CS Science with quality
e-ISSN: 2177-5133
www.comunicatascientiae.com

DOI: 10.14295/CS.v7i4.1545

Comunicata Scientiae 7(4): 494-503, 2016

Article

Identification of biomarkers associated to 'Gala' apples ripening and postharvest quality

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Abstract

Apple is, sociocultural and economically, on of the most important species in the world, and stands out for its high storage potential. However, the monitoring of factors that could result in fruit quality modifications during postharvest is essential to ensure the acceptability and for the development of new storage technologies in order to increase fruit shelf life. Approaches focused on molecular biology, such as RT-qPCR have been used to better understand the mechanisms involved in fruit development and maturation. In this study the use of RT-qPCR to monitoring apple quality during ripening and development in different postharvest conditions such as room temperature, cold storage with or without control of atmosphere and the 1-methylcyclopropene usage were proposed. The potential of genes involved in ethylene biosynthesis and response, cell wall modification and degradation, sugar and aroma metabolisms for employment as biomarkers of fruit development and quality were evaluated. Thus *MdEXP4* is highlighted as biomarker for development and *MdACO1*, *MdPG1*, *MdAF1*, *MdAF3* and *MdAAT2* as potential biomarkers for ripening. *MdACO1* and *MdPG1* appear as suitable markers for quality, conservation technologies and storage time in apples. This work suggests that the study of gene expression by RT-qPCR may be an alternative for a better fruit characterization during the development and postharvest period.

Keywords: Cold storage, gene expression, *Malus* x *domestica*, molecular analysis

Identificação de biomarcadores associados ao amadurecimento e qualidade pós-colheita em maçãs 'Gala'

Resumo

A maçã é, sociocultural e economicamente, uma das espécies mais importantes do mundo, destacando-se por seu alto potencial de armazenamento. Contudo, o monitoramento dos fatores que alteram a qualidade dos frutos durante a pós-colheita é essencial para garantir a aceitabilidade dos mesmos, e para o desenvolvimento de novas tecnologias de armazenamento com o intuito de aumentar a durabilidade dos frutos. Técnicas de Biologia Molecular, como a RT-qPCR têm sido empregadas para melhor entendimento dos mecanismos envolvidos com o desenvolvimento e maturação dos frutos. Este estudo propõe a utilização de RT-qPCR para o monitoramento da qualidade de maçãs durante o desenvolvimento e amadurecimento em diferentes condições de pós-colheita, como temperatura ambiente, armazenamento refrigerado com ou sem controle da atmosfera, e uso do 1-metilciclopropeno. Para isso foi avaliado o potencial de genes envolvidos com a biossíntese e resposta ao etileno, modificação e degradação da parede celular e metabolismo de açúcares e aromas para uso como marcadores de desenvolvimento e qualidade dos frutos. Assim, destacaram-se os genes *MdEXP4* como bom marcador de desenvolvimento e *MdACO1*, *MdPG1*, *MdAF1*, *MdAF3* e *MdAAT2* como potenciais marcadores de amadurecimento. *MdACO1* e *MdPG1* aparecem ainda como bons marcadores de qualidade, tecnologias de conservação e tempo de armazenamento. Sugere-se o estudo da expressão gênica como alternativa para melhor caracterização dos frutos durante os períodos de desenvolvimento e pós-colheita.

Palavras-chave: Armazenamento refrigerado, expressão gênica, *Malus* x *domestica*, análises moleculares

Received: 17 September 2015
Accepted: 11 February 2016

Introduction

The technological advances have allowed whole genome sequencing of several species. A large number of genome sequences from fruit species, such as apple, is currently available (Gapper et al., 2014). The increasing availability of genome sequences of fruit species has paved the way for functional genomics approaches including analyses of fruit transcriptome, proteome and metabolome.

The transcription analyses are an important tool to investigate the molecular changes during the postharvest period. Different technologies are available for transcriptome exploration, such as reverse transcription quantitative polymerase chain reaction (RT-qPCR) which is characterized by its rapidity, high sensitivity, specificity, precision, reproducibility (Gachon et al., 2004) and lower cost in comparison with other transcription analyses techniques (VanGuilder et al., 2008).

In others fields of study, transcriptional analyses are been used with classical techniques or replacing them in phenotyping analysis (Valdés et al., 2013). Likewise, RT-qPCR can be employed in postharvest with classic techniques for fruit quality determination or replacing more expensive biochemical approaches that would require specific equipment, not always available in molecular biology laboratories.

In this context, the use of biomarkers is promising in monitoring fruit quality. RT-qPCR has been shown an important tool for biomarker monitoring, where, previously identified targets can be assayed in a very large numbers of samples (VanGuilder et al., 2008).

Apple (*Malus* x *domestica* Borkh.) is one of the most important fruit species, socially and economically (Both et al., 2014; Zhu et al., 2013). Molecular biology tools have allowed scientific advances in the field and have been applied to numerous studies in apples (Ireland et al., 2014; Muñoz-Bertomeu et al., 2013; Nobile et al., 2011; Visser et al., 2014; Zheng et al., 2013). Some of these studies correlated gene expression patterns with biochemical and physiological changes taking place in fruit development (Atkinson et al., 2012, Dandekar et al., 2004). Thus, genes associated to fruit ripening can be used as biomarkers for physiological disorders, storage time and marketing decisions. The transcriptional profile of these genes can be used to monitor fruit development and to propose novel alternatives for storage, increasing fruit shelf life.

In this context, the current study aimed to identify biomarkers associated to the ripening process, condition and period of storage in 'Gala' apples. Apple genes with known behavior in fruit ripening and during postharvest storage, along with novel genes, for which the transcriptional profile is less studied, were analyzed under standard conditions, such as ripening at room temperature (RT) and under cold storage (CS). Moreover, fruit development and ripening under controlled atmosphere (CA) conditions, where the mechanisms responsible for fruit modifications are not well established, were analyzed. The current study highlights the use of RT-qPCR to monitor fruit quality.

Material and Methods

Plant Material

'Gala' apples from the clone Baigent, on M9 rootstocks, from a commercial five-year old orchard in the region of Caxias do Sul, RS, Brazil, during the 2013/2014 growing season were used. In Experiment I – 'Apple developmental stages', the samples were harvested with intervals of 15 days , starting at full bloom up to 105 days after anthesis (DAA).

For Experiment II – 'Ripening evolution in apples', fruit were harvested at physiological maturity (an average of 87 N of firmness) and split into two lots. One group was submitted to treatment with 1 μL L^{-1} of 1-methylcyclopropene (1-MCP) and the other remained without treatment. Subsequently, treated and control fruit were kept at room temperature (25°C) for 12 days to investigate ripening in the presence and absence of the ethylene inhibitor. Fruit were sampled at two days intervals.

In Experiment III – 'Influence of storage conditions on apples quality', the fruit were harvested when physiologically ripe and, subsequently, split into two groups, one receiving treatment with 1 ppm of 1-MCP and the other, remaining untreated. Fruit treated with 1-MCP

and untreated controls were submitted to cold storage (0ºC ±0.5ºC, 90% UR ± 5%) and distinct controlled atmosphere conditions (0.5% and 1.5% O_2, with 2% CO_2) for 9 months. Fruit were evaluated at removal from cold storage and after seven days.

For the treatment with the ethylene inhibitor the commercial product SmartFresh™ (Agro Fresh, Rohm and Haas, PA, USA) was used, which contains 0.14% of the active principle. The product was diluted in water and the released gas was applied to the fruit for 24 hours in hermetically sealed boxes.

Physicochemical analyses

Flesh firmness (FF) was investigated using an automatic penetrometer (Fruit Texture Analyzer), with cylindrical tip of 11 mm. The measurements were taken from the equatorial region of the fruit, at opposite sides, after the skin removal. Soluble solids content (SSC) was determined using a digital refractometer (PR 101 Atago) with automatic temperature compensation.

Statistical analyses

Physicochemical data were submitted to statistical analyses with the aid of the software WinStat v. 2.0 (Machado & Conceição, 2003). For the experiment III, the effect of each storage condition was evaluated by Tukey´s test ($p \leq 0.05$), the effect of each treatment was investigated by t test ($p \leq 0.05$) and the effect of days at room temperature was evaluated by the t test ($p \leq 0.05$).

Gene expression

A group of 10 genes associated to fruit ripening was investigated (Table 1). The sequences were obtained from *Malus* x *domestica* (Borkh.) coding sequences available at Genome Database for Rosaceae (GDR). Primers were designed using the software Primer3Plus (Untergasser et al., 2007). Primers were selected according to Applied Biosystems™ recommendations. Total RNA was extracted from a pool of ten fruit, using the protocol described by Zeng and Yang (2002), with minor modification. After nucleic acid isolation, RNA quality was verified spectrophotometrically (BioTekEpoch™, TAKE3™) and with the aid of 1% agarose gel electrophoresis. After treatment with *DNAse* I (Invitrogen™) and confirmation of DNA elimination by PCR using primers for constitutively expressed genes, cDNA was synthesized from 1 µg of RNA, using the SuperScript III/RNAse Out Mix (Invitrogen™).

Amplification by qPCR was carried out using StepOne™ Real-Time PCR Systems (Applied Biosystems™), employing the reagent *SYBR Green PCR* Master Mix (Applied Biosystems™). A pool of cDNA from all samples was used to validate the primer pairs, with a 1:10 dilution and five points. Expression analyses were carried out exclusively using primers with efficiency close to 100% and dissociation curves with a single peak. Reference genes were chosen based on the comparison of transcription stability throughout the investigated experimental conditions, as described by Storch et al. (2015a). Thus, the reference genes employed for Experiment I were *MdACT*, *MdPDI* and *MdUBC*, for Experiment II *MdH1*, *MdUBC* and *MdACT*, and the Experiment III was normalized with the reference genes *MdUBC*, *MdPDI* and *MdH1*. The amplification conditions for the genes of interest were: 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s and 60ºC for 1 min, followed by dissociation curve generation. The relative expression level was obtained for each gene. Expression levels at harvest were employed as calibrators for gene expression experiments investigating ripening evolution and fruit quality after different storage conditions. For the experiment involving developmental stages, the expression levels at 105 DAA were used as calibrators.

Table 1. Characteristics of the primers used in gene expression analyses

Metabolism	Gene description/acronym	*M. domestica* locus	Primers sequence		Melting Tm (°C)		Amplicon size (bp)
			Forward	Reverse	Forward	Reverse	
Ethylene	1-aminocyclopropane-1-carboxylate oxidase 1 (ACO1)	MDP0000195885	CAATGCACCACTCCATTGTC	TCCCATCCGACTGAGCTATC	60	58	128
	Ethylene Insensitive 2 (EIN2)	MDP0000152033	GCACACCAGCTGAAATCAAG	CCCTTTCGACAAGAGATTGC	57	58	140
	Ethylene Insensitive 3 (EIN3)	MDP0000136668	AAGACAAACATGGCCACACA	TGTTTCTTTCGCCTCTCCAT	58	58	89
Cell wall	Endo Polygalacturonase (PG1)	MDP0000326734	TCACGGTAACTGCACCAGAG	CTTTGGGACCCACTCACAAT	62	60	158
	α-L-arabinofuranosidase 1 (AF1)	MDP0000055078	TGAGATGGCAAGCTATGCACCAC	CACCGCCTGTCATGGGTATTGAC	70	72	51
	α-L-arabinofuranosidase 3 (AF3)	MDP0000140483	ATTTCACAAGGTCCATATCG	CAGGTCACCAATTTCCAG	56	54	142
	Expansin4 (EXPA4)	MDP0000681724	ACCTGGTCCTCATCACAAATG	ACCCCTTGATGGAAACAGAGT	59	59	63
Flavor	Alcohol dehydrogenase (ADH)	MDP0000594290	CGATTTCGTCTTTCGGTTTT	CTGTCCAAACAGAAGCAAACA	59	59	86
	Alcohol acyl-transferase2 (AAT2)	MDP0000166457	CGTAATGCACTTTCTGCAATGT	CATTCAAACCTTTGGATAACAGC	60	59	81
Carbohydrate	Sucrose synthase (SUSY3)	MDP0000126946	AGTGAGCAACGGTGAGCTTT	CATAAAATGCAGGCTGAGCA	60	60	71
Reference genes	Actin (ACT)	MDP0000170174	GGCTCTATTCCAACCATCCA	TAGAAGCAGTGCCACCACAC	60	62	140
	Histone 1 (H1)	MDP0000223691	CATATTTGGCAGCAGAGCAA	CTCGTTAGCCAACTGCATCA	58	60	89
	Nucleosome assembly 1 protein (NAP1)	MDP0000272485	CAAACTTGCCCCTCCATTTA	CCAGCCTTCGTGATGAATTT	58	58	117
	Protein Disulfide isomerase (PDI)	MDP0000233444	TGCTGTACACAGCCAACGAT	CATCTTTAGCGGCGTTATCC	60	60	120
	Ubiquitin conjugating enzyme E2 (UBC)	MDP0000205182	TTGCTGGTGATCTCTGCATC	AGACCCACCTACTCCCGTCT	60	60	117

Results and Discussion

Experiment I

The expression profiles of ten genes, whose function is associated to ripening, storage quality, condition and period were investigated during apple development (Table 1).

The expression of *MdACO1* was low throughout the investigated developmental stages (Figure 1), suggesting that its transcriptional regulation is exclusively associated to ethylene biosynthesis during fruit ripening (Yang et al., 2013). Thus, the absence of *MdACO1* transcription during apple development indicates that it can be used as a biomarker for ripening studies in apple. The genes *MdEIN2* and *MdEIN3* coding for proteins associated to ethylene signaling, exhibit variable expression throughout fruit development (Figure 1) that appears to be associated to basal ethylene production during the period.

The expression of *MdPG1* was increased during anthesis and during the subsequent 15 days. The absence or low level of *MdPG1* transcription in the remaining investigated developmental stages demonstrates that, although the gene codes for an essential enzyme in cell wall degradation during ripening (Wang et al., 2009), it is not associated to posterior developmental fruit changes. However, the observation of the increase in *MdPG1* transcription during anthesis is recent and appears to be associated to the formation of the flower abscission zone and pollen maturation (Iglesias-Fernández et al., 2007). The transcriptional behavior of *MdPG1* reinforces the use of the gene as biomarker in apple ripening studies.

The gene *MdAF3* exhibits a transcriptional behavior similar to that of *MdPG1* (Figure 1), suggesting that they may act concomitantly during anthesis. Due to its expression profile, the first is an important biomarker candidate, associated to apple ripening.

In contrast to the observations for *MdPG1* and *MdAF3*, the expression levels of *MdAF1* were low during fruit development. Therefore, it can also be used as a ripening marker. The transcription of *MdEXP4* increased after full bloom and lasts during the entire period of fruit development (Figure 1), suggesting its involvement in cell wall changes during development, as previously shown by Wakasa et al. (2003). Due to its transcriptional behavior, *MdEXP4* is not considered a suitable biomarker for ripening. However, the gene can be used as biomarker for fruit development.

The expression of *MdSUSY3* was low throughout fruit development (Figure 1). The activity of the enzyme coded by the gene is likely to be more important at the postharvest period, where sugar catabolism occurs in the absence of photosynthesis, making it necessary to cleave sucrose in the cytosol where it is converted to fructose and UDP-glucose by SUSY. The expression profile of *MdSUSY3* suggests that it is a suitable marker for ripening in apple.

The levels of transcripts for *MdAAT2* and *MdADH* increased in the final stages of fruit development (Figure 1). This profile may be associated to the biosynthesis of volatiles occurring in the later stages of development and initial stages of fruit ripening. Thus, these genes can be used as biomarkers associated to fruit ripening.

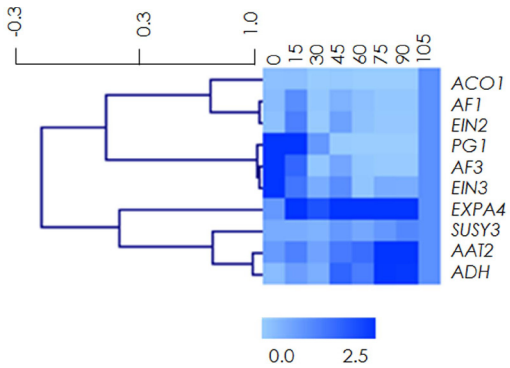

Figure 1. Relative mRNA abundance in 'Gala' apple (*Malus* x *domestica*) fruit in developmental stages. The samples were harvested at 15 days intervals, starting at full bloom (0 day) up to 105 days after anthesis (DAA).

Experiment II

In control fruit, the expression of *MdACO1* was as described previously, with increased expression along maturation (Figure 2) (Binnie & McManus, 2009; Ireland et al., 2012; Wiersma et al., 2007; Yang et al., 2013; Zhu et al., 2008), where the authors have demonstrated the association of the transcription of the gene with ethylene biosynthesis during apples ripening. In contrast, the treatment with 1-MCP repressed the transcription of *MdACO1* (Figure 2), in agreement with the effective role of the inhibitor in preventing ethylene biosynthesis in apples (Watkins, 2006, 2008). The transcriptional profile of

MdACO1 demonstrates that it is a potent marker associated to ripening in apple, as suggested by its transcriptional behavior during fruit development. Therefore, the gene expression pattern can be used to replace ethylene chromatographic analyses in laboratories of molecular analyses with lack of chromatography platforms. Additionally, in a study evaluating the effect of 1-MCP in apples, it was observed that chromatographic methods were not able to detect very low ethylene contents, whereas the transcription of *MdACO1* by RT-qPCR was detected in fruit with low amounts of ethylene (Storch et al., 2015b). Although the use of 1-MCP is known to reduce ethylene synthesis, basal levels of the hormone may occur (Hiwasa et al., 2003).

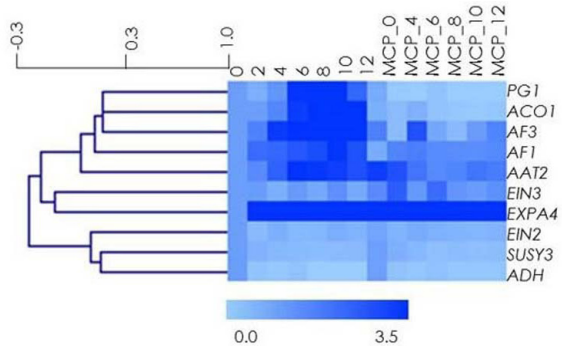

Figure 2. Relative mRNA abundance in 'Gala' apple (*Malus* x *domestica*) fruit in evolution of ripening. One group was treated with 1 ppm of 1-methylcyclopropene (1-MCP) and the other, remained untreated. Subsequently, treated and control fruit were kept at room temperature (25°C) for 12 days. Fruit were sampled at two days intervals.

Distinctly from *MdACO1* patterns, the expression profiles of *MdEIN2* and *MdEIN3* were variable throughout ripening (Figure 2), suggesting that they are not suitable biomarkers for ripening.

The transcription of a gene associated to cell wall metabolism *MdPG1* was similar to that of *MdACO1* under our experimental conditions (Figure 2), confirming the positive regulation of their transcription by ethylene (Tatsuki et al., 2011; Wakasa et al., 2006). Similar results were obtained for *MdAF3* and *MdAF1* (Storch et al., 2015b). The expression of *MdPG1*, *MdAF*3 and *MdAF1* was inversely correlated to pulp firmness, so that higher accumulation of *MdPG1*, *MdAF1* and *MdAF3* transcripts coincided with lower firmness (Storch et al., 2015b). This behavior suggests that the enzymes coded by *MdPG1*, *MdAF1* and *MdAF3* may act in concert to degrade the cell wall, resulting in firmness loss during the apples ripening. The trend observed for *MdPG1*, *MdAF1* and *MdAF3* evidences their suitability to be used as biomarkers for apple ripening, as shown previously.

In contrast, the expression of *MdEXPA4* occurred throughout fruit ripening, regardless the 1-MCP application (Figure 2), suggesting that the transcription of the gene is not regulated by ethylene. These findings are interesting, since the majority of the expansins associated to fruit ripening are characterized by their ethylene dependent transcriptional regulation (Gaete-Eastman et al., 2009; Sane et al., 2005; Trivedi and Nath, 2004; Wakasa et al., 2003). Previous studies carried out by Wakasa et al. (2003) demonstrated the importance of *MDEXP1* (hereby referred as *MdEXPA4*) in fruit development, also showing its ethylene independent transcription. In the current study, we have demonstrated that, regardless its ethylene independent regulation, *MdEXP4* may play a relevant role during fruit ripening. Due to the involvement of expansins in fruit cell wall modifications leading to tissue softening (Hiwasa et al., 2003; Sane et al., 2005), we suggest that the biological function of the product of *MdEXPA4* is associated to ethylene independent softening (Guis et al., 1997). The transcriptional behavior of *MdEXPA4* rules out its use as ripening biomarker, as shown previously.

The transcription of *MdSUSY3* remained low throughout ripening, regardless the 1-MCP application. It may be associated to the lack of changes in soluble sugars under these conditions. Although the transcription of *MdSUSY3* is correlated to sugar metabolism, the gene is not considered a suitable marker for ripening, since its expression remained constant throughout ripening, in disagreement with the proposed behavior.

The gene *MdATT2* was expressed in all investigated conditions, however, in untreated fruit the transcript accumulation was higher, suggesting a possible inductive role of ethylene, as shown by previous works (Zhu et al., 2008). The observed profile suggests that the gene *MdATT2* functions in the production of esters, the main volatiles during apple ripening (López, 1997). The observed behavior of *MdATT2* suggests that the

gene can be used as a suitable biomarker for ripening. In contrast, the expression of *MdADH* was low throughout the ripening process in treated and control fruit, thus, ruling out its use as a biomarker for ripening, as previously hypothesized.

Experiment III

Low levels of *MdACO1* transcription were observed at removal from cold storage in fruit submitted to CA with 0.5% of O_2. A slight increase in transcript accumulation was detected in fruit submitted to CA with 1.5% of O_2, although in fruit treated with 1-MCP, the increase was smaller. Higher levels of *MdACO1* transcripts were observed in fruit stored at CS (Figure 3). The higher expression of *MdACO1* at the removal from CS suggests that the condition is not effective to reduce ethylene production to basal levels, which can be associated to the intense loss of quality of the fruit stored at room temperature (Table 2). After 7 days, the accumulation of *MdACO1* transcripts increased in untreated fruit kept at CA. The increase was slighter in 1-MCP treated fruit (Figure 3). The presence of *MdACO1* transcripts observed in fruit treated with the hormone inhibitor may be derived from the synthesis of new ethylene receptors, recovering the responsiveness to the hormone. The transcriptional profile of *MdACO1* suggests that the gene is potentially a suitable biomarker for fruit quality, condition and time of apple storage.

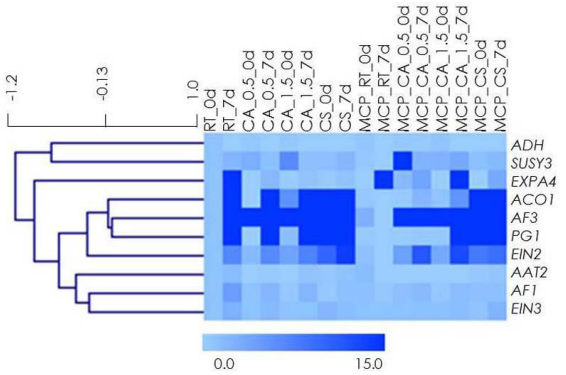

Figure 3. Relative mRNA abundance in 'Gala' apple (*Malus* x *domestica*) fruit in different storage conditions. One group received treatment with 1 ppm of 1-MCP and the other remained untreated. Fruit treated with 1-MCP and the untreated controls were submitted to room temperature (25ºC) (RT) for 7 days; cold storage (0ºC ±0.5ºC, 90% UR ± 5%) (CS) and distinct controlled atmosphere conditions (0.5% and 1.5% of O_2, with 2% of CO_2) (CA_0.5 and CA_1.5) for 9 months. Fruit were evaluated at removal from cold storage (0d) and after 7 days (7d).

The expression of *MdEIN2* was variable for different storage conditions and expression of *MdEIN3* was low in all conditions tested, discarding the use of these genes as biomarkers.

Immediately upon fruit removal from cold storage, significant differences in fruit FF were observed (Table 2). These changes are due to 1-MCP treatment and the conditions used in CA. The most drastic reduction in FF was observed in fruit kept under regular atmospheric conditions (CS). However, 7 days after storage, the period of time for the fruit to reach the consumer markets, FF was not maintained for fruit kept under CA, regardless the 1-MCP treatment or O_2 concentration. The effect of 1-MCP on the retention of FF was only observed for fruit kept under CS (Table 2).

The expression profile of *MdPG1* was similar to that of *MdACO1*, clearly demonstrating that the upregulation by ethylene is retained even after long periods of storage. The high expression levels of *MdPG1* at the removal from CS may account for the reduction in fruit firmness (Table 2) and reduced crunchiness, observed under these conditions. Similarly, the profile found in fruit stored under CA at the removal from storage and 7 days afterwards, explains the FF observed under these conditions. The transcriptional behavior of *MdPG1* after long periods of storage indicates that the gene is a potent biomarker for quality, conservation technologies and storage time in apples.

The expression of *MdAF1* was low at removal from cold storage and throughout all investigated conditions. Seven days after storage, an increase in its transcription levels was observed. In contrast, *MdAF3* was highly expressed in all tested conditions, including at the removal from cold storage. The high levels of transcription after long term cold storage indicate that other factors, besides ethylene, also contributed to the regulation of *MdAF3* expression. The expression profiles observed for *MdAF1* and *MdAF3* rule out the possibility of using these genes as quality and storage time biomarkers in apple.

Upon the removal from storage, the expression of *MdEXPA4* was low for the majority of conditions tested. Seven days afterwards the transcription increased for all investigated

Table 2. Physicochemical properties of 'Gala' apples clone 'Baigent' treated and untreated with 1-MCP, submitted to cold storage for 9 month under distinct conditions

		Control				1-MCP treatment			
		RT	CS	CA 0.5	CA 1.5	RT	CS	CA 0.5	CA 1.5
FF	0 day	84.9aA^{ns}	43.2bB^{ns}	79.2aA^{ns}	77.2abB^{ns}	90.4aA^{ns}	69.9cA^{ns}	76.6bA^{ns}	84.6abA^{ns}
	7 days	80.4aA	41.6bB	73.7aA	73.6aA	82.4aA	60.1bA	73.2aA	78.9aA
SSC	0 day	12.5bcA*	12.2cB^{ns}	13.8aA*	13.4abA^{ns}	12.7bA*	14.3aA^{ns}	13.8aA^{ns}	12.9bA^{ns}
	7 days	14.1aA	12.6bB	15.1aA	14.2aA	13.7abA	14.1aA	13.6abB	12.9bB

[1/] Means followed by the same lower case letter in line are not statistically different according to Tukey´s test (p≤0.05) comparing the storage conditions within each treatment (with or without 1-MCP). Means followed by the same capital letter in line are not statistically different according to t test (p≤0.05) comparing each treatment (with or without 1-MCP) within each storage condition for each time (0 and 7 days). * and ^{ns} corresponds to significant and non significant, respectively, by t test (p≤0.05) comparing each time (0 and 7 days) within each treatment (with or without 1-MCP) within each storage condition. [2] CS: cold storage: 0°C ±0.5°C, 90% UR ± 5%). [3] CA: controlled atmosphere conditions (0.5% and 1.5% O_2, with 2% CO_2). [4] FF: Flesh firmness expressed in Newton. [5] SSC: Soluble solids content expressed in °Brix.

conditions, with higher levels found in 1-MCP treated fruit. These results suggest that the low temperatures negatively affect the transcription of the gene, and that the expression of *MdEXPA4* is higher for green fruit, indicating that the gene product functions at early stages of fruit ripening. Moreover, the ethylene independent transcriptional regulation of the gene is also confirmed. Its expression profile demonstrates that *MdEXPA4* is not a suitable biomarker for fruit quality and storage time.

Immediately upon removal from storage and seven days afterwards, untreated fruit kept under CS exhibited the lowest values of SSC (Table 2). The low contents of soluble solids in fruit submitted to CS may be associated to the later ripening stages of the fruit, since at this point the sugars have been degraded to generate energy for other cellular processes and for the formation of intermediary compounds. In contrast, the low soluble solids content in fruit treated with 1-MCP may be associated to ripening delay, due to the inhibition of ethylene action caused by storage conditions. The gene *MdSUSY3* was slightly expressed after long term storage. The profile suggests that the regulation of *MdSUSY3* is independent of ethylene action and that the enzyme coded by the gene does not have a crucial role in sugar metabolism in apples after harvest. Thus, *MdSUSY3* is not potentially useful as a biomarker for apple postharvest conservation.

The investigation of the expression profile of *MdAAT2*, a key gene in esters biosynthesis, revealed low expression levels for all tested conditions, suggesting that the gene is not suitable as a biomarker for postharvest apples.

The accumulation of *MdADH* transcripts in apples was low upon removal from storage. Seven days later, the profile remained unchanged, suggesting that the gene is not indicated to be used as a biomarker for ripening and long-term storage.

Biomarkers may have different applications in fruit postharvest. In laboratories that do not have capacity for acquiring and maintaining chromatographs, the ethylene synthesis during storage can be monitored by *MdACO1* biomarker expression analysis. Similarly, the synthesis of esters in fruits submitted to different storage technologies may be accompanied by *MdAAT2* biomarker expression analysis. Changes in stored fruit pulp firmness may be checked by analysis of *MdACO1* and *MdPG1* biomarkers.

Conclusions

The results of the current study indicate that the genes *MdACO1*, *MdPG1*, *MdAF3* and *MdAAT2* are suitable biomarkers associated to ripening due to their function in ethylene biosynthesis, flesh firmness loss and esters biosynthesis, important quality attributes for apples. In contrast, after long term storage, ethylene synthesis is associated to quality loss and the genes *MdACO1* and *MdPG1* can be used as biomarkers of quality, storage technology and time for apples. *MdEXP4* is highlighted as biomarker for development.

Acknowledgements

The authors would like to thank Coordination for Improvement of Higher Education Personnel (CAPES) and The National Council for Scientific and Technological Development (CNPq) for scholarships and The Brazilian Agricultural Research Corporation (EMBRAPA) for research funding.

References

Atkinson, R.G., Sutherland, P.W., Johnston, S.L., Gunaseelan, K., Hallett, I.C., Mitra, D., Brummell, D.A, Schroder, R., Johnston, J.W., Schaffer, R.J. 2012. Down-regulation of POLYGALACTURONASE 1 alters firmness, tensile strength and water loss in apple (*Malus* x *domestica*) fruit. *BMC Plant Biology* 12: 129.

Binnie, J.E., McManus, M.T. 2009. Characterization of the 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase multigene family of *Malus domestica* Borkh. *Phytochemistry* 70: 348–360.

Both, V., Brackmann, A., Thewes, F.R., Ferreira, D.D.F., Wagner, R. 2014. Effect of storage under extremely low oxygen on the volatile composition of "Royal Gala" apples. *Food Chemistry* 156: 50–57.

Dandekar, A.M., Teo, G., Defilippi, B.G., Uratsu, S.L., Passey, A.J., Kader, A.A., Stow, J.R., Colgan, R.J., James, D.J. 2004. Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. *Transgenic Research* 13: 373–384.

Gachon, C., Mingam, A., Charrier, B. 2004. Real-time PCR: What relevance to plant studies? *Journal of Experimental Botany* 55: 1445–1454.

Gaete-Eastman, C., Figueroa, C.R., Balbontín, C., Moya, M., Atkinson, R.G., Herrera, R., Moya-León, M.A. 2009. Expression of an ethylene-related expansin gene during softening of mountain papaya fruit (*Vasconcellea pubescens*). *Postharvest Biology and Technology* 53: 58–65.

Gapper, N.E., Giovannoni, J.J., Watkins, C.B. 2014. Understanding development and ripening of fruit crops in an "omics" era. *Horticulture Research* 1: 14034.

Guis, M., Botondi, R., Ben-Amor, M., Ayub, R., Bouzayen, M., Pech, J.C., Latchè, A. 1997. Ripening-associated biochemical traits of cantaloupe Charentais melons expressing an antisense ACC oxidase transgene. *American Society for Horticultural Science* 122: 748-751.

Hiwasa, K., Rose, J.K.C., Nakano, R., Inaba, A., Kubo, Y. 2003. Differential expression of seven alpha-expansin genes during growth and ripening of pear fruit. *Physiologia Plantarum* 117: 564–572.

Iglesias-Fernández, R., Matilla, A.J., Rodríguez-Gacio, M.C., Fernández-Otero, C., de La Torre, F. 2007. The polygalacturonase gene PdPG1 is developmentally regulated in reproductive organs of *Prunus domestica* L. subsp. i*nsititia*. *Plant Science* 172: 763–772.

Ireland, H.S., Guillen, F., Bowen, J., Tacken, E.J., Putterill, J., Schaffer, R.J., Johnston, J.W. 2012. Mining the apple genome reveals a family of nine ethylene receptor genes. *Postharvest Biology and Technology* 72: 42–46.

Ireland, H.S., Gunaseelan, K., Muddumage, R., Tacken, E.J., Putterill, J., Johnston, J.W., Schaffer, R.J. 2014. Ethylene regulates apple (*Malus* x *domestica*) fruit softening through a dose x time-dependent mechanism and through differential sensitivities and dependencies of cell wall-modifying genes. *Plant Cell Physiology* 55: 1005–1016.

Machado, A.A, Conceição, A.R. 2003. Sistema de análise estatística para windows. WinStat. Pelotas.

López, M.L., Lavilla, M.T., Riba, M., Venderell, M. 1997. Comparison of volatile compounds in two seasons in apples: Golden Delicious and Granny Smith. *Journal of Food Quality* 21: 155–166.

Muñoz-Bertomeu, J., Miedes, E., Lorences, E.P. 2013. Expression of xyloglucan endotransglucosylase/hydrolase (XTH) genes and XET activity in ethylene treated apple and tomato fruit. *Journal of Plant Physiology* 170: 1194–1201.

Nobile, P.M., Wattebled, F., Quecini, V., Girardi, C.L., Lormeau, M., Laurens, F. 2011. Identification of a novel α-L-arabinofuranosidase gene associated with mealiness in apple. *Journal of Experimental Botany* 62: 4309–4321.

Sane, V.A., Chourasia, A., Nath, P. 2005. Softening in mango (*Mangifera indica* cv. Dashehari) is correlated with the expression of an early ethylene responsive, ripening related expansin gene, *MiExpA1*. *Postharvest Biology and Technology* 38: 223–230.

Storch, T.T., Pegoraro, C., Finatto, T., Quecini, V., Rombaldi, C.V., Girardi, C.L. 2015a. Identification of a novel reference gene for apple transcriptional profiling under postharvest conditions. *PLoS One* 10: e0120599.

Storch, T.T., Finatto, T., Pegoraro, C., Dal Cero, J., Laurens, F., Rombaldi, C.V., Quecini, V., Girardi, C.L. 2015b. Ethylene-dependent regulation of an α-l-arabinofuranosidase is associated to firmness loss in "Gala" apples under long term cold storage. *Food Chemistry* 182: 111–119.

Tatsuki, M., Hayama, H., Yoshioka, H., Nakamura, Y. 2011. Cold pre-treatment is effective for 1-MCP efficacy in "Tsugaru" apple fruit. *Postharvest Biology and Technology* 62: 282–287.

Trivedi, P.K., Nath, P. 2004. *MaExp1*, an ethylene-induced expansin from ripening banana fruit. *Plant Science* 167: 1351–1358.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M. 2007. Primer3Plus,

an enhanced web interface to Primer3. *Nucleic Acids Research* 35: 71–74.

Valdés, A., Ibáñez, C., Simó, C., García-Cañas, V. 2013. Recent transcriptomics advances and emerging applications in food science. *Trends in Analytical Chemistry* 52: 142–154.

VanGuilder, H.D., Vrana, K.E., Freeman, W.M., 2008. Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques* 44: 619–626.

Visser, M., Van Der Walt, A.P., Maree, H.J., Rees, D.J.G., Burger, J.T. 2014. Extending the sRNAome of apple by next-generation sequencing. *PLoS One* 9: e95782.

Wakasa, Y., Hatsuyama, Y., Takahashi, A., Sato, T., Niizeki, M., Harada, T. 2003. Divergent expression of six expansin genes during apple fruit ontogeny. *European Journal of Horticultural Science* 68: 253–259.

Wakasa, Y., Kudo, H., Ishikawa, R., Akada, S., Senda, M., Niizeki, M., Harada, T. 2006. Low expression of an endopolygalacturonase gene in apple fruit with long-term storage potential. *Postharvest Biology and Technology* 39: 193–198.

Wang, A., Tan, D., Tatsuki, M., Kasai, A., Li, T., Saito, H., Harada, T. 2009. Molecular mechanism of distinct ripening profiles in "Fuji" apple fruit and its early maturing sports. *Postharvest Biology and Technology* 52: 38–43.

Watkins, C.B. 2006. The use of 1-methylcyclopropene (1-MCP) on fruit and vegetables. *Biotechnology Advances* 24: 389–409.

Watkins, C.B. 2008. Overview of 1-methylcyclopropene trials and uses for edible horticultural crops. *HortScience* 43: 86–94.

Wiersma, P.A., Zhang, H., Lu, C., Quail, A., Toivonen, P.M.A. 2007. Survey of the expression of genes for ethylene synthesis and perception during maturation and ripening of "Sunrise" and "Golden Delicious" apple fruit. *Postharvest Biology and Technology* 44: 204–211.

Yang, X., Song, J., Campbell-Palmer, L., Fillmore, S., Zhang, Z. 2013. Effect of ethylene and 1-MCP on expression of genes involved in ethylene biosynthesis and perception during ripening of apple fruit. *Postharvest Biology Technology* 78: 55–66.

Zeng, Y., Yang, T. 2002. RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. *Plant Molecular Biology Reporter* 20: 417–417.

Zheng, Q., Song, J., Campbell-Palmer, L., Thompson, K., Li, L., Walker, B., Cui, Y., Li, X. 2013. A proteomic investigation of apple fruit during ripening and in response to ethylene treatment. *Journal of Proteomics* 93: 276–294.

Zhu, Y., Rudell, D.R., Mattheis, J.P. 2008. Characterization of cultivar differences in alcohol acyltransferase and 1-aminocyclopropane-1-carboxylate synthase gene expression and volatile ester emission during apple fruit maturation and ripening. *Postharvest Biology and Technology* 49: 330–339.

Zhu, Z., Liu, R., Li, B., Tian, S. 2013. Characterisation of genes encoding key enzymes involved in sugar metabolism of apple fruit in controlled atmosphere storage. *Food Chemistry* 141: 3323–3328.