity between the *Mal d 1.0201* (AY827654) allele in 'Prima' and the *Mal d 1* gene cloned from 'Gala', which differed from that in 'Florina' by a single nucleotide polymorphism (SNP) which resulted in a synonymous amino acid substitution.

The *Mal d 2* sequences of 'Florina' and 'Gala' differed by one SNP from the *Mal d 2.01B* (AY792602) gene in 'Prima', resulting in a non-synonymous amino acid substitution (Q<sup>63</sup> in 'Prima' and R<sup>63</sup> in 'Florina' and 'Gala'). The 'Gala' and 'Florina' sequences differed from each other at two other SNPs, leading to non-synonymous substitutions in the predicted protein sequences: F<sup>75</sup> → L<sup>75</sup> for 'Florina' and S<sup>141</sup> → Y<sup>141</sup> for 'Gala'. The *Mal d 3.01* and 3.02 sequences from 'Gala' and 'Florina' were 100% identical to the *Mal d 3.0101* allele of 'Prima' (AY572500) and to the *Mal d 3.0201* allele of 'Fiesta' (AY572517), respectively.

The three *Mal d 4* genes were sequenced from the 'Florina' BAC library. The complete DNA sequence of *Mal d 4.01* (ID: FM887030) that we obtained was found to be 99% identical to the sequence of 'Fiesta' profilin (AY792607), with two SNPs in the introns and a non-synonymous amino acid substitution (L<sup>131</sup> in 'Florina' → V<sup>131</sup> in 'Fiesta'). The partial DNA sequence of *Mal d 4.02* (ID: FM887031) was 98% identical to 'Fiesta' profilin (AY792613), with three SNPs in the introns and four in the exons, resulting in one synonymous and three non-synonymous (D<sup>53</sup> → E<sup>53</sup>, L<sup>67</sup> → F<sup>67</sup>, and Q<sup>76</sup> → P<sup>76</sup>) substitutions. The partial DNA sequence of *Mal d 4.03* (ID: FM887032) was 99% identical to *Mal d 4.0302* in 'Jonathan' (AM283512), with two SNPs in the introns and four SNPs in the exons, resulting in two non-synonymous substitutions (G<sup>29</sup> → D<sup>29</sup> and V<sup>96</sup> → L<sup>96</sup>).

### Gene expression analysis

End-point PCR analyses revealed that all four of the major allergens were transcribed in apple skin and flesh at different stages of growth, regardless of genotype. While amplified cDNAs for *Mal d 1*, 2, 3.01, 3.02, and

TABLE II  
Sequences of primers used for qRT-PCR of apple allergen transcripts

Allergen gene	Primer Code	Sequence (5' – 3')	Product size (bp)	Ta (°C)
<i>Mal d 1.02</i>	Mald102RTF	CACACCAAGGGTGATGTTGAGA	75	58
	Mald102RTR	CTTGAACAAACCATGAGCCTTCT		
<i>Mal d 2</i>	Mald2RTF	GCTTGCCITGCGTTTGGT	66	58
	Mald2RTR	ACATGTCTCCGGCGTATCATT		
<i>Mal d 3.01</i>	Mald301RTF	CCGCTGACCGCCAGAC	85	58
	Mald301RTR	AGCCCTGCTGCATTGTTAGG		
<i>Mal d 3.02</i>	Mald302RTF	GCTGCTTGCAACTGCCTGA	70	58
	Mald302RTR	AGCGATTCCGGCATTGCC		
<i>Mal d 4.01</i>	Mald401RTF	GGCCAAGCTTTGGTITTTTC	99	58
	Mald401RTR	GCCTTGATCAATCAGGTAGTCT		
<i>Mal d 4.02</i>	Mald402RTF	CTCCGACCGGGTTGTAT	126	58
	Mald402RTR	TCAGCAAAGCCATTGTGTC		
<i>Mal d 4.03</i>	Mald403RTF	CAGCCAGGCCCTGTTAATC	104	58
	Mald403RTR	AGAGACCTGCTCAATAAGGTAA		

Ta = annealing temperature.

4.03 were clearly visible on agarose gels, the bands for *Mal d 4.01* and 4.02 appeared faint, suggesting a lower level of expression (data not shown).

qRT-PCR analyses confirmed the results of end-point PCR, and enabled a more reliable quantification of gene expression levels. qRT-PCR showed a large variation in the abundance of allergen transcripts during fruit development, and between different samples. In ripe fruit, the most abundant allergen transcripts were from *Mal d 1.02* in the skin and *Mal d 2* in the flesh (Figure 1). The high expression level of *Mal d 1.02* supported the results reported by Puehringer *et al.* (2003). *Mal d 2* has already been reported as one of the most abundant proteins in mature apple fruit, a finding supported by our data at the transcriptome level. *Mal d 2* was also the only allergen with a higher level of expression in apple flesh than in skin. Even if the role of this protein has not been fully elucidated, it is reported to be involved in conferring the sweet taste to ripe fruit (Oh *et al.*, 2000). Both gene isoforms of *Mal d 3* were analysed and found to be more highly, although differentially, expressed in apple skin. In fact, *Mal d 3.01* was more highly expressed than *Mal d 3.02*, suggesting that the former may be largely responsible for allergenicity to LTPs in apple.

The low levels of expression found for all *Mal d 4* genes still revealed some differences. While the *Mal d 4.03* gene was transcribed in apple skin at similar levels to *Mal d 3.02*, expression levels of both *Mal d 4.01* and 4.02 were close to zero (data not shown). This finding was unexpected, since profilin is conserved in many plant species and is known to be an important allergen in many foods (Radauer and Breiteneder, 2007).

All the allergens tested showed similar expression profiles in 'Gala' and 'Florina', albeit with substantial

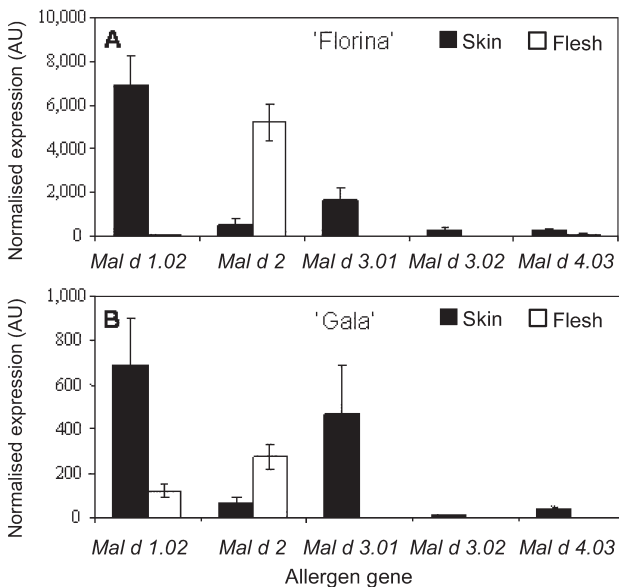


FIG. 1

Quantification, by qRT-PCR, of transcript levels of the *Mal d 1.02*, *Mal d 2*, *Mal d 3.01*, *Mal d 3.02*, and *Mal d 4.03* genes in apple fruit flesh and skin, at harvest. Relative levels of allergen expression in 'Florina' (Panel A) and 'Gala' (Panel B) apple. The levels of expression were calculated using the standard curve method, and transcript accumulation is reported as mean normalised expression, in arbitrary units (AU). The values in the charts resulted from three technical replicates, and by using two independently extracted total RNA samples ( $\pm$  SE). Solid bars represent relative gene expression in apple skin and open bars relative gene expression in apple flesh

differences in overall levels of expression. 'Florina' showed higher levels of transcripts than 'Gala', the ratio being 1:10 (Figure 1A, B). While 'Gala' was reported to be a 'high allergenic' cultivar (Bolhaar *et al.*, 2005),

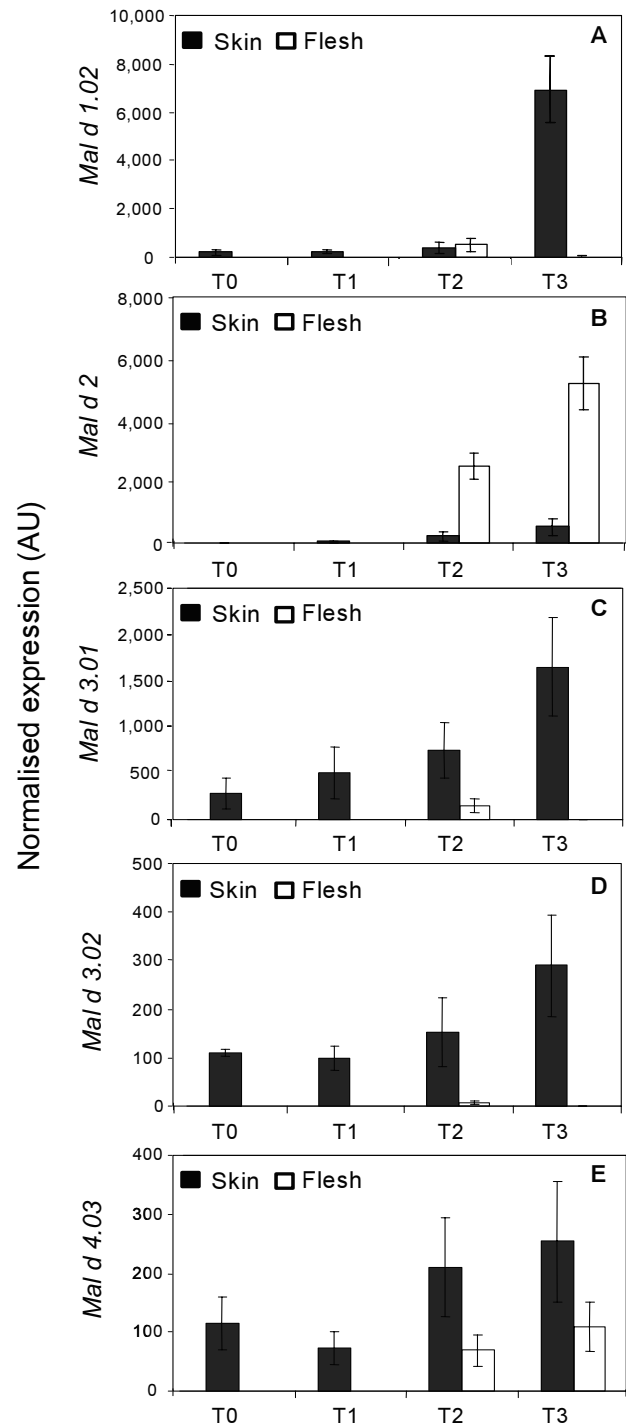


FIG. 2

Quantification, by qRT-PCR, of the levels of expression of various allergen genes in 'Florina' apple during fruit development: after June drop (T0), in July (T1), in August (T2), and in September (T3). Panel A, the *Mal d 1.02* gene. Panel B, the *Mal d 2* gene. Panel C, the *Mal d 3.01* gene. Panel D, the *Mal d 3.02* gene. Panel E, the *Mal d 4.03* gene. The levels of expression were calculated using the standard curve method, and transcript accumulation is reported as mean normalised expression, in arbitrary units (AU). The values in the charts resulted from three technical replicates, and by using two independently extracted total RNA samples ( $\pm$  SE). Solid bars represent relative gene expression in apple skin and open bars relative gene expression in apple flesh.

'Florina' has not yet been studied for its allergenic potential. If we assume a correlation between expression at the transcriptomic and proteomic levels, it seems likely that 'Florina' will be more allergenic than 'Gala', even if allergic potential is not always linearly dependent on the abundance of the allergenic protein(s) (Sancho *et al.*, 2006).

Almost all allergens showed a trend of increasing expression during fruit development in both 'Florina' (Figure 2) and 'Gala' (data not shown). Most notably, *Mal d 1.02* gene expression increased greatly at harvest (Figure 2A), whereas expression of *Mal d 2, 3.01*, and *3.02* showed a more gradual increase from T0 to T3 (Figure 2B – D). It is known that fruit ripening is characterised by processes that modify texture and flavour, and by a dramatic increase in susceptibility to pathogens (Cantu *et al.*, 2008). As allergens accumulate in ripe fruit, they could play an important role in protecting against biotic stresses. For instance, the localisation of *Mal d 1* and *Mal d 3.01* proteins in the skin suggests that these PR proteins may play a role in plant defence responses to pathological situations, reinforcing the plant cell wall as lipid carriers (Markovic-Housley *et al.*, 2003; van Loon *et al.*, 2006). A putative anti-fungal activity cannot be excluded for *Mal d 2*, a PR-5 protein (Krebitz *et al.*, 2003). In contrast, expression of *Mal d 4.03*, a gene coding for a non-PR protein, showed only a slight increase during fruit development (Figure 2E). This may be explained by the putative actin-binding role reported for profilins, which are involved in

cell growth and stability of the cytoskeleton (Schluter *et al.*, 1997). This function could be important throughout fruit development and explain the more stable pattern of expression of *Mal d 4.03* from June-drop until fruit harvest.

Our results are in agreement with published data proposing qRT-PCR as a reliable and relatively simple method to infer allergen abundance in fruit tissues. While analyses of gene expression levels also made it possible to distinguish between the highly similar sequences in the *Mal d 3* and *Mal d 4* gene families, more work is still needed to resolve the complexity in the *Mal d 1* gene family. We included 'Florina' in our tests because of its scab-resistance. Our data on allergen gene expression suggests that this cultivar is potentially highly allergenic. Although variations in allergen content have been proposed as a criterion for the breeding of new hypo-allergenic cultivars, breeders must also consider that a reduction in allergen levels in fruit might compromise plant defence systems, since these proteins may also be involved in plant responses to biotic and abiotic stress.

The ISAFRUIT Project is funded by the European Commission under Thematic Priority 5 – Food Quality and Safety of the 6th Framework Programme of RTD (Contract No. FP6-FOOD-CT-2006-016279).

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