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**Estudos transcricionais relacionados às alterações de qualidade pós-colheita
de maçãs, cv. Gala**

Tatiane Timm Storch

Pelotas, 2014

TATIANE TIMM STORCH

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de maçãs, cv. Gala**

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Comitê de Orientação:

Cesar Luis Girardi, PhD – Embrapa Uva e Vinho

Cesar Valmor Rombaldi, PhD – PPGCTA, UFPel

François Laurens, PhD – IRHS, INRA

Vera Quecini, PhD – Embrapa Uva e Vinho

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Banca examinadora:

Cesar Luis Girardi, PhD – Embrapa Uva e Vinho

Cesar Valmor Rombaldi, PhD – PPGCTA, UFPel

François Laurens, PhD – IRHS, INRA

Renar João Bender, PhD – Departamento de Horticultura e Silvicultura, UFRGS

Vanessa Galli, Dra – PPGCTA, UFPel

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e à minha irmã, cunhado e sobrinhos, pelo amor e apoio durante todos
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Resumo

STORCH, Tatiane Timm. **Estudos transcricionais relacionados às alterações de qualidade pós-colheita de maçãs, cv. Gala.** 2014. 125f. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Programa de Pós-graduação em Ciência e Tecnologia de Alimentos, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, 2014.

Análises transcricionais têm sido empregadas em vários estudos, incluindo aqueles relacionados ao desenvolvimento de frutos. No âmbito da pós-colheita, o conhecimento do comportamento transcricional de genes pode complementar as informações obtidas por análises convencionais da qualidade dos frutos, como firmeza de polpa. Assim, o presente trabalho empregou as técnicas de transcrição reversa – reação em cadeia da polimerase em tempo real (RT-qPCR) e microarranjos para avaliar o comportamento transcricional de genes que podem estar envolvidos nos processos que alteram a qualidade dos frutos de maçã ‘Gala’ durante o período em que estes são mantidos em armazenamento refrigerado (AR). Além da resposta ao AR, a influência do etileno sobre o perfil transcricional também foi estudada, através do tratamento com o inibidor do hormônio, o 1-metilciclopropeno (1-MCP). Considerando a necessidade de uma correta normalização dos dados obtidos por RT-qPCR, um estudo de estabilidade de expressão de genes referência para os experimentos testados foi realizado, onde um gene codificador de histona 1 (*MdH1*) mostrou boa estabilidade de expressão nos experimentos de pós-colheita. Uma vez estabelecido o protocolo de normalização dos resultados, um estudo da transcrição de um gene codificador de uma α -L-arabinofuranosidase (*MdAF3*), enzima atuante na degradação da parede celular, foi realizado, apontando para a associação do mesmo ao amolecimento de polpa dos frutos. Por fim, um terceiro estudo avaliou o perfil transcricional de polpa de maçã sob a influência do AR e do 1-MCP. Através deste último estudo verificou-se um grande número de genes diferencialmente expressos que respondeu principalmente ao frio. Destes genes, foi dado destaque àqueles participantes nos metabolismos de parede celular e hormônios e nos mecanismos de oxido-redução e regulação. Embora o frio tenha resultado em um maior número de genes diferencialmente expressos, observou-se que alguns genes apresentaram um comportamento de dependência ao 1-MCP, que os tornam potenciais marcadores de qualidade dos frutos. Assim, através dos três capítulos que compõem este trabalho, foi demonstrada a complexidade dos mecanismos associados às alterações que ocorrem durante a pós-colheita dos frutos de maçã, as quais envolvem diferentes vias metabólicas. Essas informações vêm a complementar os conhecimentos já estabelecidos sobre os fatores que influenciam na qualidade dos frutos armazenados.

Palavras-chave: *Malus x domestica* Borkh.; etileno; armazenamento refrigerado; parede celular

Abstract

STORCH, TatianeTimm. **Transcriptional studies related to postharvest changes in quality of apples, cv. Gala.** 2014. 125f. Thesis (Doutorado em Ciência e Tecnologia de Alimentos) – Programa de Pós-graduação em Ciência e Tecnologia de Alimentos, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, 2014.

Transcriptional analysis has been employed in many studies, including those related to fruit development. In the postharvest scope, the knowledge of the transcriptional gene profile may be complementary to the information obtained by conventional analysis of fruit quality such as flesh firmness. Thus, the present work has used the reverse transcription – quantitative polymerase chain reaction (RT-qPCR) and microarrays to evaluate the transcriptional behavior of genes that may be involved in the processes that change the fruit quality of ‘Gala’ apples during the cold storage (CS). Additionally, the ethylene influence on the transcriptional profile has been also studied through the treatment with 1-methylcyclopropene (1-MCP). Considering the need for a proper RT-qPCR data normalization, a study of gene expression stability of putative reference genes in samples from different experiments involving *Malus x domestica* has been performed, where a gene coding for a histone 1 (*MdH1*) showed high expression stability in the postharvest experiments. Once established the normalization protocol, a transcriptional study of a gene coding for cell wall degradation enzyme α -L-arabinofuranosidase (*MdAF3*) has been performed. The results show that *MdAF3* is associated with the flesh softening of apple fruits. Finally, a third study evaluated the transcriptional profile of apple flesh under influence of CS and 1-MCP. Through this latter study it was found a large number of differentially expressed genes that respond primarily to cold. Among these, emphasis was given to those participating in the cell wall and hormone metabolism as well as redox and regulation mechanisms. Although the cold has resulted in a higher number of differentially expressed genes than 1-MCP treatment, it was observed that some genes showed a behavior of dependency to 1-MCP, which makes them potential markers of fruit quality. Thus, through the three chapters from this work, we demonstrated the complexity of the mechanisms associated with the changes that occur during apple postharvest, which involve different metabolic pathways. These results complement the established knowledge about the factors that influence the quality of stored apple fruits.

Keywords: *Malus x domestica* Borkh.; ethylene; cold storage; cell wall

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Lista de Abreviaturas e Siglas

1-MCP	1-Methylciclopropene
18S	Subunit of ribossomal RNA
ABA	Abscisic acid
AC	Atmosfera controlada estática
ACC	1-Aminociclopropane-1-carboxylate
ACO	1-Aminociclopropane-1-carboxylate oxidase
ACO1, ACO2, ACO3	Coding genes for 1-aminociclopropane-1-carboxylate oxidase
ACS	1-Aminociclopropane-1-carboxylate synthase
ACS12	Coding gene for 1-aminociclopropane-1-carboxylate synthase 12
ACT	Actin
AD	Atmosfera controlada dinâmica
AF	α -L-arabinofuranosidase enzyme
AF1	Coding gene for α -L-arabinofuranosidase belonging to the Glycosyl Hydrolases 51 family
AF3	Coding gene for α -L-arabinofuranosidase belonging to the Glycosyl Hydrolases 3 family
ANOVA	Analysis of variance
APX3	Coding gene for ascorbate peroxidase 3
AR	Armazenamento refrigerado
aRNA	Amplified RNA

AryANE	Array Apple Nimblegen Expression
AsA-GHA	Ascorbate-glutathione cycle
<i>At</i>	<i>Arabidopsis thaliana</i>
β-GAL	β-Galactosidase
BH	Benjamini & Hochberg method of p-value correction
bp	Base pairs
CA	Controlled atmosphere
CAT2	Coding gene for catalase 2
CCR1	Coding gene for cinnamoyl CoA reductase (CCR1)
cDNA	Complementary DNA
CDs	Coding sequences
COBRA	Coding gene for a glycosylphosphatidylinositol-anchored protein
Cq	Quantification cycle (in qPCR)
CS	Cold storage
CV	Coefficient of variation
Cy3	Cyanine-3
Cy5	Cyanine-5
d	Days
DAA	Days after anthesis
DNA	Deoxyribonucleic acid
ERF1	Coding gene for ethylene response factor
EXP4, EXPA1, EXPA9, EXLA2	Coding genes for different expansins

EXPA, EXPB, EXLA, EXLB	Subfamilies of expansin family
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GH3	Glycosyl hydrolases 3 family
GH51	Glycosyl hydrolases 51 family
H1	Histone 1
H2A, H2B, H3, H4	Histones belonging to the octamer of histones that participate in the folding of DNA within the nucleus of eukaryotic cells
H ₂ O ₂	Hydrogen peroxide
JA	Jasmonic acid
KI	Potassium iodide
LiCl	Lithium chloride
LOX	Lipoxygenase
LOX2	Coding gene for lipoxygenase 2
LRR-RLKs	Leucine-rich repeat receptor like kinases family
<i>Md</i>	<i>Malus x domestica</i>
MDHAR4	Coding gene for monodehydroascorbate reductase 4
mRNA	Messenger RNA
M value	Transcriptional stability value generated by geNorm software
NaOH	Sodium hydroxide
NAP1	Nucleosome assembly 1 protein
NH ₄ OH	Ammonium hydroxide
NTC	No template control (in qPCR)

PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
PG	Polygalacturonase
PG1	Coding gene for endopolygalacturonase
PL	Pectate lyase
PME	Pectin methylesterase
pNP	<i>p</i> -nitrophenol
qPCR	Quantitative polymerase chain reaction
<i>r</i>	Pearson coefficient of correlation
<i>rin</i>	Tomato mutant ripening inhibitor
RLKs	Receptor-like kinases
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Reverse transcription – quantitative polymerase chain reaction
SA	Salicylic acid
SD	Standard deviation
<i>Sl</i>	<i>Solanum lycopersicum</i>
T0	Harvest sample (in chapter III)
T1	Control sample stored for two months in cold storage (in chapter III)
T2	1-MCP treated sample stored for two months in cold storage (in chapter III)

TA	Titrateable acidity
TSS	Total soluble solids
TUB	Tubulin
UBC	Ubiquitin-conjugating enzyme E2
Vv	<i>Vitis vinifera</i>
w/v	Weight / volume

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Introdução

Maçãs (*Malus x domestica* Borkh.) são altamente apreciadas devido às características sensoriais como aparência, sabor, aroma e textura, sendo essa última objeto alvo de estudo dessa tese. Neste sentido, frutos mais firmes, suculentos e crocantes são os que apresentam a preferência do consumidor (JOHNSTON et al., 2002). Dentre as variedades de maçãs, a 'Gala', com seus clones, é a mais cultivada devido à sua capacidade de adaptação tanto em regiões de clima temperado como em regiões de clima subtropical. Além disso, essa variedade apresenta características requeridas pelos consumidores e relativamente bom potencial de conservação (BAPAT et al., 2010; BOYLSTON et al., 1994; COSTA et al., 2012). Porém, essa capacidade de armazenamento depende do método de conservação empregado, sendo que tecnologias como o uso de atmosfera controlada estática (AC) ou dinâmica (AD) aliadas ou não ao uso do 1-metilciclopropeno (1-MCP) têm permitido o armazenamento de maçã 'Gala' por até 9 meses (PEGORARO et al., 2014). Por outro lado, quando o armazenamento se faz sob refrigeração, comumente denominado de armazenamento refrigerado (AR), a vida útil desses frutos é limitada a no máximo três ou quatro meses (BRACKMANN, 1992; KONOPACKA et al., 2007). Neste caso, a redução da qualidade dos frutos deve-se principalmente às alterações de textura como o amolecimento excessivo e à farinosidade de polpa (BRACKMANN, 1992). Em frutos carnosos, como a maçã, a textura é um dos principais atributos de qualidade, chegando a ser mais importante do que as propriedades aromáticas. Assim, mesmo que consumidores em diferentes países apresentem diferentes preferências quanto aos atributos de qualidade, há uma busca universal por frutos firmes, livres de desordens fisiológicas e podridões decorrentes do ataque de patógenos (GOULAO; OLIVEIRA, 2008). É por isso que as principais tecnologias de conservação utilizadas atualmente são eficientes em manter os frutos firmes durante longos períodos. Para tal, a estratégia utilizada consiste em reduzir o acúmulo e/ou ação do etileno (BRACKMANN et al., 2005; JOHNSTON et al., 2002; TATSUKI; ENDO; OHKAWA, 2007).

O etileno é um fitohormônio importante em diferentes processos do crescimento e desenvolvimento de plantas (SALTVEIT, 1999). Nos frutos, principalmente climatéricos, apresenta ressaltada importância no amadurecimento (YANG; HOFFMAN, 1984). Frutos climatéricos são aqueles que exibem, no início do amadurecimento, um aumento na respiração e na produção auto catalítica de etileno. Neste grupo de frutos, que inclui a maçã, o etileno produzido resulta no aumento da sua própria síntese, processo conhecido como produção auto catalítica, o que os diferencia dos frutos não-climatéricos (BOUZAYEN et al., 2010).

Neste contexto, destaca-se o uso do 1-MCP como tecnologia de conservação (SOZZI; BEAUDRY, 2007). O 1-MCP é um inibidor da ação do etileno devido à sua capacidade de ligação aos receptores do hormônio, impedindo assim as respostas desencadeadas pelo etileno. O produto comercial é utilizado na forma de pó e sua aplicação é realizada no período de pós-colheita (BLANKENSHIP, 2001). O 1-MCP não é tóxico, não deixa resíduos e é ativo em baixas concentrações. Seu uso no Brasil foi liberado em 2005. Além da maçã, o inibidor do etileno é também utilizado em outros frutos como tomate, kiwi, pera e pêsego (WATKINS, 2006). Seu emprego na pós-colheita de maçãs deve-se principalmente à manutenção da firmeza de polpa dos frutos (BAI et al., 2005; LU; MA; LIU, 2012).

As alterações de textura dos frutos como a perda de firmeza e o aparecimento de farinosidade de polpa devem-se às modificações que ocorrem nos carboidratos da parede celular, sendo a pectina o polissacarídeo que mais sofre alterações durante a maturação dos frutos (BILLY et al., 2008; GWANPUA et al., 2014). Nesses processos estão envolvidas diferentes enzimas com diferentes funções na modificação e degradação da parede celular (GOULAO et al., 2007; GWANPUA et al., 2014).

Destaca-se assim, a enzima poligalaturonase (PG), extensivamente estudada e relacionada ao amolecimento de frutos, incluindo a maçã (ATKINSON et al., 2002, 2012; BARTLEY, 1977; LONGHI et al., 2013; TACKEN et al., 2010; WAKASA et al., 2006). A PG tem a capacidade de clivar as ligações α -1,4 entre os resíduos de ácido galacturônico da cadeia homogalacturonana (BENNETT; LABAVITCH, 2008). Porém, nos últimos anos tem sido também enfatizada a importância de enzimas atuantes nas cadeias laterais da pectina, liberando açúcares como arabinose e galactose (NOBILE et al., 2011; WEI et al., 2010). A perda desses açúcares, principalmente arabinose, tem sido relacionada à perda da qualidade de textura de

frutos como maçã (GWANPUA et al., 2014; NARA; KATO; MOTOMURA, 2001; PEÑA and CARPITA, 2004) e pêssego (BRUMMELL et al., 2004; DI SANTO; PAGANO; SOZZI, 2009).

Neste contexto, a enzima α -L-arabinofuranosidase foi estudada em maçã (GOULAO et al., 2007; NOBILE et al., 2011; WEI et al., 2010), bem como a transcrição de genes codificadores da enzima, agrupados nas famílias GH51 (GOULAO; OLIVEIRA, 2008) e GH3 (NOBILE et al., 2011). A enzima pertencente à família GH3 isolada de pera apresentou atividade bifuncional α -L-arabinofuranosidase/ β -xilosidase contra substratos artificiais (TATEISHI et al., 2005). Em maçã, um gene codificador de α -L-arabinofuranosidase pertencente à família GH3, chamado de *MdAF3*, teve sua expressão relacionada ao distúrbio da farinosidade de polpa em uma população segregante (NOBILE et al., 2011). Porém, o trabalho não deixa clara a influência do etileno sobre a transcrição do gene. Assim, considerando que as alterações da textura de frutos de maçã têm sido relacionadas à ação do etileno (BAPAT et al., 2010), um dos objetivos do presente trabalho é apresentar a influência do etileno sobre a transcrição de *MdAF3*. Para este estudo, foi utilizada a técnica de reação em cadeia da polimerase em tempo real (RT-qPCR).

O uso de RT-qPCR para o estudo da transcrição de genes é de grande importância para diversas áreas de estudo, incluindo a maturação e pós-colheita de frutos. Em maçã, diversos trabalhos utilizando essa técnica são reportados. Uma atenção especial tem sido dada ao estudo de genes que participam de modificações da parede celular e da síntese e respostas ao etileno (IRELAND et al., 2014; MANN et al., 2008; MUÑOZ-BERTOMEU; MIEDES; LORENCES, 2013; WEI et al., 2010; YANG et al., 2013a). Porém, é importante ressaltar que para a obtenção de alta confiabilidade nos resultados de RT-qPCR é necessário um adequado processo de normalização dos dados (ANDERSEN; JENSEN; ØRNTOFT, 2004; PFAFFL et al., 2004; VANDESOMPELE et al., 2002).

Um método bastante eficiente de normalização é o uso de genes referência que devem apresentar estabilidade de expressão nas amostras a serem estudadas. Para tal, genes clássicos como gliceraldeído-3-fosfato desidrogenase (GAPDH), actina (ACT) e 18S foram empregados como referência em diferentes estudos (MANN et al., 2008; NOBILE et al., 2011; WEI et al., 2010). Porém, é necessário observar que mesmo estes genes podem ter sua expressão influenciada por

determinados tratamentos, fazendo-se necessário uma seleção adequada dos genes referência mais estáveis.

Esta seleção deve ser realizada para cada experimento a ser estudado (SCHMITTGEN; ZAKRAJSEK, 2000). Embora diversos trabalhos de RT-qPCR tenham sido feitos enfatizando as alterações que ocorrem em frutos de maçã durante a pós-colheita, nenhum trabalho foi publicado relatando a escolha de genes referência mais estáveis nesses experimentos. Assim, anterior à análise do acúmulo de transcritos do gene *MdAF3*, foi realizado um estudo de validação de genes candidatos a referência em diferentes experimentos com maçã, incluindo o período de pós-colheita.

Além da RT-qPCR, outras técnicas de biologia molecular têm se mostrado úteis para o estudo de diferentes fases do crescimento e desenvolvimento dos frutos. Estas técnicas incluem o estudo do perfil transcricional por microarranjos, que consiste na hibridização dos genes presentes na amostra de estudo com sondas de oligonucleotídeos arranjadas em um suporte sólido (*chip*). Essa técnica permite a análise da expressão de genes em ampla escala, já que um único chip pode proporcionar a hibridização de centenas ou até milhares de genes (SINGH; KUMAR, 2013). Através desta técnica, um grande número de genes foi descoberto como diferencialmente expresso em frutos como pêssigo, pera e maçã em diferentes estádios de desenvolvimento (NISHITANI et al., 2010; TOSETTI et al., 2014; ZHU et al., 2012). Assim, o uso desta técnica pode trazer grandes avanços no conhecimento de genes que codificam proteínas atuantes em diferentes vias metabólicas envolvidas nas alterações de frutos de maçã durante a pós-colheita. Neste sentido, a terceira parte desta tese apresenta os principais resultados de um experimento com o uso de microarranjos para o estudo de genes diferencialmente expressos em resposta ao AR com ou sem o uso de 1-MCP.

A interpretação dos resultados das três partes que compõem a tese traz avanços para o conhecimento dos processos transcricionais envolvidos com as alterações que ocorrem no período de pós-colheita de maçã 'Gala' e que resultam na perda de qualidade dos frutos, problema este que limita a comercialização dos mesmos após avançado tempo de armazenamento.

Assim, a tese está estruturada em três seções, cada uma na forma de artigo, seguindo as recomendações das revistas a que se destinarão os manuscritos. Os artigos são:

Artigo 1 - Identification of a novel reference gene for apple transcriptional profiling under postharvest conditions. Artigo preparado de acordo com as normas da revista PloS ONE, eISSN-1932-6203.

Artigo 2 – Ethylene-dependent regulation of a novel α -L-arabinofuranosidase is associated to firmness loss in apples under long term cold storage. Artigo preparado de acordo com as normas da revista Food Chemistry, ISSN 0308-8146.

Artigo 3 – Several genes involved in response to stress and regulatory processes are differentially transcribed in cold stored apple. Artigo preparado de acordo com as normas da UFPel para elaboração de dissertações e teses.

Os objetivos gerais de cada experimento que, por consequência, gerou o respectivo artigo-texto, são:

Artigo 1 – Caracterizar genes que possam ser utilizados como de referência, pela boa estabilidade transcricional, em estudos de pós-colheita de maçãs.

Artigo 2 – Estudar a influência do etileno na regulação de genes AF em maçãs.

Artigo 3 – Estabelecer o perfil transcricional diferencial em maçãs frente à ação do frio e do etileno.

Capítulo 1. Este capítulo consiste no manuscrito enviado à revista PLoS ONE no dia 15 de setembro de 2014. Deste modo, o mesmo segue a estrutura proposta pela revista. O manuscrito foi enviado para avaliação dos revisores no dia 22 de setembro do corrente ano.

Title: Identification of a novel reference gene for apple transcriptional profiling under postharvest conditions

Authors: Tatiane Timm Storch^{a,b}, Camila Pegoraro^a, Taciane Finatto^{a#}, Vera Quecini^a César Valmor Rombaldi^b, César Luis Girardi^{a,*}

^aEmbrapa Uva e Vinho - Caixa Postal 130, 95700-000, Bento Gonçalves, RS - Brazil

^bUniversidade Federal de Pelotas, Faculdade de Agronomia Eliseu Maciel, Departamento de Ciência e Tecnologia Agroindustrial –s/nº caixa postal 354, 96010-900, Pelotas, RS – Brazil

[#]CurrentAddress: Universidade Tecnológica Federal do Paraná, Campus Pato Branco -Via do Conhecimento - km 01, CEP: 85503-390, Pato Branco – PR

*Corresponding author: cesar.girardi@embrapa.br (CLG)

Abstract

Reverse Transcription quantitative PCR (RT-qPCR) is one of the most important techniques for gene expression profiling due to its high sensibility and reproducibility. However, the reliability of the results is highly dependent on data normalization, performed by comparisons between the expression profiles of the genes of interest against those of constitutively expressed, reference genes. Although the technique is widely used in fruit postharvest experiments, the transcription stability of reference genes has not been thoroughly investigated under these experimental conditions. Thus, we have determined the transcriptional profile, under these conditions, of three genes commonly used as reference; *ACTIN (MdACT)*, *PROTEIN DISULPHIDE ISOMERASE (MdPDI)* and *UBIQUITIN-CONJUGATING ENZYME E2 (MdUBC)*,

along with two novel candidates; *HISTONE 1 (MdH1)* and *NUCLEOSSOME ASSEMBLY 1 PROTEIN (MdNAP1)*. The expression profile of the genes was investigated throughout five experiments, with three of them encompassing the postharvest period and the other two, consisting of developmental and spatial phases. The transcriptional stability was comparatively investigated using four distinct software packages; BestKeeper, NormFinder, geNorm and DataAssist. Gene ranking results for transcriptional stability were similar for the investigated software packages, with the exception of BestKeeper. The classic reference gene *MdUBC* ranked amongst the most stably transcribed in all investigated experimental conditions. Transcript accumulation profiles for the novel reference candidate gene *MdH1* were stable throughout the tested conditions, especially in experiments encompassing the postharvest period. Thus, our results present a novel reference gene for postharvest experiments in apple and reinforce the importance of checking the transcription profile of reference genes under the experimental conditions of interest.

1.1 Introduction

Currently, reverse transcription quantitative PCR (RT-qPCR) is considered one of the most sensitive and reproducible techniques to detect specific mRNAs, being employed in a wide range of applications (NICOT et al., 2005). Frequently, the technique requires the use of two or more constitutively expressed reference genes that are unresponsive to the sampled tissue, exogenous stimuli and experimental conditions (SCHMITTGEN; ZAKRAJSEK, 2000; VANDESOMPELE et al., 2002). However, the transcription of several commonly used reference genes has been demonstrated to be unstable under certain experimental conditions (CHANDNA; AUGUSTINE; BISHT, 2012; DEKKERS et al., 2012; GIMENO et al., 2014; LING et al., 2014; NICOT et al., 2005). Thus, the identification of novel, stably expressed genes is necessary for each given experimental condition (GUTIERREZ et al., 2008).

Several statistic analysis tools are available to access transcription stability in qPCR experiments, including geNorm (VANDESOMPELE et al., 2002), DataAssist (Life Technologies, USA), NormFinder (ANDERSEN; JENSEN; ØRNTOFT, 2004) and BestKeeper (PFAFFL et al., 2004), which employ distinct or similar algorithms to identify the most stably expressed genes under certain conditions.

Transcriptional profiling during postharvest conservation is instrumental to the identification of novel regulatory genes associated to physiological and metabolic conditions affecting viability of a wide range of fruits, including apple (*Malus x domestica* Borkh.), where several modifications responsible for quality losses occur during the period (GOULAO; OLIVEIRA, 2008; WEI et al., 2010).

Transcriptional profiling by RT-qPCR in postharvest conditions is dependent on the use of reference genes that are stably transcribed under the distinct experimental conditions for ex planta development and storage. The induction of ethylene production during the period leads to several responses, such as; cell wall degradation and the autocatalytic production of the hormone (BAPAT et al., 2010; BENNETT; LABAVITCH, 2008). Moreover, changes in the respiratory pattern are also observed (WEI et al., 2010), culminating, in the latter months of storage in fruit senescence, which is accompanied by alterations in a wide range of cellular functions. These metabolic changes make it the reference genes choice a very difficult step in RT-qPCR studies.

In apple, commonly used reference RT-qPCR genes include *TUBULIN (TUB)*, *ACTIN (ACT)*, *UBIQUITIN (UBI)*, *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (GAPDH)*, *PROTEIN DISULPHIDE ISOMERASE (PDI)*, and the coding sequence for the 18S subunit of ribosomal RNA (CIN et al., 2005; ESLEY et al., 2007; HARB et al., 2012; MANN et al., 2008; MERCHANTE; ALONSO; STEPANOVA, 2013; NOBILE et al., 2011; SCHAFFER et al., 2007a; VIMOLMANGKANG et al., 2014; YANG et al., 2013b). However, their transcriptional profile can potentially be affected by many factors as the postharvest metabolic and physiological changes that occur in this period. Thus, novel candidate reference genes are highly sought after for fruit development, ripening and storage transcriptional analyses. The coding sequences for proteins involved in nuclear DNA organization and cell cycle control are promising candidates as reference genes, due to the constant maintenance of these vital and essential functions throughout development. Among these proteins, the histones are responsible for DNA condensation, organization and regulation in eukaryotic cells, being responsible in the maintenance of chromatin structure and regulation of DNA replication and repair, cell proliferation and gene expression by dynamically modulating the interaction between the nucleic acid and transcription factors (HARSHMAN et al., 2013; YI et al., 2006). The formation of the basic structure of DNA packing, the nucleosome, is

mediated by histones H2A, H2B, H3 and H4 (OUDET; GROSS-BELLARD; CHAMBON, 1975). Higher order chromatin structures, also known as stabilized nucleosomes, are produced via linker histone H1 (CARRUTHERS et al., 1998). Molecular chaperones, such as NUCLEOSOME ASSEMBLY PROTEIN 1 (NAP1) (HAUSHALTER; KADONAGA, 2003), act to prevent improper associations between histones and other proteins, or between histones and DNA. The chaperone NAP1 is an integral component of chromatin establishment, maintenance and dynamics in eukaryotes, helping nucleosome assembly and promoting chromatin fluidity, which in turn, control gene expression.

Members of the NAP family have been demonstrated to interact with a wide range of cellular factors and are likely to perform additional functions, besides histone assembly (PARK; LUGER, 2006). Thus, considering the vital role of the proteins encoded by *H1* and *NAP1*, they are hypothesized to be constitutively transcribed in a wide range of conditions, which prompted us to investigate their transcriptional profile in postharvest experiments with apple to evaluate their potential as RT-qPCR reference genes.

In the current study, we have investigated the transcriptional stability of apple reference genes employing distinct plant organs, fruit developmental stages and ripe fruits kept at room temperature and under long term cold storage, combined with treatments with exogenous ethylene, its inhibitor, 1-methylcyclopropene (1-MCP), and distinct controlled atmosphere conditions. Moreover, two novel candidate reference genes, *HISTONE 1 (MdH1)* and *NUCLEOSOME ASSEMBLY PROTEIN 1 (MdNAP1)*, were proposed and their transcription stability investigated. Comparative analyses of the results from four software packages for transcription stability determination were performed for all tested experimental conditions. Our results demonstrate that *MdH1* is stably transcribed in a wide range of postharvest conditions and is considered a suitable reference gene for RT-qPCR studies employing ripe apples.

1.2 Material and Methods

1.2.1 Plant material

Apple biological samples were harvested from a commercial orchard of 'Gala' cultivar, clone Baigent, grafted on M.9 rootstock, located in Caxias do Sul, RS, Brazil. Samples were collected as described for each experimental condition, immediately

frozen in N₂ and conserved at -80°C until further processing. For all experiments only one biological sample was assessed for each sampling point. This biological sample consisted in a pool of five fruits or other organs, depending of the experiment. The RT-qPCR was performed in a three technical replicates for each sample.

1.2.2 Experimental conditions

Transcription stability for the candidate genes was investigated in five independent experiments (I to V), consisting of developmental and postharvest conditions. In Experiment I (Plant organs) - fully expanded leaves, flowers at anthesis and green fruits, 60 days after anthesis (DAA), were sampled. For Experiment II (Fruit developmental stages) - samples were harvested from 0 up to 105 DAA, when firmness corresponded to 85 Newton, at 15 day intervals. In Experiment III, transcription profiling was obtained for fruit ripening at room temperature, using apples submitted to 1-methylcyclopropene (1-MCP) treatment and control, untreated fruits, kept at room temperature (RT, 25°C) for 12 days, sampled at two day intervals. For Experiment IV - Ethylene treatment on cold stored apples - the fruits were harvested at physiological maturity, separated into three equivalent samples; one treated with ethylene, one, with 1-MCP and the other, maintained as untreated control. Treated and untreated fruits were subsequently stored under cold storage (CS, temperature of $0 \pm 0.5^\circ\text{C}$ and relative humidity of $90 \pm 5\%$) for 180 days, sampled at two month intervals after a period of seven days at RT. Experiment V - Cold storage conditions - employed 1-MCP treated and control fruits submitted to CS combined with distinct controlled atmosphere (CA) conditions (0.5% O₂, 1.0% O₂ and 1.5% O₂, supplemented with 2% CO₂ in all conditions) for nine months. Apples were sampled immediately after removal from CS/CA and after seven days of RT incubation.

1.2.3 Ethylene and 1-MCP treatments

Exogenous ethylene application in Experiment IV was carried out by supplying fruits contained in hermetically closed flasks with 10 ppm (10 $\mu\text{L} \cdot \text{L}^{-1}$) of ethylene. In Experiments III, IV and V, 1-MCP treatments were carried out by the application of 1 ppm (1 $\mu\text{L} \cdot \text{L}^{-1}$) of the commercial product (AgroFresh™), which contains 0.14% (m/m) of the inhibitor. For 1-MCP application, the commercial product in powder was diluted

in water into a Becker and this solution was kept in the same closed container where the fruits were maintained by 24 hs.

1.2.4 Total RNA isolation and first strand cDNA synthesis

Total RNA was isolated as described by Zeng and Yang (2002), with minor modifications. Quality and quantity of the RNA were analyzed by absorbance ratio at A_{260}/A_{280} and A_{260}/A_{230} using an Epoch Micro-volume (BioTek, VT, USA) spectrophotometer. The integrity of the total RNAs was investigated by 1% agarose gel electrophoresis, stained with Gel Red (Biotium, CA, USA). Genomic DNA was eliminated by DNase I (Invitrogen, MA, USA) treatment, as recommended by the manufacturer. The efficiency of DNA removal was checked by qPCR using *MdH1* and *MdUBC* primers. Subsequently, cDNA synthesis was carried out from 1 μ g of total RNA using Oligod(T) (Invitrogen) primers and SuperScriptIII/RNase Out Mix (Invitrogen), according to the manufacturer recommendations.

1.2.5 Primer design and RT-qPCR conditions

Primers were designed for coding sequences (CDs) of the candidate reference genes of *Malus x sylvestris var domestica* genome, available at the Genome Database for Rosaceae (GDR, - <http://www.rosaceae.org/>), using the default parameters of the software Primer3Plus (UNTERGASSER et al., 2007), with amplicon sizes ranging from 80 to 150 base pairs (Table 1.1). The primers were validated for each experiment by the analyses of the amplification curve employing a pool of cDNAs from all tested conditions, at five distinct concentrations. Oligonucleotide specificity and absence of primer dimers were checked by analyses of the post transcriptional dissociation curve. All primers used in the current study exhibited amplification efficiency close to 2.0 and a single dissociation temperature peak (Table 1.2, Appendix A).

Quantitative PCR (qPCR) was carried out using the equipment StepOne™ Real Time PCR Systems (Life Technologies, MA, USA) and the SYBR™ Green PCR Master Mix (Life Technologies). The reactions were performed in a total volume of 15 μ L, consisting of 7.5 μ L of SYBR™ Green PCR Master Mix, 1 μ L of cDNA and 400nM each, primer pair, and started with a denaturation step at 95°C for 10 minutes, followed by 40 cycles consisting of 15 seconds at 95°C and 1 minute at

60°C, finalized by the dissociation curve with denaturation at 95°C for 15 seconds, cooling at 60°C for 1 minute and gradual heating, at 0.3°C steps, up to 95°C. A negative, no template control (NTC), was used to check the absence of DNA contamination.

We investigated the transcriptional stability of five candidate reference genes, consisting of three commonly used normalizers; *ACTIN* (*MdACT*), *UBIQUITIN-CONJUGATING ENZYME E2* (*MdUBC*), and *PROTEIN DISSULFIDE ISOMERASE* (*MdPDI*), and two novel proposed candidates; *HISTONE 1* (*MdH1*) and *NUCLEOSOME ASSEMBLY 1 PROTEIN* (*MdNAP1*).

1.2.6 Statistical analyses of transcriptional stability

Quantification cycle (Cq) data from RT-qPCR were recovered using the software StepOne v 2.2.2 (Applied Biosystems™, MA, USA) and submitted to analyses using four software packages, namely DataAssist v3.01 (Life Technologies™), geNorm (Biogazelle qbase+®), NormFinder (MDL®) and BestKeeper (Bestkeeper software®).

The BestKeeper algorithm employs raw Cq values to calculate the standard deviation (SD) and coefficient of variation (CV) for each candidate under the investigated experimental conditions. In this step, only the genes with a SD lower than 1.0 are considered stable. Subsequently, it calculates a 'BestKeeper Index' for each sample, consisting of the geometric mean of the Cq values for each candidate reference gene. Finally, the program calculates a Pearson coefficient of correlation (r) between the candidate reference gene and the BestKeeper Index, where high r values correspond to genes exhibiting stable transcriptional profile under the investigated experimental conditions (PFAFFL et al., 2004).

The geNorm package ranks the transcriptional stability of the candidate genes by the M value, which is calculated by the pairwise variation in the transcript levels of a given gene in comparison to the levels of the remaining reference genes in test. Raw Cq values were converted to linear data for M calculations (VANDESOMPELE et al., 2002). Genes exhibiting high M values are considered transcriptionally unstable under the tested conditions. The M value is then used to stepwise exclude the genes exhibiting the most unstable transcriptional profiles and recalculate the M

values for the remaining genes. Genes with M values inferior to 1.5 are considered stably transcribed in the investigated conditions.

The package DataAssist employs an algorithm similar to that of geNorm to evaluate the transcription stability of the candidate genes, by generating a score for each candidate reference gene. However, the software does not impose a cut-off for the stability values, but the lower the score, the more stable the gene.

In contrast, NormFinder uses an ANOVA based model to investigate the transcriptional behavior of a candidate reference throughout the tested experimental conditions. Inter and intragroup variation may be used to rank the transcriptional stability of the candidate reference genes. Thus, lower variation values correspond to the most stably transcribed candidates (ANDERSEN; JENSEN; ØRNTOFT, 2004).

1.3 Results

In the current work, we have investigated the transcriptional behavior of five candidate reference genes for RT-qPCR, consisting of three commonly used genes (*MdACT*, *MdUBC* and *MdPDI*) and two novel proposed (*MdH1* and *MdNAP1*) normalizers (Table 1.1). The transcriptional stability of the candidates in five independent experiments was evaluated using four software packages; geNorm, Normfinder, BestKeeper and DataAssist.

1.3.1 Amplification efficiency of the selected primers

Specific amplification was confirmed by the presence of a single temperature peak in the dissociation curves of all tested primers (Appendix A). The efficiency of amplification for the primers in RT-qPCR reactions ranged from 1.90 to 2.34 for all investigated experimental conditions, with most values close to 2.0 (Table 1.2). Because of this, in cases where it was necessary to transform the Cq values in Q, we use 2.0 as efficiency value.

1.3.2 Expression levels of candidate reference genes

Transcriptional stability is dependent on the Cq values of the candidate reference genes throughout the tested experimental conditions. For novel candidate *MdNAP1*, Cq values were highly variable, ranging from 28 to 37 in the investigated experimental conditions (Figure 1.1). The most variable levels of transcription for

MdNAP1 were observed in response to ethylene treatment applied to cold stored apples (Experiment IV) (Figure 1.1). Transcript accumulation for *MdACT*, a commonly employed normalizer gene in RT-qPCR experiments, was also variable for all investigated experimental conditions (Figure 1.1). The Cq values for these candidates were also higher than those observed for the remaining potential references, demonstrating the low transcription rates of the genes in the evaluated samples (Figure 1.1). In a similar manner, the highest Cq values of 37 and 36 for *MdNAP1* and *MdACT*, respectively, were observed in response to ethylene treatment of cold stored apples (Experiment IV) (Figure 1.1). In contrast, the smallest variation of Cq values in the tested experimental conditions were observed for *MdH1* and *MdUBC* (Figure 1.1). The highest transcription activation was observed for *MdPDI*, in response to fruit ripening stage (Experiment II), with Cq ranging from 24 to 27 (Figure 1.1).

1.3.3 Transcription stability analyses

1.3.3.1 BestKeeper

The results of BestKeeper analyses indicate distinct sets of genes as the most suitable references for each experimental condition tested. The transcription of the candidates was highly responsive to the type of plant organ investigated (Experiment I), with the exception of *MdH1* that exhibited SD smaller than 1 and was considered a suitable reference gene for transcriptional profiling in plant organs (Table 1.3). The values of CV were also high for *MdNAP1*, *MdACT*, *MdPDI* and *MdUBC* expression in distinct plant parts (Table 1.3). In contrast, for fruit developmental stage (Experiment II), SD values for all investigated candidate genes were inferior to 1.0, with the smaller variations being found in *MdUBC* and *MdNAP1* transcript accumulations (Table 1.3). A similar trend of low SD values was also observed for apple fruits ripening at room temperature (Experiment III), with *MdH1* and *MdUBC* exhibiting the lowest SDs (Table 1.3). The transcription accumulation of *MdACT* and *MdNAP1* was responsive to ethylene treatment in cold stored fruits (Experiment IV), which exhibited SDs superior to 1.0 (Table 1.3). As observed for Experiment III, *MdH1* and *MdUBC* were the least responsive genes in Experiment IV (Table 1.3). Similarly, the transcript accumulation profile for both genes was also the most stable in response to distinct controlled atmosphere conditions in cold stored fruits (Experiment V). In

contrast, SD value for *MdACT* expression in Experiment V was higher than 1.0 (Table 1.3); therefore, the gene was considered unsuitable as reference for studies of apple fruits under controlled atmosphere combined with cold storage (Table 1.3).

As recommended, the genes with SD greater than 1.0 were eliminated from further analyses. Thus, *MdH1* was the sole gene exhibiting acceptable SD value in response to plant organ types (Experiment I) and therefore the correlation analysis were not realized in this experiment. The correlation between the expression and BestKeeper indices was strong and significant for *MdPDI*, *MdACT* and *MdUBC* in response to the fruit developmental stage, whereas for *MdH1*, r was the lowest observed in Experiment II (Table 1.3). High coefficients of correlation were also observed for *MdUBC*, *MdPDI* and *MdH1* in response to ethylene treatment of cold stored fruits (Experiment IV). Under distinct CA conditions for fruits kept in CS (Experiment V), the most stably transcribed genes were *MdH1*, *MdPDI* and *MdUBC*, and the least stable transcription levels were observed for *MdNAP1* (Table 1.3, Table 1.4).

1.3.3.2 geNorm

The results of M value analyses using geNorm software demonstrated that not all candidates are suitable reference genes in all tested experimental conditions (Table 1.4). Genes considered transcriptionally unstable ($M > 1.5$) were *MdACT* in Experiments I, IV and V, and *MdNAP1* and *MdH1* in Experiment I (Table 1.4). The recommended stepwise exclusion of unsuitable reference genes reduced M values for all investigated genes in all tested experimental conditions (Figure 1.2).

The initial classification, based on M values, and the final classification, after the stepwise exclusion of the genes exhibiting higher M values, resulted in slightly different order of the recommended normalizers for Experiments II, III, IV and V (Table 1.4, Figure 1.2).

1.3.3.3 DataAssist

The analysis algorithm for DataAssist is similar to that of geNorm, with the recommendation of most stably transcribed genes based on a calculated score. No cut off values are admitted in this software, but a lower score indicating a stable transcription pattern. For Experiments I and II (plant organs and fruit developmental

stages), the genes exhibiting the most variable patterns of transcript accumulation were *MdH1* and *MdNAP1*, whereas *MdPDI*, *MdACT* and *MdUBC* were considered to have the most stable transcript accumulation levels (Table 1.4). The least responsive genes to 1-MCP treatment of room temperature stored apples (Experiment III) were *MdH1*, *MdUBC* and *MdACT*. Similarly, *MdUBC* and *MdH1*, along with *MdPDI*, were stably transcribed in fruits treated with ethylene and cold stored (Experiment IV) (Table 1.4). The same three candidate reference genes were also recommended as the most adequate normalizers for fruits kept under CS in different CA conditions (Experiment V), however, in a different order (*MdUBC*<*MdPDI*<*MdH1*) (Table 1.4).

1.3.3.4 NormFinder

The ANOVA based algorithm of NormFinder generated a similar rank of recommended reference genes for transcriptional analyses using distinct plant organs (Experiment I) (Table 1.4). The classification of the most stably transcribed genes in response to the fruit developmental stages (Experiment II) was similar for all employed software packages, except that the recommended order by geNorm and DataAssist was slightly different than the order recommended by NormFinder and BestKeeper (Table 1.4). The genes exhibiting the most unresponsive transcriptional profiles to 1-MCP treatment and storage at RT (Experiment III) according to NormFinder analyses were *MdH1*, *MdACT* and *MdUBC*, in agreement with the results from the other tested packages, which also detected the transcription instability of *MdNAP1* and *MdPDI* under these experimental conditions (Table 1.4). The least stably transcribed genes in response to ethylene treatment under cold storage in NormFinder analyses were *MdNAP1* and *MdACT* and similar results were generated by the other packages (Table 1.4). Under distinct CA conditions in cold storage, the most stable levels of transcript accumulation were observed for *MdUBC*, *MdPDI* and *MdH1*, in agreement with the results with DataAssist (Table 1.4).

1.4 Discussion

The transcriptional stability of five candidate genes, consisting of three commonly used and two novel proposed references, for transcriptional profiling was investigated in apple by the comparative analyses of the results generated using four software packages with data from five experiments consisting of developmental and postharvest conditions. Recently, the transcriptional stability of a wide range of

reference genes has been investigated in plants (BOROWSKI et al., 2014; CHANDNA; AUGUSTINE; BISHT, 2012; DEKKERS et al., 2012; FAN et al., 2013; GIMENO et al., 2014; IMAI et al., 2014; LING et al., 2014; ZHU et al., 2014). In summary, the results suggest that transcriptional stability is dependent on the biological material and the experimental conditions it has been submitted to, and that the use of two or more reference genes is recommended for adequate normalization. To our knowledge, the present work is the first to investigate the transcriptional behavior of reference genes in apple postharvest studies.

Distinct calculation tools are available to investigate the transcriptional stability of candidate reference genes (ANDERSEN; JENSEN; ØRNTOFT, 2004; PFAFFL et al., 2004; VANDESOMPELE et al., 2002). We have employed four software packages, namely; geNorm, NormFinder, BestKeeper and DataAssist, to study the transcript accumulation profile of the candidates in five independent experiments. Although some packages recommend a certain number of reference genes to be used for a given experimental situation (VANDESOMPELE et al., 2002), the use of three reliable references is suitable to normalize a wide range of qPCR applications (BUSTIN et al., 2010; PFAFFL et al., 2004). The comparative analysis of the transcription stability results by the four software packages has demonstrated minor differences in candidate gene ranking (Table 1.4), which is a consequence of the algorithms used by each program. Although the ranking order was not always identical, the three genes considered the most stably transcribed under a given experimental condition were similar. The noteworthy exception was the rank generated by BestKeeper for fruits treated with 1-MCP and stored at RT (Experiment III), which classified *MdNAP1* as the gene exhibiting the most stable transcription pattern, whereas the results obtained from the remaining packages ranked the gene amongst the least transcriptionally stable under those experimental conditions. Moreover, the initial ranking by BestKeeper, based on SD, suggests *MdH1* as a suitable reference gene for transcriptional profiling using distinct plant organs (Experiment I) (Table 1.3), in contrast with the ranks generated by the other packages that considered *MdH1* differentially regulated in response to the experimental conditions. Thus, in general, the most discrepant results in gene stability ranking were obtained with BestKeeper. The most critical difference between BestKeeper algorithm and those from the other packages is that it employs the correlation analyses between the candidate genes Cq and an index derived from the

candidate geometric mean. In contrast, the algorithms used by geNorm, DataAssist and NormFinder employ variation measures to calculate the stability of gene transcription (ANDERSEN; JENSEN; ØRNTOFT, 2004; PFAFFL et al., 2004; VANDESOMPELE et al., 2002). Under our experimental conditions, the software BestKeeper was not suitable to precisely determine the most stably transcribed genes, since no results were generated for Experiment I (plant organs) and the results obtained for Experiment III (fruit ripening at room temperature) were divergent from the results given by the other packages. A comparative analyses of transcriptional stability in distinct tissues of pear also reported similar results obtained by geNorm and NormFinder analyses (IMAI et al., 2014).

Fruit storage and postharvest periods are characterized by metabolic alterations distinct from those occurring during other developmental stages. Moreover, several particular metabolic aspects are typical of fruits and absent from vegetative tissue and flower development. Thus, the transcriptional profile of the candidate reference genes was investigated in distinct plant organs and throughout fruit development.

In agreement with these observations, the transcript accumulation of the gene *MdH1* was stable in all experimental conditions involving ripe, harvested fruits (Experiments III, IV and V), whereas, its transcription was differentially regulated in the tested plant organs and during fruit development, before physiological maturity (Experiments I and II) (Figure 1.1, Table 1.4). Although histone coding sequences, such as those coding for H2 and H3, have been used as reference genes in qPCR studies with several organisms (DE VEGA-BARTOL et al., 2013; DONG et al., 2012; GU et al., 2014; IMAI et al., 2014), the current work is the first one to propose an H1 coding sequence as reference. Histone 1, also called Histone 5, is classified as a linker histone due to its function in the association of nucleosome core particles during chromatosome formation. The linker function is distinct from that of H2A, H2B, H3 and H4 that constitute the core histone octamer around which the DNA is wrapped during chromatin condensation (HARSHMAN et al., 2013).

Recently, the occupancy of histone variants in nucleosomes has been associated to environmental and developmental control in higher plants; including processes associated to temperature and senescence responses (AY; JANACK; HUMBECK, 2014; RÍOS et al., 2014). The observed transcriptional stability of *MdH1* suggests that this gene does not change her transcription in these processes in

mature apples. Future studies investigating the transcriptional regulation of *H1* coding sequences in other fruit species or even apple genotypes may provide further insight into its biological role and usefulness as reference gene in transcription profiling investigation.

The transcriptional stability of *MdACT*, a commonly employed reference gene, was observed in the majority of the investigated experimental conditions, except for experiments IV and V, which use cold storage for postharvest fruit conservation. Similarly, previous studies have demonstrated that *ACT* transcription in tissues submitted to cold is less stable (LOPEZ-PARDO; RUIZ DE GALARRETA; RITTER, 2012; SAHA; VANDEMARK, 2012), suggesting that low temperatures influence its transcriptional activity. The transcription stability of the gene *MdUBC* was observed for all investigated experimental conditions; thus, it can be considered a suitable reference gene for a wide range of biological samples and experimental conditions. Moreover, results from the analyses with the four software packages tested included *MdUBC* among the three most stably transcribed genes throughout the experimental conditions. The novel proposed candidate *MdNAP1* ranked amongst the least stably transcribed genes in the majority of the investigated experimental conditions. Therefore, it was considered inadequate as a reference gene for the tested conditions. The low transcriptional stability may be associated to the low levels of transcript accumulation detected under our experimental conditions.

1.5 Conclusions

The gene *MdH1* constitutes a novel, viable, alternative reference gene and may be associated with other genes for RT-qPCR data normalization in postharvest studies with apple fruits.

The ranking results of transcription stability analyses employing the software BestKeeper were the most divergent, among those from the investigated packages.

Taking together, the results from the present work reinforce the importance of the determination of transcription stability for the experimental conditions and candidate reference genes of interest.

Tables

Table 1.1 - Genome and amplification information on the candidate reference genes

Gene description/ acronym	Accession code ^a	Primer Sequence (5' to 3')		Amplicon size (bp)
		Forward	Reverse	
<i>PROTEIN DISULFIDE ISOMERASE (PDI)</i>	MDP0000233444	TGCTGTACACAGCCAACGAT	CATCTTTAGCGGCGTTATCC	120
<i>HISTONE 1 (H1)</i>	MDP0000223691	CATATTTGGCAGCAGAGCAA	CTCGTTAGCCAACTGCATCA	89
<i>NUCLEOSSOME ASSEMBLY 1 PROTEIN (NAP1)</i>	MDP0000272485	CAAACCTTGCCCCTCCATTTA	CCAGCCTTCGTGATGAATTT	117
<i>UBIQUITIN- CONJUGATING ENZYME E2 (UBC)</i>	MDP0000205182	TTGCTGGTGATCTCTGCATC	AGACCCACCTACTCCCGTCT	117
<i>ACTIN (ACT)</i>	MDP0000170174	GGCTCTATTCCAACCATCCA	TAGAAGCAGTGCCACCACAC	140

^aAccession codes correspond to unigene numbers from *M. x sylvestris* var *domestica* genome.

Table 1.2 - Efficiency of primer pairs used for RT-qPCR amplification in each experiment

Gene	RT-qPCR efficiency				
	Plant organs	Fruit developmental stages	Fruit ripening at room temperature	Ethylene treatment on cold stored apples	Cold storage conditions
<i>ACT</i>	1.98	2.08	2.10	2.01	2.03
<i>H1</i>	1.96	2.09	2.04	2.05	2.00
<i>NAP1</i>	1.95	1.92	1.94	2.10	2.22
<i>PDI</i>	1.90	2.09	2.07	2.08	2.34
<i>UBC</i>	1.91	2.01	2.07	2.00	1.98

The genes were investigated under five independent conditions, using a pool of the cDNAs for all experimental conditions, at five distinct concentrations.

Table 1.3 - Descriptive statistic analyses of the transcriptional regulation of five candidate reference genes tested in five independent experimental conditions in apple, using the software BestKeeper. Absent data (-) corresponds to unviable calculations due to differentially regulated transcription under the given experimental conditions

Experiment	Plant organs		Fruit developmental stage		Fruit ripening at room temperature		Ethylene treatment on cold stored apple		Cold storage conditions	
	SD*	CV**	SD	CV	SD	CV	SD	CV	SD	CV
Candidate reference genes										
<i>ACT</i>	1.62	5.01	0.67	2.34	0.55	1.86	1.71	5.22	1.32	4.32
<i>H1</i>	0.55	1.86	0.66	2.39	0.41	1.47	0.42	1.50	0.70	2.64
<i>NAP1</i>	2.04	5.90	0.59	1.98	0.78	2.60	1.34	3.89	0.85	2.63
<i>PDI</i>	1.45	4.94	0.69	2.70	0.58	2.09	0.57	2.01	0.82	3.10
<i>UBC</i>	1.07	3.63	0.45	1.70	0.41	1.54	0.51	1.82	0.64	2.31
Candidate genes reference SD<1	coeff. of corr. [r]	p-value	coeff. of corr. [r]	p-value	coeff. of corr. [r]	p-value	coeff. of corr. [r]	p-value	coeff. of corr. [r]	p-value
<i>ACT</i>	-	-	0.957	0.001	0.734	0.003	-	-	-	-
<i>H1</i>	-	-	0.795	0.001	0.748	0.002	0.853	0.002	0.883	0.001

<i>NAP1</i>	-	-	0.817	0.001	0.916	0.001	-	-	0.754	0.001
<i>PDI</i>	-	-	0.974	0.001	0.611	0.020	0.913	0.001	0.856	0.001
<i>UBC</i>	-	-	0.949	0.001	0.741	0.002	0.986	0.001	0.817	0.001

*Standard deviation

**Coefficient of variation

Table 1.4 - Ranking of the five candidate reference genes according to the transcription stability

Software	BestKeeper	geNorm	DataAssist	NormFinder				
	Coeff. of. corr. [r]	Ranking	M value	Ranking	Score	Ranking	Stability value	Ranking
I. Plant organs	-	-	1.058	<i>PDI</i>	1.16	<i>PDI</i>	0.178	<i>PDI</i>
	-	-	1.076	<i>UBC</i>	1.17	<i>UBC</i>	0.178	<i>UBC</i>
	-	-	1.586	<i>ACT</i>	1.52	<i>ACT</i>	0.930	<i>ACT</i>
	-	-	1.653	<i>NAP1</i>	2.00	<i>NAP1</i>	0.964	<i>NAP1</i>
	-	-	1.823	<i>H1</i>	2.04	<i>H1</i>	1.129	<i>H1</i>
II. Fruit developmental stages	0.974	<i>PDI</i>	0.459	<i>ACT</i>	0.45	<i>ACT</i>	0.073	<i>PDI</i>
	0.957	<i>ACT</i>	0.463	<i>PDI</i>	0.46	<i>PDI</i>	0.087	<i>ACT</i>
	0.949	<i>UBC</i>	0.489	<i>UBC</i>	0.48	<i>UBC</i>	0.155	<i>UBC</i>
	0.817	<i>NAP1</i>	0.666	<i>NAP1</i>	0.66	<i>NAP1</i>	0.417	<i>NAP1</i>
	0.795	<i>H1</i>	0.732	<i>H1</i>	0.73	<i>H1</i>	0.467	<i>H1</i>
III. Fruit ripening at room temperature	0.916	<i>NAP1</i>	0.688	<i>H1</i>	0.65	<i>H1</i>	0.242	<i>H1</i>
	0.748	<i>H1</i>	0.726	<i>ACT</i>	0.65	<i>UBC</i>	0.309	<i>ACT</i>
	0.741	<i>UBC</i>	0.732	<i>UBC</i>	0.69	<i>ACT</i>	0.343	<i>UBC</i>
	0.734	<i>ACT</i>	0.885	<i>NAP1</i>	0.83	<i>NAP1</i>	0.501	<i>NAP1</i>
	0.611	<i>PDI</i>	0.891	<i>PDI</i>	0.85	<i>PDI</i>	0.501	<i>PDI</i>
IV. Ethylene treatment on cold stored apples	0.986	<i>UBC</i>	0.980	<i>UBC</i>	1.42	<i>UBC</i>	0.308	<i>UBC</i>
	0.913	<i>PDI</i>	1.085	<i>H1</i>	1.50	<i>H1</i>	0.461	<i>H1</i>
	0.853	<i>H1</i>	1.148	<i>PDI</i>	1.57	<i>PDI</i>	0.618	<i>PDI</i>
	-	-	1.434	<i>NAP1</i>	2.03	<i>ACT</i>	0.756	<i>NAP1</i>
	-	-	1.660	<i>ACT</i>	2.57	<i>NAP1</i>	1.033	<i>ACT</i>
V. Cold storage conditions	0.883	<i>H1</i>	1.015	<i>UBC</i>	1.01	<i>UBC</i>	0.065	<i>UBC</i>

0.856	<i>PDI</i>	1.102	<i>PDI</i>	1.10	<i>PDI</i>	0.343	<i>PDI</i>
0.817	<i>UBC</i>	1.110	<i>H1</i>	1.11	<i>H1</i>	0.490	<i>H1</i>
0.754	<i>NAP1</i>	1.274	<i>NAP1</i>	1.27	<i>NAP1</i>	0.708	<i>NAP1</i>
-	-	1.690	<i>ACT</i>	1.69	<i>ACT</i>	1.097	<i>ACT</i>

Ranking parameter is presented for each software. Absent data (-) corresponds to the lack of suitable ranking parameters, indicating unstable transcription under the tested conditions.

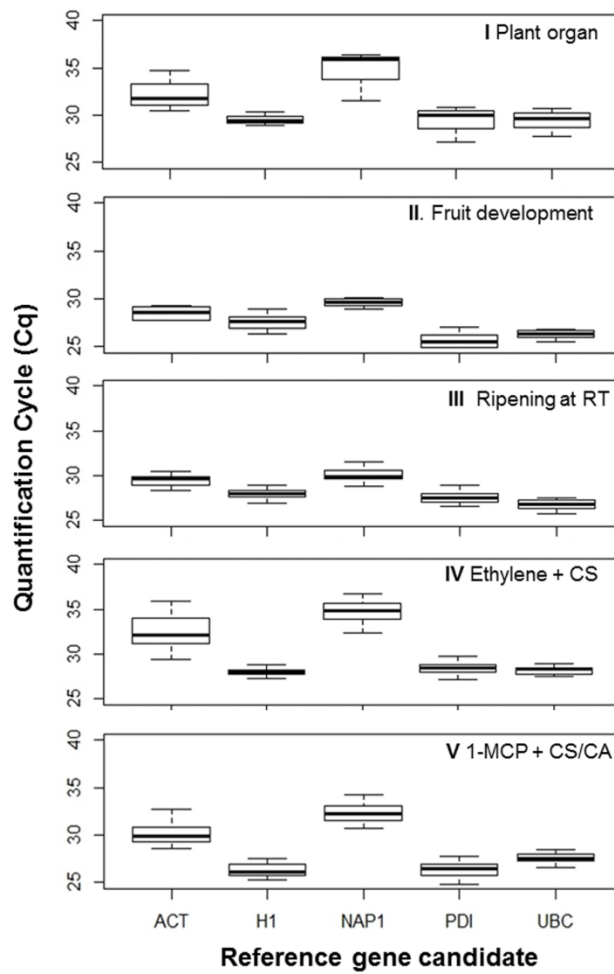


Figure 1.1 - Gene expression levels of the candidate reference genes in apple. The genes, *MdACT* (*ACTIN*), *MdH1* (*HISTONE1*), *MdNAP1* (*NUCLEOSSOME ASSEMBLY PROTEIN 1*), *MdPDI* (*PROTEIN DISULPHIDE ISOMERASE*) and *MdUBC* (*UBIQUITIN-CONJUGATING ENZYME E2*) were evaluated in five independent experiments consisting of plant organs (I), fruit developmental stages (II), fruit ripening at room temperature (III), ethylene treatment on cold stored apples (IV) and long term cold storage combined with distinct controlled atmosphere conditions (V). The box indicates the 25th and 75th percentiles. Horizontal line represents Cq median and whiskers represent highest and lowest values.

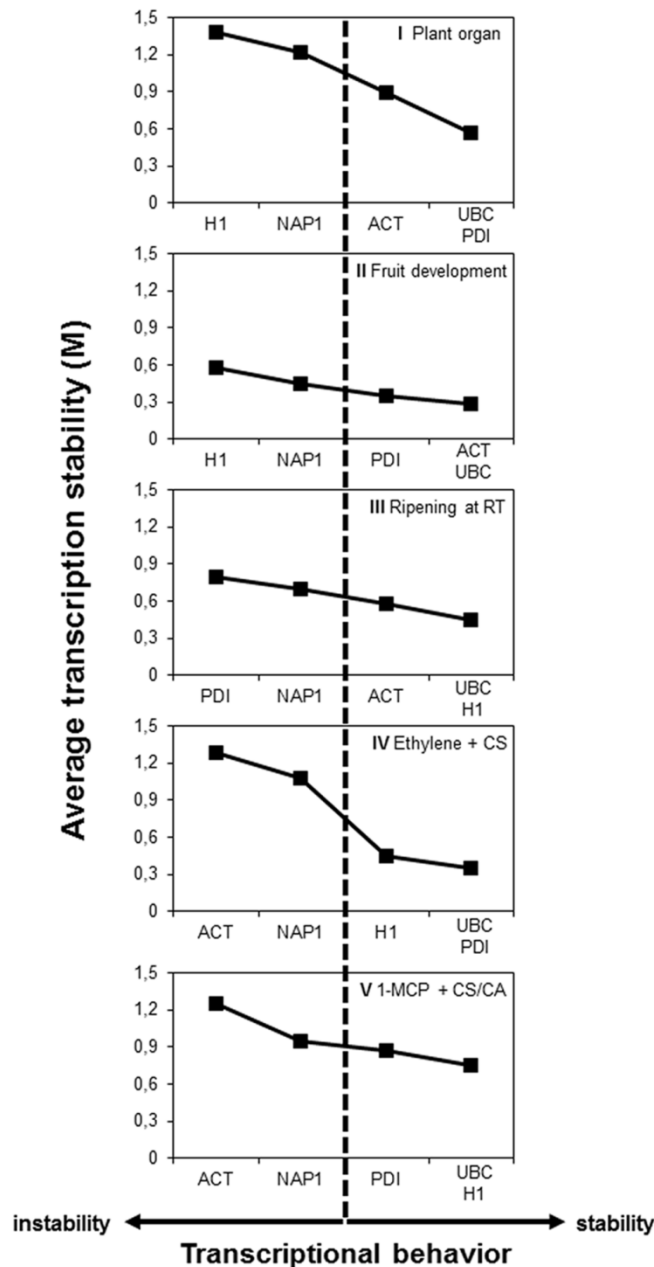


Figure 1.2 - Transcriptional stability of the candidate reference genes investigated by geNorm. The candidate reference genes, *MdACT* (*ACTIN*), *MdH1* (*HISTONE1*), *MdNAP1* (*NUCLEOSSOME ASSEMBLY PROTEIN 1*), *MdPDI* (*PROTEIN DISULPHIDE ISOMERASE*) and *MdUBC* (*UBIQUITIN-CONJUGATING ENZYME E2*) were submitted to five experimental conditions, consisting of plant organs (I), fruit developmental stages (II), fruit ripening at room temperature (III), ethylene treatment on cold stored apples (IV) and long term cold storage combined with distinct controlled atmosphere conditions (V). Low M values indicate genes with more stable transcript accumulation under the given conditions.

Capítulo 2. Este capítulo é constituído pelo manuscrito enviado para a revista Food Chemistry no dia 19 de setembro de 2014. Deste modo, a estrutura do capítulo segue as recomendações da revista.

Title: Ethylene-dependent regulation of a novel α -L-arabinofuranosidase is associated to firmness loss in apples under long term cold storage

Running Title: Ethylene regulates α -L-arabinofuranosidases in stored apples

Authors: Tatiane Timm Storch^{a,b}, Taciane Finatto^{a&}, Camila Pegoraro^a, Joceani Dal Cero^{a#}, François Laurens^d, César Valmor Rombaldi^b, Vera Quecini^a, Cesar Luis Girardi^{a,*}

Affiliations:

^aEmbrapa Uva e Vinho - Caixa Postal 130, 95700-000, Bento Gonçalves, RS - Brazil

^bUniversidade Federal de Pelotas, Faculdade de Agronomia Eliseu Maciel, Departamento de Ciência e Tecnologia Agroindustrial – s/nº caixa postal 354, 96010-900, Pelotas, RS – Brazil

^dInstitut National de la Recherche Agronomique, Institut de Recherche en Horticulture et Semences, IRHS - INRA - Bâtiment B

42, rue Georges Morel - BP 60057 - 49071 Beaucouze Cedex - France

&CurrentAddress: Universidade Tecnológica Federal do Paraná, Câmpus Pato Branco - Via do Conhecimento - km 01, CEP: 85503-390, Pato Branco, PR - Brazil
Pato Branco – PR

[#]CurrentAddress: Emater/RS – ASCAR

Rua Botafogo, 1051, Porto Alegre - RS - Brazil.

*Corresponding author: cesar.girardi@embrapa.br

Corresponding author

César L. Girardi

CNPUV (National Center for Grapevine and Wine Research)
Embrapa (Brazilian Agricultural Corporation)
Rua Livramento, 515
Bento Gonçalves, RS 95700-000
Brazil
Phone: +55 (54) 3455-8000
Fax: +55 (54) 3451 2792
email: cesar.girardi@embrapa.br

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 action. We have investigated the activity of α -L-arabinofuranosidase (AF), a glycosyl hydrolase acting on the side chains of pectin in the cell wall and middle lamella, during postharvest storage. The transcription of *MdAF1* and *MdAF3* was investigated in plant organs and in response to ethylene, applying exogenous ethylene and 1-methylcyclopropene (1-MCP). The transcription of AF genes is not restricted to fruits, although upregulated by ripening and ethylene. Transcripts of *MdAF1* and *MdAF3* were detected under cold storage up to 180 days. Similarly, AF activity increased with rising levels of ethylene and under cold storage. Levels of *MdAF3* transcript were higher than those of *MdAF1*, suggesting that the first is an important contributor to AF activity and texture changes during cold storage.

Keywords

1-Methylcyclopropene; Glycosyl Hydrolases; Cell Wall; Gene Expression; *Malus x domestica* Borkh.; Fruit Softening

2 Ethylene-dependent regulation of a novel α -L-arabinofuranosidase is associated to firmness loss in apples under long term cold storage

2.1 Introduction

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; MUÑOZ-BERTOMEU; MIEDES; LORENCES, 2013; 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; SCOSSA; FERNIE, 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).

The ethylene produced is perceived by a family of histidine kinase, copper binding transmembrane receptors, which are inactivated by the association with the hormone and inactive the Raf kinase-like 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; VILLALOBOS-ACUÑA et al., 2011; YANG et al., 2013b).

In apples, the activity of several pectin modifying enzymes has been associated to fruit softening during ripening and postharvest shelf life, including polygalacturonase (PG), pectin methylesterase (PME), β -galactosidase (β -GAL) and α -L-arabinofuranosidase (AF) (GOULAO; SANTOS; SOUSA; OLIVEIRA, 2007; GOULAO&OLIVEIRA, 2008; 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. However, the ethylene influence in this transcriptional pattern was not clear, since no treatment or condition that altered ethylene production is included in the study (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 of α -L-arabinofuranosidase 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.

2.2 Material and methods

2.2.1 Plant Material

Apples (*Malus x domestica* Borkh.) 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 to 0.5°C, 90 to 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 figure 2.1.

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*.

2.2.2 Ethylene and 1-MCP application

Exogenous ethylene application was carried out as described in Asif et al.(2009) with modifications. Thus, the exogenous ethylene at 10 ppm (10 μ L .L⁻¹) was kept in contact with 50 Kg of apples for 4 hours at 20°C, in 370 L containers.

The recommended concentration of 1ppm (1 μ L .L⁻¹) of 1-MCP (AgroFresh™, Dow Chemical Company) (DEELL; AYRES; MURR, 2008) was applied for 24 hours to 370 L containers where 50 kg of apples were maintained at 20°C. After treatments, the fruits were transferred to room temperature or cold storage, as described.

2.2.3 Fruit quality parameters

Total soluble solids (TSS) were analyzed employing a refractometer (PR 101 Atago) (0 to 45%), with temperature correction, and the values are presented as °Brix. Titratable acidity (TA) 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 (TR Italy P830075) equipped with a probe of 11 mm.

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 I₂ and 24 g of KI in 1000 mL of distilled water) for 40 seconds. Starch presence was evaluated after immersion by comparison to reference values presented by Girardi, Sanhueza and Bender (2002), ranging from 1 (maximum starch contents) to 5 (starch absence). The absence of starch indicates advanced ripening stages.

2.2.4 Ethylene production determination

Ethylene production by the fruits was determined by gas chromatography using Varian CG 3537-5 equipped with stainless steel column packed 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 a head space sample was collected using hypodermic 1 mL syringe. 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.

2.2.5 Reverse Transcription -quantitative PCR

Total RNA was extracted from 6 g of pulverized fruit tissue according to the protocol described by Zeng & 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 x 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 *Malus x sylvestris var 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 Table 2.1. Primer sequences for gene expression normalization were chosen by evaluating the transcription of *MdACT* (β -ACTIN), *MdUBC* (UBIQUITIN-CONJUGATING ENZYME E2), *MdPDI* (BISULFIDE ISOMERASE), *MdNAP1* (NUCLEOSOME 1 BINDING PROTEIN) and *MdH1* (HISTONE 1) for all tested RNA samples, employing the software DataAssist v 3.01 (Life Technologies), as shown by Storch et al., (Unpublished¹). 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.

¹Manuscript submitted to PLoS ONE journal in 15/09/2014

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 as the normalizing sample. The calculation of relative quantitation was made employing the $2^{-\Delta\Delta CQ}$ method.

2.2.6 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 *Malus x sylvestris* var. *domestica* genome (Apple Genome V1.0), *Vitis vinifera* 12X (http://www.genoscope.cns.fr/cgi-bin/blast_server/projet_ML/blast.pl) and *Solanum lycopersicum* V.2.3 (<http://solgenomics.net/tools/blast/>) genomes using the tBLASTn algorithm (ALTSCHUL et al., 1997). 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 et al., 2013), with default parameters. Re-sampling bootstrap trees containing 1000 random samples were constructed using PSIGNFIT software (<http://www.bootstrap-software.org/>).

2.2.7 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.

2.2.8 Enzyme activity

Protein extraction for enzyme activity analyses was adapted from Wei et al. (2010). Briefly, 3 g of pulverized apple pulp without the peel 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 x g for 10 min at 4°C. The supernatant was discarded and the pellet, washed with 1 mL of 0.2 % (w/v) sodium bisulfate 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 p-nitrophenol from the artificial substrate p-nitrophenyl α -L-arabinofuranoside, as described by Brummell et al. (2004). Briefly, 40 μ L of the protein extract were transferred to a microtube containing 400 μ L of 0.1M sodium acetate pH 5.0 and 80 μ L of the substrate (16 μ L of p-nitrophenyl α -L-arabinofuranoside 50mg.mL⁻¹ in 64 μ L of water). The reaction mixture was incubated at 30°C for 90 min and an aliquot of 250 μ L was removed and added to 250 μ L of 1.0M of NH₄OH 1M. Absorbance values were subtracted from the blank readings prepared with protein extract inactivated by boiling. Protein extraction and enzyme activity quantification were carried out as duplicates.

2.3 Results

2.3.1 Fruit quality analyses

The conservation of apple fruits 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 2.2). As part of the ripening process, apples stored at room temperature (RT) for short periods exhibited a slight increase in total soluble solids and no differences in titratable acidity (Table 2.3). 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 2.3). 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 2.2). In contrast, fruit firmness was significantly affected by ethylene and 1-MCP treatments under long term cold storage and short term room temperature conservation (Table 2.2, Table 2.3). Fruit firmness was shown to decrease with time for all tested conditions (Table 2.2, Table 2.3, Figure 2.2). Although pulp resistance was higher for 1-MCP treated fruits, softening increased linearly up to 180 days under CS, with an estimated reduction in firmness of 2.77 N per month (Figure 2.2). Control and ethylene-treated fruits exhibited linear decreases in firmness at higher rates under CS in comparison to those treated with the inhibitor. For control fruits, the quadratic negative value indicates the storage period of minimum firmness is estimated to occur at 62.24 N after 2.5 months of storage under CS (Figure 2.2). Ethylene treatment exponentially reduced fruit firmness with a trough at 59.39 N after 2.45 months under CS (Figure 2.2).

2.3.2 Ethylene production

Ethylene production was investigated by time course analyses of the fruits treated or not with ethylene or 1-MCP and conserved for 12 days at RT or 180 days at 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 in fruits at RT or CS (Figure 2.3). At RT, exogenous ethylene application did not alter the pattern of the hormone accumulation, although total levels were slightly inferior in control fruits (Figure 2.3). After 60 days under CS, ethylene accumulation increased for ethylene treated and control fruits; however, exogenous hormone application did not further increase ethylene levels in fruits under CS (Figure 2.3).

2.3.3 The α -L-arabinofuranosidase gene family in apple

In the apple genome, we have identified 11 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 (Figure 2.4). 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 two additional closely related sequences, coding for putative β -D-xylosidases 1 and 2, indicating the occurrence of gene duplication events throughout evolution in apple (Figure 2.4).

2.3.4 Gene expression profiling

The transcription profiles were determined for two apple α -L-arabinofuranosidases encoded by *MdAF1* and *MdAF3* (Table 2.1) during storage (Figure 2.5). 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 (Figure 2.6).

Reference genes for the experimental conditions are presented in table 1 and were described by Storch et al. (Unpublished).

The genes coding for *AF3* and *AF1* were expressed in all investigated conditions (Figure 2.5, Figure 2.7), including under cold storage and in the presence of ethylene inhibitor 1-MCP (Figure 2.5). Steady-state levels of *MdAF3* transcripts were higher than those of *MdAF1* throughout the investigated conditions. A quantitation of *MdAF3* relative to *MdAF1* demonstrates this higher transcription of the first gene. This difference is highlighted in the fruits conserved at cold storage (Appendix B). The expression of *MdAF3* was strongly inhibited in the presence of ethylene inhibitor 1-MCP (Figure 2.5), as observed for *MdACO1* (Figure 2.6) and demonstrated by the high positive correlation (Table 2.4). Similarly, *MdAF1* expression regulation was also positively correlated to *MdACO1*, however at lower levels (Table 2.4). Prolonged exposition 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) (Figure 2.5).

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 (Figure 2.5, Figure 2.7). Similar results were observed in tomato, where the transcription of *SIAF3* (probe Les.4445.1.S1_at) is higher in ripening fruits (Figure 2.8). In grape berries, *VvAF3* (probe 1615005_at) expression is independent of the ripening stage (Figure 2.9). In contrast, *MdAF1* is expressed in flowers and immature fruits and not induced at later stages of ripening (Figure 2.5, Figure 2.7). A similar non fruit specific pattern is observed for *SIAF1* (probe Les.3991.1.S1_at) that is highly induced in vegetative tissues, such as hypocotyls, cotyledons and leaves (Figure 2.8). In tomato fruits, *AF1* transcription is higher in initial ripening stages in the pulp and peel (Figure 2.8). 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 (Figure 2.9).

The transcription of *MdPG1* was repressed in 1-MCP treated fruits throughout the experiment and did not increase with the application of exogenous ethylene (Figure 2.6). The highest levels of *MdPG1* transcript accumulation were detected in untreated control fruits, as observed for *MdACO1* under cold storage up to 120 days (Figure 2.6). As observed for *MdAF3*, the transcription pattern of *MdPG1* also exhibited a significant correlation to *MdACO1* profile (Table 2.4). The high standard deviation values for *MdPG1* expression is a consequence of its high transcription levels.

2.3.5 Enzyme activity

The activity of α -L-arabinofuranosidase increased with storage time under RT and CS for control fruits and all tested treatments (Figure 2.10). Application of ethylene and its inhibitor did not significantly alter enzyme activity in fruits conserved for short periods at RT (Figure 2.10). 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 (Figure 2.3, Figure 2.10). After six months of cold storage, the application of 1-MCP promoted a two-fold reduction in α -L-arabinofuranosidase activity, whereas exogenous ethylene increased 1.23 times 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 (Figure 2.10).

2.4 Discussion

In the current work, an integrative approach combining transcriptional profiling, quality characterization and biochemical analyses was employed to investigate the role of the coding genes of α -L-arabinofuranosidase, *MdAF1* and *MdAF3*, in texture changes undergone by 'Gala' apples during storage and in response of ethylene.

2.4.1 Storage induced changes in quality traits

The main changes associated with the ripening process in fleshy fruits include color, firmness, taste, and flavor (OSORIO; SCOSSA; FERNIE, 2013). The reduction in pulp firmness is dependent on cell wall degradation and alterations in cuticle properties, whereas the modification in taste is due to an increase in sugar and concomitant decline in organic acids contents (OSORIO; SCOSSA; FERNIE, 2013).

The most significant change occurring during storage in 'Gala' apples was pulp softening, mainly detected in the fruits at room temperatures, as soon as four days after harvest. The treatment with 1-MCP reduced these changes, thus confirming the role of ethylene in apple softening. The cold also reduced these changes in fruits maintained in CS, where no statistical differences were observed up to 180 days. Nevertheless slight differences in the firmness pulp were observed even in 1-MCP treated fruits. Fruit softening in apple was proportional to the length of the storage period mainly in fruits conserved in RT, even in the presence of 1-MCP, suggesting that other developmental factors besides ethylene contribute to the processes control.

The changes associated to fruit flavor were not significantly affected by storage, for short periods at room temperature or long term conservation under cold 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 apple fruits under storage confirmed that most carbohydrates and organic acids are not appreciably affected (LEE et al., 2011).

2.4.2 Effect of ethylene on quality traits of stored apples

Physiologically, fruits are generally classified into two ripening groups, climacteric and non-climacteric, according to their respiratory activity and pattern of ethylene biosynthesis. Ethylene synthesis in climacteric fruits, such as apple, is essential to induce ripening, as shown by several studies with natural and transgenic mutants (OSORIO; SCOSSA; FERNIE, 2013). Ethylene function and synthesis is competitively inhibited by 1-MCP by the occupation of the binding site of ethylene receptors, an irreversible process in the presence of high dosages of the inhibitor (BLANKENSHIP; DOLE, 2003). The exogenous application of ethylene inhibitor did not affect taste parameters, such as sugar accumulation and acidity reduction, in 'Gala' apples during 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 investigated responses. These observations are 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).

2.4.3 Gene expression profiling under storage

The enzyme ACO catalyzes the oxidation of ACC to ethylene (LIN; ZHONG; GRIERSON, 2009) and is encoded by a multigene family, *MdACO1* to *MdACO3*, in apple (BINNIE; MCMANUS, 2009). *MdACO1* transcription is repressed in 1-MCP treated fruits lacking ethylene, as described in a previous work (YANG et al., 2013b).

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). In contrast, the authors have demonstrated that the transcription of the other members of the gene family, *MdACO2* and *MdACO3*, was up regulated in 1-MCP-treated fruit; thus, suggesting the most important contributor to ACO activity in ethylene production in apples is *MdACO1* (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; YANG 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 et al., 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 -L-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. To further confirm the hypothesis, analyses of PG activity and composition of cell wall polysaccharides are required.

In a previous work using distinct genetic backgrounds, the ethylene dependence of *MdAF3* was not clear (NOBILE et al., 2011). Our data showed that in 'Gala' apples, the transcription of *MdAF3* is regulated by the hormone, as observed for *MdACO1*. These results were confirmed by microarray data analyses for climacteric tomato fruits, where *SIAF3* transcription is also ethylene dependent during ripening and in *rin* mutants, that fails to ripen due a mutation in the *ripening-inhibitor* locus. Thus, the increase in transcription of *SIAF3* in *rin* mutants only in the breaker and red ripe stages when a higher ethylene production occur, demonstrate the ethylene dependence of *SIAF3*. 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 et al. (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; COSGROVE; OLIVEIRA, 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).

2.4.4 α -L-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; PAGANO; SOZZI, 2009; GWANPUA et al., 2014; NOBILE et al., 2011; TAKIZAWA et al., 2014; 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 -L-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 et al., 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 considerable levels of *MdAF1* transcript in immature fruits suggest that this gene may play a role in growth associated cell wall modification processes. In contrast, the role of *MdAF3* appears is more likely related to cell wall degradation during postharvest texture alteration in apple.

2.5 Conclusions

In the current work, we have demonstrate 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. *MdAF3* 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.

Tables

Table 2.1 -*Malus x sylvestris* var *domestica* primers used in gene expression analyses by RT-qPCR

Gene	<i>M. x domestica</i> locus	Primer sequence (5' to 3')		Annealing Temperature (°C)	
		Forward	Reverse	Forward	Reverse
Ethylene metabolism					
1-aminocyclopropane-1-carboxylic acid oxidase (<i>ACO1</i>)	MDP0000195885	CAATGCACCACTCCATTGTC	TCCCATCCGACTGAGCTATC	60	58
Cell wall metabolism					
α -L-Arabinofuranosidase3 (<i>AF3</i>)	MDP0000140483	ATTCACAAGGTCCATATCG	CAGGTCACCAATTTCCAG	56	54
α -L-Arabinofuranosidase1 (<i>AF1</i>)	MDP0000055078	TGAGATGGCAAGCTATGCACCAC	CACCGCCTGTCATGGGTATTGAC	70	72
Endopoligalacturonase (<i>PG1</i>)	MDP0000326734	TCACGGTAACTGCACCAGAG	CTTTGGGACCCACTCACAAT	62	60
Endogenous control					
Histone1 (<i>H1</i>)	MDP0000223691	CTCGTTAGCCAACCTGCATCA	CATATTTGGCAGCAGAGCAA	58	60
Ubiquitin-Conjugating Enzyme E2 (<i>UBC</i>)	MDP0000205182	AGACCCACCTACTCCCGTCT	TTGCTGGTGATCTCTGCATC	60	60
Actin(<i>ACT</i>)	MDP0000170174	GGCTCTATTCCAACCATCCA	TAGAAGCAGTGCCACCACAC	60	62
Protein Disulfide Isomerase (<i>PDI</i>)	MDP0000233444	TGCTGTACACAGCCAACGAT	CATCTTTAGCGGCGTTATCC	60	60

Table 2.2 - Time course analyses of apple fruit quality traits under cold storage treated with exogenous ethylene, 1-MCP and untreated. Statistical significance within treatments, calculated by Tukey test ($p \leq 0.05$), is represented by lowercase (lines) and uppercase (columns) lettering

Trait	Values at		Cold storage (days)		
	Harvest	Treatment	60	120	180
Firmness (N)	83.85	Control	59.86 aB	61.91 aA	56.94 aB
		1-MCP	79.24 aA	73.11 aA	69.99 aA
		Ethylene	66.96 aB	61.88 aA	64.46 aAB
TSS (°Brix)	12.53	Control	13.18 aA	13.08 aA	12.80 aA
		1-MCP	13.86 aA	14.08 aA	13.46 aA
		Ethylene	13.82 aA	14.24 aA	13.66 aA
TA (% of malic acid)	5.5	Control	4.16 aAB	3.48 bA	3.22 bB
		1-MCP	4.30 aA	3.87 aA	3.80 aA
		Ethylene	3.76 aB	3.78 aA	3.24 bB

Table 2.3 - Time course analyses of apple fruit quality traits stored at room temperature, treated with 1-MCP and untreated. Statistical significance within treatments, calculated by Tukey test ($p \leq 0.05$), is represented by lower- (lines) and uppercase (columns) lettering

Trait	Treatment	Room temperature storage (days)					
		2	4	6	8	10	12
Firmness (N)	Control	84.72 aA	78.26 bB	77.91 bB	76.85 bB	75.34 bB	70.27 cB
	1-MCP	86.38 aA	84.63 abA	83.02 abA	81.40 abA	80.89 bA	80.39 bA
TSS (°Brix)	Control	12.90 cA	13.33 bcA	13.4 abcA	14.13 abA	14.25 aA	14.13abA
	1-MCP	12.70 cA	13.33 bcA	13.30 bcA	13.73 abA	13.87 abA	14.20 aA
TA (% of malic acid)	Control	4.82 aA	4.67 aA	4.52 aA	4.36 aA	4.30 aA	4.07 aA
	1-MCP	5.25 aA	5.16 aA	5.04 aA	4.73 aA	4.64 aA	4.51 aA

Table 2.4 - Spearman rank correlation of transcript accumulation of *MdACO1* and *MdAF3*, *MdAF1* and *MdPG1* in apple fruits during storage

Correlation between <i>MdACO1</i> transcript level to	Spearman's rank correlation coefficient	p-value
<i>MdAF3</i>	0.92	2.04e-10
<i>MdAF1</i>	0.47	0.0206
<i>MdPG1</i>	0.84	2.02e-7

Figures

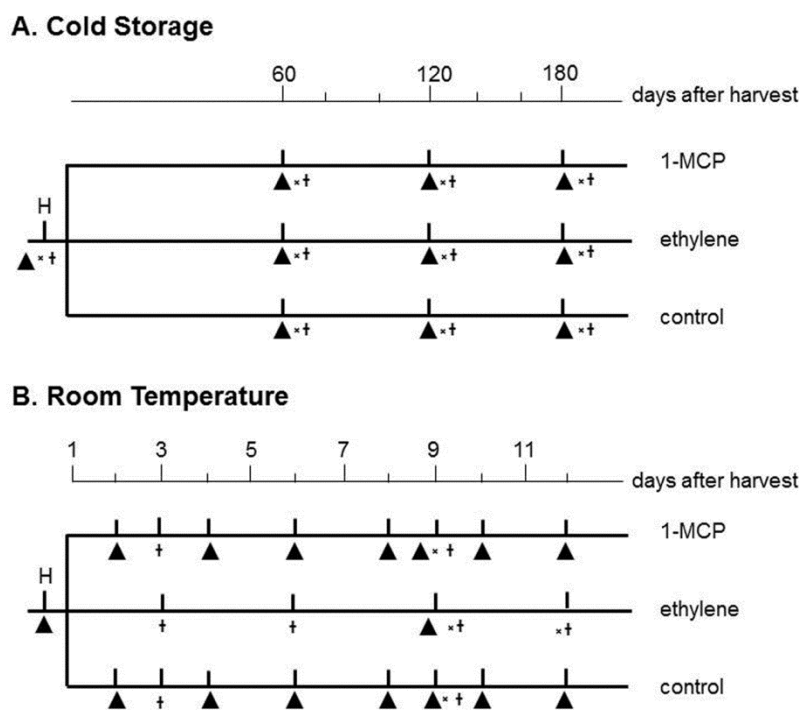


Figure 2.1 - Schematic representation of the fruit sampling points used in the current study. Sampling of the fruits under cold storage (CS) started after seven days at room temperature (RT). Long term cold storage samplings were carried out in 2009 (A) and room temperature time course samples were taken in 2013 (B). Triangles represent sampling points for RNA extraction. * for enzymatic activity and †, for ethylene production analysis.

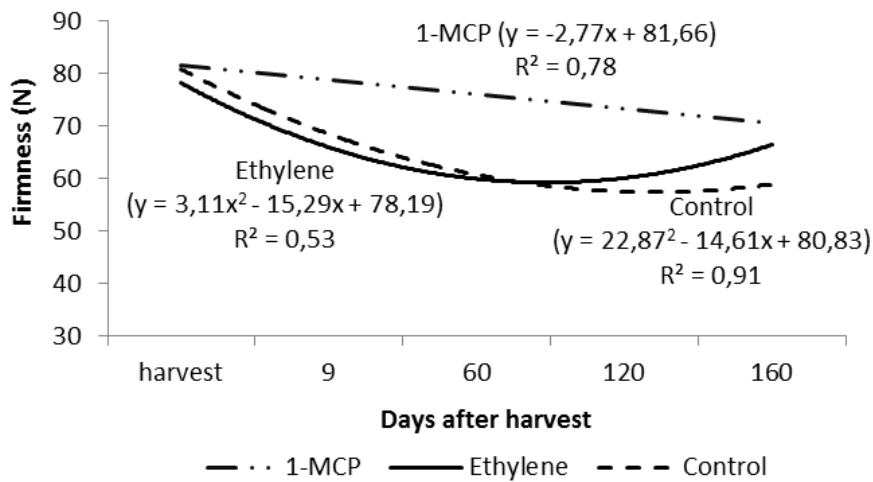


Figure 2.2 - Adjusted regression analyses for pulp firmness of 'Gala' apples at harvest, control conditions, ethylene and 1-MCP treatments after nine days under RT and 60, 120 and 180 days under cold storage.

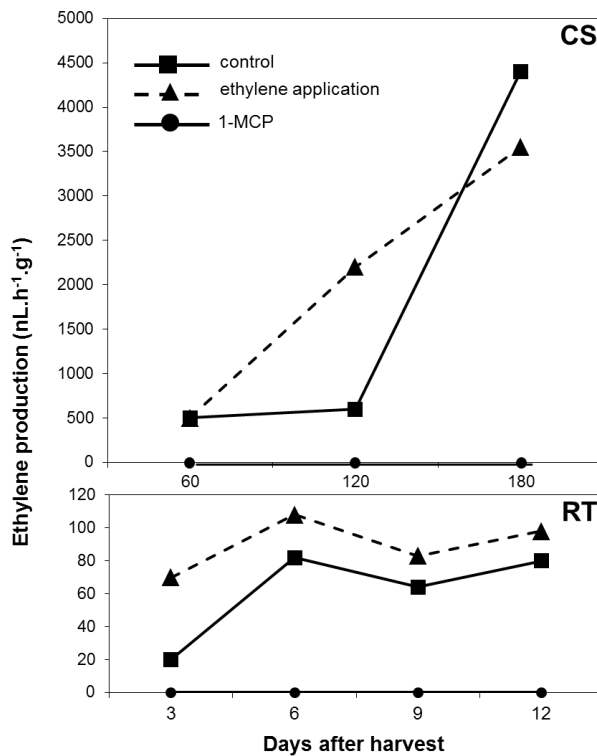


Figure 2.3 - Time course analysis of ethylene production in control, ethylene and 1-MCP treated fruits under cold storage (CS) and room temperature (RT). Magnitude differences in the hormone production levels are due to changes from pre climacteric to post climacteric phase.

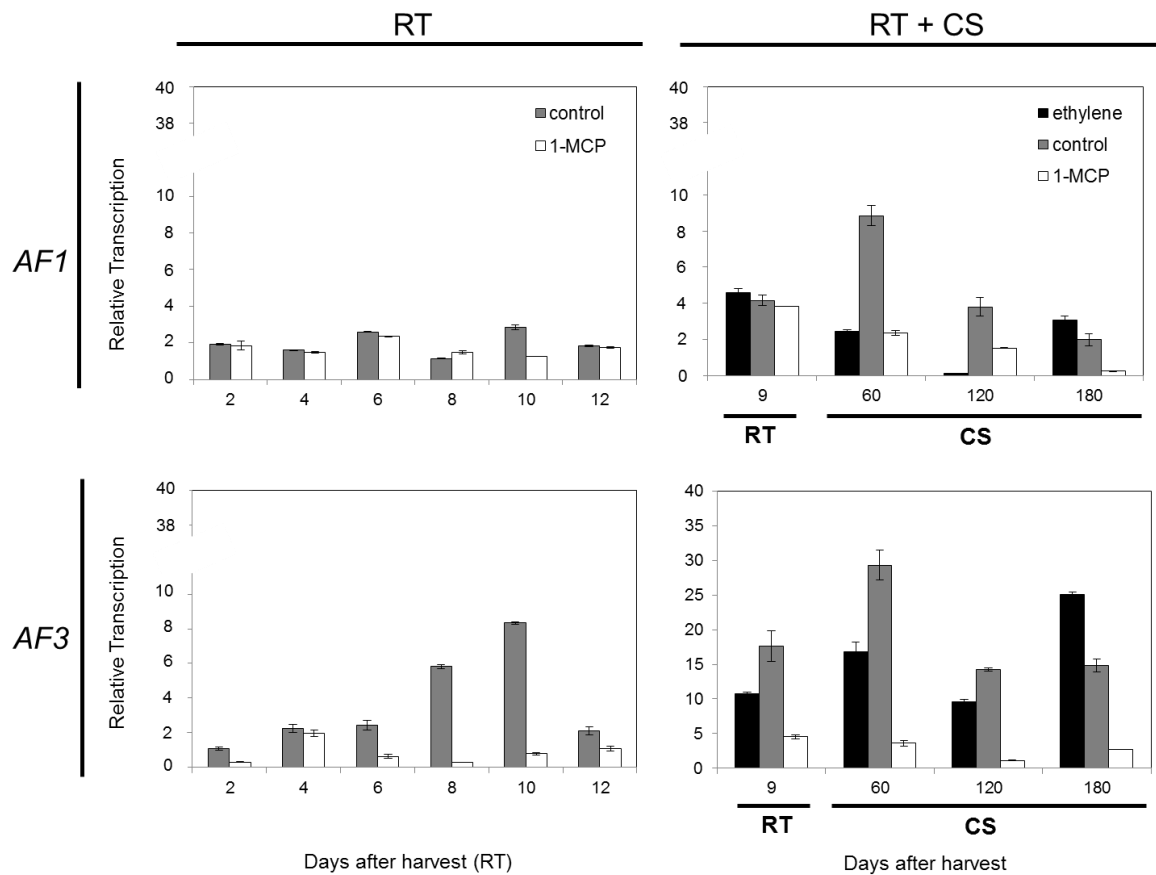


Figure 2.5 - Relative expression of *MdAF1* and *MdAF3* in fruits under control conditions, 1-MCP and ethylene application treatments, kept under room temperature and cold storage conditions. The calculations were performed using the harvest sample as the normalizing sample. Relative transcription corresponds to average values for three technical replicates. Error bars represent \pm standard deviation.

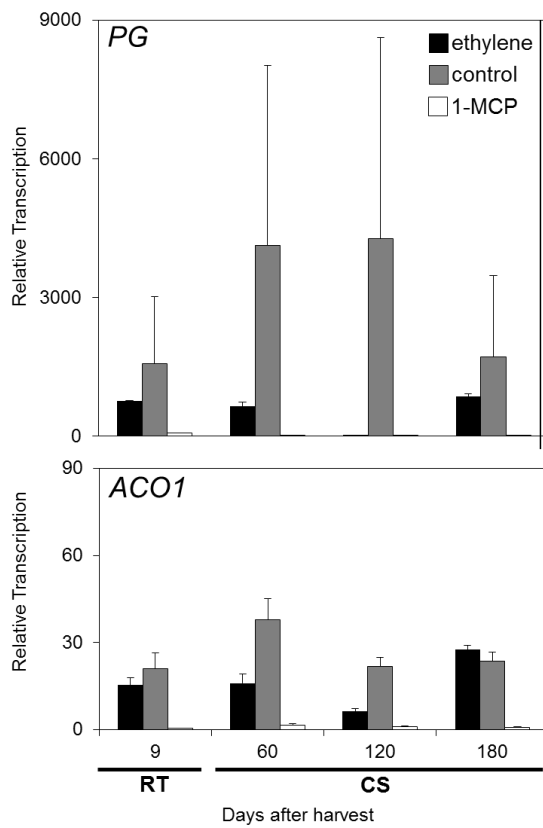


Figure 2.6 - Relative expression of *MdPG1* and *MdACO1* in fruits under control conditions, 1-MCP and ethylene application treatments, kept under room temperature and cold storage conditions. The calculations were performed using the harvest sample as the normalizing sample. Relative transcription corresponds to average values for three technical replicates. Error bars represent + 2 x standard deviation.

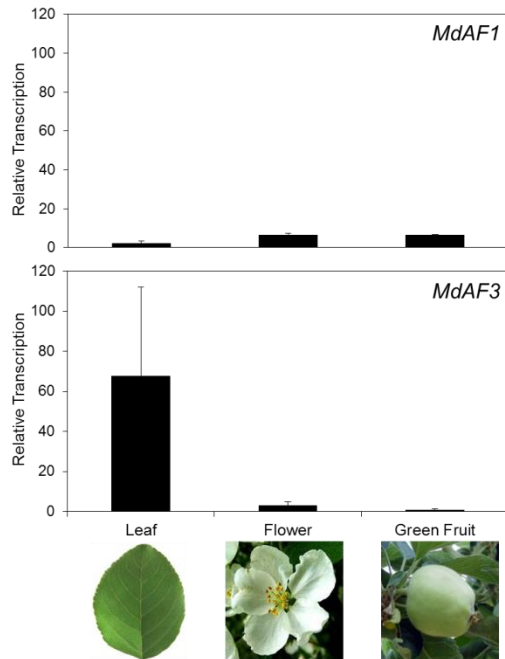


Figure 2.7 - Relative expression of *MdAF1* and *MdAF3* in plant organs of 'Gala' apples. The calculations were performed using the mature fruit at harvest as the normalizing sample. Relative transcription corresponds to average values for three technical replicates. Error bars represent + 2 x standard deviation. Representative apple plant organs are depicted.

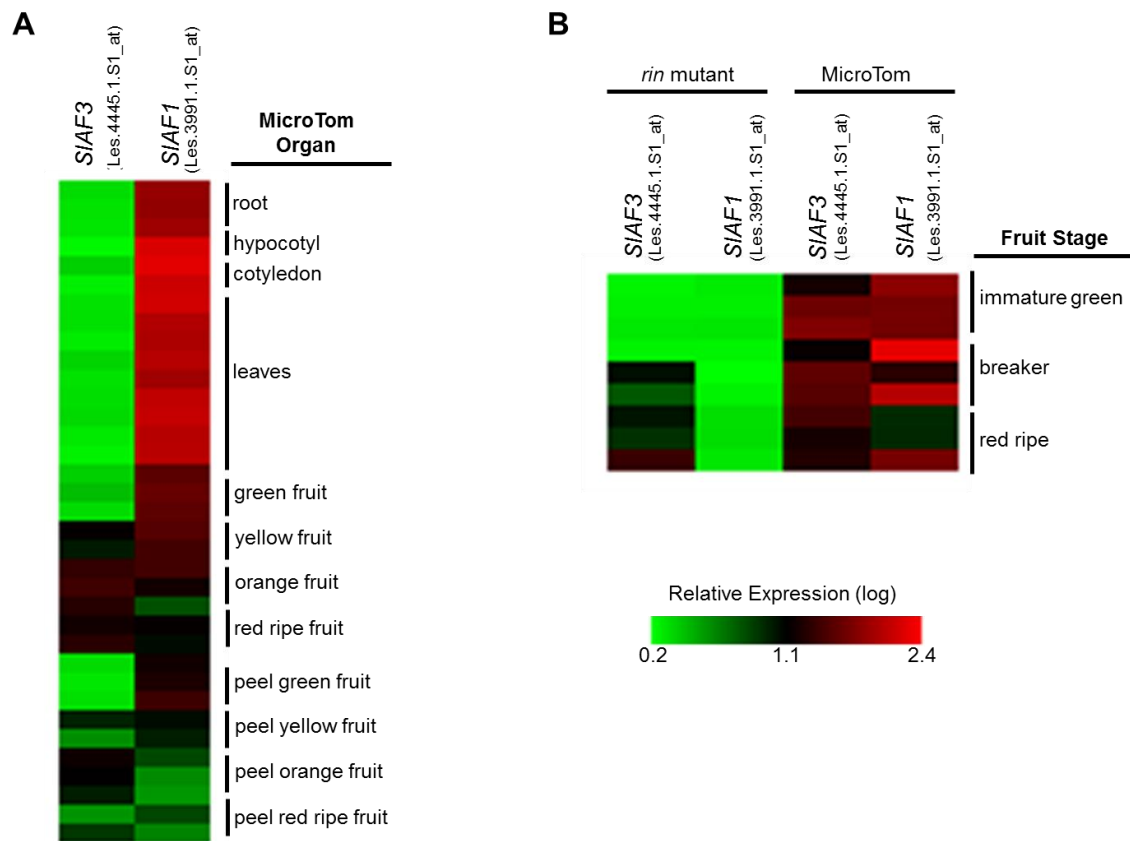


Figure 2.8 - Expression profile of tomato *SIAF3* (Les4445.1.S1_at) and *SIAF1* (Les3991.1.S1_at) genes in 'MicroTom' plant organs (A) and in 'MicroTom' and *rin* mutant fruit developmental stages (B). Color scale represents relative expression is shown above (green refers to low expression; black refers to medium expression and red refers to high expression).

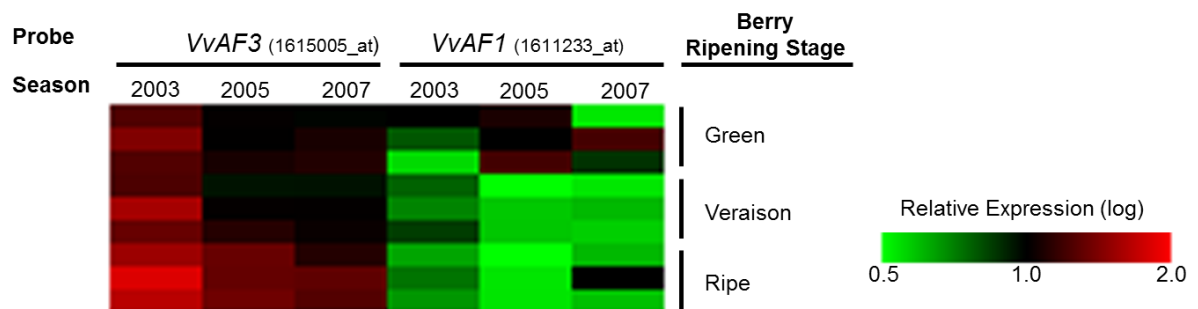


Figure 2.9 - Expression profile of grapevine *VvAF3* (1615005_at) and *VvAF1* (1611233_at) genes in ripening berries during in three harvest seasons. Color scale represents relative expression is shown above (green refers to low expression; black refers to medium expression and red refers to high expression).

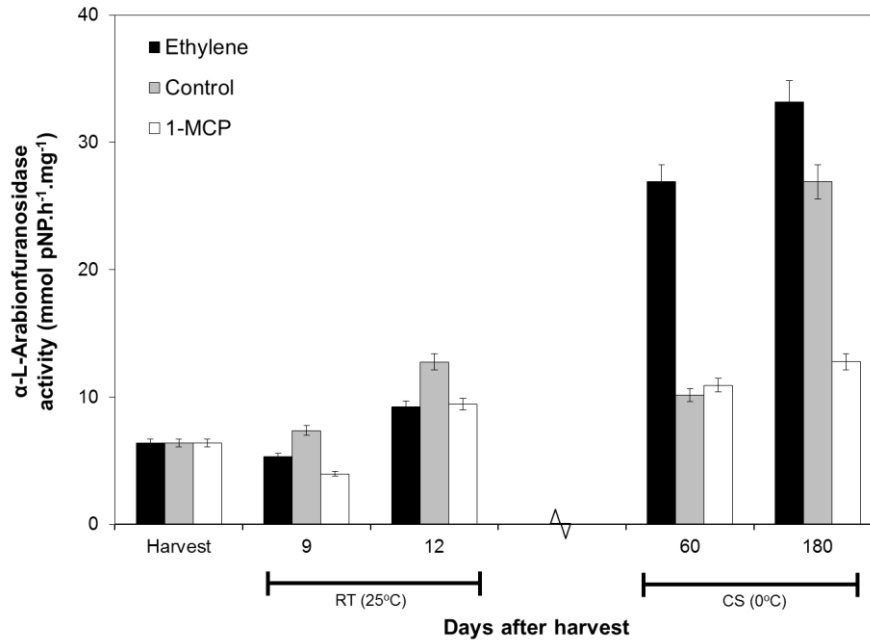


Figure 2.10 - Activity of α -L-arabinofuranosidase in apple fruits under control conditions and 1 MCP and ethylene treatments. Time course starts at harvest, followed by room temperature conservation (9 and 12 d) and subsequent cold storage sampled at 60 and 180 d. Enzyme activity is represented as mM of p nitrophenol (pNP) release per hour per mg of protein extract. Error bars represent \pm standard deviation.

Capítulo 3: Este capítulo consiste em uma proposta de artigo. A revista para a qual o manuscrito será submetido ainda não foi definida, estando o mesmo sujeito a alterações antes da submissão. A estrutura em que o mesmo se encontra segue o manual para elaboração de dissertação e tese da UFPel, seguindo a estrutura de capítulo.

Title: Several genes involved in response to stress and regulatory processes are differentially transcribed in cold stored apple

3.1 Introduction

Fruit ripening is a complex phenomenon that involves several molecular, biochemical and physiological changes and leads to more attractive and palatable fruits (BAPAT et al., 2010; GIOVANNONI, 2001). The changes consist in fruit softening, color changes, volatile compounds biosynthesis and the conversion of starch and organic acids into sugar and other flavor components (BOUZAYEN et al., 2010). In climateric fruits, such as apple (*Malus x domestica* Borkh.), ripening alterations also occur after harvest and many of them are affected by the hormone ethylene. Although some of the postharvest changes are desirable, such as the production of volatile compounds influencing aroma (BAI et al., 2005), others are responsible for fruit deprecation, such as excessive pulp softening (GOULAO; OLIVEIRA, 2008) that limits shelf life. Consumer acceptability of apple fruits is reduced after two months under storage, mainly due to excessive pulp softening (KONOPACKA; RUTKOWSKI; PŁOCHARSKI, 2007).

In order to increase the fruits shelf life, 1-methylcyclopropene has been successfully used, especially due to its effect on pulp firmness maintenance (BAI et al., 2005; LU; MA; LIU, 2012). The compound binds to ethylene receptors, thus, impairing hormone triggered responses, such as excessive loss of pulp firmness (BLANKENSHIP, 2001). The commercial use of 1-MCP has been approved in many countries, including China, USA, Turkey, Italy, France and Brazil (SOZZI; BEAUDRY, 2007). Thus, the technological use of 1-MCP is widespread in the conservation of several fruits, including apple, tomato, kiwi, peach and pear. Moreover, 1-MCP is also

an important investigation tool in understanding ethylene-dependent events in climacteric and non-climacteric fruits (WATKINS, 2006).

Recently, research has employed molecular tools to further understand the processes associated to fruit ripening. Among these techniques, transcriptional profiling of the genes involved in ripening processes, such as cell wall modification and/or degradation, ethylene biosynthesis and perception and aroma production (GOULAO; COSGROVE; OLIVEIRA, 2008; NOBILE et al., 2011; SCHAFFER et al., 2007b; WEI et al., 2010). Previous studies have employed microarrays to investigate the transcriptional profile of apple fruits during ripening (JANSSEN et al., 2008; SCHAFFER et al., 2007b; SOGLIO et al., 2009; YU et al., 2007; ZHU et al., 2012), although, to the best of our knowledge, no study has focused on further ripening stages of fruits under long term cold storage (CS). The elevated conservation ability of apples under long term cold storage and the importance of the alterations occurring during storage for postharvest quality have prompted us to investigate the transcriptional profile of the pulp of 'Gala' apples kept under CS for 60 days in comparison to fruits at harvest. Moreover, the influence of ethylene on conservation has also been investigated in the current study by employing its inhibitor, 1-MCP.

In nectarine, 1-MCP was used to further understanding of the molecular mechanisms involved in ripening and to identify genes exhibiting ethylene-regulated transcription 72 hours after harvest (ZILIOTTO et al., 2008). Similarly, Costa et al. (2010) have employed microarrays to elucidate the mechanisms of 'Mondial Gala' fruits for nine days at room temperature (RT). The inhibitor 1-MCP was also used to investigate the influence of ethylene on the transcription of genes involved in ripening processes. Genes associated to hormone and cell wall metabolism have been demonstrated to be differentially regulated in apple fruits from cultivars Honeycrisp and Cripps Pink during the initial stages of ripening (ZHU et al., 2012).

The current work is the first to investigate the influence of CS, in the presence and absence of 1-MCP, on the whole genome transcriptional profile of apple fruits. The conservation period of two months (60 days) was chosen due to its critical importance in the maintenance of the quality of 'Gala' fruits under CS. Thus, our goal is to associate the changes in fruit quality with the transcriptional changes influenced by ethylene and the cold in stored fruits.

3.2 Material and methods

3.2.1 Plant material and fruit physiological characterization

Apple fruits from the cultivar Gala were harvested from commercial orchards located in Caxias do Sul-RS, during the harvesting period of 2009, and in Vacaria-RS, during the harvesting period of 2012. At harvest (T0), five fruits were submitted to iodine-starch index, pulp firmness, titratable acidity (TA) and total soluble solids (TSS) evaluations in order to characterize the physiological stage of the fruits.

Subsequently, the fruits were divided into two groups each with 50 Kg: the first, was treated with 1 ppm of 1-MCP for 24 hours, and the second group, remained as untreated control. Control and treated samples (T1 and T2, respectively) were conserved for 60 days under CS (0 ± 0.5 °C, 90 ± 5 % RU). After CS conservation, the fruits were kept for seven days under room temperature (RT) for simulate the commercialization period. Following, the fruits were submitted to pulp firmness analysis. Fruits at harvest (T0) and after storage (T1 and T2) were peeled and pieces of pulp were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction that was proceeded in a pool of five fruits per sampling point.

Iodine-starch index was evaluated in fruits sectioned at the equatorial zone and immersed in iodine solution (12 g of I₂ and 24 g of KI in 1000 mL of distilled water) for 40 s. The results were obtained by visual comparison to the scale described by Girardi et al. (2002), ranging from 1 (immature fruit) to 5 (ripe fruit).

Contents of TSS were determined using a digital refractometer with temperature correction (model PR 101; Atago Co. Tokyo, Japan) from fresh juice extracted from the fruits. The analyses of TA was carried out in 10 mL of juice diluted in 90 mL of distilled water titrated with 0.1 M NaOH. Pulp firmness was determined from opposite sides of the fruits, where the peel had been previously removed, using a manual penetrometer equipped with a 11 mm probe.

3.2.2 RNA extraction and microarray transcriptional profiling

Total RNA was extracted as described (ZENG; YANG, 2002) from 6 g of frozen pulp material. Two biological samples were utilized for each sampling point; the first one refers to the year 2009 and the second to the year 2012. After isolation, total RNA was spectrophotometrically quantified using ND-1000 NanoDrop

(NanoDrop Technologies, DE, USA) and the integrity was checked by agarose gel electrophoresis.

The preparation of amplified RNA (aRNA) was carried out from 400 ng of total RNA, reverse transcribed into cDNA using the Ambion messageAmp II aRNA Amplification kit (Ambion, Austin TX). Resulting cDNA was purified using the DNAClear kit (Ambion – Invitrogen, Austin TX) and transcribed to aRNA employing the Ambion messageAmp II aRNA Amplification kit. Amplified RNAs were purified using Ambion MEGA clear kit and quantified by NanoDrop, as described.

Hybridization targets were prepared from 10 µg of each aRNA transcribed using 400 U of Superscript II reverse-transcriptase (Invitrogen Corp., Carlsbad, CA) and labeled with 1.5 µM of cyanine-3 (Cy3) or cyanine-5 (Cy5) (Interchim, France). Subsequently, the labeled targets were purified using the QIAquick PCR Purification kit (Qiagen, Hilden). Reactions and purification procedures were carried out as described by the manufacturers' instructions, except for the RNA amplification step that was optimized using a fourth of the recommended reagent quantities.

Labeled cDNAs were quantified and combined to 30 pmol of each fluorescent dye and co-hybridized to AryANE 12x135K microarrays (CELTON et al., 2014).

Hybridizations were conducted at a NimbleGen Hybridization System 4 (mix mode B) at 42°C overnight. Subsequently, the slide was washed, dried and scanned at a resolution of 2 µm. Fluorescence data was extracted from the scanned images using NimbleScan Software v2.4. The sampling points (T0, T1 and T2) were interrelated in three comparisons (T1-T0; T2-T0; T2-T1) (Figure 3.1). Each comparison was hybridized to four microarrays, with each one of the biological samples (2009 and 2012) repeated in a dye-swap experiment.

Statistical analyses of gene expression were carried out using the R language (R DEVELOPMENT CORE TEAM, 2011). Thus, intensity data were normalized using the Lowess method and differential expression analyses employed the lmFit and Bayes moderate t test functions using the LIMMA package (SMYTH, 2005) at the Bioconductor project.

3.2.3 Gene expression validation by RT-qPCR

In order to validate the microarray results, 14 genes were chosen based on their expression profile and putative biological function for validation by RT-qPCR using the same RNAs employed for the microarray analysis. Therefore, genes

associated to the most important biological pathways affected by our experimental conditions were chosen. Primers were designed based on the coding sequences (CDs) of *Malus x sylvestris* var. *domestica* available at GDR (Genome Database for Rosaceae - <http://www.rosaceae.org/>) using the software Primer3Plus (UNTERGASSER et al., 2007), to generate amplicons ranging from 80 to 150 base pairs (Table 3.1). Primer amplification was validated for each experiment using a pool of all cDNAs and standard curve for five concentrations of the cDNA mixture. Oligonucleotide specificity and primer dimer absence were investigated by post transcriptional dissociation curve analyses. Primers chosen exhibited amplification efficiency ranging from 1.85 to 2.0 and a single dissociation temperature. Real time quantitative PCR (qPCR) was carried out using the Opticon Monitor (Bio-Rad) equipment and the qScript 1-Step qRT-PCR Kit (Quanta Biosciences). Reactions were performed in a total volume of 15 μ L (7.5 μ L of qScript 1-Step qRT-PCR Kit, 1 μ L of cDNA and 400nM forward/reverse primers). Results were normalized employing the commonly used reference gene *MdUBI* and the genes corresponding to identifiers MDP0000217860 and MDP0000271281, which showed a stable transcription pattern in our microarray analysis.

3.3 Results

3.3.1 Physiological analyses

Pulp firmness has been shown to decrease after harvest (T0) for cold stored 1-MCP treated (T2) and untreated (T1) fruits. In 2012, fruit softening was more significant, since firmness loss was observed for T1 and T2, which did not differ from each other. In contrast, in 2009, 1-MCP treatment (T2) promoted the retention of fruit firmness, as observed by the non-significant softening in comparison to that at harvest (T0). In 2012, iodine-starch indices indicate that fruits were harvested at further ripening stages, in comparison to 2009 (Table 3.2).

Total acidity (TA) was not affected by 1-MCP treatment during the investigated years (Table 3.2). In contrast, in 2009, acidity was higher in fruits at harvest (T0) in comparison to the observed TA for cold stored apples (T1 and T2), thus, indicating an effect of the cold on the parameter (Table 3.2).

In 2009, the contents of TSS were similar for all treatments (Table 3.2), differently from the observations for 2012, where higher levels of soluble solids were

observed in untreated cold stored apples (T1) in comparison to the contents at harvest (T0).

3.3.2 Whole genome transcription profiling

In 2009, a total of 9418 differentially expressed genes were observed in the comparison between cold stored (T1) and harvest (T0) fruits, 7040 between cold stored 1-MCP treated (T2) and harvest (T0) and 11067, in the comparison between cold stored 1-MCP treated (T2) and untreated (T1) apples. In 2012, the comparison between the transcriptional profile at T1 and T0 evidenced 5385 differentially expressed genes, whereas comparing T2 and T0 7402 exhibited distinct expression patterns and 1330 were differentially regulated in the comparison between T2 and T1. These results represent probes with p-value (BH) correction of ≤ 0.05 . For the subsequent analyses, the genes exhibiting distinct transcriptional patterns in 2009 and 2012 were removed, thus, considering genes displaying $BH \leq 0.1$.

The genes meeting the parameters were classified into metabolic classes employing the software MapMan (Figures 3.2 –3.7). A wide range of differentially expressed genes were attributed to several metabolic pathways, being noteworthy those associated to cell wall metabolism, photosynthesis and specialized metabolism (Figures 3.2 –3.4). Concerning regulatory processes, a large number of differentially expressed genes were associated to hormone metabolism and cellular reduction-oxidation (redox) system (Figures 3.5 –3.7). Therefore, the differentially regulated genes related to cell wall processes (Table 3.3), hormone metabolism (Table 3.4), redox system and regulatory mechanisms (Table 3.5) and photosynthesis (Table 3.6) are presented.

The interactions among the differentially expressed genes in all three comparisons are schematically represented by the Venn diagram, generated by the MapMan software (Figure 3.8). Genes exhibiting $BH \leq 0.1$ and displaying a conserved profile for the investigated years were considered in further analyses. Thus, only 125 genes common to all three comparisons, met the parameters. The number of shared genes was higher for the comparisons T1-T0 and T2-T0. The comparison between cold storage (T1) and fruits at harvest (T0) revealed the highest number of differentially expressed genes, namely 3836. In contrast, the comparison

between 1-MCP treated (T2) and untreated (T1) fruits under cold storage had the smallest number of differentially regulated genes, 1215 (Figure 3.8).

3.3.3 Gene expression profile validation by RT-qPCR

In order to validate the profiles observed by microarray analyses, the profiles of 14 genes were investigated, based on their levels of differential expression and putative biological function. The vast majority of the profiles investigated by RT-qPCR (86%, 12 genes) was similar to the one obtained by the microarray analyses, in two independent experiments for all three comparisons in both investigated years (Figure 9). Slight differences were observed for the profiles of *MdEXP4* and *MdCCR1*, although it is noteworthy that the distinct profile for *MdEXP4* in the comparison T2-T1 in 2012 was not significantly different in the microarray analysis (Figure 3.9), thus, accounting for the observed divergence. Therefore, a distinct profile between the microarray and RT-qPCR analyses was only observed for the gene *MdCCR1*.

3.4 Discussion

Fruit softening is the most important alteration taking place in apple fruits under cold storage (GOULAO; OLIVEIRA, 2008). Although 1-MCP is effective in attenuating fruit softening, firmness loss still occurs in treated apples (STORCH et al., Unpublished²). The phenomenon was observed for the two harvesting seasons investigated in the current work, since all conditions of cold stored fruits exhibit some extent of softening, although reduced for those treated with 1-MCP (Table 3.2). The decrease in pulp firmness was more intense in 2009, probably due to the harvest of fruits in more advanced ripening stages in 2012. Thus, softening onset had already started for fruits at harvest in 2012 (Table 3.2). Among the factors affecting fruit firmness loss, the action of several enzymes on the cell wall is a critical one (ATKINSON et al., 2012; GOULAO et al., 2007; WEI et al., 2010), along with the signaling events triggered by ethylene, as shown for several fleshy fruits, such as apple, peach and melon (LI et al., 2011; MARONDEDZE; THOMAS, 2012; TATSUKI et al., 2013). Some studies have shown that softening is also influenced by low temperatures employed in cold storage (MWORIA et al., 2012; TACKEN et al., 2010).

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Thus, in the current work, our goal was to dissect the role of ethylene and cold in apple quality by analyzing whole genome transcriptional profiles to determine the relative contribution of each factor on the complex biological processes leading to quality decay, mainly due to the firmness loss. The experimental design allowed the investigation of the role of ethylene, due to 1-MCP mediated signaling blocking, in the comparison T2-T1, whereas the effect of low temperatures could be isolated in the comparison T1-T0. The joined effect of ethylene and cold was investigated in the comparison T2-T0.

In general, low temperatures induced transcriptional changes in a larger number of genes in comparison to the absence of ethylene signaling (Figure 3.8). A similar trend is also observed for the genes associated to the pathways studied, since transcription of the vast majority of the genes was not significantly affected in the comparison T2-T1, including for the hormone biosynthesis and signaling pathway (Table 3.4). The regulation of a wide range of genes associated to hormone metabolism was not differentially expressed in the comparison T2-T1, indicating that, in this experiment, they were not responsive to ethylene, including those involved in brassinosteroid and cytokinin metabolisms (Table 3.4). In contrast, low temperatures have been demonstrated to affect the transcription of these genes. Ethylene has been shown to affect gene expression in ripening apples stored under room temperature (COSTA et al., 2010). To the best of our knowledge, our results are the first investigating the transcriptional profile of the apple fruits kept in cold storage. Our results indicate that the suppression of ethylene signaling does not significantly affect the transcription of genes associated to hormone responses during apple cold storage when the fruits are harvested in late ripening stage. In this case, the influence of low temperatures appears to be the dominant factor controlling gene expression. Similarly, it is noteworthy to observe that the transcription of only three genes, previously characterized as ethylene controlled, was repressed by 1-MCP. These genes correspond to the coding sequences for 1 aminocyclopropane 1 carboxylic acid synthase 12 (ACS12), an ethylene-responsive element-binding protein and the coding sequence for an ethylene response factor, similar to *AtERF1*, which was repressed by cold and 1-MCP. The transcription of *ACS12* was induced under cold storage (T1-T0) and repressed by the treatment with ethylene repressor under cold storage (T2-T1) and its transcription was not affected in the comparison T2-T0. Considering the importance of delaying fruit ripening to increase shelf life

(BAPAT et al., 2010), the transcriptional profile suggests that this gene may be of biotechnological interest, since even after two months under storage, its transcription level remained similar to that observed at harvest. However, the *Arabidopsis thaliana* ortholog of *MdACS12*, At5g51690, has been demonstrated to code for an aminotransferase and not an enzyme with 1-aminocyclopropane-1-carboxylic acid synthase activity (YAMAGAMI et al., 2003). Thus, its role in ethylene biosynthesis remains unknown.

A similar transcriptional behavior was observed for a lipoxygenase 2 (*LOX2*), associated to the jasmonic acid metabolism by the software MapMan. Lipoxygenases have been demonstrated to be involved in the biosynthesis of volatile compounds in several fruits, such as apple, kiwi and olive (LI et al., 2006; MUZZALUPO et al., 2012; ZHANG et al., 2009). In these fruits, LOX transcription and/or activity has been shown to be ethylene induced during later stages of ripening or senescence. Thus, the down regulation of *LOX2* by 1-MCP (Table 3.4) may be associated to the loss of aroma in stored apples treated with 1-MCP (BAI et al., 2005; LI et al., 2006). However, further studies aiming the association of *LOX2* transcription with contents of aromatic compounds in apple treated with 1-MCP must be made to prove this hypothesis.

The coding sequence of an α -L-arabinofuranosidase, *AF3*, also exhibited a transcriptional profile similar to that of *ACS12* and *LOX2*. This gene has been previously demonstrated to be involved in apple texture changes, such as softening (STORCH et al., unpublished) and pulp mealiness (NOBILE et al., 2011). In our results, it was the sole gene associated to cell wall degradation to be negatively regulated by 1-MCP, confirming its ethylene dependent transcriptional induction as shown by Storch et al., (Unpublished) and reinforcing its role in the changes during storage. The decreased softening observed in 1-MCP treated fruits reinforces its involvement in firmness loss processes under cold storage. The substrate specificity of *AF3* remains unclear, since it has been classified, by sequence comparison, to a family of bifunctional α -L-arabinofuranosidase/ β -xilosidase enzymes (NOBILE et al., 2011).

A gene coding for a pectate lyase (*PL*) exhibited transcriptional induction in the comparison T1-T0, however, its profile was not significantly affected in the comparisons including 1-MCP, namely T2-T0 and T2-T1 (Table 3.3). Similarly, a kiwifruit gene coding for a *PL* was not responsive to 1-MCP in fruits maintained under

cold storage and to the endogenous ethylene levels, thus, suggesting that it is preferentially regulated by cold instead of ethylene (MWORIA et al., 2012). The endopolygalacturonase coding sequence, *PG1*, which has been previously described to exhibit an ethylene dependent transcriptional induction, was down regulated by 1-MCP in 2009 (Figure 3.9), although, in 2012, the transcription was not significantly affected in the comparisons T2-T0 and T2-T1. The behavior was further confirmed by RT-qPCR analyses (Figure 3.9) and is probably due to the fact that the fruits were harvested at advanced ripening stages in 2012 (Table 3.2). Thus, it is likely that, at harvest, endogenous ethylene production high enough to induce gene expression at harvest, so that the application of 1-MCP was only able to impair gene expression to reach even higher levels. Similarly, in 2012, pulp firmness was not significantly different between fruits treated with 1-MCP and untreated apples under cold storage for 60 days (Table 3.2). Taken together, the results confirm the predominant role of *MdPG1* in fruit softening. In contrast, another transcript, with predicted endopolygalacturonase activity, was down regulated by cold and remained constant in the presence of 1-MCP (T2-T1) (Table 3.3).

Among the genes associated to cell wall modifications, the transcription of two sequences coding for expansins, *EXPA1* and *4*, was induced in T1-T0 and T2-T0. The first gene was negatively regulated by 1-MCP in T2-T1, whereas the transcription of *EXP4* was not affected (Table 3.3). Recently, a work on expansins in apple has demonstrated that *EXPA1*, labeled *MdEXPA9*, is highly transcribed in the stem and fruits (ZHANG et al., 2014). Similarly, we have also observed high expression of the gene in fruits at later stages of ripening, which is compatible with a role in the modifications of cell wall brought about by storage. Moreover, its transcription has also been demonstrated to be dependent on ethylene signaling, in contrast to the behavior of *EXP4* that was transcribed in the absence of ethylene triggered responses (Table 3.3). Proteins from the EXPA and EXPB subfamilies function in cell wall loosening, which may allow the access of other enzymes to their substrates (ZHANG et al., 2014), promoting softening. A transcript coding for EXLA2 exhibit a contrary profile to *EXPA1* and *EXPA4*, that is, it was down regulated by cold (Table 3.3). In *Arabidopsis* and rice, the expansin family is divided into four subfamilies; EXPA, EXPB, EXLA and EXLB, where the biological function of the latter two is unknown (ZHANG et al., 2014).

The genes involved in cellular reduction-oxidation processes were also differentially regulated in the tested conditions, including those associated to ascorbate metabolism, dismutase/catalase system, thioredoxin, glutaredoxin, heme groups, receptor kinases and calcium signaling. Among these, the coding sequences for Monodehydroascorbate reductase 4 (*MDHAR4*) and Ascorbate Peroxidase 3 (*APX3*), associated to ascorbic acid metabolism, were up regulated in the comparisons T1-T0 and T2-T0, suggesting a role of low temperatures in their induction. In contrast, 1-MCP application only affected the transcription of *MDHAR4*, which was repressed in the comparison T2-T1. A catalase 2 coding sequence (*CAT 2*) exhibit a profile similar to that of *APX3*, that is, the transcription was induced in the comparisons T1-T0 and T2-T0 and remained unchanged in T2-T1 (Table 3.5). Considering the importance of the enzymes in reactive oxygen species (ROS) removal, our results suggest that the low temperatures during storage induced a state of oxidative stress in the fruits. The enzymes ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR) are components of the ascorbate-glutathione (AsA-GHA) cycle, where the first is responsible for the oxidation of ascorbic and monodehydroascorbic acids to deplete the medium of H₂O₂. The regeneration of ascorbic acid from monodehydroascorbic acid is catalyzed by the enzyme MDHAR (LATOWSKI et al., 2010) and the process is considered an effective way of H₂O₂ detoxification. During several ripening stages of acerola fruits, the transcription of a *MDHAR* coding sequence peaked at the over ripe stage coinciding with the highest levels of enzyme activity (ELTELIB et al., 2011). Thus, its transcriptional association to later stages of ripening may be explain the transcriptional repression of the gene in apples treated with 1-MCP that delays the ripening process. The presence of transcripts corresponding to *MDHAR* may also indicate the initial stages of senescence stress and function as senescence marker in apple. In tomato, the peak of MDHAR activity occurred in fruits stored under cold temperatures (STEVENS et al., 2008). Similarly, in peach the increase in the activity of enzymes involved in antioxidation processes, such as APX, MDHAR and catalase, was associated to increased chilling tolerance in cold stored fruits, as a consequence of salicylic acid and ultrasound treatments (YANG et al., 2012). Thus, transcriptional down regulation or unaffected transcripition of these genes in the comparison T2-T1 indicates that 1-MCP reduces or has no effect on ROS production. Moreover, the induction of the transcription of these genes in the comparison T2-T0, where 1-MCP

and low temperatures act in concert, indicates that, even in the presence of the ethylene inhibitor, the most relevant factor inducing oxidative stress in the fruits is cold.

Several regulatory mechanisms are present in plants, including receptor-like kinases (RLKs), which regulate a wide range of growth, development and biotic and abiotic stress responses (OSAKABE et al., 2013). In the current work, several genes coding for RLKs were differentially regulated, mainly those representing the large family of leucine-rich repeat receptor like kinases (LRR-RLKs) (Table 3.5). LRR-RLKs have been associated to abiotic stress responses to cold, salt, heat and gamma irradiation (PARK et al., 2014; SHI et al., 2014; YANG et al., 2014). In rice, LRR-RLKs have also been demonstrated to be regulated by the hormones salicylic acid (SA), abscisic acid (ABA) and jasmonic acid (JA) (PARK et al., 2014). Although several expression profiles were found for RLKs in our work, the vast majority of their coding sequences were not differentially regulated by 1-MCP treatment (T2-T1) (Table 3.5). Thus, it is inferred that 1-MCP application alone is not able to induce transcriptional changes for these genes. In contrast, some RLK genes exhibit distinct transcriptional profiles only in the comparison T2-T0 (Table 3.5). The transcription of these genes is up regulated by the interaction between cold and 1-MCP treatment, so that both factors are essential for their differential transcription.

The low temperatures are also likely to be the predominant factor controlling the transcription of the genes associated to Ca^{2+} regulatory processes (Table 3.5). Ca^{2+} functions as a secondary messenger and it is released in response to several stimuli, such as biotic and abiotic stresses (STEINHORST; KUDLA, 2013). More recently, the combined role of Ca^{2+} and other signaling molecules, mainly protein kinases (FURUYA; MATSUOKA; NANMORI, 2013; KANWAR et al., 2014; LI et al., 2009; RAORANE et al., 2013) and ROS (GILROY et al., 2014) has been demonstrated. Ca^{2+} and ROS waves are associated to signal transmission between distinct tissues (systemic signaling pathways) via cell-to-cell communication (GILROY et al., 2014; STEPHAN; SCHROEDER, 2014). Although our results are not conclusive to demonstrate the coaction of these gene products in signaling pathways, the observed differential expression in comparisons where the influence of low temperatures is investigated suggests that a wide range of signaling and stress response mechanisms are triggered in fruits under cold storage. Thus, although the transcription of genes associated to fruit quality processes has been demonstrated to

be dependent on ethylene, a larger number of genes was responsive to low temperatures.

The current study points out to the cold stress in fruits, which is represented by the large transcriptional program shifts occurring in low temperatures (comparisons T1-T0 and T2-T0) but absent from the comparisons employing the ethylene inhibitor (T2-T1). Senescence processes may also account for the transcriptional shifts, since these responses are minimized by the use of 1-MCP. Genes exhibiting cold induced and 1-MCP repressed transcription, which are not differentially regulated in the comparison T2-T0, indicate a constant transcriptional activity after two months under cold storage, at levels similar to those observed for 1-MCP treatment and, are likely to be adequate quality markers for stored fruits. Among the genes meeting these requirements, *ACS12*, *AF3* and *LOX2* are the most promising. Genes associated to the redox metabolism and upregulated by cold, such as *MDHA4*, *APX3* and *CAT2* could provide oxidative stress markers in fruits and function as senescence indicators.

Our results suggest the participation of expansins in the processes leading to fruit texture changes during storage and confirm the role of *AF3* in fruit softening.

Finally, the presence of differentially expressed genes associated to the light reactions of photosynthesis was also observed (Table 3.6). Currently, the possible function of these genes in fruit quality loss during storage is unknown. Considerable transcriptional changes were observed for these genes in the investigated comparisons; the majority of the genes was induced by 1-MCP and repressed by cold. The fact that these genes were up regulated in the comparison T2-T0 indicates that ethylene is the prevalent regulator in comparison to cold. Although, their role in postharvest fruits is not determined, the ethylene dependent transcriptional induction of the photosynthesis light reaction genes may allow their use as markers for other events controlled by the hormone, such as the excessive loss of firmness.

The experimental design of the current work comprising the late stage of ripening in 2012 allowed to evaluate the ethylene and cold influence on the transcriptional profile of the fruits harvested after the rise of ethylene production. In these conditions, we observed that the cold effect was higher than ethylene effect. This behavior was expected since due to the advanced maturity at harvest, many genes responsive to ethylene had already changed their transcription before the storage of the fruits. However, even in these conditions, many genes were

differentially transcribed, mainly in response to cold or some developmental control. Thus, emphasis is given to genes involved in regulatory processes and stress response that may be associated to the changes that lead the fruit to the senescence stage.

3.5. Conclusions

The current work has investigated the influence of 1-MCP and cold storage on the transcription of genes associated to cell wall metabolism, hormone biosynthesis and signaling and cellular redox system in apple pulp. A wide range of genes were differentially regulated in response to the investigated factors, mainly cold storage, which affected the transcription of a larger number of genes in comparison to those influenced by 1-MCP treatment. However, several genes, especially *AF3*, *ACS12* and *LOX2*, exhibited a clear ethylene dependent regulation pattern, thus, being considered potential quality markers during the cold storage period of 'Gala' apples.

Our results also highlight the differential regulation of genes associated to stress responses, especially under cold storage. Similarly, genes associated to the cell redox system, such as *MdHAR4*, *APX3* and *CAT2*, are likely to be useful as fruit senescence markers.

Finally, the large number of differentially expressed genes for all investigated comparisons of the current work indicates the complexity of the processes that affect fruit quality in cold stored apples.

Tables

Table 3.1 - Primer information for the genes validated by RT-qPCR. Biological function is represented by annotation information, ID represents *M. x sylvestris* var. *domestica* genome identification. Forward and reverse primer sequences are given from 5' to 3'

Biological Function	ID	Forward	Reverse
Expansin 4 (EXP4)	MDP0000410264	ATGGTTCCAGAGGCGTCGTT	CTTCCTTCGCGTGCTCTAC
α -L-Arabinofuranosidase 3 (AF3)	MDP0000140483	CTGGTGGGATTTACAAGGT	GCAGGTCACCAATTTCCAGT
Endo-polygalacturonase 1 (PG1)	MDP0000326734	TCACGGTAACTGCACCAGAG	CTTTGGGACCCACTCACAAT
Endoxyloglucantransferase (EXGT)	MDP0000398765	GACGGCGTAGGAGTGGTAAG	AAACAACGAACACGACGAGA
COBRA (COB)	MDP0000094767	ACACAGCTTCCTGGGTCTGT	CATGCACGTACTIONCGCAGTTT
Ethylene Response Ractor 1 (AtERF1)	MDP0000299277	TTCGCCTTCTTTCTCCGCTT	TTGTTGAATTTCCCGCTCCG
Auxin Response Factor 11 (ARF11)	MDP0000134824	AAGTATGCCACCCGAAGAGA	ATTCAGGGATGCCAGTCAAG
Cinnamoyl CoA Reductase 1 (CCR1)	MDP0000268045	ACCAACTGTCAATGCCAGCA	CCAAAATGTGAGCCAGTGCG
Chlorophyll a/b Binding Protein (Cab2)	MDP0000182265	TCAGCTGTCCAAGGAAACGG	CCGGAAAATGGGCCAAGGTA
Light harvest complex (LHCB4)	MDP0000757636	TCTTCAGCTGGCCGAAATCA	AGGCGTCAATAATGGTGGTGT
Light Harvest Complex (LHCB5)	MDP0000943426	ACCTCAGCAACAACAGCGAA	GGCCTTCAACAAATTTGGAGCT

Light Harvest Complex (LHCB6)	MDP0000327940	AGGTGACTACGGTTTTGATCCA	AACAAAGATACCAACGACTGCC
Photosystem II Subunit O-2 (PSBO1)	MDP0000248920	TGCTAACCAATGCCCAACCA	TGGTGGAGCGTTCTTGTTCA
Photosynthetic Electron Transfer C (PETC)	MDP0000194460	TCCCTTTCCCATCATCTGTGTC	TATGTCCTGCTACCACTTCCCA
Ubiquitin 2 (UBI)	MDP0000498429	TTGATCTTTGCTGGGAAACAG	CACCACCATCATTCAACACC
Drought-Responsive Family Protein	MDP0000217860	ACGCAAGAGGAAGACAAGGA	GGGTGGCATGCTAACAAAAT
Porin	MDP0000271281	CGCCCAAATTATTGAAGCA	ATCCCCAGCCATTGATTTTT

Table 3.2 - Results of the physical-chemical analyses of the fruits at the harvest (T0) and after cold storage as untreated control (T1) and 1-MCP treated fruits (T2) in 2009 and 2012. Distinct lettering represents statistical differences at 5% by Tukey test

		Firmness (N)	Total Acidity (cmol/L)	Total Soluble Solids (°Brix)	Iodine-Starch Index (1-5)
2009	T0	83.85 a	8.29 a	12.68 a	2.33
	T1	59.86 b	6.21 b	13.18 a	
	T2	79.24 a	6.42 b	13.86 a	
2012	T0	75.00 a	4.97 a	12.47 b	4.21
	T1	63.48 b	4.54 a	13.43 a	
	T2	67.31 b	5.24 a	13.19 ab	

*Analyzed at harvest (T0) to evaluate fruit ripening stage.

Table 3.3 - Summary of the differentially expressed genes associated to cell wall metabolism. Gene ID represents *M. x sylvestris* var. *domestica* genome (GDR) and *Arabidopsis thaliana* (TAIR) identification

Functional Annotation	Gene ID		Harvest Year/Comparison					
	GDR	TAIR	2009	2012	2009	2012	2009	2012
			T1-T0	T1-T0	T2-T0	T2-T0	T2-T1	T2-T1
Proteins								
RGP1 (reversibly glycosylated polypeptide 1)	MDP0000156837	At3g02230	1.91	1.18	1.41	0.9	NS	NS
	MDP0000204345		1.98	1.04	1.34	0.81	NS	NS
Reversibly glycosylated polypeptide	MDP0000461829	At5g16510	NS	NS	-1.29	-1.21	-1.14	-0.94
Synthesis precursor								
UGE1 (UDP-D-glucose/UDP-D-galactose 4-epimerase 1)	MDP0000021376	At1g12780	2.05	1.13	2.16	0.84	NS	NS
MUR4 (galactose metabolic process)	MDP0000280845	At1g30620	-2.82	-1.17	-3.16	-1.35	NS	NS
GAE1 (UDP-D-glucuronate 4-epimerase 1)	MDP0000444614	At4g30440	-0.96	-0.93	-1.23	-0.71	NS	NS
Modification								
Pectin esterases PME	MDP0000222620	At1g53830	NS	NS	-2.78	-1.98	-2.88	-1.18
EXPA1 (expansin)	MDP0000193127	At1g69530	4.76	3.91	0.75	3.92	-4.69	-1.17
EXPA4 (expansin)	MDP0000410264	At2g39700	3.49	1.35	2.97	1.86	NS	NS
	MDP0000743239		2.49	0.86	2.13	1.62	NS	NS
	MDP0000817926		2.9	1.26	2.5	1.55	NS	NS
XTR7 (xyloglucan endotransglycosylase 7)	MDP0000158544	At4g14130	-3.26	-2.53	-3.72	-2.56	NS	NS
Pectin esterases.acetyl esterase	MDP0000273242	At4g19420	-1.67	-1.61	-1.4	-2.04	NS	NS
	MDP0000313863		-1.85	-0.7	-1.61	-1.08	NS	NS
Pectin esterases PME	MDP0000420628	At4g33220	NS	NS	-2.95	-0.71	-3.11	-1.33
EXLA2 (expansin-like A2)	MDP0000126245	At4g38400	-3.04	-2.96	-3.89	-1.43	NS	NS

Pectin esterases PME	MDP0000212502	At5g19730	-3.67	-0.77	-3.45	-1.06	NS	NS
Xyloglucan endotransglycosylase	MDP0000333317	At5g65730	-2.56	-2.94	-0.92	-1.72	1.88	1.43
Degradation								
Glycosyl hydrolase family 9 protein	MDP0000147635	At1g19940	-1.9	-1.65	-2.18	-1.87	NS	NS
	MDP0000405831		-1.54	-1.44	-2.26	-1.88	NS	NS
Glycosyl hydrolase family 9 protein	MDP0000178787	At1g75680	-1.80	-1.45	-2.00	-1.56	NS	NS
	MDP0000230169		-1.23	-0.69	-1.4	-1.2	NS	NS
	MDP0000295518	At2g28100	-1.96	-1.51	-1.51	-1.24	NS	NS
AtFUC1 (alpha-L-fucosidase 1)	MDP0000295518	At2g28100	-1.96	-1.51	-1.51	-1.24	NS	NS
Pectate lyase family protein	MDP0000539270	At3g55140	0.8	1.03	NS	NS	NS	NS
ADPG1 (endo-polygalacturonase 1)	MDP0000168416	At3g57510	-2.15	-1.21	-1.53	-1.46	NS	NS
Glycoside hydrolase family 28 protein	MDP0000292963	At4g23820	NS	NS	-1.27	-0.73	NS	NS
	MDP0000581832		NS	NS	-1.41	-0.89	NS	NS
Glycosyl hydrolase family 3 protein	MDP0000154790	At5g04885	-1.88	-1.1	-1.52	-1.03	NS	NS
	MDP0000190086		-2.23	-1.37	-2.05	-1.91	NS	NS
	MDP0000580762		-1.73	-1.19	-1.67	-1.52	NS	NS
Glycosyl hydrolase family 3 protein	MDP0000062066	At5g20950	-2.47	-1.95	-3.32	-1.87	NS	NS
	MDP0000153761		-2.71	-2.67	-3.36	-1.82	NS	NS
AF3 (α -L-Arabinofuranosidase/ β -xylosidase)	MDP0000140483	At5g49360	2.19	1.61	NS	NS	-1.72	-1.31

*NS- Statistically non significant (BH \geq 0.1)

Table3.4 - Summary of the differentially expressed genes associated to hormone metabolism and signaling. Gene ID represents *M. x sylvestris* var. *domestica* genome (GDR) and *Arabidopsis thaliana* (TAIR) identification

Functional Annotation	Gene ID		Harvest Year/Comparison					
	GDR	TAIR	2009	2012	2009	2012	2009	2012
			T1-T0	T1-T0	T2-T0	T2-T0	T2-T1	T2-T1
Abscisic Acid								
AtHVA22A (ABA- and stress-inducible gene)	MDP0000312509	At1g74520	-1.99	-0.79	NS	NS	1.94	1.18
AtHVA22D (ABA- and stress-inducible gene)	MDP0000324254	At4g24960	-3.91	-2.46	-2.31	-1.51	2.6	0.96
SMT1 (sterol methyltransferase 1)	MDP0000720292	At5g13710	-1.85	-1.24	-1.81	-1.28	NS	NS
Auxin								
ILR1 (IAA-leucine resistant 1)	MDP0000281064	At3g02875	2.42	1.5	NS	NS	-3.04	-1.41
AFB5 (auxin f-box protein 5)	MDP0000809218	At5g49980	1.14	0.81	1.2	1.39	NS	NS
Auxin-responsive protein-related	MDP0000186167	At1g17345	2.04	1.08	NS	NS	-2.03	-1.88
Auxin-responsive protein	MDP0000318568	At4g38840	3.59	1.48	3.26	2.09	NS	NS
Auxin-responsive family protein	MDP0000786165	At2g46690	-0.68	NS	-1.13	-0.9	NS	NS
Brassinosteroid								
3-oxo-5-alpha-steroid 4-dehydrogenase family protein	MDP0000140225	At2g16530	-1.8	-2.08	-1.48	-1.9	NS	NS
	MDP0000165170		-1.73	-0.93	-1.81	-1.58	NS	NS
	MDP0000171374		-1.71	-1.18	-1.83	-1.69	NS	NS
	MDP0000268406		-1.47	-1.27	-1.22	-1.54	NS	NS
	MDP0000271970		-1.65	-1.18	-1.84	-1.53	NS	NS
Sterol methyltransferase 1	MDP0000267815	At5g13710	-1.75	-1.19	-1.75	-1.57	NS	NS
	MDP0000164160		-1.87	-0.83	-1.44	-0.85	NS	NS
	MDP0000720292		-1.85	-1.24	-1.81	-1.28	NS	NS
	MDP0000903076		-1.85	-1.84	-1.56	-1.65	NS	NS

Brassinosteroid signalling positive regulator-related	MDP0000130173	At1g78700	3.04	1.25	2.73	1.74	NS	NS	
	MDP0000344348		1.66	0.79	1.8	1.55	NS	NS	
Brassinosteroid signalling positive regulator-related	MDP0000203462	At3g50750	-1.81	-1.35	-1.57	-1.54	NS	NS	
Cytokines									
AtIPT1 (isopentenyltransferase 1)	MDP0000189484	At1g68460	-1.69	-1.77	-1.75	-1.44	NS	NS	
AtIPT1 (isopentenyltransferase 1)	MDP0000285240	At1g68460	-1.56	-1.86	-1.85	-1.47	NS	NS	
CKX7 (cytokinin oxidase 7)	MDP0000634311	At5g21482	-1.34	-0.93	NS	NS	NS	NS	
Ethylene									
SRG1 (senescence-related gene 1)	MDP0000113336	At1g17020	0.76	0.78	1.08	1.31	NS	NS	
2OG-Fe(II) oxygenase family protein	MDP0000156478	At5g05600	1.6	1.24	2.07	1.64	NS	NS	
	MDP0000225939		1.67	2	2.08	2.56	NS	NS	
	MDP0000621569		1.76	2.3	2.01	2.19	NS	NS	
2-oxoglutarate-dependent dioxygenase	MDP0000283867	At1g06620	NS	NS	NS	NS	-1	-0.82	
GA4 (gibberellin 3-beta-dioxygenase)	MDP0000316943	At1g15550	NS	NS	2.79	1.74	1.85	1.47	
	MDP0000716315		-1.01	-1.24	3.65	1.58	4.25	2.21	
ACS12 (1-Amino-cyclopropane-1-carboxylate synthase 12)	MDP0000408853	At5g51690	1.91	1.59	NS	NS	-1.65	-1.34	
Ethylene-responsive element-binding protein	MDP0000297646	At5g25190	4.75	2.77	0.73	1.79	-4.16	-1.86	
ATERF-1 (ethylene responsive element binding factor 1)	MDP0000299277	At4g17500	-1.16	-1.48	-2.15	-2.94	-1.24	-1.59	
Ethylene-responsive protein	MDP0000148221	At1g05710	-1.17	-1.14	-0.72	-0.86	NS	NS	
Gibberellin									
2OG-Fe(II) oxygenase family protein	MDP0000321690	At1g52800	3.51	0.77	NS	NS	-3.14	-1.1	

	MDP0000739070		2.77	1.35	NS	NS	-3.01	-1.3
GA4H (gibberellin 3 beta-hydroxylase)	MDP0000822659	At1g80340	-3.85	-0.79	3.66	1.36	4.41	1.99
Jasmonate								
LOX2 (lipoxygenase 2)	MDP0000300321	At3g45140	2.67	1.05	NS	NS	-3.22	-0.98
OPR2 (12-oxophytodienoate reductase 2)	MDP0000119756	At1g76690	-1.37	-1.26	-1.62	-1.9	NS	NS
	MDP0000120430		-1.6	-0.84	-1.19	-1.26	NS	NS
	MDP0000137042		-1.82	-1.02	-1.24	-1.91	NS	NS
	MDP0000158694		-1.28	-1.22	-1.75	-1.74	NS	NS
	MDP0000182695		-1.55	-0.96	-1.34	-1.06	NS	NS
	MDP0000283089		-1.36	-0.95	NS	NS	NS	NS
	MDP0000395101		-1.24	-1.15	-1.74	-1.71	NS	NS
	MDP0000658829		-1.4	-0.9	-1.1	-1.36	NS	NS
	MDP0000658830		-1.04	-1.22	-1.69	-0.77	NS	NS
	MDP0000690110		-1.35	-1.04	-1.47	-1.32	NS	NS
OPR1 (12-oxophytodienoate reductase 1)	MDP0000180773	At1g76680	-1.15	-0.9	NS	NS	NS	NS
12-oxophytodienoate reductase	MDP0000268819	At1g09400	-1.58	-1.15	-0.99	-1.12	NS	NS
kelch repeat-containing protein	MDP0000397221	At3g07720	NS	NS	1.09	0.95	1.29	1.57
Salicylic Acid								
JMT (jasmonic acid carboxyl methyltransferase)	MDP0000275210	At1g19640	NS	NS	-0.85	-0.66	NS	NS

*NS- Statistically non significant (BH \geq 0.1)

Table 3.5 - Summary of the differentially expressed genes associated to cellular reduction-oxidation (redox) system and regulation mechanisms. Gene ID represents *M. x sylvestris* var. *domestica* genome (GDR) and *Arabidopsis thaliana* (TAIR) identification

Functional Annotation	Gene ID		Harvest Year/Comparison					
	GDR	TAIR	2009	2012	2009	2012	2009	2012
			T1-T0	T1-T0	T2-T0	T2-T0	T2-T1	T2-T1
Ascorbate								
AtMP2 (putative progesterone-binding protein homolog)	MDP0000264722	At3g48890	-2.11	-1.25	-1.87	-0.91	NS	NS
APX3 (ascorbate peroxidase3)	MDP0000169497	At4g35000	1.6	1.24	1.29	1.81	NS	NS
Monodehydroascorbate reductase4 (MDHAR4)	MDP0000320539	At3g27820	0.67	0.79	NS	NS	-0.62	-1.29
VTC5 (Vitamin C defective5)	MDP0000191488	At5g55120	NS	NS	-0.97	-0.73	NS	NS
Dismutase/Catalase								
CAT2 (catalase2)	MDP0000678891	At4g35090	1.48	0.92	1.2	0.79	NS	NS
Thioredoxin								
AtPDIL5-2 (Protein disulphide isomerase-like 5-2)	MDP0000157499	At1g35620	NS	NS	NS	NS	-0.75	-1.28
AtPDIL5-3 (Protein disulphide isomerase-like 5-3)	MDP0000143707	At3g20560	NS	NS	-0.65	-0.69	NS	NS
RTH (RTE1-homolog – Reverse to ethylene sensitivity 1)	MDP0000272268	At3g51040	-1.88	-1.03	-1.74	-0.75	NS	NS
	MDP0000317051		-1.91	-1.32	-1.78	-1.09	NS	NS
Thioredoxin family protein	MDP0000288395	At1g08570	1.35	1.57	0.99	1.33	NS	NS
Thioredoxin family protein	MDP0000397437	At4g04950	NS	NS	0.74	0.81	NS	NS
Phosphoadenosine phosphosulfate reductase family protein	MDP0000318969	At5g03430	NS	NS	-0.63	-0.67	NS	NS

AtHX (thioredoxin X)	MDP0000431533	At1g50320	-0.84	-0.72	NS	NS	NS	NS	
Thioredoxin-related	MDP0000912059	At5g06430	NS	NS	0.8	0.62	NS	NS	
Glutaredoxin									
Glutaredoxin putative	MDP0000284842	At5g40370	-1.59	-1.06	-1.24	-1.14	NS	NS	
Glutaredoxin family protein	MDP0000292940	At5g58530	NS	NS	NS	NS	1.95	1.03	
Heme									
SOUL heme-binding family protein	MDP0000284838	At2g37970	NS	NS	-2.24	-1.12	-0.99	-1.14	
Receptor Kinases									
Leucine-rich repeat protein kinase putative	MDP0000120486	At1g51805	2.37	0.68	NS	NS	-2.51	-0.85	
Receptor serine/threonine kinase	MDP0000184292	At4g18250	NS	NS	1.06	0.69	NS	NS	
BAM1 (Big apical meristem 1)	MDP0000618819	At5g65700	-2.42	-0.81	NS	NS	2.82	1.22	
Lectin protein kinase putative	MDP0000207437	At5g55830	1.53	1.43	1.47	1.89	NS	NS	
Leucine-rich repeat family protein	MDP0000207774	At3g20820	-3.28	-1.07	-4.38	-1.53	NS	NS	
	MDP0000261548		-4.28	-1.63	-4.15	-1.97	NS	NS	
	MDP0000585188		-4.08	-1.59	-4.21	-1.63	NS	NS	
BAK1 (Brassinosteroid insensitive 1-associated receptor kinase)	MDP0000218840	At4g33430	NS	NS	0.69	0.79	0.73	1.14	
33 kDa secretory protein-related	MDP0000276361	At2g01660	NS	NS	-1.43	-0.8	NS	NS	
Leucine-rich repeat family protein	MDP0000283397	At1g56130	1.11	1.46	NS	NS	NS	NS	
Leucine-rich repeat transmembrane protein kinase putative	MDP0000313102	At4g23740	2.45	2.66	0.98	1.3	-1.93	-0.97	
DRT100 (DNA-damage repair/tolerance 100)	MDP0000315498	At3g12610	-3.79	-1.79	-3.74	-2.35	NS	NS	
RLK (receptor lectin kinase)	MDP0000316360	At2g37710	-3.11	-0.79	-3.7	-0.67	NS	NS	
Serine/threonine protein kinase putative	MDP0000693156	At1g66920	NS	NS	0.91	0.68	NS	NS	
Leucine-rich repeat transmembrane protein kinase	MDP0000721832	At3g47090	NS	NS	0.63	1.61	NS	NS	

Leucine-rich repeat family protein	MDP0000760156	At5g25930	NS	NS	-0.87	-0.73	NS	NS
Calcium								
Calcium-binding protein putative	MDP0000143036	At1g18210	-2.6	-1.74	-2.64	-1.48	NS	NS
	MDP0000225910		-2.84	-2.04	-2.81	-1.85	NS	NS
Calcium ion binding	MDP0000143147	At3g20290	NS	NS	-0.66	-0.67	NS	NS
IQD32 (IQ-domain 32 - calmodulin binding)	MDP0000143754	At1g19870	1.11	1.48	0.93	1.35	NS	NS
ACA10 (autoinhibited Ca ²⁺ -ATPase 10) calmodulin binding	MDP0000230664	At4g29900	NS	NS	-2.36	-0.74	-2.23	-1.43
Polcalcin putative	MDP0000240032	At1g24620	-2.54	-2.07	-2.36	-2.29	NS	NS
Calcium ion binding	MDP0000295196	At3g10300	2.32	0.93	1.34	1.05	NS	NS
Calcium-binding EF hand family protein	MDP0000499214	At4g27280	-3.04	-0.95	-2.34	-1.19	NS	NS
Calcium-binding EF hand family protein	MDP0000163333	At4g38810	-2.62	-1.18	-2.12	-1.65	NS	NS
AtCP1 (CA ²⁺ binding protein 1)	MDP0000785886	At5g49480	-3.39	-0.96	-3.61	-1.57	NS	NS

*NS- Statistically non significant (BH ≥ 0.1).

Table 3.6 -Summary of the differentially expressed genes associated to photosynthesis light reactions. Gene ID represents *M. x sylvestris* var. *domestica* genome (GDR) and *Arabidopsis thaliana* (TAIR) identification

Functional Annotation	Gene ID		Harvest Year/Comparison					
	GDR	TAIR	2009	2012	2009	2012	2009	2012
			T1-T0	T1-T0	T2-T0	T2-T0	T2-T1	T2-T1
LHCA1 (component of the light harvesting complex associated with photosystem I)	MDP0000151095	At3g54890	NS*	NS	1.99	1.40	2.23	1.30
	MDP0000259706		-3.85	-0.87	2.45	0.88	3.47	1.53
CAB1 (chlorophyll a/b binding protein 1)	MDP0000204678	At1g29930	-3.85	-0.90	2.38	1.27	3.59	2.08
	MDP0000217215		-3.85	-0.67	2.30	1.50	3.66	2.27
	MDP0000293052		-3.85	-1.46	2.02	1.04	3.20	2.49
	MDP0000468881		-0.77	-0.69	2.24	1.10	2.32	1.54
	MDP0000875642		-3.85	-1.14	2.42	1.24	3.62	2.32
NDF2 (NAD(P)H dehydrogenase-dependent cyclic electron flow)	MDP0000225351	At1g64770	NS	NS	1.67	1.00	1.81	1.30
LHB1B1 (Photosystem II light harvesting complex gene)	MDP0000235175	At2g34430	-3.85	-0.85	2.11	1.03	3.46	1.82
	MDP0000309425		-3.85	-1.12	2.2	1.12	3.64	1.59
	MDP0000417927		-3.85	-0.90	2.03	0.86	3.48	1.69
	MDP0000417929		-3.85	-1.46	2.00	1.21	3.54	2.18
	MDP0000601491		-3.85	-1.44	1.91	1.62	3.36	2.96
	MDP0000656152		-3.85	-1.13	2.08	1.16	3.52	2.08
	MDP0000656154		-3.85	-1.01	2.15	0.83	3.55	1.86
Ferredoxin-related	MDP0000247361	At1g02180	-4.1	-0.75	-4.0	-0.7	NS	NS
PSBO-2/PSBO2 (photosystem II subunit O-2)	MDP0000248920	At3g50820	-3.85	-0.90	2.71	0.88	4.01	1.73
PSBP-1 (oxygen-evolving enhancer protein 2)	MDP0000361338	At1g06680	NS	NS	2.75	1.9	2.82	1.07
PSAN (photosystem I reaction center subunit	MDP0000419945	At5g64040	-1.31	-1.15	-0.90	-1.89	NS	NS

PSI-N)									
Chlorophyll A-B binding protein putative/ LHCI type II CAB putative	MDP0000813922	At5g28450	NS	NS	2.47	0.86	3.05	1.11	
LHCB5 (light harvesting complex of photosystem II 5)	MDP0000943426	At4g10340	-3.85	-1.23	1.75	0.63	3.10	1.81	

*NS- Statistically non significant (BH \geq 0.1).

Figures

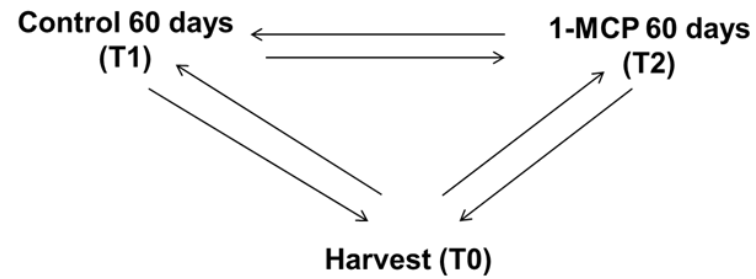


Figure 3.1 - Schematic representation of the transcriptional profile comparisons by microarray analyses. Arrow heads in both directions represent dye-swap experiments.

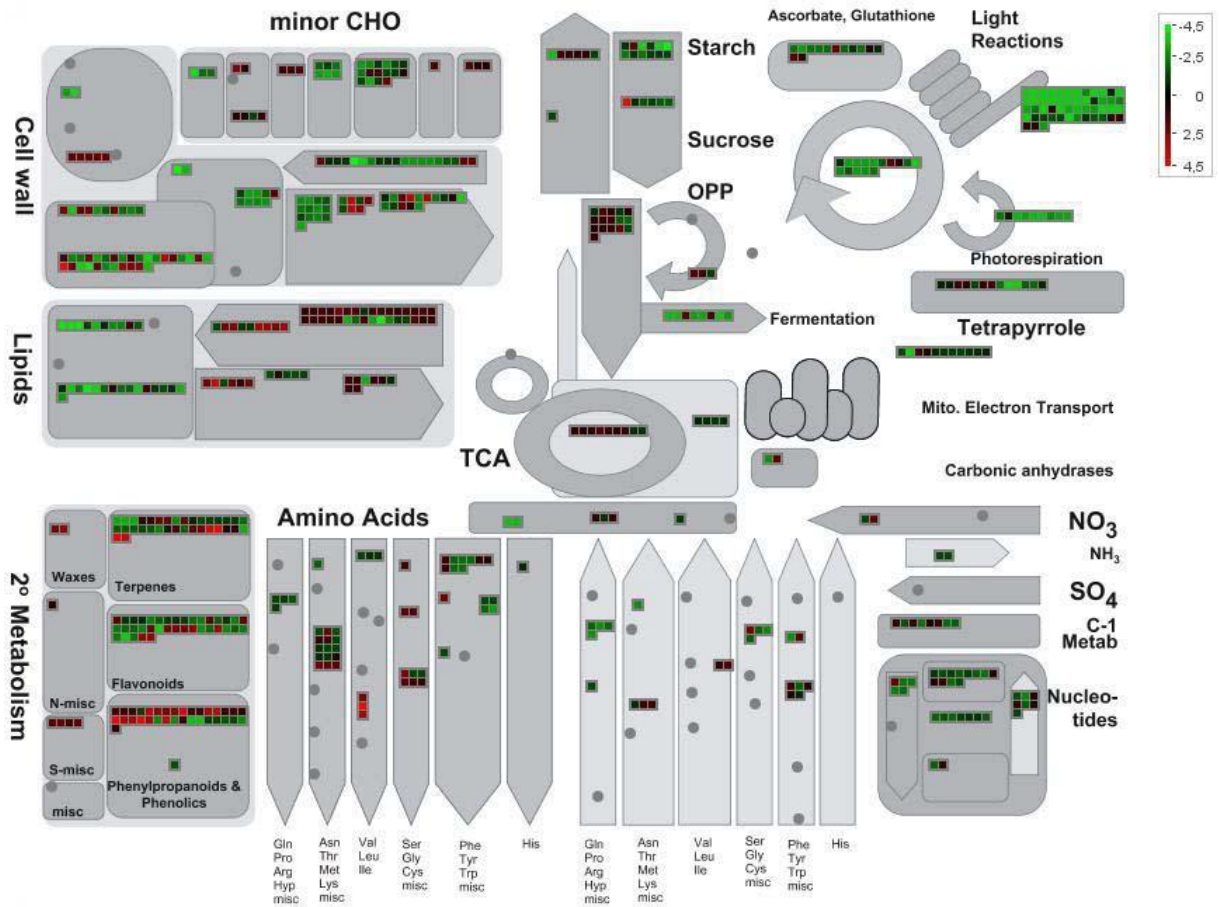


Figure 3.2 - Graphical representation of the metabolism overview distribution of the differentially expressed genes in reciprocal comparisons between fruits at harvest (T0) and untreated cold stored (T1), using the software MapMan. Gray boxes represent metabolic pathways and colored squares represent the genes, according to the transcriptional fold change in each given comparison. Up regulated genes are shown in red and down regulated ones in green according to the color scale.

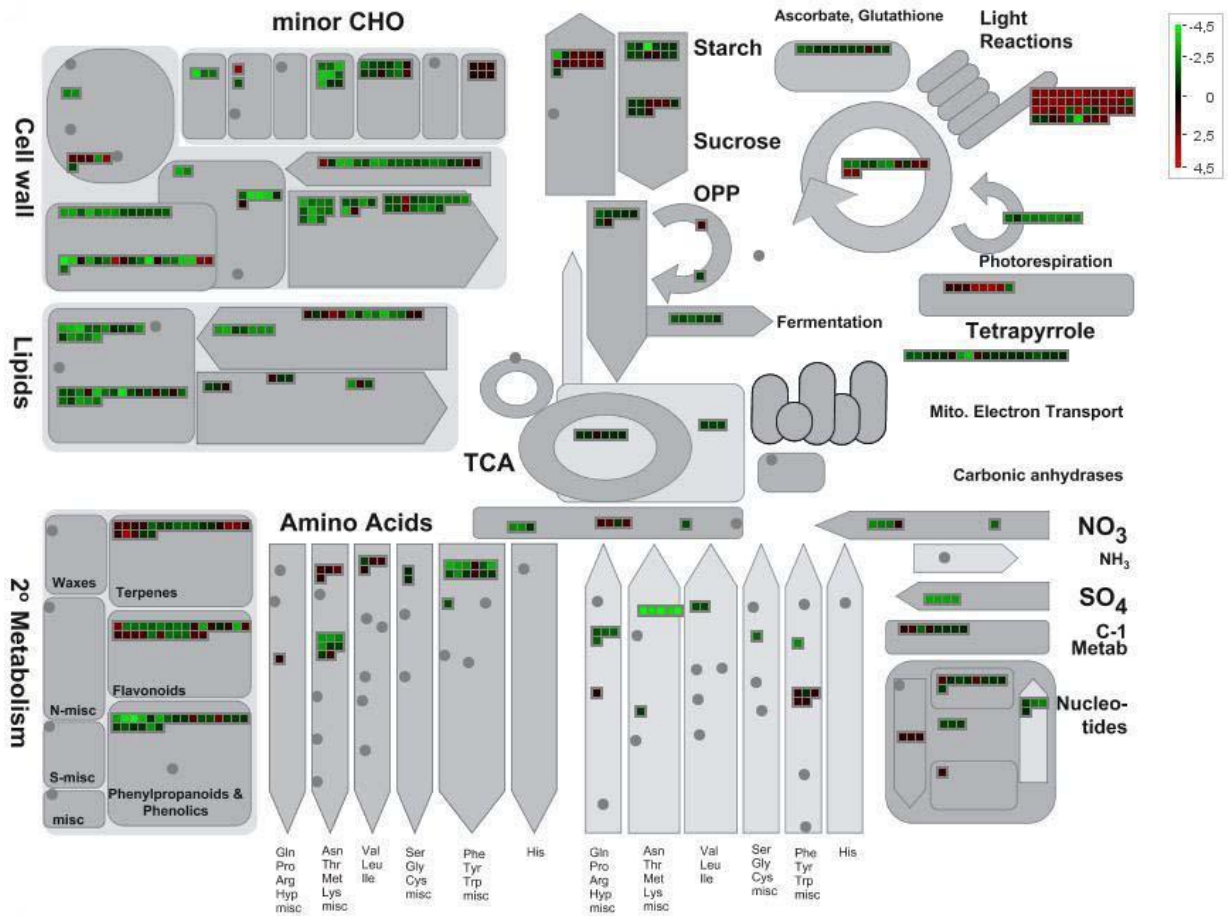


Figure 3.3 - Graphical representation of the metabolism overview distribution of the differentially expressed genes in reciprocal comparisons between fruits at harvest (T0) and cold stored 1-MCP treated fruits (T2), using the software MapMan. Gray boxes represent metabolic pathways and colored squares represent the genes, according to the transcriptional fold change in each given comparison. Up regulated genes are shown in red and down regulated ones in green according to the color scale.

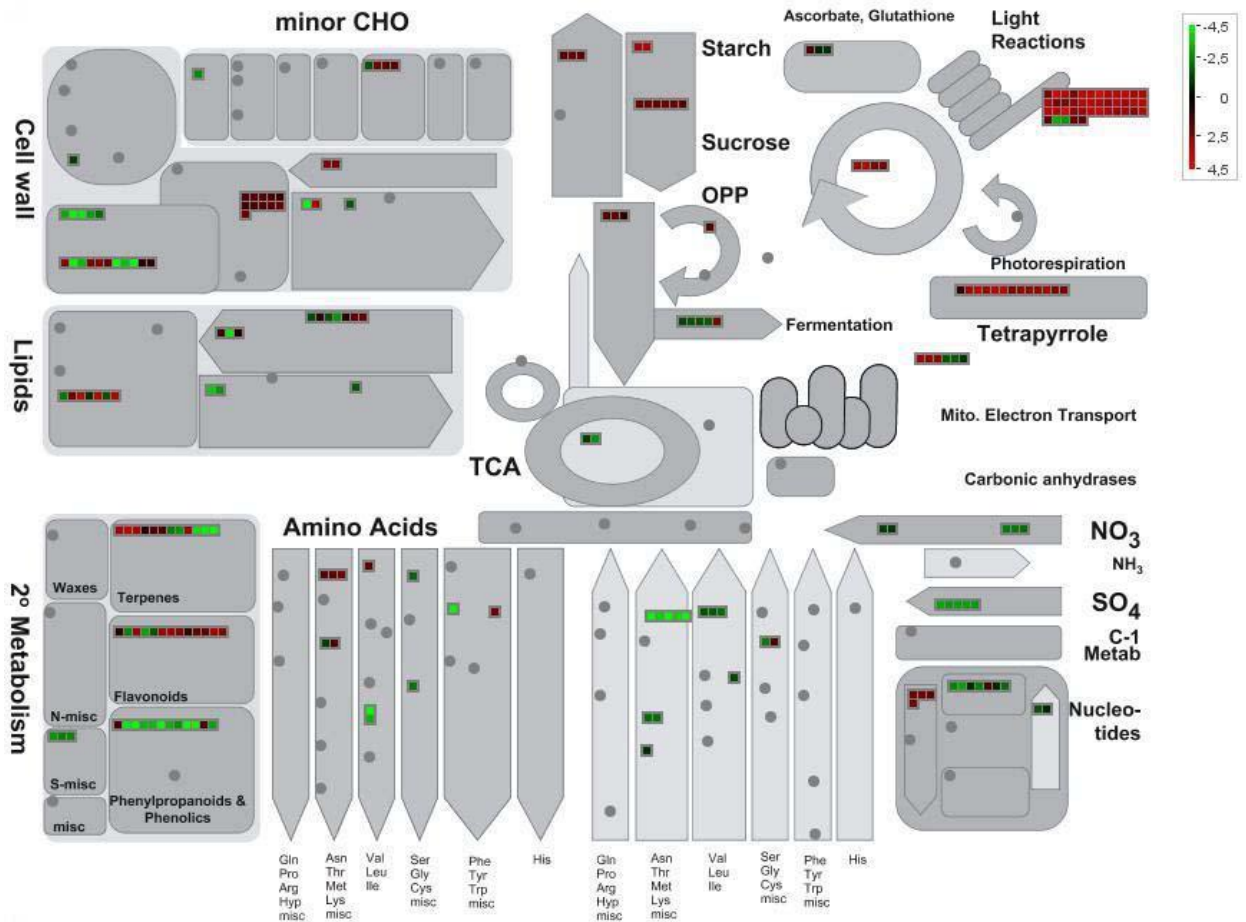


Figure 3.4 - Graphical representation of the metabolism overview distribution of the differentially expressed genes in reciprocal comparisons between untreated cold stored (T1) and 1-MCP treated fruits (T2), using the software MapMan. Gray boxes represent metabolic pathways and colored squares represent the genes, according to the transcriptional fold change in each given comparison. Up regulated genes are shown in red and down regulated ones in green according to the color scale.

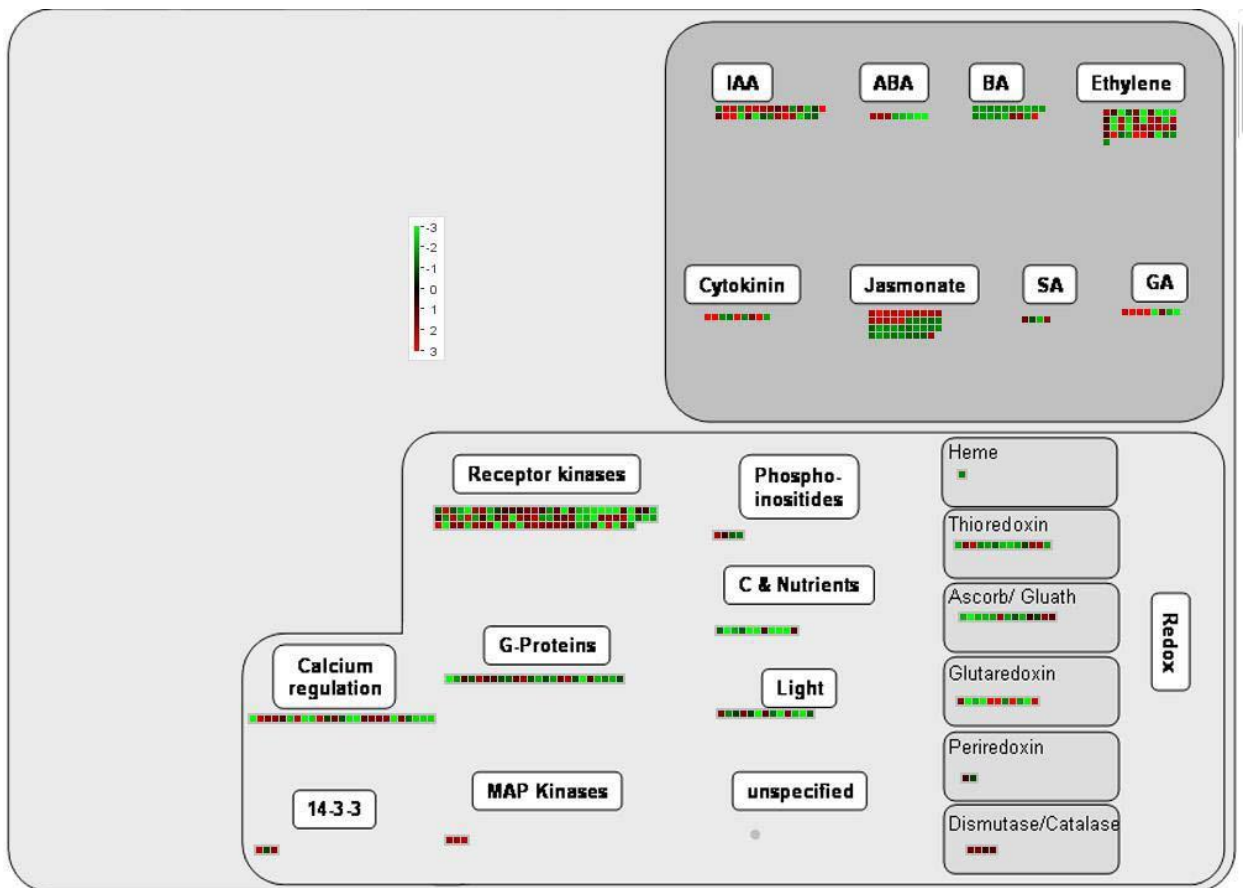


Figure 3.5 - Graphical representation of the distribution of the differentially expressed genes throughout the regulatory processes in reciprocal comparisons between untreated cold stored (T1) and fruits at harvest (T0), using the software MapMan. Gray boxes represent metabolic pathways and colored squares represent the genes, according to the transcriptional fold change in each given comparison. Up regulated genes are shown in red and down regulated ones in green according to the color scale.

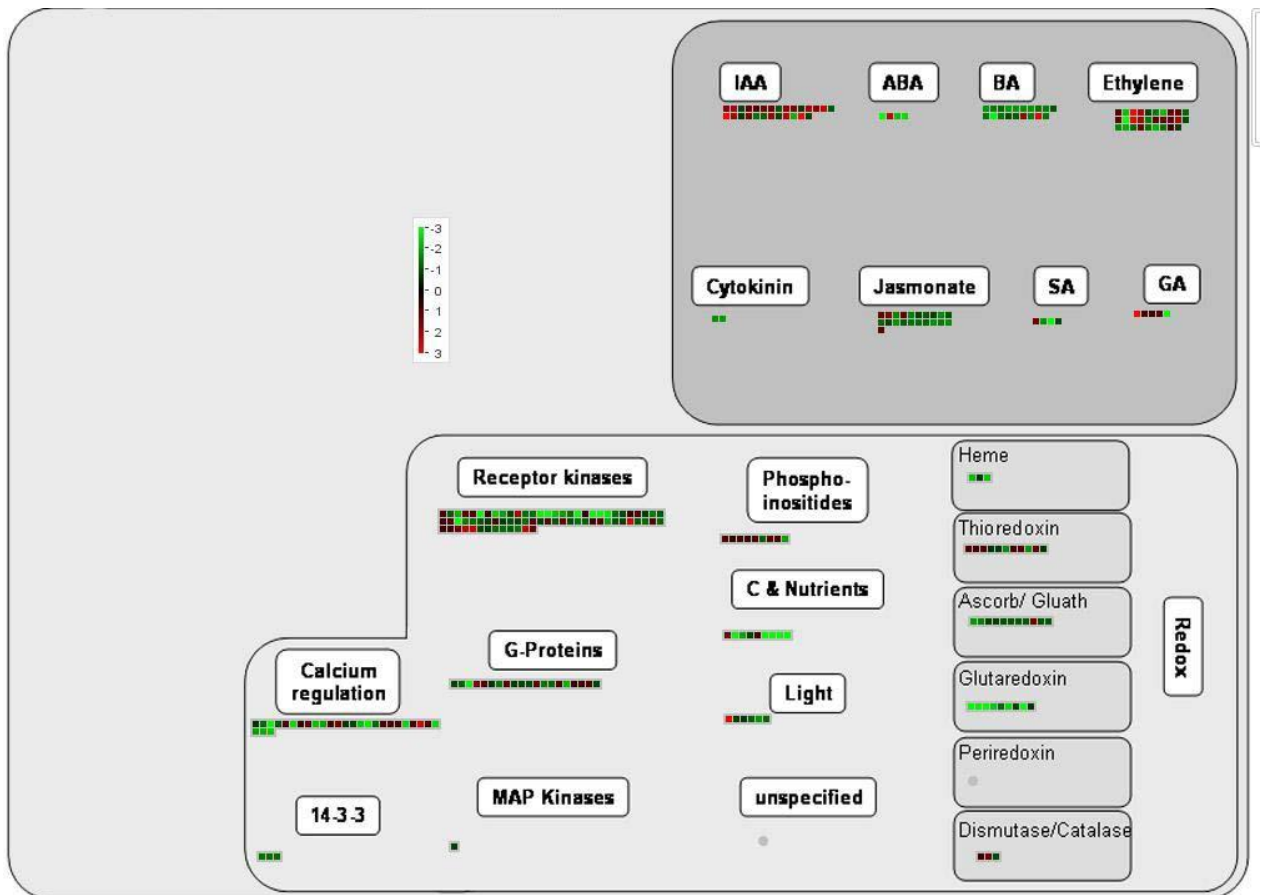


Figure 3.6 - Graphical representation of the distribution of the differentially expressed genes throughout the regulatory processes in reciprocal comparisons between 1-MCP treated cold stored (T2) and fruits at harvest (T0), using the software MapMan. Gray boxes represent metabolic pathways and colored squares represent the genes, according to the transcriptional fold change in each given comparison. Up regulated genes are shown in red and down regulated ones in green according to the color scale.

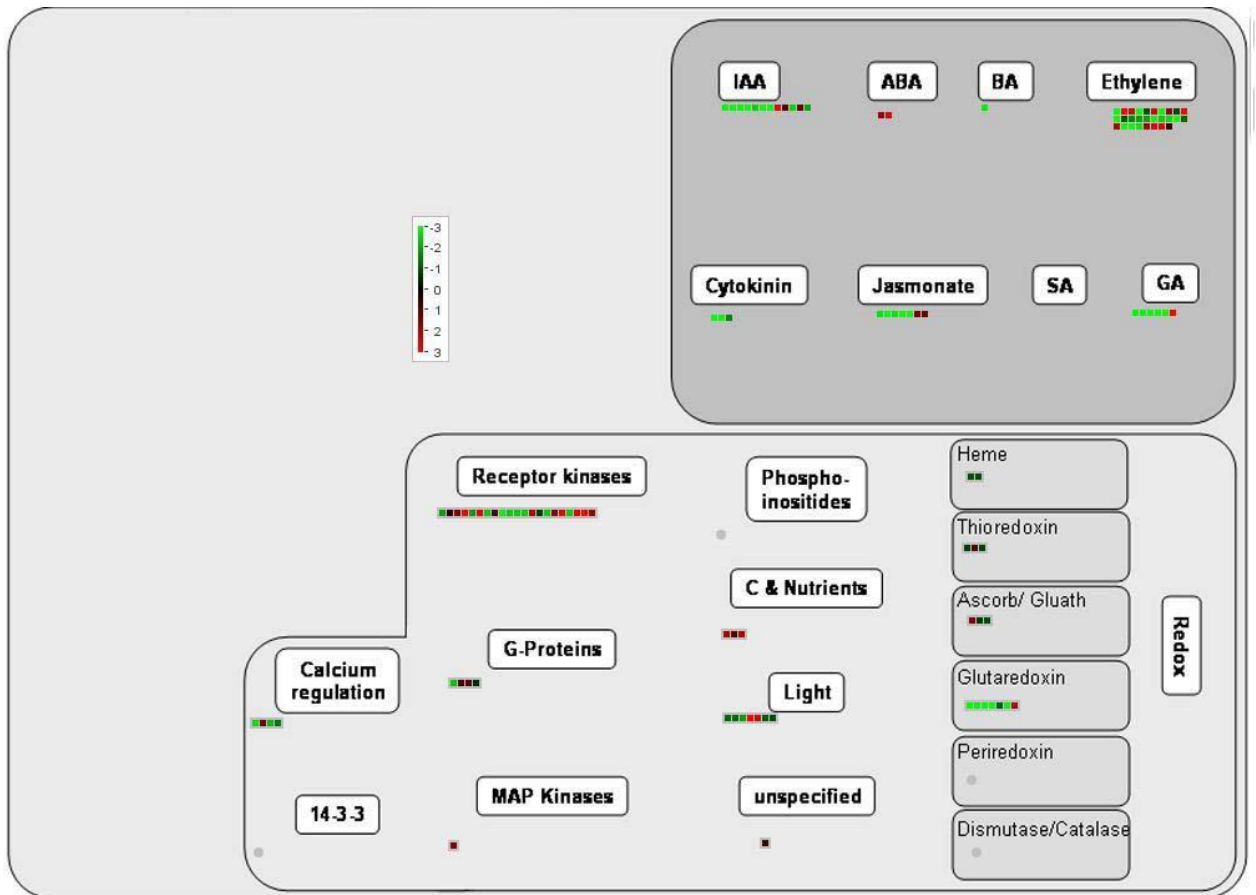


Figure 3.7 - Graphical representation of the distribution of the differentially expressed genes throughout the regulatory processes in reciprocal comparisons between cold stored 1-MCP treated (T2) and untreated (T1) fruits, using the software MapMan. Gray boxes represent metabolic pathways and colored squares represent the genes, according to the transcriptional fold change in each given comparison. Up regulated genes are shown in red and down regulated ones in green according to the color scale.

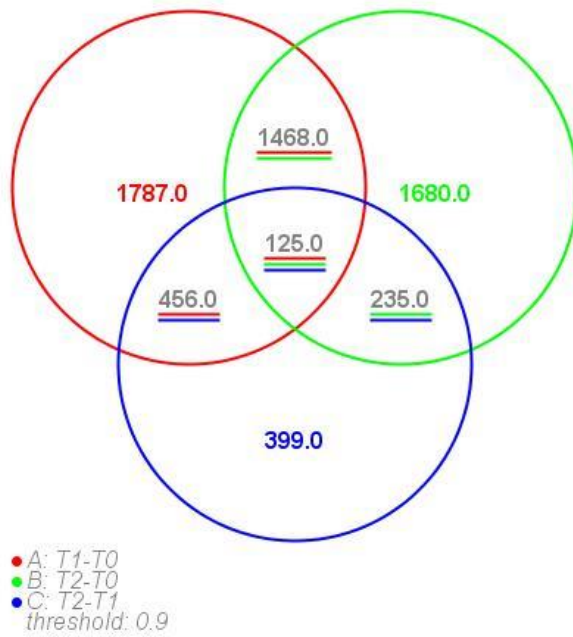


Figure 3.8 - Venn diagram representing the number of differentially expressed genes in reciprocal comparisons between fruits at harvest (T0), untreated cold stored (T1) and 1-MCP treated fruits (T2). Genes were chosen based on BH \leq 0.1 and identical expression profile in 2009 and 2012.

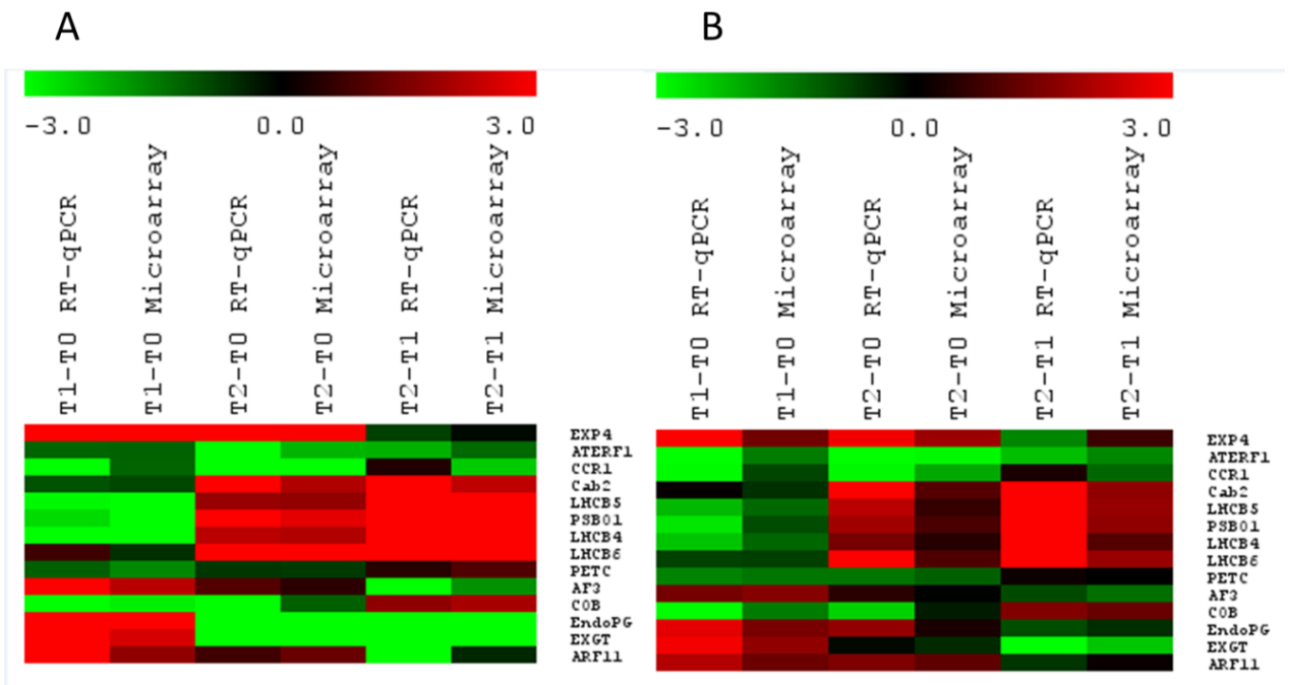


Figure 3.9 - Validation of microarray gene expression profiles by RT-qPCR for 14 genes associated to biological functions of interest during ripening in reciprocal comparisons between fruits at harvest (T0), untreated cold stored (T1) and 1-MCP treated fruits (T2). Transcriptional fold change is represented by color scale. A. samples harvested in 2009; B. samples harvested in 2012.

Considerações finais e perspectivas

É amplamente sabido que a maturação de frutos é um evento complexo, à semelhança do que ocorre com todos os demais estádios de desenvolvimento vegetal. Isso não é diferente no caso de maçãs. No entanto, é importante além da estratégia de estudos do tipo *top-bottom*, nos quais faz-se uma ação e tem-se a resposta tecnológica, às vezes sem ter-se o mecanismo, que se iniciem estudos numa estratégia *bottom-top*, ou seja, partindo de bases científicas, buscando explicações para possíveis eventos relacionados com a maturação. Embora esse seja o intento, nessa tese, isso não foi atingido na plenitude, mas trata-se de um início.

Assim, num conjunto de experimentos concatenados e em paralelo, realizaram-se três estudos. O primeiro, embora se apresente como experimento de padronização de método, também demonstra a complexidade dos eventos que ocorrem nos frutos durante a pós-colheita. Tal conclusão é baseada no fato de que os genes avaliados como candidatos à referência mostraram comportamento diferenciado nos experimentos que abordaram o período de pós-colheita. Um gene nunca antes mencionado como referência, denominado *MdH1*, apresentou-se estável nos experimentos de pós-colheita testados neste trabalho. Essa contribuição é importante, pois esse gene será usado nos cálculos de acúmulo diferencial de transcritos em estudos dessa categoria.

Ainda, é sabido que há alterações fisiológicas e tecnológicas significativas quando se armazenam maçãs sob AR, destacando-se a redução de firmeza de polpa. Esse assunto é amplamente abordado na pós-colheita de maçãs e de outros frutos, mas os achados ainda deixam dúvidas de como isso ocorre. O fato é de que há envolvimento de enzimas clássicas, como Expansinas, PG e PMEs. Mais recentemente, foi caracterizada uma enzima α -L-arabinofuranosidase em maçãs, que neste experimento apresentou alta atividade em frutos sob AR. A transcrição do gene *MdAF3*, relacionado a essa enzima, também manteve elevado nível de transcritos durante todo o período avaliado. Foi enfatizada neste capítulo a clara dependência da expressão desse gene ao etileno. Assim, a hipótese de que seja um

gene-enzima putativamente envolvido com a redução da firmeza de polpa é fortemente provável. O estudo de mutantes ou a geração de mutantes com supressão desse gene permitirá testar essa hipótese. Esse trabalho está em andamento junto à equipe de pesquisa.

Por fim, confirmou-se a complexidade de eventos durante a maturação, através do grande número de genes diferencialmente expressos entre as comparações que evidenciam o uso do frio, e a ação do etileno, avaliada através do uso de seu inibidor, o 1-MCP. O uso do frio resultou em maior número de genes diferencialmente expressos do que o 1-MCP. Esse trabalho ressaltou ainda a importância do gene *MdAF3* para as alterações de textura relacionadas ao etileno, já que este foi o único gene de degradação de parede celular que apresentou um aumento de transcrição em AR nos frutos que não receberam o tratamento com 1-MCP, ao mesmo tempo em que houve uma redução na transcrição do gene nos frutos tratados quando comparados aos frutos controle armazenados. Alguns genes classificados no metabolismo de hormônios também apresentaram este mesmo comportamento, podendo ser bons marcadores de qualidade dos frutos mantidos em AR. Por fim, ressalta-se a presença de um grande número de genes diferencialmente expressos envolvidos com os mecanismos de oxi-redução e sinalização, o que pode ser resultado de um estado de estresse dos frutos.

A partir das informações geradas nesse trabalho, algumas questões podem ser levantadas referentes ao período de armazenamento de frutos de maçã. Entre elas destacam-se a importância de enzimas atuantes nas cadeias laterais da pectina no processo de amolecimento pós-colheita e a expressiva influência do frio na indução de genes de resposta ao estresse. Essas informações devem ser relacionadas mais nitidamente às alterações bioquímicas e fisiológicas observadas neste período. Para tal, novas avaliações devem ser incorporadas ao estudo, incluindo análises estruturais e composicionais da parede celular, análises do perfil protéico que acompanha este período e análise sensorial com foco na resposta do consumidor aos fatores abordados neste estudo, a saber, etileno e uso do frio. Além disso, os genes apontados neste trabalho como possíveis marcadores de qualidade devem ser avaliados em diferentes tempos e condições de armazenamento aliados à redução da produção de etileno pelos frutos. Finalmente, um estudo do comportamento transcricional destes genes deve ser aliado às respostas fisiológicas

obtidas pelas avaliações sugeridas a fim de validar o potencial dos mesmos como marcadores de qualidade.

Ainda, um estudo de transformação com o gene *MdAF3*, em tomate e *Arabidopsis thaliana*, já está sendo conduzido a fim de caracterizar o gene quanto à função.

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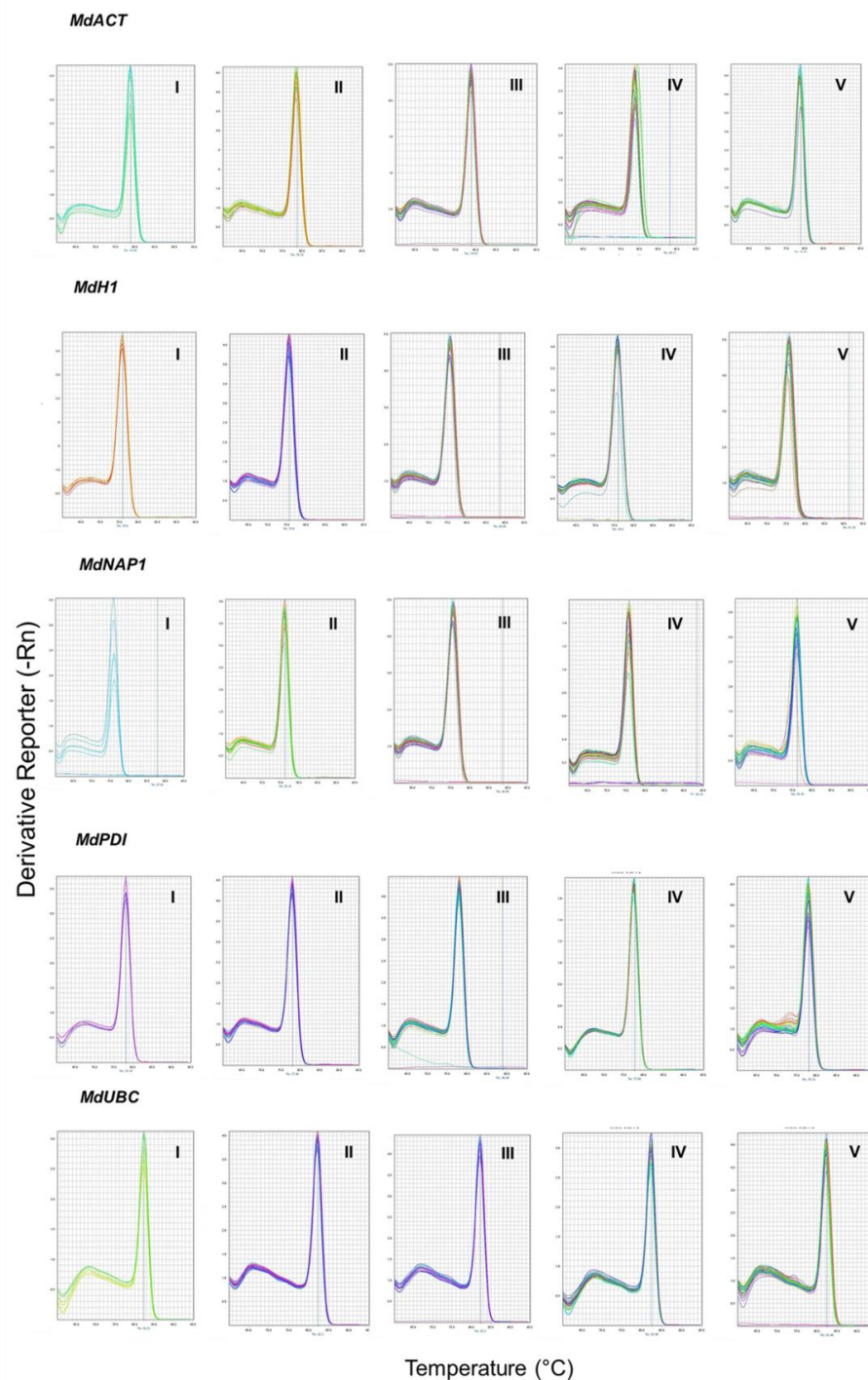
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Apêndices

Apêndice A: Amplification specificity, indicated by RT-qPCR dissociation curves, for the primers used to investigate the transcription of the candidate reference genes in five independent experiments (I to V): (I) plant organ; (II) fruit developmental stages; (III) fruit ripening at room temperature; (IV) ethylene treatment on cold stored apples and (V) long term cold storage combined with distinct controlled atmosphere conditions.



Apêndice B: Transcript accumulation of *MdAF3* relative to *MdAF1* transcripts in different apple organs and in the fruit at room temperature and at cold storage under 1-MCP and ethylene treatments.

Storage Condition	Treatment	Sample	Relative Quantitation*	Standard Deviation
-	Control	Leaf	544.358	205.663
	Control	Flower	7.633	0.598
	Control	Green Fruit	2.153	0.116
	Control	Mature Fruit at Harvest	7.998	0.729
Room Temperature	Control	2 days	4.547	0.915
	Control	4 days	11.201	2.508
	Control	6 days	7.433	1.176
	Control	8 days	40.607	2.902
	Control	10 days	23.324	1.251
	Control	12 days	9.220	2.278
Cold Storage	Control	60 days	127.070	46.268
	Control	120 days	142.366	13.933
	Control	180 days	286.857	30.872
Room Temperature	1-MCP	2 days	1.437	0.384
	1-MCP	4 days	10.787	2.786
	1-MCP	6 days	2.141	0.593
	1-MCP	8 days	1.517	0.095
	1-MCP	10 days	4.923	0.606
	1-MCP	12 days	4.904	1.434
Cold Storage	1-MCP	60 days	11.505	1.238
	1-MCP	120 days	5.706	1.057
	1-MCP	180 days	86.536	17.689
	Ethylene	60 days	190.177	33.383
	Ethylene	120 days	2656.147	694.907
	Ethylene	180 days	231.370	52.832

*The calculation of relative quantitation was made for *MdAF3* utilizing for each sampling point the corresponding Cq of *MdAF1* analysis as the normalizing sample.