



Allergen related gene expression in apple fruit is differentially controlled by ethylene during ripening

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ABSTRACT

Certain proteins or families of proteins present in plants and in many fruits can act as allergens that may cause an overreaction of the human immune system. Recent findings showed that apple consumption can cause allergic reactions in some consumers, especially among northern and central European populations, due to the presence of allergenic proteins. Investigations of apple fruit allergens have indicated that production of allergens is influenced by many biotic and abiotic factors. To better understand the regulation of allergen production during fruit ripening, and to examine the influence of ethylene on expression of genes encoding allergens in fruit, apples harvested at the pre-climacteric stage were allowed to ripen naturally, or ripening was either stimulated by treatment with $36 \mu\text{L L}^{-1}$ ethylene for 24 h or inhibited with 1-MCP treatment ($1 \mu\text{L L}^{-1}$ for 24 h). As an indicator of physiological status, ethylene production, was monitored up to 21 d after ethylene treatment or up to 47 d after 1-MCP treatment. Real-time qPCR was used to identify allergen genes that were differentially expressed in ethylene- and 1-MCP-treated fruit. Sixteen allergen genes representing four gene families were investigated. Transcript abundance of several genes was found to change significantly during ripening. Genes encoding Mal d1.01, Mal d1b, Mal d1d, Mal d1e, Mal d1-associated protein (MdAP), and Mal d4.01 were significantly up-regulated in fruit during ripening, and further enhanced after ethylene treatment. By contrast, expression of Mal d1.04, Mal d2.01, Mal d2.02, Mal d2.03, Mal d3.01, and Mal d4.03 genes decreased. Declining expression of Mal d3.01, Mal d4.02, and Mal d4.03 during fruit ripening was also found. Treatment with 1-MCP and ethylene generally produced opposite effects, which provides additional evidence that regulation of these genes is ethylene dependent. Overall, changes in the transcriptional profiles of genes encoding apple fruit allergens during ripening and senescence, and in response to ethylene, were complex and dynamic. Characterization of changes in these allergens during storage will potentially aid in the control of quality and safety of apple fruit.

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1. Introduction

Apple fruit (*Malus domestica* Borkh.) is a well known nutritious fruit and its consumption is important for a healthy diet. Recent findings showed that apple consumption can cause allergic reactions in some people, especially in northern and central European populations, because of the presence of allergens. Allergens are the unique proteins present in plants and many fruit that may cause an overreaction of the human immune system. Accumulation of allergens during development and ripening is identified as an undesirable health attributes in many fruits (Breiteneder and Ebner, 2000). This adverse reaction to the consumption of fruit is caused by an IgE-response and manifested through

pathophysiological response to food (Hoffmann-Sommergruber, 2000). Four main classes of allergens have been found in apples, namely, Mal d1, Mal d2, Mal d3, and Mal d4, according to the rules of the WHO/IUIS Allergen Nomenclature Subcommittee (www.allergen.org). Prior investigations of only a certain groups of allergens in apple fruit have focused on tests of allergenicity, comparisons of cultivars and cultural practices (Sancho et al., 2006b; Zuidmeer et al., 2006). For example, consumption of apples can provoke severe allergic reaction due to the major apple allergen Mal d1. Mal d1, a 17.5 kDa protein, is similar to the major birch pollen allergen, Bet v 1, and shares allergenic epitopes leading to IgE cross-reactivities (Hoffmann-Sommergruber, 2002; Midoro-Horiuti et al., 2001; Van Loon and Van Strien, 1999). Mal d1 protein increased in apple during storage at 2 °C for 14 weeks (Matthes and Schmitz-Eiberger, 2009). Mal d2, was also identified as a major allergen in apple (Breiteneder and Ebner, 2000). This 31 kDa apple protein is a thaumatin-like protein (TLPs, PR-5 protein), which has been

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reported to react with almost 75% of the apple allergic patient sera (Hsieh et al., 1995). Mal d2 is equally distributed in apple peel and pulp, and food processing does not destroy its molecular stability (Marzban et al., 2009). Storage enhances expression of Mal d2 in 'Golden Delicious' (Botton et al., 2008). Mal d3, a non-specific lipid transfer protein (nsLTPs, PR-14 proteins), was found mainly in the fruit skin and can provoke severe allergic reactions (Salcedo et al., 2007). As the apples matured, Mal d3 levels increased but then decreased during storage (Sancho et al., 2006a). Mal d4 allergens belong to the profilin protein family (PRF) and are also involved in allergic reactions to fruit (Van Ree et al., 1995). Three Mal d4 genes were observed, which were clearly down-regulated during storage in apple (Botton et al., 2008). There is some evidence that allergenicity is apple cultivar dependent, resulting from differences in the quantities and types of allergenic proteins in each cultivar (Beuning et al., 2004).

The relationship between allergens and fruit ripening process has also been studied in other fruit. For example, a 9 kDa allergen was found in peach fruit two weeks before the commercial ripening stage (Brenna et al., 2004) and the majority of allergen-encoding genes are expressed at their maximum level during the ripening stage (Botton et al., 2009). Two possible tomato fruit allergens have also been reported to be higher in red-ripe than in mature green fruit (Kitagawa et al., 2006).

In apple, ethylene plays a central role in ripening and senescence and leads to diverse ripening processes such as color development, texture changes, and flavor and aroma formation. Treatment with ethylene induces ethylene production and triggers downstream pathways leading to changes in various quality attributes (Schaffer et al., 2007). The roles of allergens as defence-associated proteins in response to biotic and abiotic stresses have been well-established (Krebitz et al., 2003). Many ethylene-perception mutants, including *etr1* in *Arabidopsis* (Pantelides et al., 2010a) and *etr 4* in tomato (Pantelides et al., 2010b), show reduced pathogen symptoms and induced synthesis and accumulation of defence-associated proteins, such as PR-1 and PR-5. Mal d1 is not only structurally related to PR-10 proteins, but also fulfills the criteria of a PR protein, i.e. is induced by pathogen and stress factors (Pühringer et al., 2000). It was reported that gene expression of a protein sharing 90% sequence homology with Mal d1 was up-regulated in response to ethylene (Atkinson et al., 1996). Recently, it was reported that the prevention of plant ethylene perception resulted in reduced *PR1-like* gene expression, suggesting that ethylene treatment may mediate plant resistance responses (Kesari et al., 2010), and therefore may be involved in accumulation of plant allergenic proteins. Nevertheless, the role of ethylene in gene expression of allergens during apple fruit storage has not been demonstrated to date.

1-Methylcyclopropene (1-MCP), an ethylene action inhibitor, is a potent competitor for ethylene receptor binding sites and recently has been used in many post-harvest practices. The application of 1-MCP maintains the freshness of various fruits, vegetables, and flowers, prolongs shelf-life and delays ripening and senescence (Blankenship and Dole, 2003). Apple is a climacteric fruit, and the ripening of harvested apple fruit is delayed by 1-MCP treatment (Watkins, 2006). Besides preventing ethylene-dependent responses by binding to ethylene receptors, 1-MCP can also inhibit apple ethylene production by inhibiting the expression of genes involved in ethylene biosynthesis (Tatsuki and Endo, 2006; Wakasa et al., 2006). The use of 1-MCP is proving to be supplemental to molecular approaches for identifying and understanding the spectrum of senescence and ripening processes under the direct control of ethylene perception (Huber, 2008). Application of 1-MCP could reveal additional information on the relationship between allergens and ethylene mediated fruit ripening, and may provide a means of maintaining low allergen levels in apple fruit.

In the present work, we investigated the gene expression of allergens in apple fruit during ripening and in response to both ethylene and 1-MCP treatments. The results provide new insights into the role of ethylene in changes in apple allergen gene expression during fruit ripening and senescence.

2. Materials and methods

2.1. Plant materials, treatments and storage

Apple fruit (*Malus x domestica* Borkh) 'Golden Delicious' were harvested from a commercial orchard in Berwick, Nova Scotia before the climacteric stage, with internal ethylene concentration $0.1\text{--}0.2\ \mu\text{L L}^{-1}$ (ca. 1 week before commercial harvest). Internal ethylene concentration was determined on 14 apples (Fan et al., 2011). One group was placed in glass chambers and ethylene treatment was carried out by ventilating with ethylene gas at $36\ \mu\text{L L}^{-1}$ for 24 h at 20°C to initiate ripening; the control group was placed at 20°C for 24 h without any ethylene treatment. After treatments, fruit were allowed to ripen in air at 20°C for 22 d. The study with ethylene treatment was conducted in the year 2004 and repeated in 2005. Experiments involving treatment with 1-MCP were conducted over two seasons, using fruit harvested in 2006 and 2007 at the same pre-climacteric stage and divided into two groups. One group was exposed to $1\ \mu\text{L L}^{-1}$ of 1-MCP (EthylBloc, 0.14%, Rohm and Haas Company, Philadelphia, PA) in sealed containers for 12 h; the control group was held at 20°C for 12 h without any 1-MCP treatment. At each sampling time, five fruit were randomly selected from each group, quickly frozen into liquid N_2 , grounded into powder, and stored immediately at -85°C until further use. For ethylene treatment, tissue from days 0, 7, 13, and 21 of each year was used for real-time PCR analysis. For the 1-MCP treatments, ground tissue from days 0, 7, 14 and 22 for control fruit and days 0, 7, 14, 22, 39 and 43 for treated fruit, was used for real-time PCR analysis.

2.2. Ethylene production

Ethylene production was determined on samples of five apples that were sealed in five 4 L glass jars with a constant flow rate of $0.58\ \text{mL s}^{-1}$ of fresh air and equilibrated overnight at 20°C . One mL samples of headspace gas were withdrawn and injected into a gas chromatograph equipped with a flame ionization detector (Carle Instruments, Inc., Anaheim, CA) equipped with a $1.9\ \text{m} \times 3.2\ \text{mm}$ (o.d.) activated alumina column with a helium carrier flow of $0.83\ \text{mL s}^{-1}$, for the measurement of ethylene production. Quantification was done by comparing the gas chromatography response of the samples with certified standards (Praxair Canada Inc.).

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from apple tissues according to the hot borate method (Wan and Wilkins, 1994) with some modifications to the extraction buffer, which contained 200 mM sodium tetraborate decahydrate, 30 mM EGTA, 1% deoxycholic acid sodium salt, 10 mM DTT, 2% PVP 40, and 1% NonidetTM P-40 (octyl phenoxy-polyethoxyethanol). All RNA extracts were treated with DNase I using a DNA-free kit and following the manufacturer's recommendations (Applied Biosystems, USA). Quantity and quality of RNA were determined spectrophotometrically by measuring the $OD_{260/280}$ and $OD_{260/230}$. RNA integrity was assessed by visual inspection after electrophoresis on a formaldehyde agarose gel in the presence of ethidium bromide. First-strand cDNA synthesis was performed on $2\ \mu\text{g}$ total RNA, using oligo dT as a primer and reverse transcriptase from a RETROscript Kit (Applied Biosystems, USA).

Table 1
Real time PCR primer sets.

Genes	Primers	Sequence (5'–3')	Access No.
<i>Mal d1.01</i>	Md1-1.01F	AAGCTGAAATCCTTGAAGGAA	AJ417551
	Md1-1.01R	GTGCTCTTCTTGATTTCATG	
<i>Mal d1.03</i>	Md1-1.03F	ACCTCCGTCATCCCCCTG	AY186248
	Md1-1.03R	TCTTCTCAATTGTCTCAGAGAT	
<i>Mal d1.04</i>	Md1-1.04F	CATCGAAGCGGATGGAGGT	Z72428
	Md1-1.04R	CCTTAGCAYGGTAGTGGCTA	
<i>Mal d1b</i>	Mal d 1b F	CTTTTGGTGAAGGTAGCCAA	AY428578
	Mal d 1b R	CACACAACCTTCGACTGAAG	
<i>Mal d1d</i>	Mal d 1d F	CTTTTGGTGAAGGCGCCAG	AY428580
	Mal d 1d R	CCTTCGACTGACCATATAGT	
<i>Mal d1e</i>	Mal d 1e F	TTGTTGCCAGATGGATGGTC	AY428581
	Mal d 1e R	TTGATGCTGACAATCTCATT	
<i>Mal d1g</i>	Mal d 1g F	AAGATTGTTGCCATATGGAA	AY428583
	Mal d 1g R	ACAATGCCCTTGTCTTGAC	
<i>MdAP</i>	MdAP-F	CCTCCTGACGAGGTGCTCAA	AY262166
	MdAP-R	GGAAAAGCTTCTGCTGATG	
<i>Mal d2.01</i>	Mal d 2.01 F	GTGTGCCCGGCTCCACTT	AJ243427
	Mal d 2.01 R	TTCGAATCACCAACGCAAG	
<i>Mal d2.02</i>	Mal d 2.02 F	CCCGGCTGAGTTACAAGTGA	CO904477
	Mal d 2.02 R	TACTTCGGCTCACCGAAAGC	
<i>Mal d2.03</i>	Mal d 2.03 F	TGGCAGCAAATTGAAGAAGTG	CO866703
	Mal d 2.03 R	ATGTGCACTGCGAAGAAGA	
<i>Mal d3.01</i>	Mal d 3.01F	GTGACCAGCAGCCTTGCG	AF221502
	Mal d 3.01R	TTCAAGCAGTTGCAAGCAGT	
<i>Mal d3.02</i>	Mal d 3.02 F	AACATGTGGCCAGGTGAGATC	AY572517
	Mal d 3.02 R	TGATTCCATTGCAAGCAGC	
<i>Mal d4.01</i>	Mal d 4.01F	GCTCTGTGGCGTAACGTG	AF129426
	Mal d 4.01R	CCTGGAGTCAAAGGCTCCTC	
<i>Mal d4.02</i>	Mal d 4.02 F	CTCCGACCGGTTGTATCTT	AF129427
	Mal d 4.02 R	GCCCTCTTCTCTCGAATCA	
<i>Mal d4.03</i>	Mal d 4.03F	GTCTCAGAGCGCCTCTTCC	AF129428
	Mal d 4.03R	GGTTCACCTGGATCACCAT	
<i>MdUBI</i>	UBI-F	TGATCTTTGCTGGGAAACAG	CN491263
	UBI-R	CACCACCATCATCAACACC	
<i>MdActin</i>	Actin-F	TGACCGAATGAGCAAGGAAATTACT	CN938023
	Actin-R	TACTCAGCTTTGGCAATCCACATC	

as per the manufacturer's instructions. Controls with no RETROscript reverse transcriptase (NRT) were used to assess for potential genomic DNA contamination. The concentration of cDNA used for real-time qPCR was measured and each sample was diluted to 0.248 g L⁻¹ with Tris–EDTA buffer (pH 8.0).

2.4. Optimization of real-time qPCR analysis

The oligonucleotide primers used for real-time quantitative PCR (qPCR) analysis were adopted from the literature (Botton et al., 2008; Espley et al., 2007; Pühringer et al., 2003; Wiersma et al., 2007) (Table 1). Conditions for all PCRs were optimized in an Eppendorf Mastercycler EP gradient PCR Thermocycler (Eppendorf, USA) and Stratagene MX3005p (Agilent, USA) with regard to forward and reverse primers, various annealing temperatures (50–65 °C) and cDNA concentration. Amplification products were separated on 1% agarose gel electrophoresis and analyzed with the PharosFX Molecular Imager (Bio-Rad, Canada). Optimized results were used on further RT-qPCR analysis for all genes. For measurement of primer efficiency, amplification of a 6 log of ten-fold serial dilutions made from cDNA of fruit sample on day 0 and ethylene treated fruit on day 21 was used. For each dilution, a qPCR was run in triplicate. Only genes with primer efficiency above 80% were further used. The efficiency value for each gene in apple was as shown in Table 2. Two reference genes, MdActin and MdUBI were analyzed in each real-time qPCR to normalize the expression patterns. Normalization factors were calculated by taking the geometric mean of the two reference genes (Vandesompele et al., 2002). Raw quantification cycles (C_q) were converted to quantities representing relative expression levels using a modified comparative cycle threshold method (Pfaffl, 2001) and with correction for different

Table 2
Gene efficiency in real time PCR.

Gene	Efficiency (%)
<i>Mal d1.01</i>	85.0
<i>Mal d1.03</i>	89.6
<i>Mal d1.04</i>	90.4
<i>Mal d1b</i>	83.4
<i>Mal d1d</i>	85.0
<i>Mal d1e</i>	81.5
<i>Mal d1g</i>	93.6
<i>MdAP</i>	89.0
<i>Mal d2.01</i>	85.8
<i>Mal d2.02</i>	93.5
<i>Mal d2.03</i>	102.8
<i>Mal d3.01</i>	84.1
<i>Mal d3.02</i>	83.3
<i>Mal d4.01</i>	90.5
<i>Mal d4.02</i>	85.7
<i>Mal d4.03</i>	86.3
<i>MdActin</i>	86.1
<i>MdUBI</i>	85.6

amplification efficiencies (Ramakers et al., 2003). In these methods, samples from day 0 (assigned an arbitrary quantity of “1”) were used as a calibrator to calculate relative quantity of the results.

2.5. Real-time qPCR analysis

Real-time qPCR reactions were repeated three times in a Stratagene MX3005p (Agilent, USA) using 1 µL of dilute cDNA, 1.5 µL primers, and 12.5 µL MaximaTM SYBR Green QPCR Master Mix (Fermentas, USA). For all genes, cycling conditions included an initial hot start at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 58–60 °C for 1 min and 72 °C for 1 min. Each real-time PCR was ended by the addition of a dissociation curve analysis of the amplified product, involving denaturation at 95 °C for 1 min, cooling to 55 °C for 30 s and then gradual heating at 1 °C per cycle to a final temperature of 95 °C. Individual PCR products were separated on 1% agarose gels and stained with SYBR green to examine their size and ensure that a single PCR product was detected for each primer pair.

2.6. Statistical analysis

The ethylene experiment was a set of 6 treatment combinations with 2 replicates (2004 and 2005). These were analyzed in a randomized block model using an ANOVA procedure. The 6 treatments tested were control (day 7), control (day 13), control (day 21), ethylene (day 7), ethylene (day 13) and ethylene (day 21). To evaluate treatment differences orthogonal contrast were used for the difference between control vs. ethylene, linear and quadratic response across control and linear and quadratic response across ethylene. The 1-MCP experiment was a combination of 8 treatments with 2 replicates (years). These were analyzed in a randomized block model using ANOVA but the treatments were grouped as just control, just 1-MCP, and overlap of control and 1-MCP. The 8 treatments were control (day 7), control (day 14), control (day 22), 1-MCP (day 7), 1-MCP (day 14), 1-MCP (day 22), 1-MCP (day 39), 1-MCP (day 43). Due to the longer shelf-life of apples stored with 1-MCP treatment, it was possible to measure more time points. Each analysis included a polynomial contrast to evaluate the change across the stages for 1-MCP, control and the interaction for the first 3 stages. EPCLUST software (<http://www.bioinf.ebc.ee/EP/EP/EPCLUST/>) allowed clustering genes according to their expression profile. A complete linkage hierarchical clustering (Euclidean distance) was demonstrated for both experiments.

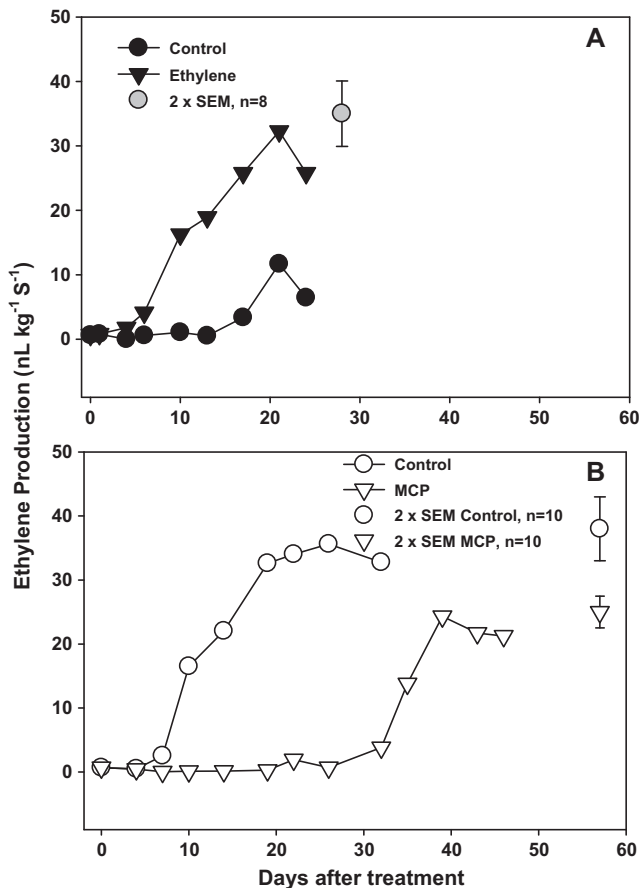


Fig. 1. Ethylene production of apple fruit during ripening, with and without ethylene or 1-MCP treatment at harvest. (A) Ethylene production after ethylene treatment (Study 1). Six treatments tested were control (day 7), control (day 14), control (day 21), ethylene (day 7), ethylene (day 14) and ethylene (day 21). (B) Ethylene production after 1-MCP treatment (Study 2). Eight treatments were control (day 7), control (day 14), control (day 22), 1-MCP (day 7), 1-MCP (day 14), 1-MCP (day 22), 1-MCP (day 39), 1-MCP (day 43).

3. Results

3.1. Ethylene production and expression of allergens genes during fruit ripening and in response to ethylene treatment (Study 1)

To relate the gene expression study on allergen profiles in apple fruit during ripening and senescence to fruit physiology, biological measurements including fruit ethylene production (Fig. 1A) and respiration (data not shown) were conducted. Pre-climacteric stage of fruit at harvest was confirmed by both low internal ethylene concentration $<0.1 \mu\text{L L}^{-1}$. At 20°C , typical climacteric changes in ethylene production were shown at around 20 d. Ethylene treatment accelerated the onset of fruit ethylene production and showed a maximum at about 7 d (Fig. 1A).

A significant effect of fruit ripening on the transcription of apple allergen related genes is shown in Fig. 2. Up-regulation of several Mal d1 related genes, such as Mal d1.01, d1.04, d1b, d1d and d1e can be seen (Fig. 2A, C–F). Also, induced fruit ripening stimulated the expression of Mal d2.03 at day 13 (Fig. 2K). The level of expression of the Mal d3 stayed stable during fruit ripening (Fig. 2L and M). Compared with the relatively high abundance of Mal d1 transcripts, MdAP, a putative binding partner for apple Mal d1, showed a consistent low expression level during ripening (Fig. 2F). Fruit ripening had no effect on gene expression of Mal d1g and Mal d3. (Fig. 2G, L and M). Similar to Mal d3, ripening had no effect on expression of

Mal d4.01, Mal d4.02 and Mal d4.03 genes (Fig. 2N and O), but did reduce that of Mal d2.02 (Fig. 2P).

Ethylene treatment not only increased the expression of Mal d1 genes Mal d1.01, Mal d1b, d1d, d1e and d1g (Fig. 2A and D–G), but also increased the expression of MdAP and Mal d4.01 (Fig. 2H and N). Among the Mal d1-related genes which were examined in the present study, the Mal d1e transcript was increased the most compared with the day 0 control (Fig. 2F). An increased transcription level was also observed for MdAP and Mal d4.01 in ethylene treated tissue samples, although the differences between the control and treatment sample became less significant at the later stage. The three Mal d2 genes were analyzed for expression during the ripening and with ethylene treatment. Results showed suppressed the expression of Mal d2.02 and d2.03 (Fig. 2I–K).

Treatment with ethylene had no effect on gene expression of Mal d.3. Three genes of the Mal d4 class were investigated. Ethylene treatment caused a significant increase in the expression level of Mal d4.01 and Mal d4.02 at the early stage of fruit ripening, while no significant difference in expression was observed between control and ethylene-treated sample at other stages of ripening (Fig. 2N and O). Expression of Mal d4.03 was slightly down-regulated by ethylene treatment (Fig. 2P). Cluster analysis indicates that Mal d1e, Mal d1d, Mal d1.01 and Mal d1b are closely related and induced by fruit ripening and ethylene treatment (Fig. 4). Also, ethylene treatment reduced the expression of all Mal d.2 genes as compared with the control.

3.2. Ethylene production and expression of allergen genes in response to 1-MCP treatment (Study 2)

1-MCP treatment significantly delayed fruit ripening and senescence, and ethylene production was inhibited by at least 23 d, finally increasing at about 30 d after harvest. Control fruit showed a typical climacteric increase of ethylene production around 7 d after harvest (Fig. 1B).

Within Mal d1 and its related genes, control fruit showed a similar trend as in study 1 with significant increase of expression level of Mal d1.01, Mal d1.03, Mal d1b, Mal d1d and Mal d1e during fruit ripening. 1-MCP treatment significantly reduced the expression of Mal d1.01, Mal d1b, Mal d1d, Mal d1e, Mal d1g, and MdAP at early stages of ripening (Fig. 3A, D–H). When 1-MCP treated fruit were at the climacteric stage later during ripening, the expression levels were slightly lower than the maximum levels reached in control fruit. In contrast, gene expression of Mal d1.04 at the early stage of ripening was significantly induced by 1-MCP treatment (Fig. 3C). 1-MCP also enhanced expression of Mal d2 group genes (Fig. 3I–K). No significant difference was found between 1-MCP-treated and control fruit in Mal d3 genes (Fig. 3L, M). 1-MCP treatment reduced Mal d4.01 and Mal d4.02 gene expression, while it induced Mal d4.03 gene expression, compared with untreated fruit (Fig. 2N–P). Cluster analysis demonstrated that 1-MCP treatment enhanced the expression of Mal d2.03 as compared with control, while Mal d3 and d4 gene transcripts were reduced by the 1-MCP treatment. Also, 1-MCP treatment showed an inhibitory effect on gene expression of Mal d1e, Mal d1.01, Mal d1d, and d1b at the early stage of ripening (Fig. 5).

4. Discussion

The identification of fruit allergens from both genomic and proteomic data (Marzban et al., 2008; Zheng et al., 2007) demonstrates the need for more intensive investigations on regulation of fruit allergens. Up to now, studies on apple fruit allergens have mainly focused on how environmental conditions, harvest maturity, storage and cultivars affect the allergenic potential of allergen Mal d1

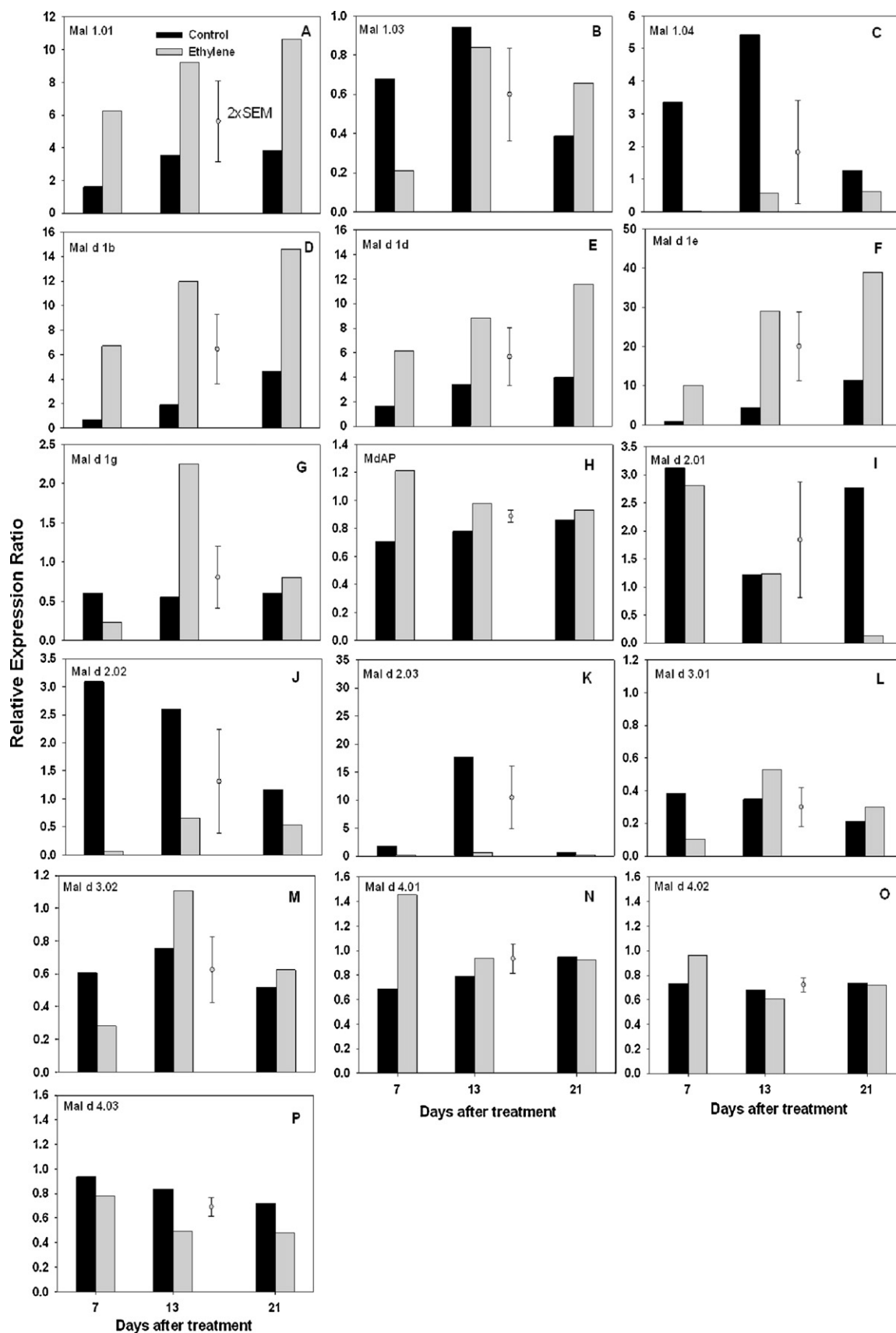


Fig. 2. Expression of apple allergen genes during 22 d of storage at 20 °C. Quantitative real-time PCR was used to analyze the mRNA changes as described in Section 2. The y axis represents the relative difference in mRNA abundance and was calculated using a modified 2^{-DDCT} formula with *Mdactin* and *MdUBI* as references. The values presented were obtained from 2 biological repeats and within each repeat each data point is the mean of values from qPCR reactions performed in triplicate. Error bars indicate standard error of means.

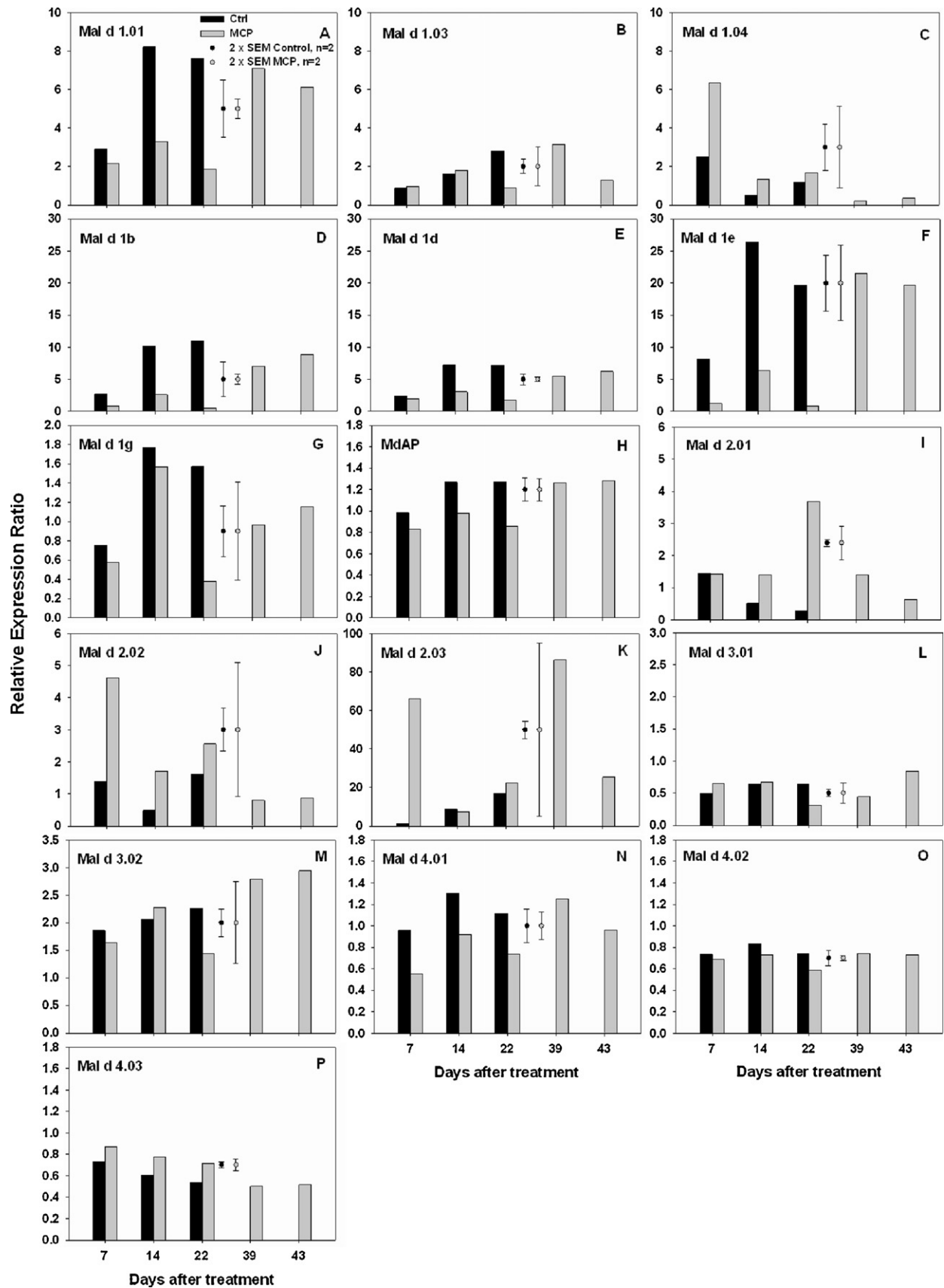


Fig. 3. Expression of apple allergen genes after 1-MCP treatment during five stages of ripening at 20 °C. Quantitative real-time PCR was used to analyze the mRNA changes as described in Section 2. The y axis represents the relative difference in mRNA abundance and was calculated using a modified $2^{-\Delta\Delta C_t}$ formula with *Mdactin* and *MdUBI* as references. The values presented were obtained from 2 biological repeats and within each repeat each data point is the mean of values from qPCR reactions performed in triplicate. Error bars indicate standard error of means.

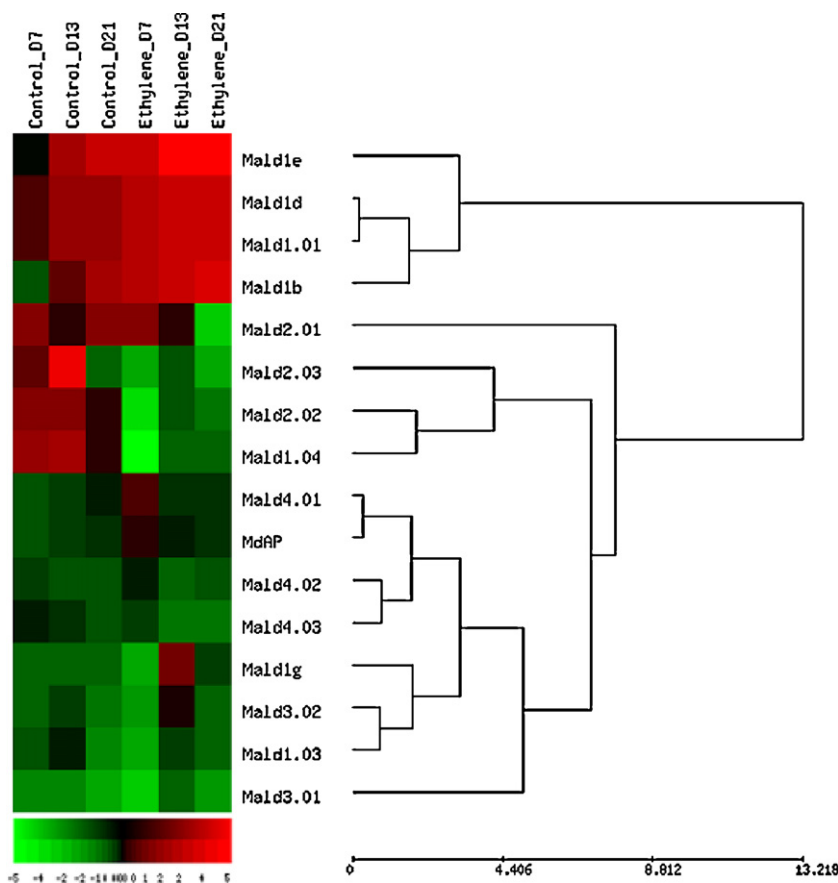


Fig. 4. Hierarchical cluster analysis (EPCLUST software) of transcript levels from allergen genes differentially expressed during apple fruit ripening with or without ethylene treatment. Red boxes mean high levels of expression compared to Day 0, and green boxes mean lower expression levels compared to Day 0. The color brightness is directly proportional to the expression ratio. Black boxes are genes not significantly differentially expressed compared to Day 0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Botton et al., 2008; Matthes and Schmitz-Eiberger, 2009; Sancho et al., 2006a,b). Previous studies carried out on certain group allergens at both protein and gene expression levels indicated that there were clear links between fruit ripening and formation of allergens in apples and discrepancies in the quantification of different groups of allergic proteins (Sancho et al., 2006b). More information on ethylene and its regulation of all classes of allergens at both the protein and gene expression levels are necessary. Despite the difficulties in assessing the protein level for each isoform, it has been reported that genes expression (transcript accumulation) and protein levels of allergens are closely related (Botton et al., 2008; Sancho et al., 2006b). In the present study, the effects of ethylene and 1-MCP on transcript levels of apple fruit allergen are demonstrated, with implications for general postharvest management and fruit ripening regulation.

Fruit harvested at the similar date of each year at pre-climacteric stage showed variation of the time of onset of climacteric stage during ripening, which may cause the variations of physiological results. Despite the physiological variation between years, our gene expression results indicate that changes of many allergens in apple fruit coincide with ethylene regulated fruit ripening. Expression correlated well with the process of ripening and they were up-regulated by ethylene treatment and down-regulated by 1-MCP (Figs. 2 and 3). In their expression patterns, Mal d1.01, Mal d1b, Mal d1d and Mal d1e fell into one cluster that were rapidly induced during ripening and by ethylene, and were suppressed by 1-MCP (Figs. 3I and 4I), while Mal d1.03 and Mal d1.04 fell into another cluster where ripening or ethylene exposure has no effect (Figs. 4 and 5). Enhanced expression level of a Mal d1 related gene

was observed in ripening apple fruit (Atkinson et al., 1996). Using mRNA differential display technique, Mal d1 was confirmed to be up-regulated during 'Mondial Gala' apple fruit ripening (Goulao and Oliveira, 2007). Those results are also in accord with the report that Mal d1 apple allergens are enhanced by fruit ripening (Botton et al., 2008). The gene expression of Md MAP is under the ethylene influence, which can be inferred from effects of ethylene and 1-MCP treatments (Figs. 2H and 3H). MdAP protein interacted with Mal d1 proteins and could act to stabilize a transport complex within the plant, (Pühringer et al., 2003).

Information of storage and treatment effects on Mal d2 is limited. In the present study, fruit ripening and ethylene had a negative effect on the expression of Mal d2.01 and Mal d2.02 based on observations from the ethylene and 1-MCP treatment (Figs. 2I, J and 3I, J). The expression of Mal d2.03 was closely correlated with the initiation of ethylene production, but was reduced in ethylene-treated apple and induced in 1-MCP treated apple (Figs. 2K and 3K). In hierarchical cluster analysis, Mal d2.01 and 2.02 genes showed a significant ethylene effect in both ethylene and 1-MCP treated apple and had high similarity to Mal d1.04. This family of genes fell into a subgroup for which ethylene had a negative effect on gene expression (Figs. 4 and 5). Interestingly, only Mal d2.03 was induced by 1-MCP treatment. It is suggested that under short-term postharvest conditions with less biotic and abiotic stress, apple has a reduced ability for Mal d2 formation and ethylene-treated apple produced even less Mal d2. No significant changes or a slight decrease of both Mal d 3.01 and Mal d 3.02 gene expression for apple stored at room temperature was observed (Figs. 2L, M and 3L, M). Through clustering analysis, Mal d 3 genes, which were consistently

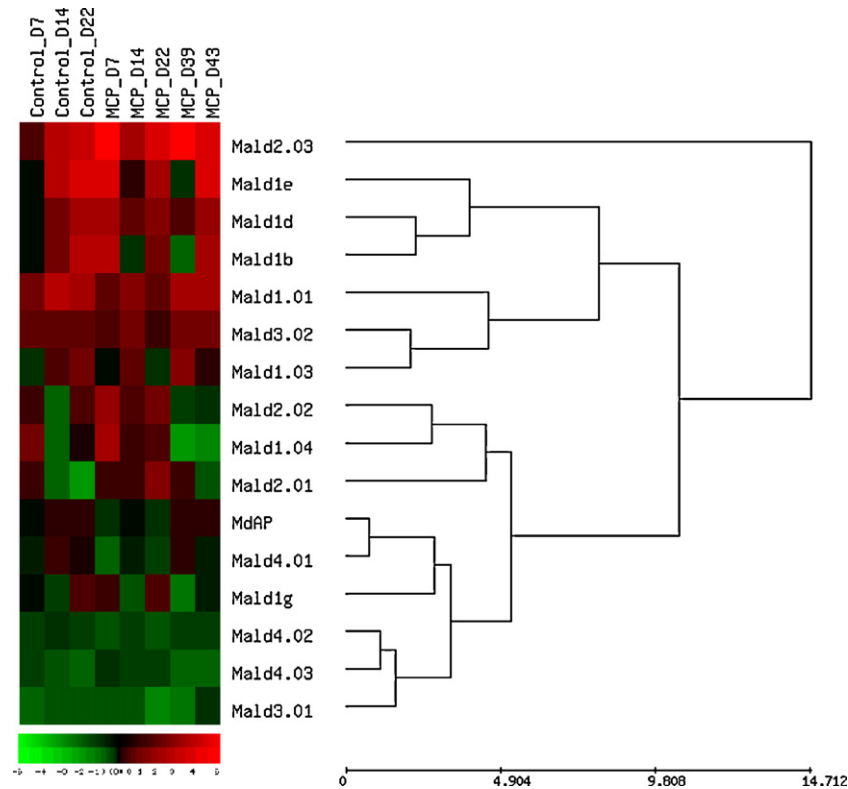


Fig. 5. Hierarchical cluster analysis (EPCLUST software) of transcript levels from allergen genes differentially expressed during apple fruit ripening with or without 1-MCP treatment. Red boxes mean high levels of expression compared to Day 0, and green boxes mean lower expression levels compared to Day 0. The color brightness is directly proportional to the expression ratio. Black boxes are genes not significantly differentially expressed compared to Day 0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

expressed at a low level fell into a cluster of genes for which generally ethylene has no significant effect (Figs. 4 and 5). However, when 1-MCP treated apple fruit were at the delayed climacteric stages, Mal d3.02 genes recovered to a higher expression level compared with the untreated fruit (Fig. 3M).

Mal d3 belongs to a large non-specific lipid transfer protein (nsLTPs) family (Sancho et al., 2006a). The role of nsLTPs in plant defence mechanisms against phytopathogens and in human allergic reaction to consumption of rosaceous fruits is well discussed (Borges et al., 2006, 2008; Salcedo et al., 2007). nsLTP genes are developmentally regulated and accumulation of gene transcripts and encoded protein was seen during fruit developing in several fruits, such as apple (Sancho et al., 2006a), peach (Botton et al., 2002, 2006, 2009; Brenna et al., 2004), grape (Salzman et al., 1998), and pepper (Liu et al., 2006). Storage reduced nsLTPs content and related gene expression in apple and peach (Botton et al., 2006, 2008; Sancho et al., 2006a). In pepper leaves, ethylene, methyl jasmonate and abscisic acid treatment induced CALTP1 and CALTPIII gene expression (Jung et al., 2003). Therefore, ethylene may have a role in activating nsLTP gene expression.

At least three distinct mRNA sequences (Mal d4.01, 4.02 and 4.03) encoding three isoforms of apple profilin have been mapped (Gao et al., 2005). Profilins are small cytosolic proteins (12–15 kDa) found in all eukaryotic cells that function as structural proteins by participating in the regulation of actin filament polymerization (Gao et al., 2005). Profilins have been found in vegetative and reproductive tissues and may play a role in cell elongation, cell shape, flowering and intracellular signalling or regulation of cell architecture in *Arabidopsis* (McKinney et al., 2001; Ramachandran et al., 2000) and may be related to defence responses in rice (Liao et al., 2009). Many fruits contain profilins and their allergenic potency has been frequently reported in cherry, pear (Scheurer

et al., 2001), peach (Rodríguez-Pérez et al., 2003), tomato (Westphal et al., 2004), orange (López-Torrejón et al., 2005b), mango (Song et al., 2008), melon (López-Torrejón et al., 2005a), and apple (Ma et al., 2006). The gene transcription rates for Pru p 4.01 and 4.02, two profilin genes in peach, are almost constant throughout the four stages of fruit development (Botton et al., 2009). Since profilins are likely an important link between signal transduction cascades and cytoskeleton organization covering essential cellular functions (Machesky and Pollard, 1993), it was assumed they would show constitutive expression like actins which is important during fruit development. However, during long-term storage, Mal d4 genes in apple were down-regulated (Botton et al., 2008). Our study showed that the expression level of all Mal d4 genes declined after harvest and slowly diminished during ripening (Figs. 2N–P and 3N–P), despite a transient response of Mal d4.01 to ethylene treatment at the early stage of ripening. In contrast, 1-MCP treatment demonstrated the opposite effect to that of ethylene, indicating that both Mal d4.01 and Mal d4.03 are under the influence of ethylene (Figs. 2N and P, and 3N and P). Specific developmental stages or specific tissues may also result in the expression of specific profilin isoforms. This may explain the differences response to ethylene among different isoforms of Mal d4.

Although the number of plant food allergens is actually rather small, understanding the biological function and regulation of each is important in order to devise specific methods to minimize the allergic reaction potential. Studies on individual allergens are therefore needed to support strategies to reduce allergens in plant food products. For example, Lyc e 3, a non-specific lipid transfer protein (ns-LTP) in tomato, was targeted for down regulation by the RNAi approach and showed reduced allergenicity in transgenic compared with wild-type fruit (Le et al., 2006; Lorenz et al., 2006). A similar technique has been applied in an effort to remove

allergens from apple (Gilissen et al., 2005). Our current study provides additional information on regulation of gene expression of apple allergens under the influence of ethylene and 1-MCP, which may be helpful for developing alternative postharvest strategies to regulate target allergens during fruit ripening and mitigate the risk caused by allergens in apple fruit.

The main objective was to examine the effect of ethylene on gene expression of allergens and these findings obtained from a short period study at 20 °C. However, they have potential merit for design of long term storage of apple. It is also fully understood that these findings are based on the gene expression not on protein itself, which is limited by the multi-detection technique available for fruit allergens. A quantitative proteomic study has been developed and conducted using the same biological samples and the results will be report in a subsequent publication.

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