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# 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.



## 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.