Ethylene-dependent regulation of an α-L-arabinofuranosidase is associated to firmness loss in 'Gala' apples under long term cold storage

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ABSTRACT

Fruit texture changes impair the quality of apples submitted to long term storage, especially under cold. The changes are due to cell wall modifications during ripening and senescence and are associated to ethylene. We have investigated the activity of α-L-arabinofuranosidase, a glycosyl hydrolase acting on the side chains of pectin in the cell wall and middle lamella. The transcription of arabinofuranosidase coding sequences 1 and 3 was investigated in plant organs and in response to ethylene, employing hormone application and 1-methylcyclopropene. The transcription of arabinofuranosidase genes is not restricted to fruits, although upregulated by ripening and ethylene. Transcripts of the genes were detected under cold storage up to 180 days. Similarly, arabinofuranosidase activity increased with rising levels of ethylene and under cold storage. Levels of *ARABINOFURANOSIDASE3* transcripts were higher than those of *ARABINOFURANOSIDASE1*, suggesting that the first is an important contributor to enzyme activity and texture changes during cold storage.

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1. Introduction

Certain apple (*Malus x domestica* Borkh.) genotypes are prone to physiological modifications in the cell wall structure and biochemistry, leading to firmness, crispness and juice losses that negatively affect consumer acceptability of the fruits, including 'Gala' and its derivations (Ng et al., 2013; Oraguzie et al., 2007). These genetically controlled modifications are regulated in a spatial, temporal and developmental manner, largely dependent on complex transcriptional programs, post translational chemical modification and enzyme activity regulation (Ireland et al., 2014; Osorio, Scossa, & Fernie, 2013). The major components of plant cell walls; pectin and hemicellulose polysaccharides, have been demonstrated to undergo solubilization and depolymerization as a consequence of the coordinated action of a wide range of cell wall modifying enzymes, releasing polymer units and altering the cellular microstructure of the fruits (Gapper, McQuinn, & Giovannoni, 2013; Osorio et al., 2013).

In climacteric fruits, such as apples, the onset of ripening is characterized by marked increases in respiration rates and ethylene production, and the critical role for the hormone on the induction of ripening has been demonstrated in tomato plants suppressed for the expression of genes coding for key enzymes in the autocatalytic biosynthesis of ethylene; 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO) and synthase (ACS) (Lin, Zhong, & Grierson, 2009). Similar results were later obtained in loss of function *acc* and *aco* apples (Dandekar et al., 2004). The ethylene produced is perceived by a family of histidine kinase transmembrane receptors that are inactivated by the association with the hormone. In turn, they inactive the repressor, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), thus inducing ethylene responses (Merchante, Alonso, & Stepanova, 2013). Several conservation techniques and scientific studies of climacteric fruits employ 1-methylcyclopropene (1-MCP), a competitive ethylene inhibitor that binds to the hormone receptors to block its action (Tacken et al., 2010; Yang, Song, Campbell-Palmer, Fillmore, & Zhang, 2013). In apple, the activity of several pectin modifying enzymes has been associated to fruit softening during ripening and

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postharvest conservation, including polygalacturonase (PG), pectin methylesterase (PME), β-galactosidase (GAL) and α-L-arabinofuranosidase (AF) (Goulao, Santos, de Sousa, & Oliveira, 2007; Gwanpua et al., 2014; Nobile et al., 2011; Wei et al., 2010). A novel AF coding sequence, MdAF3, belonging to the GH3 family, has been identified and its transcriptional levels have been associated to mealiness in a segregating apple population, where it was demonstrated to function in an ethylene independent way (Nobile et al., 2011). Genotype, ethylene action and environmental conditions are known to influence the transcriptional regulation of genes and the activity of enzymes associated to cell wall metabolism in apple; thus, resulting in distinct fruit phenotypes and sensory properties during ripening and storage (Gwanpua et al., 2014; Ng et al., 2013; Nobile et al., 2011). In order to gain further insight on the role of the α-L-arabinofuranosidase gene family in postharvest texture changes in apple, we have integrated transcription profiling, enzyme activity and physiological analyses to characterize the role of ethylene in regulating the transcription and enzymatic action throughout the storage period of 'Gala' fruits, at room temperature and long term cold storage. Our results demonstrate that the transcription of MdAF3 and α-L-arabinofuranosidase activity are induced by ethylene and contribute to fruit texture modifications, even under long periods of cold storage.

2. Material and methods

2.1. Plant material

Apples (M. x domestica Borkh.) from cultivar Gala, strain Baigent were harvested at physiological maturity (120 days after anthesis) from a commercial orchard. Samples were divided into three technical replicates consisting of five fruits each. Gene expression and fruit quality parameters were investigated under long term cold storage (0–0.5 °C, 90–95% relative humidity) for 60, 120 and 180 days, treated with exogenous ethylene, 1-methylcyclopropene (1-MCP) or as untreated cold stored controls. Fruit quality, enzyme activity and gene expression analyses were also performed for fruits kept at room temperature (23–25 °C, 75–80% relative humidity), submitted to 1-MCP treatment and as untreated control, for 12 days. Exogenous ethylene application was not investigated for fruits kept at room temperature since the endogenous hormone production was shown to saturate the investigated responses. Endogenous ethylene production was evaluated for fruits kept at room temperature at 3 day intervals after harvest up to 12 days. Sampling points are schematically represented in Supplementary Fig. 1.

Fully expanded leaves, flowers at anthesis and green fruits 60 days after anthesis were used to determine the spatial expression patterns of MdAF1 and MdAF3.

2.2. Ethylene and 1-MCP application

Exogenous ethylene application was carried out in 370 L containers, containing 50 kg of apples, at 10 ppm (10 μL L^{-1}), at 20 °C as described in Asif, Pathak, Solomos, and Trivedi (2009), using an exposure time of 4 h.

The recommended concentration of 1 ppm (1 μL L^{-1}) of 1-MCP (AgroFresh™, Dow Chemical Company) (DeEll, Ayres, & Murr, 2008) was applied for 24 h to 370 L chambers containing 50 kg of apples at 20 °C. After treatments, the fruits were transferred to room temperature or cold storage, as described.

2.3. Fruit quality parameters

Total soluble solids (TSS) were analyzed employing a refractometer (model PR 101, Atago) (0–45%), with temperature correction, and the values are presented as °Brix. Total titratable acidity was evaluated in a 10 mL sample from the juice of each replicate diluted in 90 mL of distilled water, titrated with a digital burette containing 0.1 M NaOH until pH 8.1 measured using a digital pHmeter.

Pulp firmness was measured from two opposite sides at the equatorial region of the fruits from where the epidermis had been removed. The measurements were taken using a manual penetrometer model P830075, TR Italy equipped with a point of 11 mm. Penetration depth of 8 mm was used at a 4 mm s^{-1} rate, as described by Girardi, Nachtigall, and Parussolo (2004).

Iodine–starch indices were evaluated at harvest by slicing the fruits at the equatorial region and immersing the peduncle half in an iodine solution (12 g of I$_2$ and 24 g of KI in 1000 mL of distilled water) for 40 s. Starch presence was evaluated after immersion by comparison to reference values presented by Girardi et al. (2004), ranging from 1 (maximum starch) to 5 (starch absence). The absence of starch indicates advanced ripening stages.

2.4. Ethylene production determination

Ethylene production by the fruits was determined by gas chromatography using a CG 3537-5 equipment, supplied with a stainless steel column prepared with Porapak™ Q 5 and a flame ionization detector. The temperatures used for the vaporizer, column and detector were of 140 °C, 70 °C and 142 °C, respectively. A solution of ethylene 10 ppm was employed as standard.

Fruits, with a total weight of 1 kg, were placed in hermetically closed flasks for 1 h at 25 °C and the gas atmosphere was collected using hypodermic needles. Ethylene contents were quantified by correlating the mean height of the peaks from each sample and the mean height of the peak from the ethylene standard solution.

2.5. Reverse Transcription-quantitative PCR

Total RNA was extracted from 6 g of pulverized fruit tissue according to the protocol described by Zeng and Yang (2002), with an additional precipitation step with sodium acetate 3 M pH 5.5, followed by incubation at −80 °C for 25 min and centrifugation at 20,000×g for 20 min at 4 °C, before the overnight precipitation with 10 M LiCl. Quantity and integrity of the isolated RNA were evaluated by spectrophotometric readings (Epoch Micro-volume Biotek) and 1% (w/v) gel electrophoresis. For cDNA synthesis, 2 μg of total RNA were treated with DNase I (New England Biolabs) and submitted to reverse transcription using oligo d(T) primers (Invitrogen) and MMLV-Reverse Transcriptase (Promega). The primers for expression analyses were designed based on M. x domestica coding sequences (CDs) publically available at the GDR (Genome Database for Rosaceae) database employing the software Primer3Plus (Untergasser et al., 2007). The sequences of the primers used in the current work and the optimization parameters are presented as Supplemental Table 1. Primer sequences for gene expression normalization were chosen by evaluating the transcription of MdACT (β-ACTIN), MdUBC (UBIQUITIN-CONJUGATING ENZYME E2), MdPDI (DISULFIDE ISOMERASE), MdNAP1 (NUCLEOSOME 1 BINDING PROTEIN) and MdH1 (HISTONE 1) for all tested RNA samples, employing the software DataAssist v 3.01 (Life Technologies). The genes exhibiting the most stable transcription profile were MdUBC, MdH1 and MdPDI for ripe fruits under cold storage, MdPDI, MdUBC and MdACT for immature fruits, leaf and flower tissues and MdH1, MdUBC and MdACT for ripe fruits stored at room temperature. Real time quantitative PCR was carried out in a StepOne™ Real Time PCR Systems (Life Technologies) using the SYBR™ Green PCR Master Mix (Life Technologies). Relative transcription rates were determined employing the harvest time samples.

2.6. Functional motif and phylogenetic analyses

Functionally characterized α-L-arabinofuranosidase sequences from *Arabidopsis thaliana* and the previously characterized apple AF3 and AF1 were used to query the *M. x domestica* genome (Apple Genome V1.0 pseudo haplotype – primary assembly), *Vitis vinifera* 12X (http://www.genoscope.cns.fr/cgi-bin/blast_server/projet_ML/blast.pl) and *Solanum lycopersicum* V.2.3 (http://solge-nomics.net/tools/blast/) genomes using the tBLASTn algorithm (Altschul et al., 1997). *Pyrus* sequences were obtained from tBLAST searches at the ExPaSy/Prosite database (http://prosite.expasy.org/). The retrieved sequences were validated by reverse BLAST to *Arabidopsis* and conserved domain analyses, using the HMMER-based SMART Web site (http://smart.embl-heidelberg.de). The resulting hits were filtered by a threshold e value of $1e^{-15}$ and sequences failing to retrieve the original bait or lacking conserved domains associated to enzyme function were eliminated from further analyses.

Validated sequences were translated and protein alignments were performed using maximum parsimony methods in the software MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), with default parameters. Re-sampling bootstrap trees containing 1000 random samples were constructed using PSIGNFIT software (http://www.bootstrap-software.org/).

2.7. Microarray gene expression analysis

Gene expression profiling in tomato and grapevine were analyzed from publicly available data from the Plant Compliant Gene Expression Resources for Plants and Plant Pathogens (http://www.plexdb.org/). The genes of interest were used in queries against microarray sequences, and the hits were aligned by Clustal X in MEGA 6.0 (Tamura et al., 2013). The expression values were retrieved, normalized and analyzed employing the package LIMMA (Smyth, 2005) in the computational language R v.2.5.13 (R Core Team, 2012). Hierarchical clustering was performed employing Spearman Rank correlation matrix in LIMMA. Graphic outputs were generated and are presented as color scale.

2.8. Enzyme activity

Protein extraction for enzyme activity analyses was adapted from Wei et al. (2010). Briefly, 3 g of pulverized apple pulp were evenly distributed to six 2 mL microtubes (0.5 g per microtube) and 1 mL of homogenizing solution (12% w/v polyethylene glycol, 0.2% w/v sodium bisulfite). After mixing, the samples were centrifuged at $20,000 \times g$ for 10 min at 4 °C. The supernatant was discarded and the pellet, washed with 1 mL of 0.2% (w/v) sodium bisulfite solution. The mixture was centrifuged as described previously and 1 mL of extraction buffer (0.1 M sodium acetate pH 5.2, 0.1 M NaCl, 5% (w/v) polyvinyl pyrrolidone) was added per tube. The tubes were vigorously shaken and incubated overnight at 4 °C. The supernatants were pooled as brute extract. Total protein in the extract was quantified using Bradford (1976) reagent by spectrophotometric absorbance readings at 595 nm (Evolution 60, Thermo Scientific) in comparison to a standard curve with bovine serum albumin.

The activity of α-L-arabinofuranosidase was spectrophotometrically evaluated by the production of ρ-nitrophenol from the artificial substrate ρ-nitrophenyl α-L-arabinofuranoside, as described by Brummell, Dal Cin, Crisosto, and Labavitch (2004). Briefly, 40 µL of the protein extract were transferred to a microtube containing 400 µL of 0.1 M sodium acetate pH 5.0 and 80 µL of the substrate (16 µL of ρ-nitrophenyl α-L-arabinofuranoside 50 mg mL^{-1} in 64 µL of water). The reaction mixture was incubated at 30 °C for 90 min and an aliquot of 250 µL was removed and added to 250 µL of 1.0 M of NH_4OH 1 M. Absorbance values were subtracted from the blank readings prepared with protein extract inactivated by boiling. Protein extraction and enzyme activity quantification were carried out in duplicate.

3. Results

3.1. Fruit quality analyses

The conservation of apples at 0 °C impaired soluble solids accumulation for periods as long as 180 days, although a reduction of 41.5% in titratable acidity was observed in comparison to harvest (Table 1). As part of the ripening process, apples stored at room temperature (RT) for short periods exhibited a slight increase in total soluble solids and decrease in titratable acidity (Table 1). The application of 1-MCP to fruits stored at RT reduced firmness losses to approximately 7% in 12 days, whereas in untreated fruits firmness reduction reached up to 83% during the period (Table 1). The application of exogenous ethylene and 1-MCP to fruits submitted to cold storage (CS) caused a slight change in the normal ripening patterns of soluble solids accumulation and acidity reduction (Table 1). In contrast, fruit firmness was affected by ethylene and 1-MCP treatments under long term cold storage and short term room temperature conservation (Table 1). Fruit firmness was shown to decrease with time for all tested conditions, although at distinct rates (Fig. 1). Treatment with 1-MCP under cold storage promoted a firmness increase of approximately 1.32 times, although softening rates remained similar (Fig. 1 and Table 1). Under long term CS (180 days), firmness loss was similar for control and ethylene treated fruits but slightly smaller in 1-MCP treated apples (Fig. 1).

3.2. Ethylene production

Ethylene production was investigated by time course analyses of the fruits from harvest, for 12 days at RT, after ethylene and 1-MCP treatment and for fruits under CS. The results indicate a strong inhibition of ethylene production by 1-MCP, with virtually no detection of the hormone in presence of the inhibitor (Supplementary Fig. 2). At RT, exogenous ethylene application did not alter the pattern of the hormone accumulation, although total levels were slightly inferior in control fruits (Supplementary Fig. 2). After 60 days under cold storage, ethylene accumulation increased for ethylene treated and control fruits; however, exogenous hormone application did not further increase ethylene levels in fruits under CS (Supplementary Fig. 2).

3.3. The α-L-arabinofuranosidase gene family in apple

In the apple genome, we have identified 10 putative genes coding for α-L-arabinofuranosidases using stringent search parameters. The majority of the apple genes share extensive sequence conservation with the family of bifunctional β-D-xylosidase/α-L-arabinofuranosidases of *Arabidopsis* and tomato (Supplementary Fig. 3). The grapevine gene family has undergone differential expansion, since the vast majority of the identified sequences exhibits more significant sequence conservation to β-D-xylosidases, rather than to α-L-arabinofuranosidases. Besides the three previously characterized α-L-arabinofuranosidases (*AF1*, *AF2* and *AF3*), apple genome has seven additional sequences, coding for putative β-D-xylosidases/arabinofuranosidases, indicating the occurrence of gene duplication events throughout evolution in apple (Supplementary Fig. 3).

Table 1
Time course analyses of apple fruit quality traits stored at room temperature and under cold storage, treated with 1-MCP, exogenous ethylene application and untreated. Exogenous ethylene application was not performed for fruits kept at room temperature and physiological analyses are not available (na). Statistical significance within treatments, calculated by Tukey test ($p \leqslant 0.05$), is represented by lower- (lines) and uppercase (columns) lettering.

Trait	Treatment	Storage time (days)								
		Room temperature (RT)						Cold storage (CS)		
		2	4	6	8	10	12	60	120	180
Firmness (N)	Control	84.7 aA	78.3 bB	77.9 bB	76.8 bB	75.3 bB	70.3 cB	59.9 aB	61.9 aA	56.9 aB
	1-MCP	86.4 aA	84.6 abA	83.0 abA	81.4 abA	80.9 bA	80.4 bA	79.2 aA	73.1 aA	70.0 aA
	Ethylene	na	na	na	na	na	na	67.0 aB	61.9 aA	64.5 aAB
TSS (°Brix)	Control	12.9 cA	13.3 bcA	13.4 abcA	14.1 abA	14.2 aA	14.1abA	13.2 aA	13.1 aA	12.8 aA
	1-MCP	12.7 cA	13.3 bcA	13.3 bcA	13.7 abA	13.9 abA	14.2 aA	13.9 aA	14.1 aA	13.5 aA
	Ethylene	na	na	na	na	na	na	13.8 aA	14.2 aA	13.7 aA
TA (% malic acid)	Control	4.8 aA	4.7 aA	4.5 aA	4.4 aA	4.3 aA	4.1 aA	4.2 aAB	3.5 bA	3.2 bB
	1-MCP	5.2 aA	5.2 aA	5.0 aA	4.7 aA	4.6 aA	4.5 aA	4.3 aA	3.9 aA	3.8 aA
	Ethylene	na	na	na	na	na	na	3.8 aB	3.8 aA	3.2 bB
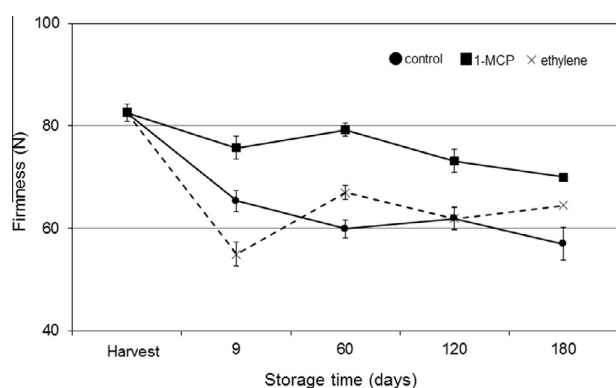
Fig. 1. Pulp firmness of 'Gala' apples at harvest and control storage conditions, ethylene or 1-MCP treatments after nine days at room temperature (RT) and 60, 120 and 180 days in cold storage (CS).

3.4. Gene expression profiling

The transcription profiles were determined for two apple α-L-arabinofuranosidases encoded by *MdAF1* and *MdAF3* (Supplementary Table 1) during storage (Fig. 2). The transcriptional regulation of the gene coding for ethylene biosynthesis enzyme 1-aminocyclopropane-1-carboxylate oxidase (ACO1) and cell wall metabolism enzyme endopolygalacturonase (PG1) (Supplementary Table 1), associated to ethylene mediated ripening processes, were also investigated (Fig. 3).

Reference genes for the experimental conditions are presented in Supplementary Table 1 and were described as Supplementary Table 2.

The genes coding for AF3 and AF1 were expressed in all investigated conditions, including under cold storage and in the presence of ethylene inhibitor 1-MCP (Fig. 2). Steady-state levels of *MdAF3* transcripts were on average 3.0 times higher than those of *MdAF1* throughout the investigated conditions (Fig. 2). The expression of *MdAF3* was strongly inhibited in the presence of ethylene inhibitor 1-MCP (Fig. 2), as observed for *MdACO1* (Fig. 3) and demonstrated by the high positive correlation of 0.92 (p-value of 2.04^{e-10}). Similarly, *MdAF1* expression regulation was also positively correlated to *MdACO1*, however at lower levels, with a Spearman rank coefficient of 0.47 (p-value of 2.06^{e-4}). Prolonged exposure to cold had stronger inhibitory effect on the transcription of *MdAF1* than on *MdAF3*, whereas the magnitude of 1-MCP inhibition was stronger on *MdAF3* (approximately 6.0-fold) than on *MdAF1* (approximately 2.0-fold) (Fig. 2).

High levels of *MdAF3* transcripts were detected in leaf tissues, whereas in immature green fruits, its expression is low and induced at later stages of ripening (Fig. 2). Similar results were observed in tomato, where the transcription of *SlAF3* (probe Les.4445.1.S1_at) is restricted to ripening fruits (Fig. 4). In grape berries, *VvAF3* (probe 1615005_at) expression is independent of the ripening stage (Supplementary Fig. 4). In contrast, *MdAF1* is expressed in flowers and immature fruits and not induced at later stages of ripening (Fig. 2). A similar non fruit specific pattern is observed for *SlAF1* (probe Les.3991.1.S1_at) that is highly induced in vegetative tissues, such as hypocotyls, cotyledons and leaves (Fig. 4). In tomato fruits, *AF1* transcripts are restricted to initial ripening stages in the pulp and peel (Fig. 4). The transcription of grapevine *AF1* (probe 1611233_at) is also repressed in the later stages of berry ripening; however, it appears to be modulated by environmental conditions since the year affects its response (Supplementary Fig. 4).

The transcription of *MdPG1* was repressed in 1-MCP treated fruits throughout the experiment and did not increase with the application of exogenous ethylene (Fig. 3). The highest levels of *MdPG1* transcript accumulation were detected in untreated control fruits, as observed for *MdACO1* under cold storage up to 120 days (Fig. 3). As observed for *MdAF3*, the transcription pattern of *MdPG1* also exhibited a significant correlation to *MdACO1* profile (Spearman rank correlation of 0.84, p-value 2.02^{e-7}). The high standard deviation values for *MdPG1* expression is a consequence of its high transcription levels.

3.5. Enzyme activity

The activity of α-L-arabinofuranosidase increased with storage time under RT and CS for control fruits and all tested treatments (Fig. 5). Application of ethylene and its inhibitor did not significantly alter enzyme activity in fruits conserved for short periods at RT (Fig. 5). Under cold storage, in control, ethylene and 1-MCP treated fruits the highest levels of enzyme activity occurred after 180 days, coinciding with higher levels of ethylene accumulation (Supplementary Fig. 2, Fig. 5). After six months of cold storage, the application of 1-MCP promoted a twofold reduction in α-L-arabinofuranosidase activity, whereas exogenous ethylene slightly increased enzymatic action in comparison to control cold stored fruits. The hormone treatment accelerated the peak of α-L-arabinofuranosidase activity to 60 days under CS, whereas in untreated fruits it was observed four months later (Fig. 5).

4. Discussion

In the current work, an integrative approach combining transcriptional profiling, quality characterization and biochemical
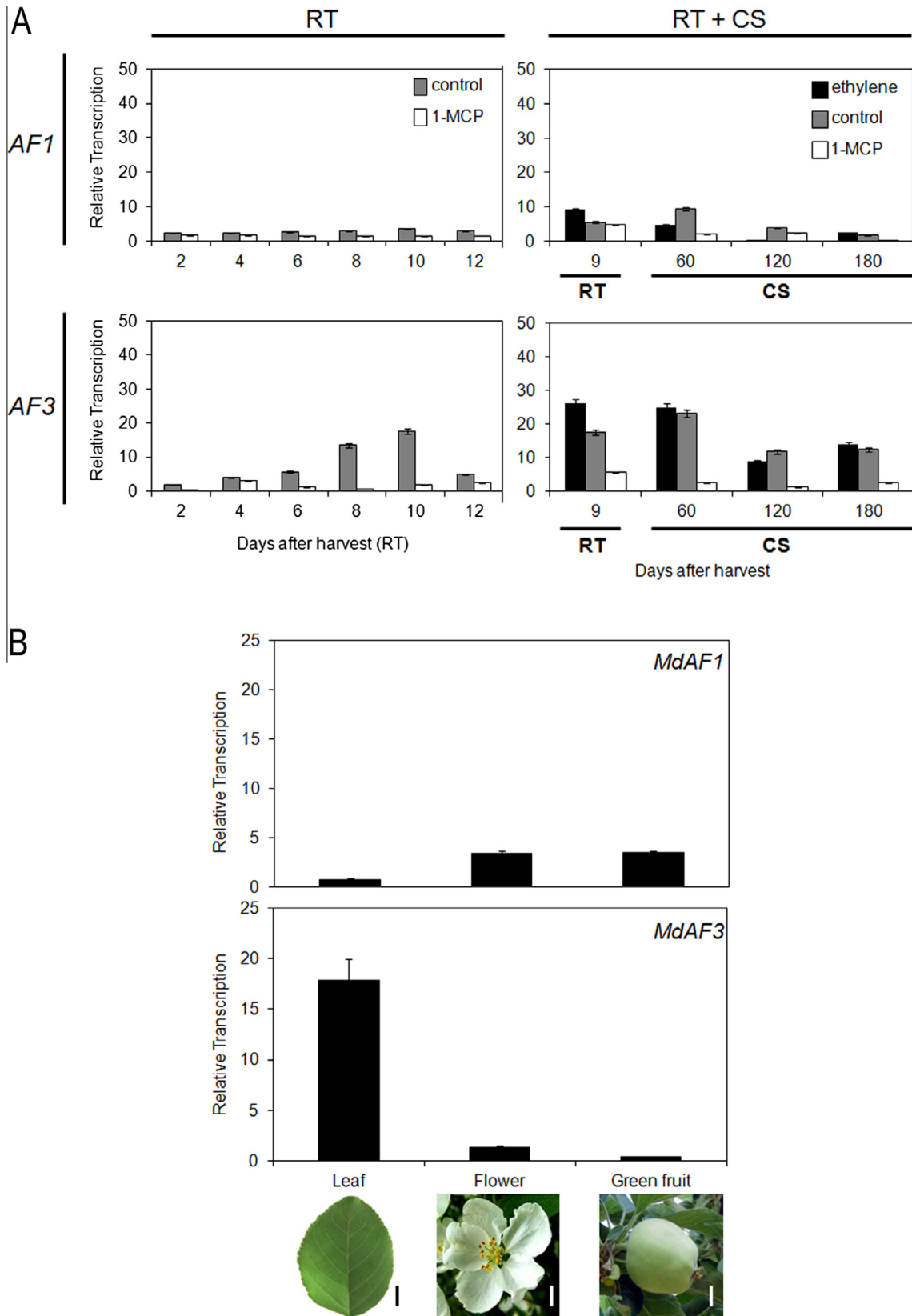
Fig. 2. Relative expression of *MdAF1* and *MdAF3* in fruits under control conditions, 1-MCP and ethylene application treatments, kept under room temperature and cold storage conditions (A) and plant organs (B). The normalization and relative transcription calculations are described in Section 2 and Supplementary Material. Relative transcription corresponds to average values for three technical replicates. Error bars represent ± standard deviation (A) and +2 × standard deviation (B). Representative apple plant organs are depicted and scale bars correspond to 1.5 cm (leaf), 0.9 cm (flower) and 0.95 cm (green fruit) (B).
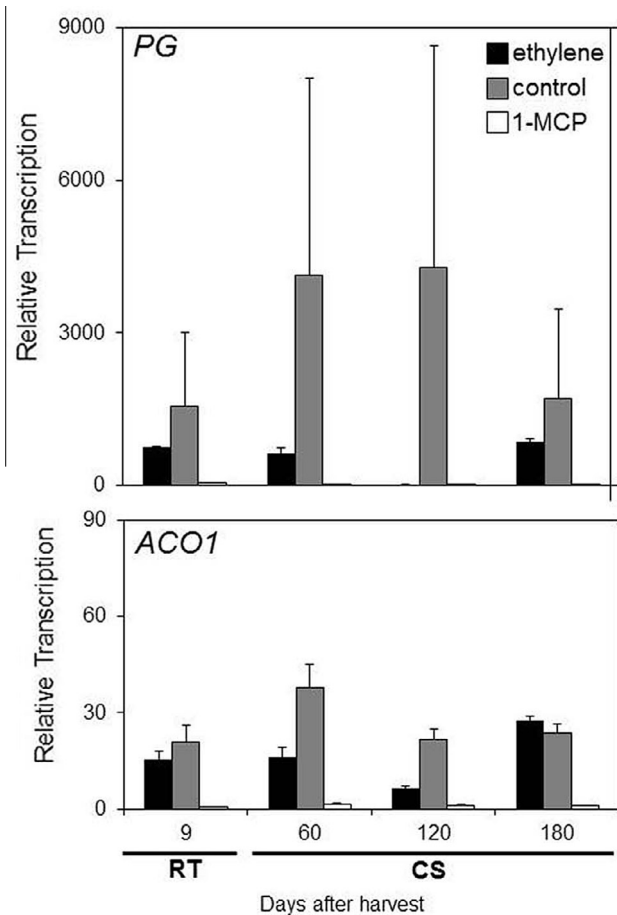
Fig. 3. Relative expression of *MdPG1* and *MdACO1* in fruits during control conditions, 1-MCP and ethylene application treatments, kept under room temperature and cold storage. The normalization and relative transcription calculations are described in Section 2 and Supplementary Material. Relative transcription corresponds to average values for three technical replicates. Error bars represent +2 × standard deviation.

analyses was employed to investigate the texture changes undergone by 'Gala' apples during storage and the role of ethylene in controlling ripening associated processes.

4.1. Storage induced changes in quality traits

The main changes associated with the ripening process in fleshy fruits include color, firmness, taste, and flavor (Osorio et al., 2013). The reduction in pulp firmness is dependent on cell wall degradation and alterations in cuticle properties leading to water loss by transpiration, apoplast composition changes and reduction in turgor pressure (Tong et al., 1999; Vicente, Saladié, Rose, & Labavitch, 2007), whereas the modification in taste is due to an increase in sugar and concomitant decline in organic acids contents (Osorio et al., 2013). The most significant change occurring during storage in 'Gala' apples was pulp softening. Fruit softening in apple was proportional to the length of the storage period, even in the presence of 1-MCP, suggesting that other developmental factors besides ethylene contribute to the processes control. The changes associated to fruit taste were not critically affected by storage, although the expected increase in total soluble solids and decrease in titratable acidity were detected at room temperature and under cold storage. Similarly, a comprehensive metabolome study of apples under storage confirmed that most carbohydrates and organic acids are not appreciably affected (Lee, Rudell, Davies, & Watkins, 2011).

4.2. Effect of ethylene on quality traits of stored apples

The exogenous application of ethylene inhibitor did not strongly affect taste parameters, such as sugar accumulation and acidity reduction in 'Gala' apples during short term room temperature storage or long term cold storage. In contrast, 1-MCP had a significant positive effect on firmness conservation for fruits at room temperature and under cold storage. For the investigated responses, the role of exogenous ethylene application was not clear, since the responses observed in untreated control fruits were higher than those in hormone treated apples. These observations suggest that endogenous ethylene production is sufficient to saturate the
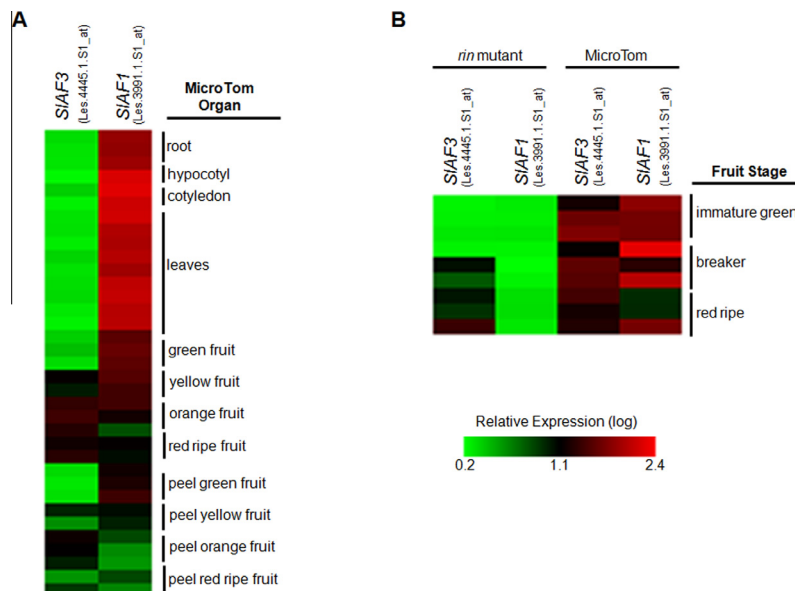
Fig. 4. Expression profile of tomato *SlAF3* (Les4445.1.S1_at) and *SlAF1* (Les3991.1.S1_at) genes in 'MicroTom' plant organs (A) and in 'MicroTom' and *rin* mutant fruit developmental stages (B). Color scale representing relative expression is shown on the right (green refers to low expression; black refers to medium expression and red refers to high expression). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
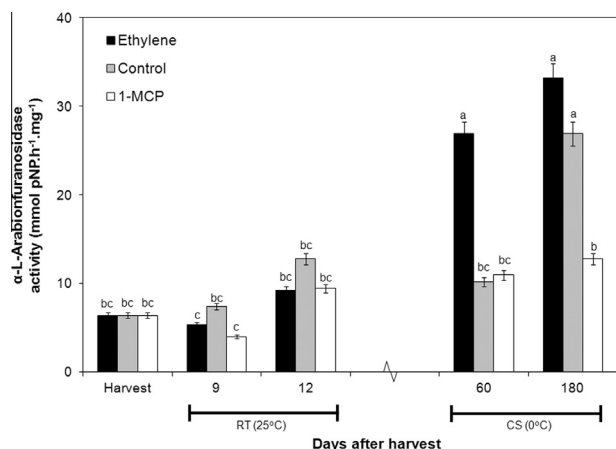
Fig. 5. Activity of α-ʟ-arabinofuranosidase in apples under control conditions and 1-MCP and ethylene treatments. Time course starts at harvest, followed by room temperature conservation (9 and 12 d) and subsequent cold storage sampled at 60 and 180 d. Enzyme activity is represented as mM of p nitrophenol (pNP) release per hour per mg of protein extract. Distinct letters represent statistical difference at 5% of probability by Tukey test in comparisons of enzyme activity among the conditions.

investigated responses, in accordance with the recent sensitivity dependency proposed model, which shows that ethylene-dependent traits can progress over time to the same degree with lower levels of ethylene (Ireland et al., 2014).

4.3. Gene expression profiling under storage

The transcriptional activation of *MdACO1* has been demonstrated to have a strong positive correlation with the enzymatic activity of ACO and ethylene production in stored 'Jonagold' apples (Bulens et al., 2014). Exogenous ethylene application did not significantly increase *MdACO1* expression under our experimental conditions, whereas treatment with 1-MCP strongly repressed the gene transcription. These observations are in agreement with other studies demonstrating non correlated effects of the application of the hormone and its competitive inhibitor on *ACO* genes expression (Bulens et al., 2014; Ng et al., 2013). The absence of transcriptional induction by the hormone application treatment may be due to sufficient endogenous ethylene produced leading to response saturation.

The expression of the single PG coding sequence in apple genome is induced during ripening, after exposure to ethylene and cold (Goulao & Oliveira, 2008; Tacken et al., 2010) and genetic studies have associated it to firmness decrease during ripening (Costa et al., 2010; Longhi, Moretto, Viola, Velasco, & Costa, 2012). A discrepancy in the transcriptional response of *MdPG1* to ethylene and 1-MCP treatments was also observed, since the first produced no significant effect on steady state mRNA levels of the gene and the second imposed a severe repression on transcription. *MdPG1* was highly expressed in apples stored at room temperature and under cold storage and gene expression was suppressed in fruits retaining firmness due to 1-MCP treatment, thus confirming previous associations between PG induction and fruit softening (Costa et al., 2010; Longhi et al., 2012; Tacken et al., 2010).

The transcription profile of the investigated *PG* and *AF* genes was similar, suggesting that the α-ʟ-arabinofuranosidase activity may contribute to the polygalacturonase activity by releasing the arabinose residues from the side chains and allowing access of PG to the principal chain. Data from previous work have demonstrated the coordinate action of cell wall degrading enzymes (Goulao & Oliveira, 2008), the removal of pectin side chains

increases cell wall porosity allowing the access of other hydrolases (Brummell, 2006).

In a previous work using distinct genetic backgrounds, the transcription of *MdAF3* has been demonstrated to be independent of ethylene (Nobile et al., 2011). Our data showed that in 'Gala' apples, the transcription of *MdAF3* is dependent of the hormone or its signal transduction, as observed for *MdACO1*. Similar results were obtained from tomato microarray analyses, where *SlAF3* transcription is also ethylene dependent during ripening and in *rin* mutants. In contrast to previous in silico analyses (Nobile et al., 2011), we have demonstrated the presence of *MdAF3* transcripts in flower and leaf tissues in apple. Similarly, in peach transcripts corresponding to arabinofuranosidases from GH51 and GH3 families were present in leaves and flowers (Di Santo, Pagano, & Sozzi, 2009).

The gene *MdAF1* exhibited low levels of transcription in leaves and high levels in flowers and immature fruits. Similarly, the expression profile for the gene described by Goulao, Cosgrove, and Oliveira (2008), employing semi quantitative PCR analyses, includes leaf, flower and petiole. The technical differences between real time quantitative and semi quantitative PCR may account for the differences in the transcription profile of *MdAF1* in the current work and the previous report (Goulao et al., 2008). The low transcription levels of *MdAF3* in immature apple and tomato fruits are in agreement with the bioinformatic analyses of Nobile et al. (2011), thus, reinforcing its role in later stage fruit ripening when texture changes occur (Gwanpua et al., 2014).

The regulation of *MdAF1* also appears to be modulated by ethylene, since it displays a profile similar to that of *MdACO1* in 1-MCP mediated repression, mainly under long term cold storage. A similar expression profile was reported in 'Golden Delicious' treated with the inhibitor at the commercial maturation point (Wei et al., 2010).

4.4. α-ʟ-Arabinofuranosidase activity and firmness loss during storage

Pulp firmness loss and other texture alterations, such as mealiness, have been associated to cell wall modifications brought about by the action of several enzymes in fleshy fruits during ripening (Goulao et al., 2007; Nobile et al., 2011; Tateishi et al., 2005). These enzymes act on polysaccharides of the cell wall promoting the depolymerization, solubilization and loss of neutral sugars in the pectic and hemicellulosic fractions, thus, disturbing the organization of the cell wall and causing chemical and microstructural alterations leading to pulp softening (Gwanpua et al., 2014; Ng et al., 2013). Enzymes associated to the depolymerization of cell adhesion homogalacturonan pectin, such as pectin methylesterase (PME) and endo-polygalacturonase (PG), are considered critical players in fruit texture changes during ripening in apple (Atkinson et al., 2012; Ng et al., 2013). Recently, several genetics and biochemical approaches have identified arabinofuranosidases/xylosidases as important contributors to the coordinated enzymatic network leading to texture changes in ripening fruits (Di Santo et al., 2009; Gwanpua et al., 2014; Nobile et al., 2011; Tateishi et al., 2005). Although the role of ethylene in fruit softening is well established, a direct correlation between the hormone accumulation and the activity of cell wall hydrolases is not always observed. The activity of two α-ʟ-arabinofuranosidase forms is regulated in an ethylene independent manner in tomato, whereas the activity of a third, distinct isoform, has been demonstrated to be controlled by the hormone (Sozzi, Greve, Prody, & Labavitch, 2002). In plants unable to produce ethylene due to silencing of a key enzyme of its biosynthesis, the low temperatures used in cold storage were shown to interfere with the regulation of fruit firmness in 'Royal Gala' apples (Tacken et al., 2010).

We were able to detect α-L-arabinofuranosidase activity throughout the investigated storage period, at higher levels under CS in comparison to RT, in agreement with previous works describing a prevalent role of the enzymatic activity during the post-harvest period (Goulao et al., 2007; Nobile et al., 2011; Wei et al., 2010). Moreover, low temperatures under CS may have also contributed for the higher rates of α-L-arabinofuranosidase activity, as shown for β-galactosidase in tomato (Rugkong et al., 2010) and PG in apple (Tacken et al., 2010). The effect of 1-MCP on the enzyme activity was more pronounced at later storage stages, suggesting the presence of a developmental control switch.

The presence of differentially regulated enzyme isoforms has been predicted in 'Mondial Gala' by Goulao et al. (2007). In tomato, two α-L-arabinofuranosidase isoforms are regulated independently of ethylene in response to gibberellic acid and synthetic auxin (Sozzi et al., 2002). The high transcriptional levels of *MdAF3*, in comparison to *MdAF1*, throughout the investigated period may suggest that the influence of the first is determinant to the enzymatic activity. The steady state levels of *MdAF1* transcript were higher than those of *MdAF3* in immature fruits, thus suggesting that *MdAF1* may play a role in growth associated cell wall modification processes. In contrast, the role of *MdAF3* is more likely related to cell wall degradation during postharvest texture alteration in apple.

5. Conclusions

In the current work, we have demonstrated the positive regulation of *MdAF3* transcription by ethylene in 'Gala' apples during postharvest storage, indicating that it is involved in fruit texture changes during prolonged cold storage. It is also suggested to represent the principal gene responsible for coding the α-L-arabinofuranosidase isoform active during prolonged storage and fruit texture changes. The transcription of *MdAF1* is likely to represent a less significant contribution to the activity resulting in fruit softening under cold storage. Our results also indicate the effectiveness of 1-MCP application as a tool to investigate ethylene regulated processes during apple ripening. In contrast, endogenous ethylene production was sufficient to saturate the investigated responses masking the effect of exogenous hormone application.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.02.123.

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