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DOCTORAL COURSE IN CROP SCIENCE

CYCLE: XXVI

A PRELIMINARY SURVEY OF MOLECULAR FACTORS INVOLVED IN APPLE (*MALUS DOMESTICA* BORKH. CV GRANNY SMITH) SUPERFICIAL SCALD DEVELOPMENT

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

January 31st, 2014 Elisabetta Zonin

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Riassunto

La conservazione delle mele per lunghi periodi è resa possibile grazie all'introduzione di tecniche quali la conservazione in atmosfera controllata e permette ai produttori di aumentare la finestra temporale di commercializzazione di questo frutto sul mercato. È una procedura costosa che può causare a seconda delle varietà di mele conservate, della stagionalità, del periodo di raccolta dei frutti e delle condizioni di conservazione applicate (bassa concentrazione di O_2, bassa/alta concentrazione di CO_2, bassa temperatura) la comparsa di diversi disordini fisiologici. Tra i disordini più comuni che colpiscono le mele prodotte e vendute in Italia vi è il riscaldo superficiale che si manifesta come un'area necrotica a livello della buccia nelle varietà sensibili quali Granny Smith e Red Delicious. Il riscaldo superficiale causa la maggior perdita di mele e di conseguenza il maggior danno economico ai produttori di tutto il mondo. Il riscaldo insorge in seguito a periodi di conservazione a basse temperature relativamente lunghi (2-4 mesi) e successiva conservazione a temperatura ambiente (ca 7 gg) dopo l'uscita dalle celle. È un disordine la cui comparsa è influenzata anche da fattori indipendenti dalla conservazione quali lo stadio di maturazione dei frutti alla raccolta, le condizioni ambientali durante la crescita, l'azione dell'etilene o ancora il contenuto minerario. Ad oggi studi fisiologici e biochimici su Granny Smith hanno evidenziato come l'α-farnesene, un volatile presente nella buccia delle mele il cui processo finale di biosintesi è influenzato dall'etilene, possa andare incontro ad un processo di ossidazione quando le mele vengono poste nelle celle di conservazione in atmosfera controllata. L'accumulo dei prodotti ossidativi derivanti, tra cui i trienoli coniugati porterebbe alla degenerazione del tessuto. Diverse strategie sono state adottate negli anni per prevenire la comparsa dei sintomi del riscaldo tra cui l'impiego dell'antiossidante difenilamina (DPA), dell'inibitore della percezione dell'etilene 1-metilciclopropene (1-MCP) o l'applicazione di un iniziale stress a basso ossigeno (ILOS - initial low oxygen stress-) durante le prime settimane di conservazione. L'impiego del DPA è stato proibito in Europa dal 2011, mentre i trattamenti con 1-MCP assicurano il controllo del riscaldo ma hanno costi elevati, infine lo stress iniziale a basso ossigeno non permette una conservazione per lunghi periodi e necessita di continui monitoraggi per evitare che le mele sviluppino disordini legati allo stress da basso ossigeno. Conoscere i meccanismi molecolari che regolano la comparsa e lo sviluppo dei sintomi del riscaldo potrebbe

permettere di identificare alla raccolta le partite di mele soggette alla manifestazione del disordine, così da individuare quali partite conservare o meno e garantire un guadagno economico al produttore. Lo scopo di questo lavoro è stato quindi cercare di caratterizzare in maniera preliminare su campioni di bucce di mele della varietà Granny Smith, trattate o meno con 1-MCP o DPA e conservate in atmosfera controllata per 1, 3 o 6 mesi, i possibili fattori molecolari coinvolti nel riscaldo, in associazione con l'attività dell'etilene e del metabolismo ROS, due agenti che dai dati in letteratura sembrano avere un ruolo nello sviluppo del riscaldo. A questo scopo sono state caratterizzate in melo le famiglie geniche coinvolte nel mantenimento dell'omeostasi dei ROS. In particolare le ROP e le proteine accessorie ROP-GEF, -GAP e -GDI, le RBOH (NADPH ossidasi coinvolte nella produzione dei ROS a livello apoplastico) e le PLDα (coinvolte nella regolazione dell'attivazione delle RBOH insieme alle ROP) in quanto è noto che in Arabidopsis, in condizioni di basso ossigeno, le cellule attivano un meccanismo regolativo a feedback negativo che coinvolge in generale ROP, ROP-GAP, RBOH e H_2O_2, e prende il nome di reostato ROP-GAP. Tramite real-time PCR sono state analizzate le espressioni trascrizionali dei geni identificati, individuando 2 ROP, 7 ROP-GEF, 8 ROP-GAP, 2 RBOH e 2 PLDα che vengono de-repressi nei campioni trattati con 1-MCP, e in maniera minore anche dal trattamento con DPA. Trattamenti di 4h e 24h con etilene esogeno hanno permesso di dimostrare che alcuni di questi geni de-repressi in presenza di 1-MCP vengono effettivamente regolati in maniera negativa dall'etilene. Successivamente le analisi effettuate sul contenuto in malonildialdeide, un marcatore della perossidazione lipidica, in H_2O_2, ascorbato e glutatione, suggeriscono che le cellule delle mele non trattate, che nel 97% dei casi hanno manifestato riscaldo alla fuoriuscita dalle celle, presentino una situazione di stress associata alla perdita dell'omeostasi dell'H_2O_2 che viene invece mantenuta nei campioni trattati con 1-MCP i quali presentano anche un aumento dei livelli trascrizionali di alcol deidrogenasi (ADH), un marcatore della risposta all'H_2O_2. La localizzazione subcellulare dell'H_2O_2, determinata col cerio cloruro tramite microscopia elettronica, ha rilevato poi maggiori livelli di H_2O_2 a livello dell'apoplasto nelle bucce dei campioni trattati con 1-MCP rispetto al controllo, confermando quindi un ruolo delle RBOH e del loro sistema regolativo nel mantenimento di livelli omoeostatici di H_2O_2 nell'apoplasto. Infine in seguito ad un'analisi RNA-seq sugli stessi campioni, è stato

possibile costruire una heatmap che ha evidenziato solo nei campioni trattati con 1-MCP una evidente co-regolazione tra i geni identificati del reostato ROP-GAP, e in generale del sistema ROP, e le sequenze geniche appartenenti alle famiglie delle ascorbato perossidasi, monodeidroascorbato reduttasi e tioredossine coinvolte rispettivamente nella detossificazione e nella protezione dei gruppi tiolici dall'azione dei ROS.

Nell'insieme i risultati ottenuti dimostrano per la prima volta che durante lo stress da freddo la presenza dell'etilene induce nei campioni che manifestano riscaldo una perdita dell'omeostasi dell'H_2O_2 causata dalla mancata regolazione del reostato ROP-GAP e delle RBOH, che porta all'attivazione di una diversa risposta trascrizionale dei geni coivolti nella detossificazione dei ROS.

Summary

Apple storage in controlled atmosphere (CA) can increase the marketing window of apples but at the same time it is an expensive practice and on the basis of cultivar, season, harvest time and storage condition (low O_2, low/high CO_2, low temperature) apples can develop different physiological disorders. It appears as a darkened area due to necrosis of hypodermal cells especially in Granny Smith and Red Delicious cultivars. Superficial scald annually causes the major economic loss to apple growers worldwide. It can arise after a relative long period (2-4 months) of cold storage with an increase in severity when apples are removed from storage and are leaved at room temperature (ca 7 dd). It is also influenced by different preharvest factors including fruit ripening at harvest, environmental conditions during growth, ethylene action or fruit mineral content. Superficial scald is associated with the accumulation of volatiles, in particular of α-farnesene, the biosynthesis of which is influenced by ethylene. This compound can be oxidized along with the storage period to a group of molecules called conjugated trienols. The accumulation of these oxidative products can induce metabolic dysfunction and cell death. Different strategies are provided to successfully inhibit scald development. The most common techniques in use combine storage in control atmosphere (CA) with treatments with the antioxidant diphenylamine (DPA), or with 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception and the most effective molecule to control scald, or application of an initial low oxygen stress (ILOS), followed by CA storage. In 2011 the use of DPA has been banned in Europe for health concerns, 1-MCP is effective but has high costs, while ILOS is difficult to apply requiring more research for its optimization and apples cannot be stored for long periods. The identification of the molecular mechanism involved in the regulation of superficial scald development may allow the possibility to predict at harvest, before CA storage, which apple batches may probably develop the disorder allowing rational storage strategies with significant economic gains. The aim of this work was to provide a preliminary characterization of the molecular factors putatively involved in scald development associated with ethylene action and ROS metabolism, two actors that seem to have a role in scald occurrence, in peels of apples, cv Granny Smith, treated or not with 1-MCP or DPA and stored in controlled atmosphere for 1, 3 or 6 months. For this purpose gene families involved in the maintenance of ROS homeostasis were identified in the apple

genome. In particular ROPs and ancillary proteins ROP-GEFs, -GAPs and -GDIs, RBOHs (NADPH oxidases involved in the generation of ROS at the apoplastic levels) and PLsDα (involved togheter with ROPs in the regulation of RBOHs activity) were identified. It is known in fact that Arabidopsis cells during oxygen deprivation activate a mechanism controlled by negative feedback regulation which involves ROPs, ROP-GAPs, RBOH and H_2O_2, and it is termed the ROP-GAP rheostat. Expression analyses on the identified genes evaluated by real-time PCR allowed the identification of 2 ROPs, 7 ROP-GEFs, 8 ROP-GAPs, 2 RBOHs and 2 PLsDα de-repressed in samples treated with 1-MCP and in most cases with a minor extent, by DPA treatment. The expression of the same genes was evaluated by qPCR on peels of apples subjected to short-time treatments with a saturating concentration (100 ppm) of ethylene for 4 and 24 hours showing that ethylene negatively regulates many of the apple ROP-GAP rheostat genes including RBOHs and PLsDα. Then analyses of content of malonyldialdehyde, a marker of lipid peroxidation, H_2O_2, ascorbate and glutathione, suggested that cells of untreated apples, which showed scald symptoms in the 97% of cases after 6 months of storage, perceived the oxidative stress associated with the loss of H_2O_2 homeostasis. On the contrary samples treated with 1-MCP showed higher levels of H_2O_2 thus maintaining the H_2O_2 homeostasis. This finding was further confirmed by the up-regulation of transcription of alcohol dehydrogenase, a marker of H_2O_2 response. The subcellular localization of H_2O_2, obtained by means of cerium chloride reaction through transmission electron microscopy, revealed higher levels of H_2O_2 in the apoplast of apple peels treated with 1-MCP compared to control ones, confirming a role of RBOHs and their regulative system on control of apoplastic H_2O_2 homeostasis. Finally RNA-seq analyses on the same samples, allowed to construct an heatmap that highlighted in samples treated with 1-MCP a co-regulation between genes of the ROP-GAP rheostat and of the ROP system, and genes belonging to the ascorbate, dehydroascorbate reductase and thioredoxin families, involved respectively in the detoxification or protection of thiol groups by ROS action. These results demonstrate for the first time that during cold stress ethylene induces in apples that develop scald a disruption of ROS homeostasis caused by the loss of ROP-GAP rheostat and RBOHs regulation, thus provoking a different transcriptional response of genes involved in ROS detoxification.

Chapter I

General Introduction

Introduction

Apple belongs to the *Rosaceae* family, subfamily *Pomoideae*, genus *Malus*, species *Malus domestica*. There are more than 7500 known cultivars in the world, most of them grown in Asia, originated from the ancestor *Malus sieversii*. *M. sieversii* has been identified as the main contributor to the *M. domestica* genepool based on similarities in fruit and tree morphology, and on genetic data (Coart *et al*., 2006; Velasco *et al*., 2010). The secondary contributor to the diversity of apples, resulting in the current varieties of *M. domestica* Borkh, is the wild European crabapple *M. sylvestris* (Cornille *et al*., 2012). Apples are popular because of the many ways they can be consumed and because of their convenience and durability. Apples from different cultivars have different uses: fresh eating, cooking or cider production. In particular, apples can be processed into sauce, slices, or juice but they can be used also for making pastries, cakes, tarts, and pies (Downing, 1989). The pulp can be processed into candies or used as a source of pectin. The juice can be consumed fresh, both natural and filtered, but also it can be fermented into alcoholic beverages such as cider or wine, moreover distilled into brandy, or finally transformed into vinegar (Janick *et al*., 1996). Recent works have shown that apple fruit and apple juice bring benefits to human health by reducing the incidence of lung cancer, viral diseases and cardiovascular disorders (Boyer & Liu, 2004; He & Liu, 2007).

Apple is the second fruit crop in importance after banana (107M tons)(FAOSTAT), with more than 75M tons of apples produced in 2011. Asia produces 55% of the total worldwide apple production, Europe 22% and America 15%. China is the biggest apple producer (ca 36M tons, 50% of the whole world production), followed by USA with a production reaching 4M tons, (6% of the whole world production). The first ten leading countries in apple production are shown in Figure1. Italy is the sixth worldwide producer with a production of 2,4M tons, for an economic value of 978 million dollars (FAO). The Italian apple production is obtained mainly from four regions: Trentino Alto Adige (accounting for almost 70% of the total Italian production, ISTAT 2011 and for 20% of the European production), Veneto (8.3%) Emilia Romagna (7.4%), and Piemonte (6.1%). The top 5 apples varieties grown in Italy include Golden Delicious, Gala, Red Delicious, Fuji and Granny Smith.

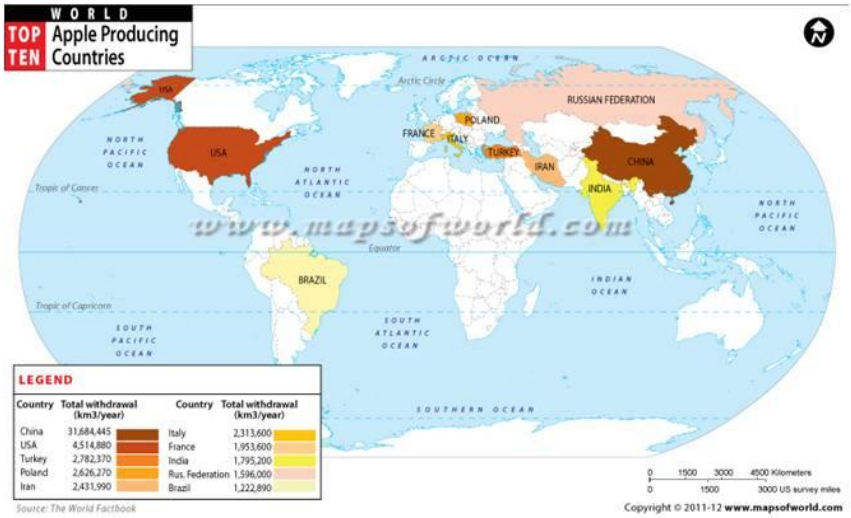

Figure 1 – The most important States leading apple fruit production are shown with different colors: China, the first world producer, is colored in brown; Brazil, the tenth world producer which is represented in light yellow (from http://www.mapsofworld.com).

In 2009 nearly 7400 tons of the worldwide apple production were exchanged for a total economic value equal to 5 million dollars (FAO). The main States and the relative imported apple quantity from 2006 to 2009 are highlighted in Table 1.

Table 1 – Apple volume (tons) and relative economic value (× 1000 dollars) of main importers, from 2006 to 2009 (FAO).

State:	2006	2007	2008	2009	Value
Russian Federation	90.322	53.897	1.062.900	1.108.210	547.500
Germany	171	917	613.288	622.564	521.320
United Kingdom	121.411	141.018	481.809	455.671	511.407
The Netherlands	18.216	626.999	396.415	360.250	382.221
Spain	44	35	227.886	238.712	201.299
France	49.408	54.543	147.827	161.085	131.346
United States of America	531.785	522.841	165.282	155.775	169.661

In the same year China was the first principal exporter with its 1,2M tons exported, for an economic value estimated in 713 million dollars (FAO), followed by USA, Poland and Italy with 732,794 tons exported, equal to 32% of total national apple production, and an overall business of 667 million dollars (FAO). In Figure 2 a graph reports the main markets served by Italy: in 2009 Germany imported from Italy 260 thousand tons of apples for an

estimated value of 250 million dollars, instead 76,4 thousand tons were exported to Spain and a minor quantity (only 30 thousand tons) was transferred to Russian Federation, Rumania, Sweden and United Kingdom. France and Libya also imported from Italy 25 thousand tons of apples (FAO).

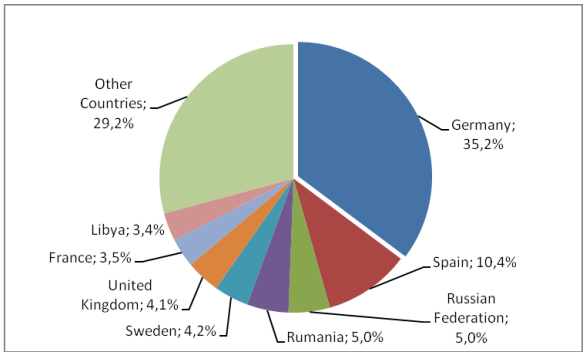

Figure 2 – The main States importing apples from Italy and the relative importing percentage (2009).

Vice versa Italy imports only a minor quantity of apples (37 thousand tons for a business of 32 million dollars in 2009, FAO). This is due probably to the fidelity that the consumer has to the Italian product and to the Italian brands (Chamber of Commerce of Cuneo, 2008). Every year the quantity of apples being sold soon after harvest for fresh consumption or stored for different periods of time changes significantly on the basis of fluctuations of market demand. If apples are harvested at the right ripening stage with optimal caliber the price of apple is profitable for growers which will push producers to sale off apples rather than storing them. In fact even if storage can increase the marketing window of apples (in 2010 for example, in Alto Adige region, 350 rooms which contained the 10% of the whole apple production, were destined to stored apples subjected to an initial low oxygen stress (Terra e Vita, 2010)) and it is an advantage for growers, at the same time it is an expensive practice and during or after storage apples can lose their quality by undergoing several storage related physiological disorders.

Apple injuries

Different strategies have been developed to extend the period for commercialization of fruits through storage after harvest. Apples can be stored for relatively long periods of time. Nevertheless, after a certain period of time, that varies among different varieties, apples can undergo a number of so-called "physiological disorders". Overall, there are different aspects that can negatively affect quality of apple fruits (and of fruits in general), and in particular they can be divided into three categories:

1. Physically-induced pre-harvest damages, that occur to apples prior to harvest and include frost, hail, bruising and sunburn damages;
2. Pathological disorders, due to pathogens, mainly develop prior to harvest and, to a lesser extent, during storage. The principal postharvest diseases are blue mold caused by *Penicillium* species and gray mold caused by *Botrytis cinerea*;
3. Physiological disorders can affect apple fruits during both pre- and post-harvest life. These disorders may result in damage of the fruits' skin, cortex or core area, alone or in combination, depending on several pre- and post-harvest factors and on variety. All physiological disorders are the consequence of abiotic stresses to which fruits had been exposed either in the field or during storage.

Apple physiological disorders

Low or high temperature, drought or high salinity are the most important abiotic stresses that adversely affect plant growth and crop production (Xiong *et al*., 2002). The most common abiotic stresses to which apples are exposed during storage are represented by low temperature, low oxygen (O_2) and/or high carbon dioxide (CO_2) concentrations. These stresses either alone or in combination can cause, on susceptible cultivars, the development of different physiological disorders. In addition, the occurrence of post-harvest physiological disorders often depends, for both initiation and severity of symptoms, on pre-storage conditions (Ferguson *et al.*, 1999). The most obvious pre-harvest factor that influences development of physiological disorders is the ripening degree of fruit at harvest. However, additional factors may have a role in determining how fruit respond to abiotic stresses during storage, such as fruiting position on the tree, seasonal temperature dynamics and availability of nutrients (e.g. calcium)(Ferguson *et al.*, 1999). In apples, the position of the fruit within the tree or the inflorescence affects pollination and cropping effects, or influences minerals and water flow into the developing fruit.

Overall, on the basis of the nature and timing at which the inductive factors of the disorder are determined, it is possible to identify two main classes of physiological disorders:

1. Physiological disorders which are predetermined on the tree and occur at pre- or post-harvest but do not depend on post-harvest conditions; this type of disorders can be also enhanced or delayed depending on post-harvest conditions;
2. Physiological disorders which are specifically induced by post-harvest conditions (storage) yet can be modulated by pre-harvest factors (Ferguson *et al.*, 1999).

The first class of disorders doesn't require post-harvest storage conditions in order to be expressed and it is associated with the fruit's physiological state depending on developmental or environmental aspects and on the degree of ripening. Storage conditions (e.g. low temperatures, controlled atmospheres with inappropriately low oxygen and/or low/high carbon dioxide) can still influence development of these disorders, ameliorate or delay in some cases or result in greater disorder expression in other cases. The interplay between fruit position and nutrition, and its responses to seasonal temperature changes

influences the appearance of different physiological disorders of this type such as, for example, *watercore* and *bitter pit*. The dysfunction in carbohydrate physiology influences the development of *watercore* (Marlow & Loescher, 1984) the presence of which is strictly connected to development of *internal breakdown*. Advanced ripening associated with warm storage temperature and *watercore* gives rise to *senescent breakdown*. The incidence of *bitter pit* is associated directly or indirectly with unbalanced calcium nutrition of the fruit during development (Ferguson & Watkins, 1989). Another physiological disorders associated with mineral imbalance that develops after fruit packing is *lenticel breakdown* (Curry 2002; Kupferman, 2005).

The second class of disorders is influenced by the post-harvest conditions applied for long term storage of apples: low temperature, low O_2 and/or high CO_2. Low temperature disorders can also be connected to the condition of the fruit at harvest. Responses to altered gas concentrations during storage may also be associated with maturity, cropping factors and skin gas diffusion properties (Ferguson *et al*., 1999). Gas-related disorders include external and internal CO_2 injuries and low O_2 injuries which depend on advanced fruit ripening and cool weather late in the growing season as pre-harvest and pre-storage factors. Disorders associated with long-term cold storage are for example *core browning*, *soft scald* and *superficial scald*.

Below a list of the main physiological disorders of apples is given with a description of symptoms and of the corresponding inductive pre- and post-harvest factors.

Watercore

Watercore is a physiological internal disorder that occurs before harvest while fruit are on the tree, in many varieties close to harvest, in others, instead, 4-6 weeks before harvest. Apple cultivars differ in *watercore* expression. Cultivars such as Red Delicious, Fuji, Jonathan, Jonagold or Granny Smith are susceptible while Golden Delicious and Cortland are less susceptible (Yamada *et al*., 1994). *Watercore* is associated with advanced fruit ripening and appears as translucent liquid-soaked tissue initially located around the vascular bundles and nearby flesh but it can extend from the core towards the skin surface (Marlow and Loescher, 1984)(shown in Figure 3A, from left to right panels). When the

disorder affects the skin it may become visible externally as translucent skin blotches on lighter pigmented apples or as very dark patches in darker fruits (Figure 3B).

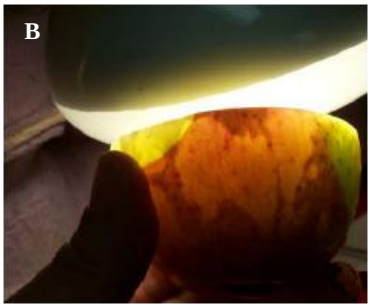

Figure 3 – A. Granny Smith affected by *watercore* at three different stages. In the early stage (left panel) *watercore* affects the tissue around vascular bundles and, at a later stage, may affect the core flesh progressively extending outwards (central and right panels) (from http://postharvest.ucdavis.edu/produce_information/Fruit_Physiological_Disorders/Apple_Watercore); **B.** Apple skin affected by *watercore*, externally visible through the appearance of translucent blotches (from http://www.apples.msu.edu/pdf/BeaudryDisordersCAClinic10.pdf).

The affected areas are characterized by a decrease in starch content and a corresponding accumulation of soluble sugars, in particular sorbitol, in intercellular spaces. Susceptible cultivars may be differently affected by *watercore*. For example, in Red Delicious the development of *watercore* is usually associated with tissue damage, since the soaked tissue undergoes anaerobiosis and cells subsequently break down during storage (Marlow & Loescher, 1984). Instead, Fuji apples seem to tolerate high levels of *watercore* without developing internal disorders during storage (Watkins *et al.*, 1993a), and inner *core browning* only occurs in severely affected fruits (Fukuda, 1984), especially during controlled atmosphere (CA) storage. Different strategies of CA have been tested to decrease the development of *watercore*. However, since the market requires apples harvested with the optimum color and blush, in order to satisfy these requirements Fuji

apples are normally harvested having *watercore* symptoms (Watkins *et al*., 1993a). Even if *watercore* is a sign of good ripening and indicates that fruit have more sugars, it is not desirable and may evolve into more severe internal disorders such as *internal breakdown*, *core browning* and *CO_2 injury* (Kweon *et al*., 2013). The severity of w*atercore* changes from season to season, with an increase in years in which apples ripen earlier. Apples of the same cultivar subjected to different growing environments and climates show different severity of symptoms (Harker *et al*., 1999). Most authors agree on that *watercore* is related to the changes in membrane integrity associated with ripening, which would account for both the accumulation of fluid in the intercellular spaces and the elevated sorbitol concentrations (Marlow & Loescher, 1984; Ferguson *et al*., 1999). Temperature directly affects *watercore* development on the tree (Yamada *et al*, 1994). Low temperatures during fruit ripening can exacerbate *watercore* associated with late harvest and advanced fruit maturity (Yamada *et al*., 1994). Low temperature accelerates leaf senescence and this may cause the movement of leaf storage sugars (primarily sorbitol) to the fruits to initiate *watercore* symptoms. Exposure of fruit to high temperatures on the tree, before fruit ripening can also induce *watercore* (Faust *et al*., 1969), probably by hastening ripening. A central role in the development of this disorder is played by the fruit position in relation to water and nutrient supply, particularly low calcium concentrations (Sharples, 1967; Perring, 1968) which can affect cell walls, membranes and the functioning of enzymes. Calcium may also be involved in earlier development of the disorder (Bowen & Watkins, 1997). Moreover fruits undergoing *watercore* generally have higher amounts of precursors and activities of enzymes involved in the ethylene biosynthetic pathway producing more ethylene than fruits not developing symptoms (Wang & Faust, 1992). In fact it was seen that application of exogenous ethylene induces *watercore* development (Greene *et al*., 1977). However, Fuji apples, which have a high incidence of *watercore*, have a relatively low ethylene production (Bowen & Watkins, 1997). Cold storage often recover tissues from mild *watercore* symptoms but at the same time in some cultivars can cause flesh browning and breakdown (Marlow & Loescher, 1984).

Storage-related disorders

The shelf-life and maintenance of quality of apple fruits can be extended significantly through storage at low temperature (lower than 5°C, usually between 0.5 and 1.5°C) and in controlled atmosphere (CA)(in which oxygen is lowered and carbon dioxide increased to delay senescence, generally to 5% or lower and 1-3% or higher, respectively). However, different disorders can develop during storage which may depend on low O_2 and/or high CO_2 and/or on low temperature, or on their respective combinations, and which are generally defined as storage-related disorders. The development of these disorders can be complex. Storage affects skin or pulp and can bring to development of "overlapping" symptoms which can make it difficult to recognize the indusing factors since they can be induced either by single stresses or combinations of CA and/or low temperature stresses. For example, *internal flesh browning* (*FB*) can occur with at least three different manifestations (De Castro *et al*., 2007) depending on the inductive factors: a) diffuse *FB*, which appears to be related to a chilling injury and comprehends *internal browning* and *low temperature breakdown* (Bramlage *et al*., 1980; James *et al*., 2005); b) radial browning which is related to *senescent breakdown* (Wilkinson & Fidler, 1973) and called *core browning*; c) CO_2-induced injury, associated with CA storage, that is also known as *brownheart* (Lau, 1998). Below a brief description is given for the major storage-related disorders, providing an overview of pre- and post-harvest inductive factors.

Senescent breakdown

Senescent breakdown in apples is correlated with over-ripening or over-storage. It appears in early storage when fruits are picked over-ripe, cooled too slowly or held at an insufficiently low temperature, and occurs in fruits stored for too long. This disorder affects all cultivars, even though with different susceptibility between them. It is correlated with low calcium content before harvest and during storage (Dewey *et al*., 1981) and it has been reported that a decrease in calcium content may precede senescent breakdown development (Saks *et al*., 1990). Even if it is difficult to distinguish between different forms of *senescent breakdown* only from visual symptoms, it is possible to characterize the disorder when flesh becomes soft and subsequently mealy, brown and dry (Figure 4A). In advanced stages

also skin is affected, with the calyx-end becoming dull and dark (Figure 4B). In Gala apples the disorder generally affects the tissue layers immediately below the skin, spreading all around the fruit before progressing inward (Figure 4C). The symptoms are aggravated by high humidity (Snowdon, 2010). Using a rapid and prompt cooling and low humidity inside storage rooms it is possible to prevent *senescent breakdown*. In recent years the disorder is becoming less common, probably thanks to the greater attention paid to picking fruits at the right ripening stage.

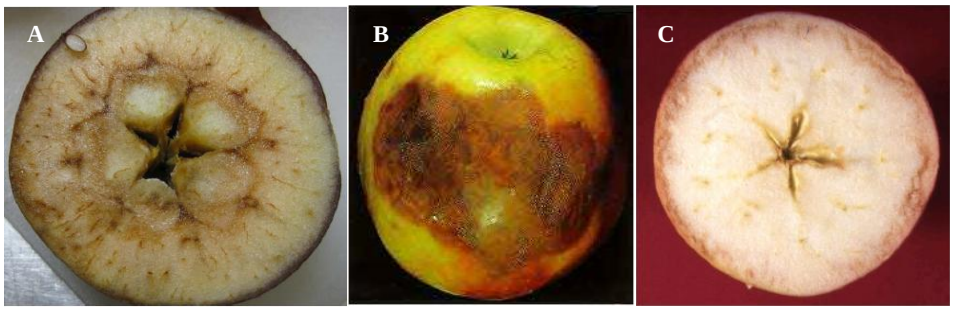

Figure 4 – A. Section of a Rome apple affected by *senescent breakdown* (modified from www.apples.msu.edu/pdf/BeaudryDisordersCAClinic10.pdf); **B.** Jonathan apple skin showing *senescent breakdown* symptoms (from http://postharvest.tfrec.wsu.edu/marketdiseases/internalbreakdown.html); **C**. cross section of a Gala apple with tissue below the skin affected by *senescent breakdown* (from http://apples.hdc.org.uk/disorders-flesh.asp).

Low oxygen injury

Low O_2 injury appears as a dark area within the apple skin, often associated with a discoloration of red or green regions of the skin. The symptoms vary with the oxygen level associated with the variety of apple and with the storage temperature. Long-term storage in adverse O_2 conditions induces development of symptoms. The susceptibility to this injury depends not only on cultivar but seems to be influenced also by pre- and post-harvest factors. The etiology of the disorder may involve increases in phenolic compounds during storage and may be also related to fruit ripening (Lougheed *et al.*, 1982). Apples with a severe *low O_2 injury* have a loss of flavor and strong fermented odor (http://postharvest.tfrec.wsu.edu/market/lowo2). If the odor is identified immediately at the beginning of its manifestation it can disappear changing storage conditions (Watkins *et al.*,

2009). McIntosh (Figure 5A) and Red Delicious (Figure 5B) apples are considered the most sensitive cultivars to this type of injury.

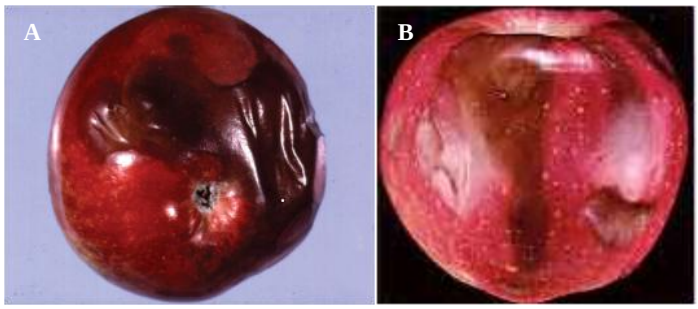

Figure 5 – McIntosh (**A**) (from http://apples.hdc.org.uk/disorders-skin.asp) and Red Delicious (**B**) (from http://postharvest.tfrec.wsu.edu/market/lowo2) apples affected by *low oxygen injury*.

Carbon dioxide injuries

CO_2 injuries can affect apple skin or flesh and are thus divided in *external* and *internal* CO_2 *injuries*, respectively. *External* CO_2 *injury* consists of wrinkled and bronze discoloration patches restricted to the skin surface which may join to form a unique big patch, often sunken, with defined edges (Blanpied *et al*., 1990)(Figure 6A). Symptoms are similar to those of *superficial scald* but, differently from *scald*, *external* CO_2 *injury* develops during early stages of controlled atmosphere and not after several months, moreover lesions caused by carbon dioxide are more sharply defined (Figure 6B). The risk of injury can be reduced maintaining low levels of CO_2 during the early period of storage, when symptoms have the highest probability to develop (Watkins *et al*., 1997a; Argenta *et al*., 2000; Fawbush *et al*., 2008). Otherwise *external* CO_2 *injury* can be prevented by delaying the application of CA storage after harvest or by using treatments with diphenylamine (DPA)(Watkins *et al*., 1997a; Wang *et al*. 2000; Fawbush *et al*., 2008), but not with the inhibitor of ethylene perception 1-methylcyclopropene (1-MCP)(DeEll & Prange, 1993; Watkins & Nock, 2004). It was proposed that DPA acts as a free radical scavenger (Yeh *et al*., 2003; Whitaker, 2004) suggesting that an oxidative reaction may be involved in development of CO_2 injury. McIntosh, Bramley's Seedling and Empire apples are prone to develop external CO_2 symptoms, particularly when treated with 1-MCP (DeEll *et al*., 2003; Fawbush *et al*., 2008). Susceptibility varies within the same cultivar, and increases when

fruits are harvested immature (Watkins & Liu, 2010). One of the latest works has shown the possibility to predict *external CO_2 injury* through studying changes in transcriptome and DNA methylation in Empire apples treated with DPA or 1-MCP (Gapper *et al*., 2012).

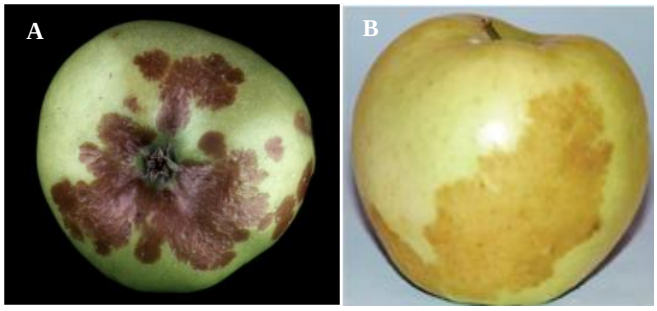

Figure 6 – Bramley (**A**) (from http://apples.hdc.org.uk/disorders-skin.asp) and Golden Delicious (**B**) (modified from http://entomology.tfrec.wsu.edu/Cullage_Site/Physiol_CO2.html) apples showing *external carbon dioxide injury* symptoms.

Internal CO_2 injury affects apple flesh and is also known as *brownheart*. Like *external CO_2 injury*, it is associated with abnormally high concentrations of carbon dioxide in the storage atmosphere, that initially causes the formation of brown necrotic areas in the cortex tissue around the vascular bundles (Figure 7). The tissue is firm and moist, than, after some weeks' of storage, it becomes dry, moisture is lost and cavities appear in the flesh.

Figure 7 – Cross section of an apple affected by *brownheart*: brown tissue around the vascular bundles and cavities in the flesh are visible (from http://entomology.tfrec.wsu.edu/Cullage_Site/Physiol_CO2.html).

Fruits affected by *brownheart* don't show any symptoms externally thus seem normal (Melville, 1963). When storage rooms are opened and affected fruits are cut a fermentation odor is perceived. Fuji apples are susceptible to develop *brownheart* during CA storage (Park & Lee, 1991). The injury appears during the first weeks of storage (Argenta *et al*, 2002a). The risk that symptoms may develop increases when rapid CA procedures are used or when the treatment with DPA, which has a protective action, is not performed (Watkins *et al*., 1997b). The mechanism by which CO_2 causes the injury in not understood. It was seen that accumulation of succinate in Bramley's Seedling apples exposed to high concentration of CO_2 (15kPa) may lead to the development of *brownheart* (Hulme, 1956). Elevated CO_2 concentrations were shown to induce an increase of acetaldehyde and ethanol contents in Red Delicious and Jonathan apples (Clijster, 1965; Smagula *et al*., 1968), products of anaerobic metabolism which accumulate as a consequence of the inhibition of pyruvate decarboxylase and the induction of alcohol dehydrogenase exerted by CO_2 stressful concentrations (Ke *et al*.,1995). In Fuji apples the development of symptoms was reported to be related with increased membrane permeability and oxidation of phenolic compounds (Choi, 1997). Even if the accumulation of acetaldehyde often correlates with CO_2 injury, probably it is not the direct cause (Argenta *et al*., 2002a). DPA treatment, which prevents the development of *brownheart* symptoms, reduces the accumulation of acetaldehyde and ethanol (Argenta *et al*., 2002a). While *external CO_2 injury* appears to be more severe in early harvested fruits (Meheriuk, 1977), *internal CO_2 injury* develops *vice versa* in apples harvested at an advanced stage of ripening (Volz *et al*., 1998, Elgar *et al*., 1999). In Fuji apples the incidence of *brownheart* is associated with *watercore*. Apples that have a severe *watercore* have low intercellular air space volume with a significant reduction of gas diffusion and an increase in internal CO_2 partial pressure (Argenta *et al*., 2002b). Delaying CA establishment or CO_2 accumulation decreases the incidence of *brownheart* and prevents *watercore* but may increase the probability that *brown core* (described in the next paragraph) may develop (Argenta *et al*., 2000). Apples prone to develop *brownheart* should be cooled to storage temperature before carbon dioxide is allowed to accumulate in CA rooms. However there is not a simple relationship between carbon dioxide and susceptibility to *brownheart*. Harvest maturity, speed of cooling, temperature of storage and severe *watercore* together with other factors, such as cultivar,

season and orchard, affect susceptibility of apples fruits to CO_2 injury (Carne, 1950; Lau, 1998; Elgar *et al.*, 1999).

Variability in development of CO_2 and also of O_2-injuries has been associated with a different respiration rate and gas permanence of the apple peel which cause an increase of internal CO_2 and a decrease of internal O_2 partial pressures (Park *et al*., 1993).

Brown core

Brown core is a form of flesh browning related to *senescent breakdown* (Lougheed *et al*., 1978), but it was proposed that this physiological disorder depends also on low temperature in storage rooms (Smock, 1946) and high levels of carbon dioxide (Scott & Wills, 1976). It is common in Granny Smith, Cox's Orange Pippin, Fuji, Bramley's Seedling, Braeburn and McIntosh apples. It develops only after a long period of cold storage and becomes more severe at room temperature (Argenta *et al*., 2000). It is characterized by affected tissues forming a partial or complete circle of yellow-brown discolored tissue surrounding the core (Wilkinson & Fidler, 1973), that becomes moist and soft, and may extend up to just below the skin (Figure 8A). It is not visible outside (Smock, 1946), except for McIntosh apples that may show brown skin and flesh at the stalk-end (Lougheed *et al*., 1978) (Figure 8B). In Fuji apples *brown core* resembles *senescent breakdown* because the discoloration of affected tissues starts from the outer portion of the cortex and then appears in vascular tissues (Argenta *et al*., 2001).

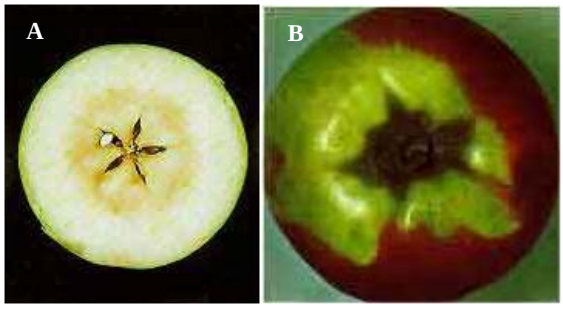

Figure 8 – A. Cross section of a Granny Smith apple in which *brown core* symptoms appear: a circular yellow discoloration is visible around the core flesh (modified from http://postharvest.tfrec.wsu.edu/market/browncore); **B.** McIntosh apple skin affected by *brown core* (from http://postharvest.tfrec.wsu.edu/market/browncore).

Susceptibility varies within the same cultivar depending on region, maturity and season in addition to adverse CA stored conditions. Generally *brown core* is aggravated by early harvesting, high water loss during storage and cool summer temperatures, but in some countries, such as Australia, fruits grown in warmer areas are more affected than fruits grown in cooler districts. *Brown core* can be prevented by harvesting apples at optimal ripening and storing them preferably with low oxygen (less than 2%) and low carbon dioxide (Little *et al*., 1985).

Internal browning

Internal browning is a low-temperature-induced storage disorder that affects only some cultivars such as Yellow Newton, Yellow Bellflower or Red Delicious apples. In the last case it is associated with *watercore* (DeEll, 2009). It affects fruits grown in a cool and cloudy or foggy climate lacking sunshine during the growing season and it develops in apple fruits stored at low temperature (0-1°C) for several months (http://postharvest.tfrec.wsu.edu/marketdiseases/internalbrowning.html).

This physiological disorder shows a diffuse browning of the flesh that appears firm, without a definite outline, but it can be confined to the core area and doesn't occur in the vascular tissue (James *et al*., 2008)(Figure 9). Symptoms can be observed only when the fruit is cut. To prevent the occurrence of *internal browning* it is possible to reduce CO_2 concentrations (1%) in combination with higher storage temperature (James *et al*., 2005; James *et al*., 2008).

Figure 9 – Apple core tissue showing typical symptoms of *internal browning* (from http://postharvest.tfrec.wsu.edu/marketdiseases/internalbrowning.html)

Low temperature breakdown

Low temperature breakdown varies according to storage conditions and cultivars. Susceptible varieties are Cox's Orange Pippin, Starking Delicious, McIntosh, Jonathan and Bramley's Seedling apples. In some cases *low temperature breakdown* shows symptoms similar to *senescent breakdown*, but flesh appears browner, firmer and moister than the dry tissue found in *senescent breakdown* (Watkins *et al*., 2009; Snowdon, 2010). Browning diffuses towards the outer cortex and doesn't occur in the zone near the skin which remains almost normal in the early stages (Figure 10). Even if initially the core tissue is not affected, at later stages the vascular strands become dark brown (Snowdon, 2010).

Figure 10 – The apple flesh in *low temperature breakdown* appears brown and doesn't affect the tissue near the skin (from http://www.storagecontrol.com/documents/Storage%20Disorders%20of%20Apples.pdf).

In advanced stages skin becomes waterlogged and discolored. This disorder is caused by low temperature (generally below 2 or 3°C) and varies between cultivars and growing conditions. The time of exposure to cold significantly influences the development of symptoms of *low temperature breakdown*. Short periods at low temperature will not cause the physiological disorder. However cool growing seasons, especially during the last part that precedes harvest, and late picking increase the probability that susceptible cultivars undergo this type of *internal browning*. Prediction of risk is possible and it is based on climatic conditions during growing season and mineral composition of the fruit at harvest. Susceptibility is related to low calcium (Perring, 1985) and low phosphorous content (Webster & Lidster, 1986) in the affected tissue. It is also associated with larger fruit size, and higher levels of humidity and carbon dioxide in storage rooms (Snowdon, 2010). To prevent the development of *low temperature breakdown* apples need to be stored at temperatures above the critical value. Within the same cultivar, in different countries, this

critical value changes. This depends not only on climate but also on mineral content, that can be influenced by treatments with phosphate and calcium compounds (Perring, 1985; Webster & Lidster, 1986).

Bitter pit

Bitter pit is a physiological disorder which affects cortical flesh and skin in apples. It usually develops after harvest during storage but sometimes bitter pit like symptoms can occur also on tree (Ferguson & Watkins, 1989; Snowdon, 2010). It is characterized by small necrotic lesions, located mostly near the calyx end, which are probably caused by loss of functionality of the plasma membrane (Fuller, 1980). The affected tissue becomes dehydrated and can collapse forming dark and depressed spots in the skin (Ferguson & Watkins, 1989)(Figure 11).

Figure 11 – Golden Delicious apple showing *bitter pit* symptoms. Spots appear lightly sunken, large and diffuse with irregular edges. Under the peel, the flesh is darkened and corky (from http://entomology.tfrec.wsu.edu/Cullage_Site/Physiol_BP.html).

It was proposed that calcium acts as a primary factor in the development of *bitter pit*, attributing the induction of this disorder to fruit calcium-deficiency. In fact, the recognition of calcium as an important factor in the development of *bitter pit* is based on two experimental evidences: 1) a relationship exists between the calcium content in the fruit flesh at harvest and the incidence of *bitter pit* after storage; 2) the incidence of this disorder is attenuated when treatments with calcium are applied during fruit growth (Ferguson & Watkins, 1989). Larger fruits are more subjected to develop *bitter pit* rather than small fruits probably because in larger fruits a "dilution" of calcium content takes place during

growth (Raese, 1988). Consistently, *bitter pit* incidence has been associated with an excessive shoot growth which causes a competition between shoots and fruits for use of available calcium (Garman & Mathis, 1956; Terblanche *et al*., 1980). Application of plant growth regulators which may reduce calcium content of fruits (also indirectly through stimulation of vegetative activity) such as gibberellins, causes an increase of *bitter pit* incidence (Stahly & Benson, 1976). *Vice versa* the use of regulators which induce an increase of calcium content by inhibiting gibberellins biosynthesis, such as paclobutrazol, may cause the reduction of *bitter pit* development (Lee *et al*., 1985; Greene, 1986; Luo *et al*., 1989; Saure, 1996). It was also proposed that gibberellins may play a direct role in *bitter pit* development, by increasing membrane permeability and enhancing fruit size (Pauls *et al*., 1982; Pharis & King, 1985). It must be noted, however, that some authors have questioned the role of calcium as the primary factor involved in the development of *bitter pit* symptoms. Perring & Jackson (1975) found that calcium concentration is unrelated to fruit mass, with *bitter pit* occurring in small apples at low calcium concentration as well as in large fruits with high calcium concentration, and *bitter pit* may not necessarily and always occur in fruits where calcium levels are quite low (Perring, 1986).

There are several pre-harvest factors which influence *bitter pit* development. These include genetic aspects (cultivar), climate and soil, mineral nutrition and orchard management (Saure, 1996). Early harvest generally results in higher risk of *bitter pit* (Ferguson & Watkins, 1989), even though ripening-related differences in terms of susceptibility to the disorder cannot be explained by differences in calcium content (Ferguson *et al*., 1993). Besides calcium (Ca^{2+}), mineral nutrients such as potassium (K), magnesium (Mg) and nitrogen (N), and the ratio between Ca^{2+} and K or Ca^{2+} and Mg, may affect *bitter pit* development (Fukumoto, 1985). The involvement of these nutrients seems to be associated with an effect on calcium availability rather than to a direct action of nutrients. The excess of N influences fruit size and reduces fruit/shoot ratio and may be somehow related to calcium distribution within the tree, as mentioned previously (Faust & Shear, 1968). Cultivars susceptible to *bitter pit* are York Imperial, Early Victoria, Baldwin, Golden Delicious or Granny Smith, while others such as Gala, Fuji or McIntosh are not susceptible. Golden Delicious apples are more susceptible to *bitter pit* in the USA rather than in

Australia, while Cox's Orange Pippin is highly susceptible in New Zealand but not in UK (Ferguson & Watkins, 1989).

As far as post-harvest (storage) conditions are concerned, rapid pre-cooling and low oxygen concentrations reduce *bitter pit* (Eksteen *et al*., 1977). Humidity in storage chambers doesn't influence *bitter pit* or rather favors calcium movement inside the fruit (Lidster *et al*., 1977). During fruit growth as well as after harvest treatments with calcium compounds inhibit *bitter pit*, to some extent, that may otherwise appear during the first one or two months of cold storage (de Freitas *et al*., 2010).

The mechanism by which calcium may regulate this physiological disorder is largely uncharacterized. It is hypothesized that calcium is involved on one hand in stabilizing the structure of cellular membranes by binding phospholipids and integral proteins and, on the other hand, in stabilizing pectin-protein complexes in the middle lamella (Dey & Brinson, 1984; Hirschi, 2004), thus calcium deficiency may increase membrane permeability leading to collapsing of cells associated with consequent pit formation. Cells need free apoplastic calcium directly available to maintain the structure of plasma membrane. A depletion of this calcium store affects the stability of plasma membrane leading to *bitter pit* development. The apparent contradiction that *bitter pit* may appear differently and erratically in fruits with comparable total contents of calcium could be thus explained by a different subcellular distribution of this element, for example by its movement from the apoplast to storage organelles (Saure, 2005). A recent work by de Freitas *et al*. (2010) has addressed some of these aspects. The authors have suggested that movement of calcium is allowed by the activity of calcium ATPases and Ca^{2+}/proton antiporter proteins (called CAXs). CAXs use the membrane's electrochemical gradient energy generated by H-pyrophosphatase (PPase) to pump calcium inside the vacuole. Pectin methyltransferase (PME) also affects the amount of free calcium available through the demethylation of pectins and the consequent formation of carboxyl groups which can bind calcium ions (Ralet *et al*., 2001). The reduction of free apoplastic calcium may derive from PME activity and may result in an increase of membrane permeability and *bitter pit* incidence (de Freitas *et al*., 2010). Later during storage, enzymes involved in the degradation of pectins can release calcium into the apoplast reducing *bitter pit* development (de Freitas *et al*., 2010). In Granny Smith apples an increase in the expression of PPase was found in the outer

cortical tissue at the calyx end of pitted fruits (de Freitas *et al*., 2010) suggesting a possible increase in activity of CAX proteins which move calcium inside the vacuole. Moreover in pitted fruits a higher expression of PMEs and a higher degree of pectin deesterification were found which can potentially enhance calcium deficiency (de Freitas *et al*., 2010). These results can explain the depletion of calcium content at the apoplastic level associated with membrane breakdown and to subsequent *bitter pit* development (de Freitas *et al*., 2010).

Lenticels blotch

Lenticels blotch is a physiological disorder related to *bitter pit*, principally differing from the latter for it consists of brown lesions on the apple skin beginning from lenticels (Fidler *et al*., 1973)(Figure 12). As for *bitter pit*, it occurs in fruits with abnormally low calcium content. This disorder affects apples from cultivars like Bramley and Cox's Orange Pippin.

Figure 12 – The image shows *lenticels blotches* occurring all over the surface of a Cox apple (from http://apples.hdc.org.uk/disorders-skin.asp).

Lenticels breakdown

Lenticels blotch shouldn't be confused with *lenticels breakdown* which affects Gala and Fuji apples and appears after packing. *Lenticels breakdown* is characterized by darkened or black lenticels and small brown spots (Figure 13). It doesn't affect fruits before packing. The risk of incidence increases in fruit with mineral imbalance (high levels of potassium, magnesium and nitrogen, and low level of calcium). *Lenticels breakdown* affects differently apples from different orchards, and its causes are not known (Curry, 2002; Kupferman, 2005 and 2009). A storage period that doesn't exceed 4 months seems to prevent the development of *lenticels breakdown* after packing in Gala apples.

Figure 13 – *Lenticels breakdown* appears as small brown spots on Gala apples skin after a period of cold storage that exceeds 4 months and after packing (from Kupferman, 2005).

Soft scald

Soft scald is a skin affecting disorder, occurring only in some cultivars including Jonathan, Rome Beauty, Honeycrisp, Golden Delicious and Red Delicious. In some cases may also damage the hypodermal tissue (DeEll, 2009) and be climate-related (Watkins *et al*., 2009). It is characterized by brown lesions sharply defined in the apple peel which are smooth and slightly sunken and generally localized to the equatorial parts of the fruits (Brooks & Harley, 1934)(Figure 14).

Figure 14 – Honeycrisp apple affected by *soft scald*. Skin has sharply defined brown and sunken lesions (from http://www.omafra.gov.on.ca/english/crops/facts/05-047.htm).

Soft scald occurs after a period of storage at low temperature, and over mature fruits seem to be more affected. DPA may reduce the incidence of the disorder in Golden Delicious apples (Watkins *et al*., 2009). A delay between picking and cooling seems to increase the development of symptoms (Gerhardt & Sainsbury, 1952) inducing the climacteric rise in respiration (Snowdon, 2010). The incidence of *soft scald* is also linked to pre-harvest factors like cool wet summers and large fruits (Snowdon, 2010).

Superficial scald

Superficial scald is a chilling-related injury that affects apple skin after a prolonged storage at low temperature (Watkins *et al*., 1995). It can arise after 2-4 months of cold storage with an increase in severity when apples are removed from storage and are leaved at room temperature (Rudell *et al*., 2005). *Superficial scald* appears as a darkened area due to necrosis of hypodermal cells (Bain & Mercer, 1963)(Figure 15). It never occurs on the tree and its development can be divided into four different stages (Bramlage, 1988). During the first stage changes occur in the fruit that create the potential for scald incidence and spans the 6-8 weeks of storage. This period is probably crucial for applying measures to control *scald*. During the second stage which comprehends the next 5 to 8 weeks changes continue to occur but even if *scald* does not yet appear it is too late to prevent its development. Only after these period *superficial scald* starts to develop slowly (third stage). The last stage is characterized by a rapid development of *scald* during post-storage (Bramlage, 1988).

Figure 15 – Granny Smith apple showing *superficial scald* symptoms: the skin appears with dark areas occurring after cells death (from http://postharvest.tfrec.wsu.edu/marketdiseases/ordinaryscald.html).

Oxidative stress occurring after a cold storage for several months leads to *scald* development (Du & Bramlage, 1995; Watkins *et al*., 1995; Rao *et al*., 1998; Watkins & Nock, 2005). Early in storage fruit accumulates at the skin level different volatiles such as esters, aldehydes, ketons and terpenes (Dimick & Hoskin, 1983). *Superficial scald* was associated with the accumulation of volatiles, in particular to the sesquiterpene α-farnesene. This compound can be oxidized during the storage period to a group of molecules called conjugated trienols (CT)(Paliyath *et al*., 1997)(Figure 16). These products may modify the

physiochemical properties of membranes of skin and hypodermal cells becoming the causative factor of *scald* development (Paliyath *et al*., 1997).

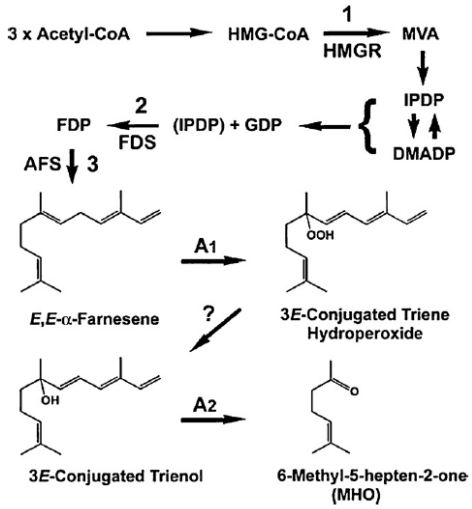

Figure 16 – A scheme of α-farnesene biosynthetic pathway and formation of its oxidative products in apple fruits: 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) is reduced by (1) hydroxymethylglutaryl-CoA reductase (HMGR) to mevalonic acid (MVA). Then isopentenyl diphosphate (IPDP) is condensed with GDP by (2) farnesyl diphosphate synthase (FDS) to form farnesyl diphosphate (FDP) and finally transformed to α-farnesene by (3) α-farnsene synthase (AFS). Step A1 and A2 represent autoxidation of α-farnesene to conjugated triene hydroperoxide and autoxidation of conjugated trienol to 6-methyl-5-hepten-2-one (MHO) respectively (from Lurie & Watkins, 2012).

In fact conjugate trienols seem to be toxic for the cell causing cell damage and death associated with brown or black discoloration (Bramlage, 1988). Conjugate trienols absorb at different length waves including 281nm and 258nm. The ratio between these two values has been associated with scald incidence: CT258/CT281 > 1 is negatively related to *scald*, vice versa CT258/CT281 < 1 is positively associated with *scald* development (Bramlage, 1988; Du & Bramlage, 1993). A close relationship between *scald* and conjugate trienols was not always found suggesting the involvement of other elements in the development of the disorder such as antioxidative molecules which may inhibit the oxidation of α-farnesene (Fernandez-Trujillo *et al*., 2003). However, the end product of α-farnesene oxidation, 6-methyl-5-heptene-2-one (MHO)(Figure 16), can be involved in the discoloration and death of hypodermal cells leading to *scald* development since treatment with exogenous MHO was shown to induce scald-like browing in peel tissue of susceptible apple cultivars (Mir &

Beaudry, 1999). Moreover at 20°C conjugate trienols autoxidize yielding MHO as the major product (Whitaker & Saftner, 2000). α-farnesene synthesis is mediated by ethylene (Watkins *et al*., 1993b; Ju & Curry, 2000): in apple peel tissue synthesis of α-farnesene derives from the mevalonic acid pathway (Ju & Curry, 2000) and the final enzyme involved in its biosynthesis is the α-farnesene synthase which is induced by ethylene, making the latter one the main hormonal regulator of *scald* (Rupasinghe *et al*., 2000; Pechous *et al*., 2005; Tsantili *et al*., 2007)(Figure 16). It is suggested that ethylene-induced α-farnesene is oxidized by reactive oxygen species (ROS) in the fruit peel leading to *superficial scald* development (Anet, 1972; Rao *et al*., 1998; Whitaker, 2004). It was found that α-tocopherol, an antioxidant molecule, can prevent *scald* and this suggests that free radicals are maybe involved in its development (Anet, 1974; Barden & Bramlage, 1994; Meir & Bramlage, 1988). ROS can cause lipid peroxidation, protein denaturation and DNA mutation (Elstner, 1987). Under normal conditions ROS are maintained at low concentrations to prevent cell damage. Cell protection is guaranteed by the action of antioxidant enzymes including catalases, peroxidases, ascorbate peroxidases, superoxide dismutase and gluthatione reductases. During fruit storage an increase in ROS production in response to low temperature stress can be associated with membrane disruption leading to chilling injury and death (Lyons, 1973; Prasad *et al*., 1994; Fernandez-Trujillo *et al*., 2003; Watkins & Rao, 2003). Anet (1972) found in *scald* resistant apples antioxidant levels sufficient to prevent oxidation of α-farnesene during storage. Since *superficial scald* derives from an oxidative stress its development can involve the antioxidant cell system (Du & Bramlage, 1995; Watkins *et al*., 1995; Rao *et al*., 1998). It has to be clarified whether compounds derived from α-farnesene oxidation are involved in *scald* induction or are byproducts of free radical reactions which are responsible for metabolic dysfunction and cell death (Whitaker *et al*., 2000). In fact, it was found by Rao *et al*. (1998) that α-farnesene and its oxidative products are high in susceptible apple cultivars at the beginning of storage but they are not elevated when apples show *scald* symptoms. The relationships between ethylene production and α-farnesene accumulation depend on cultivar but are also related to the storage temperature (Golding *et al*., 2001). Moreover, ethylene action in *scald* induction seems to be more related to its perception and signal transduction rather than to its biosynthesis, since Granny Smith apples, highly susceptible to this disorder, have low

levels of internal ethylene and high α-farnesene concentrations, while high ethylene and low α-farnesene levels are found in resistant cultivars, such as Golden Delicious (Golding *et al*., 2001). In Granny Smith apples showing *superficial scald* a lower production of MHO was found compared to the resistant cultivar Fuji (Fan *et al*., 1999) suggesting that MHO production is not sufficient alone to cause *scald* development.

Superficial scald is influenced by different pre- and post-harvest factors including cultivar susceptibility, fruit ripening at harvest, environmental conditions during growth, fruit mineral content, light exposure, storage conditions and ethylene action (Ingle & D'Souza,1989). McIntosh, Cortland, Granny Smith and Red Delicious cultivars are very susceptible to *scald* while Gala, Empire, Fuji and Golden Delicious have a low susceptibility (Paliyath *et al*., 1997). Immature fruit tend to have a higher risk of *scald* incidence than the over-mature ones. Excessive tree vigor or an inadequate pruning increase the susceptibility. Finally *scald* is influenced by climates with hot and dry summers (Bramlage, 1988).

Superficial scald annually causes the major economic loss to apple growers worldwide (Kuc *et al*., 1953). Different strategies are provided to successfully inhibit *scald* development. The most common techniques in use combine storage in controlled atmosphere (CA) with treatments with the antioxidant diphenylamine (DPA), or with 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception and the most effective molecule available to control *scald*. CA, by reducing ethylene biosynthesis, prevents *scald* development (Lau, 1990). Both low oxygen and high carbon dioxide have a positive effect on *scald* inhibition but, as previously mentioned, concentrations of CO_2 that exceed 5% cause the occurrence of other injuries in most cultivars. Thus the greatest benefit in CA derives from low O_2. Low O_2 within 1-2% inhibits the oxidation of α-farnesene (Whitaker, 2000). It is important to establish rapidly the CA conditions which vary between cultivars. The recommended CA conditions depend not only on cultivar but also on region, ripening stage, storage duration, season and interactions with environmental aspects (Thompson, 1998). CA does not block completely *scald*, especially when storage is prolonged. In Croatia *superficial scald* causes 15% of fruit loss even in CA storage (Jemric *et al*., 2006). Thus CA technique is always associated with treatment with chemical molecules to prevent this disorder. DPA has been the main way to control *scald* chemically for over 40 years

(Calvo, 2010a). DPA may block *scald* suppressing ethylene production (Lurie *et al*., 1989) but its primary action was shown to be the inhibition of α-farnesene oxidation preventing CTs accumulation (Whitaker, 2000; Rudell *et al*., 2005). Investigation of its metabolism in DPA-treated apples revealed the presence of molecules derived from a reaction between DPA and reactive oxygen species suggesting a possible activation of processes involved in ROS generation during apple storage to ameliorate the control of this disorder (Kim-Kang *et al*., 1998; Rudell *et al*., 2005). Inhibition of *scald* by DPA depends on concentration, cultivar, delay between harvest and treatment and storage temperature (Jung & Watkins, 2008). Treatment with DPA is done after harvest, by dipping apples in a solution containing the antioxidant. Excessive dosage can cause fruit injuries. In recent years it was considered toxic and potentially carcinogenic in mice (Calvo, 2010a) due to the high levels used (1500-2000 ppm depending on cultivar). Since 2011 the use of DPA has been banned in Europe for apple industries and the importation of DPA-treated apples from other States has been prohibited for human health concerns (Calvo, 2010a-b). In the last years 1-methylcyclopropene (1-MCP) has become the golden standard for prevention of *scald* in cv Granny Smith apples and in susceptible cultivars in general, as a consequence of DPA withdrawal but also because it ensures complete control of *scald*. 1-MCP is easy to apply and increases firmness, titratable acids and soluble solid content while delaying respiration rate (Fan *et al*., 1999). 1-MCP, known with its commercial name Smartfresh™ (AgroFresh, Inc.), completely blocks *scald* through the inhibition of ethylene perception, by binding to its receptors (Sisler & Serek 2003; Tsantili *et al*., 2007), at very low concentrations and after a short-time exposure (Sisler & Serek, 1999) and results in complete prevention of α-farnesene accumulation, by blocking α-farnesene synthase (Fan *et al*., 1999; Tsantili *et al*., 2007). Even if 1-MCP improves apple quality, the timing of treatment is a crucial issue since a delay between harvest and 1-MCP treatment can decrease the control of *scald* symptoms in cultivar like Cortland and Law Rome (Tsantilli *et al*., 2007). Moreover Smartfresh™ is expensive and not all markets accept apples treated with this chemical molecule (Calvo, 2010c). Finally 1-MCP cannot be used with all cultivars because it may be implicated in the development of other physiological disorders such as *external CO_2 injury*.

A recently introduced practice to control *scald*, which is free of use of chemicals, is represented by ILOS, an initial low oxygen stress (storage at 0.4% O_2, for a few weeks), followed by CA storage (Wang & Dilley, 2000). In Granny Smith apples ILOS blocks *scald* symptoms probably reducing ethylene accumulation which leads to less α-farnesene formation and accumulation of its oxidative products (Scott *et al*., 1995; Sabban-Amin *et al*., 2011). Even if this approach allows to prevent *scald* physically without use of any chemical, apples treated with ILOS cannot be stored for long periods of time as for those treated with 1-MCP. Moreover different cultivars affected by *superficial scald* such as Red Delicious and Granny Smith have different tolerance to low oxygen stress, depending on seasonal aspects thus making it difficult to apply this technique reliably and requiring more research for its optimization (Zanella, 2003). Even if different strategies are provided to inhibit development of *superficial scald* and some physiological and biochemical aspects responsible for development of *scald* symptoms seem to be known, very little is known about the molecular factors involved in the inductive phase of *superficial scald*.

Superficial scald and oxidative stress

Superficial scald develops after a cold storage for several months and a short period at room temperature which leads to oxidative stress (Du & Bramlage, 1995; Watkins *et al*., 1995; Rao *et al*., 1998; Watkins & Nock, 2005). Thus a brief overview of oxidative processes in plants is provided. Oxidative stress is a condition in which ROS are generated extra- or intra-cellularly (reviewed by Gill and Tuteja, 2010). ROS signaling strength depends on four factors: rates of production, ROS production site, rates of removal and presence of receptors that feel changes in ROS homeostasis (reviewed by Foyer & Noctor, 2009). Once stress is perceived at the apoplastic level it can provoke variation in intracellular calcium concentration that leads to specific calcium signatures (Nomura *et al*., 2012) and generation of ROS in different subcellular compartments which act as signal amplifiers (reviewed by Shapiguzov *et al*., 2012). The location of organelles close to plasma membrane may facilitate signal communication (Rivero *et al*., 2009) and changes in spatial arrangement are driven by the specific kind of stress (reviewed by Suzuki *et al*., 2012). However how the signal is transmitted from the apoplast to the organelles is still unknown. ROS are produced continuously under normal conditions at low concentrations

as byproducts of metabolic pathways in different cellular compartments such as photosynthesis in chloroplasts, respiration in mitochondria, photorespiration and fatty acid oxidation in peroxisomes and glyoxysomes respectively (Figure 17)(Foyer, 1996; Polle, 2001; Del Rio *et al*., 2006; Navrot *et al*., 2007).

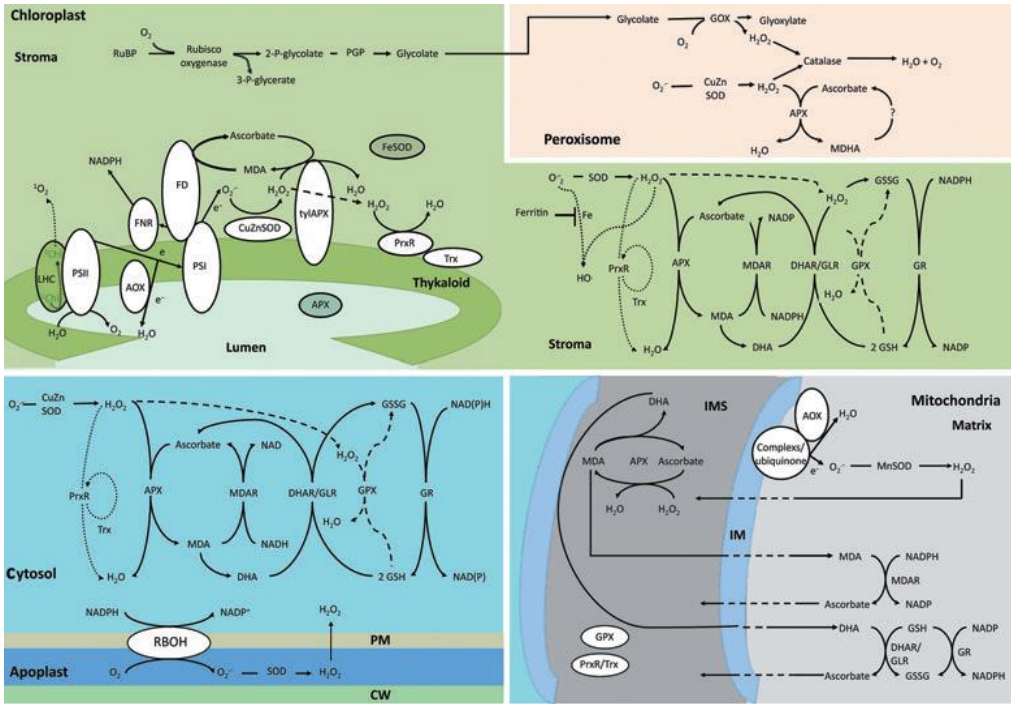

Figure 17 – Different subcellular localization of ROS scavenging pathways in plant cells. Abbreviations: DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; FD, ferredoxin; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; IM, inner membrane; IMS, inner membrane space; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; PSI, photosystem I; PSII, photosystem II; Trx, thioredoxin; tyl, thylakoid (from Miller *et al*., 2010).

All these organelles have developed specific scavenging ROS systems but when ROS accumulate at high concentrations they can cross the organelle membrane and reach the cytosol (reviewed by Suzuki *et al*., 2012). ROS cannot regulate nuclear genes but probably their oxidative damage on proteins cause a proteolytic break leading to the formation of peptides which contribute to retrograde signaling between organelles, nucleus and apoplast (Møller & Sweetlove, 2010). Cytosol becomes a sort of point of contact where cross-talk between divergent ROS signals occurs (reviewed by Suzuki *et al*., 2012). Reactive oxygen

species derive from the excitation of O_2 and comprehend radical ($O_2^{\cdot -}$ superoxide radical, $OH^{\cdot}$ hydroxyl radical, $HO_2^{\cdot}$ perhydroxy radical and $RO^{\cdot}$ alkoxy radical) and non-radical molecules (H_2O_2 hydrogen peroxide and $^1O_2$ singlet oxygen)(Figure 18)(reviewed by Gill & Tuteja, 2010). They can act in a dual manner during stress: on one hand they are used by cells as indicators of stress and become second messengers leading to stress-response signal transduction pathways, on the other hand an over accumulation of ROS is toxic and cause cell death (reviewed by Dat *et al*., 2000).

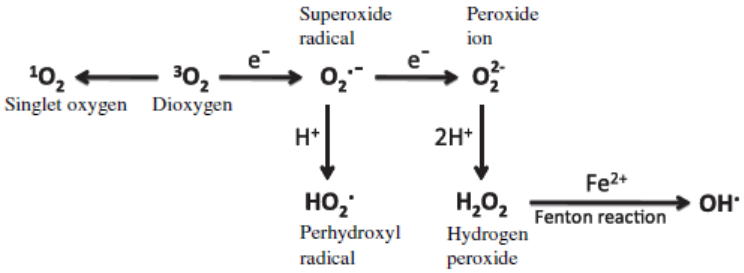

Figure 18 – scheme of generation of different reactive oxygen species (from Gill & Tuteja, 2010).

High levels of ROS damage cellular and organelle membranes causing lipid peroxidation which leads to the formation of products from polyunsaturated fatty acids such as malonyldialdehyde (MDA)(Blokhina *et al.*, 2003; reviewd by Gill & Tuteja, 2010). The aldehyde derivate products can react with proteins or DNA causing cellular damages (reviewed by Gill & Tuteja, 2010). Plant responses to changes in ROS homeostasis depend on ROS nature, signal intensity, ROS production site, plant developmental stage and interaction with other signal molecules such as nitric oxide or plant hormones (Gechev & Hille, 2005). Overall ROS are used as signal molecules for several reasons (reviewed by Mittler *et al*., 2011):

- Cells can produce and scavenge rapidly different form of ROS, thanks to an arsenal of ROS metabolizing enzymes encoded by the "ROS-gene network" (Mittler *et al*., 2004);
- ROS can be produced in a specific subcellular localization causing a specific signal and allowing a specific spatial control (Mittler *et al*., 2011);

- ROS can be auto-propagated cell by cell for long distance throughout the plant (Miller *et al.*, 2009; reviewed by Suzuki *et al*., 2013);
- ROS allow the activation of other cellular pathways through variations, for example, in internal calcium concentration (Kobayashi *et al*., 2007; Ogasawara *et al*., 2008; Suzuki *et al*., 2013);
- ROS are connected to cellular homeostasis. Changes in the homeostasis of cells cause changes of the steady-state level of ROS (Mittler *et al*., 2011).

H_2O_2 compared to $O_2^{\cdot -}$ or $^1O_2$ is considered the best ROS messenger molecule for its relative stability, in fact it has a longer life (1-2 ms instead of 2-4 μs), and it can cross membranes through specific aquaporins called peroxiporins (Neill *et al*., 2002; Bhattacharjee, 2005; Foyer & Noctor, 2005; Bienert *et al*., 2007). Intensity, duration and localization of ROS signals depend on the interplay between ROS-producing and ROS-scavenging enzymes (reviewed by Mittler *et al*., 2004). As far as the ROS scavenging system is concerned, plant cells have developed different antioxidant systems, which include the exploitation of antioxidant molecules such as ascorbate (AsA) and glutathione (GSH) and antioxidant enzymes, involved in the detoxification of ROS during stress. Ascorbate and glutathione are found in their reduced form in the apoplast and inside the cell, in chloroplasts, mitochondria, peroxisomes, vacuoles and in the cytosol (reviewed by Dat *et al*., 2000; Asada, 2006), but they can undergo a reversible oxidation. The balance between reduced GSH and its oxidized form (GSSG) is a signal of the redox state of the cell and transmits information to target molecules which may include transcription factors or metabolic enzyme (May *et al*., 1998). Electron transfer between glutathione and a target protein is mediated by glutaredoxins (GRXs)(Meyer *et al*., 2007). Glutathione is a potential scavenger of different types of ROS including $^1O_2$, H_2O_2 and $OH^{\cdot}$. Moreover it contributes to regenerate AsA. ROS scavenging enzymes belonging to the ROS-gene network comprehend superoxide dismutase (SOD), ascorbate peroxidase (APX) and enzymes involved in the Halliwell-Asada cycle, glutathione peroxidase (GPX), peroxiredoxin (PrxR) and catalase (CAT)(Noctor & Foyer, 1998; reviewed by Arora *et al*., 2002; reviewed by Mittler *et al*., 2004). In chloroplasts and mitochondria ROS production can also decrease through the action of alternative oxidases (AOXs). Dismutation of superoxide radical

formed in the apoplast by RBOH activity during stress perception into H_2O_2 is mediated by SOD (reviewed by Mittler *et al*., 2004). SODs are divided into three types on the basis of their metal cofactor: copper/zinc (Cu/Zn) SOD, manganese (Mn) SOD and iron (Fe) SOD, and they localize in different cellular compartments including mitochondria, chloroplasts, peroxisomes and in the cytosol (Alscher *et al*., 2002; reviewed by Mittler, 2002). APX and CAT have a different affinity for H_2O_2, within the µM and mM range, respectively, suggesting that the first enzyme is involved in the fine modulation of ROS for signaling while the second one can be responsible for removing exceeded ROS during stress (reviewed by Mittler 2002). APX removes H_2O_2 using AsA as the electron donor causing its oxidation in monodehydroascorbate (MDHA) and eventually in dehydroascorbate (DHA). AsA is regenerated in the Halliwell-Asada pathway from MDHA through the activity of monodehydroascorbate reductase consuming NADH or from DHA through the enzyme dehydroascorbate reductase which uses two molecules of GSH as the electron donor. The resulting GSSG is reduced by glutathione reductase (GR) using NADPH as cofactor (Figure 17). In the cytosol H_2O_2 is removed by GPX which oxidizes GSH to GSSG. Some GPXs seem to act as thioredoxin-dependent peroxidases (Herbette *et al*., 2002). PrxRs are found in chloroplast, mithocondria and cytosol and constitute a group of H_2O_2-decomposing antioxidant enzymes which use thioredoxins (TRX) as cofactors (Dietz *et al*., 2006). CATs are involved in the detoxification of H_2O_2 in the peroxisome (reviewed by Dat *et al*., 2000). H_2O_2 inactivates enzymes or creates protein damages oxidizing their thiol groups (Vranová *et al*., 2002). TRX, GRX and PrxR are involved in a reversible mechanism of oxidation and reduction of thiol groups.

As far as ROS production is concerned, plant homologs of the mammalian NADPH oxidase catalytic subunit $gp91^{phox}$, called RBOHs (Respiratory Burst Oxidase Homologs) are localized at the plasmalemma, are gaining significant attention as central players in ROS generation in the apoplast in response to biotic and abiotic stimuli (Mittler *et al*., 2011; Suzuki *et al*., 2013). They are involved in generation of $O_2^{\cdot -}$, which is rapidly dismutated in H_2O_2, in the apoplast during stress (Torres *et al*., 1998; Sagi & Fluhr, 2001; Torres *et al*., 2002). RBOHs have two EF-hand motifs, which bind Ca^{2+} ions, at the N-terminal and six transmembrane helices with two heme groups which are necessary for electron transport through the membrane to the extracellular acceptor O_2. Heme groups are

linked to the third and fifth transmembrane helices binding four histidine residues (Figure 19). RBOHs also contain a cysolic FAD- and NADPH-binding domains at the C-terminal (reviewed by Glyan'ko & Ischenko)(Figure 19). RBOH activity is regulated directly by Ca^{2+} likely through the EF-binding motifs or through the phosphorylation of the N-terminal mediated by Ca^{2+}-dependent protein kinases as well as interaction with ROPs (Kobayashi *et al*., 2007; Wong *et al*., 2007).

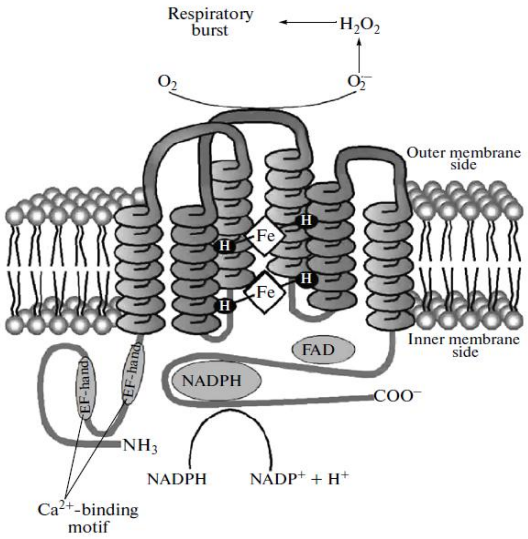

Figure 19 – Schematic representation of the structure of RBOH. EF-hand motifs at the N-terminal bind Ca^{2+} ions causing a conformational change. Transmembrane helices allow the transport of electrons to O_2. H-Fe-H bridges bind heme to histidine residues, $O_2^{\cdot-}$: superoxide, H_2O_2: hydrogen peroxide, FAD: flavin adenine dinucleotide; NADPH: reduced nicotinamide dinucleotide phosphate (from Glyan'ko & Ischenko, 2010).

Abiotic stresses induce an increase in intracellular calcium content which allows the activation of Ca^{2+}-dependent protein kinases that, by phosphorylating the N-terminal region of RBOHs, cause a conformational change. Such changes make RBOH prone to bind ROP-GTPases leading to RBOHs activation and to an enhancement of ROS production. RBOHs activity and generation of ROS induce a second accumulation of cytosolic calcium through the stimulation of Ca^{2+} channels at the plasma membrane (Wong *et al*., 2007)(Figure 20). Moreover RBOHs can be activated directly by interaction with phosphatidic acid (PA) generated by phospholipase Dα (PLDα)(Zhang *et al*., 2009), suggesting that several factors impinge on RBOHs regulation which appears to be a highly coordinated process. The phospholipase D (PLD) family is subdivided into 6 different subgroups in Arabidopsis (α,

β, γ, δ, ε and ζ) with different biochemical, regulatory and catalytic properties. PLDs are involved in the regulation of different cellular processes comprehending ABA signaling, programmed cell death, cold tolerance and other stress responses (Wang, 2005). PLDs hydrolyze phospholipids which form phosphatidic acids (PAs), important intracellular messengers in plants (Munnik, 2001). PLDα is the predominant isoform and it is activated by calcium. It presents at the N-terminal a C2-domain, the calcium/lipid-binding domain responsible for Ca^{2+} binding and Ca^{2+}-dependent activity, and two catalytic HxKxxxxD (HKD) motifs at the C-terminal which interact between each-other to promote lipase activity (Exton, 2002).

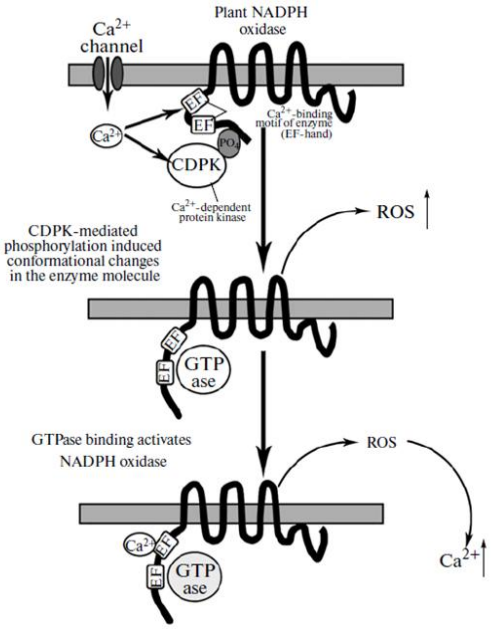

Figure 20 – Scheme of regulation of RBOH activity. Increase in cytosolic Ca^{2+} content allows the phosporylation at the N-terminal of RBOH causing a conformational change. ROP-GTPases binding activates the enzyme causing ROS generation. A second increase of intracellular Ca^{2+} is measured after ROS production by RBOH (from Glyan'ko & Ischenko, 2010).

ROPs (Rho Of Plants) are the unique group of small GTP-binding proteins belonging to the RHO family in plants, thus differing from the three subfamilies (Rho, Rac and Cdc42) characterizing the animal RHO GTPase family (Mackay & Hall, 1998). ROPs are encoded by a multigene families (Winge *et al.*, 2000; Velasco *et al.* ,2010) and display five conserved domains called G-box-motifs (G1-G5) which allow the GDP/GTP binding, an

insert region probably responsible for signal transmission and an hypervariable region at the C-terminal which targets the protein to specific membranes (Bourne *et al*., 1991). Moreover ROPs contain putative serine/threonine phosphorylation sites which may allow the binding whit RLKs (Trotochaud *et al*., 1999). ROPs can switch between an active GTP-bound conformation and an inactive GDP-bound form and this switch is strictly regulated by the action of guanine nucleotide exchange factors (GEFs) which catalyze the exchange between GDP and GTP. Activated ROPs become substrate for GTPase activating proteins (GAPs) which stimulate the GTP hydrolysis (reviewed by Berken & Wittinghofer, 2008). Guanine nucleotide dissociation inhibitors (GDIs) remove ROPs from membranes where GEFs are localized, thus preventing their activation and the GTP hydrolysis (Dovas & Couchman, 2005). ROPs for their particular capability to change from an active to inactive form are involved in different physiological roles such as root-hair elongation, pollen-tube growth, cell-shape formation, responses to hormones such as abscisic acid (ABA) and responses to abiotic stresses like low oxygen (Baxter-Burrell *et al*., 2002; Zheng *et al*., 2002; reviewed by Yang, 2002; Cheung *et al*., 2003). ROPs are probably involved in the transmission of extracellular signals both through the association with receptor-like serine/threonine kinases (RLKs)(Trotochaud *et al*., 1999)(Figure 21) and through the activation of RBOHs during oxygen deprivation (Baxter-Burrell *et al*., 2002) inducing generation of second messengers (Ca^{2+} and H_2O_2) which in turn regulate signaling cascades.

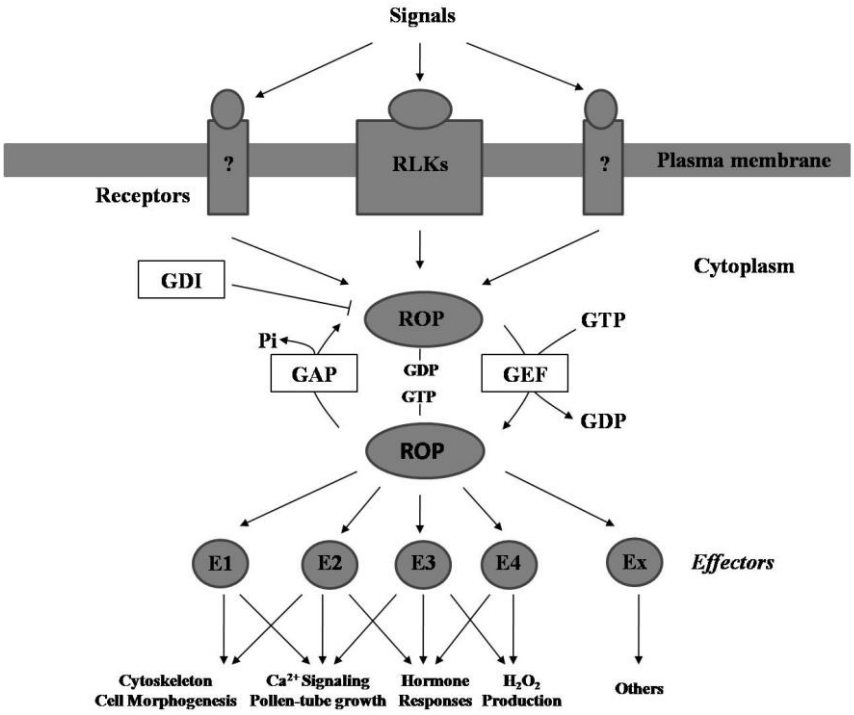

Figure 21 – Scheme of ROP signaling model. ROPs may act as a common switch in different cell physiological responses. Perception of signals at the plasmalemma, probably by RLKs and other unknown receptors, is transmitted through ROPs activated by GEFs to different effectors which give rise to specific signal responses. ROPs are then inactivated by GAPs which catalyze the hydrolyses of GTP (modified from Zheng and Yang, 2000).

As far as responses to O_2 deprivation are concerned, Baxter-Burrell *et al*. (2002) discovered that the ROP signal transduction pathway is stimulated and, concomitantly with an increase in cytolsolic free Ca^{2+}, activates an NADPH oxidase (RBOH) which in turn induces H_2O_2 generation. The H_2O_2 produced acts as a second messenger and allows the induction of the alcohol dehydrogenase (ADH) expression. The authors also showed that for plants to control low O_2 tolerance, cells need an attenuation of the ROP signal activity through a negative feedback regulation of ROP activity. In fact the H_2O_2 produced by the NAPDH oxidase stimulated the transcription of ROP-GAP4 negatively regulating ROP signaling (Figure 22). This finely tuned regulatory negative feedback loop between ROPs and ROP-GAPs was termed the ROP-GAP rheostat (Figure 22)(Baxter-Burrel *et al.*, 2002).

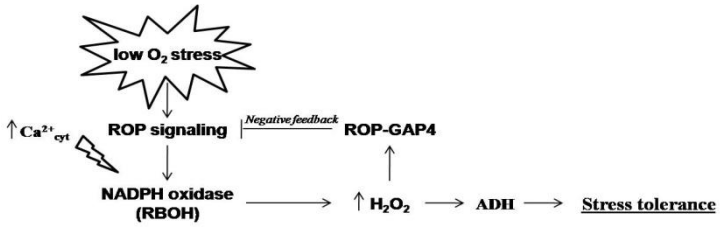

Figure 22 – Schematic view of the ROP-GAP rheostat involved in the low oxygen tolerance.

The ROP-GAP rheostat was studied only in oxygen deprivation conditions but it is possible that this mechanism may act in the presence of a range of abiotic stresses, as suggested by the authors. However this hypothesis was not further developed neither a hormonal regulation of the rheostat has been reported so far.

Aim of work

Aim of this work is to provide a preliminary characterization of the molecular factors putatively associated with superficial scald development, in an attempt to understand the early inductive factors involved in its etiology. To do so, apples from cultivar Granny Smith treated chemically, with DPA or 1-MCP, or physically, with ILOS, have been studied in time-course experiments conducted during storage. Since scald is associated with ethylene action and probably its development involves ROS generation and the cell antioxidant system, the relationships existing between ethylene, ROS homeostasis and scald induction were addressed by studying gene families involved in ROS homeostasis and indentifying genes putatively involved in ROS metabolism by adopting both targeted (qPCR) and untargeted (RNA-seq) analyses. In particular, apple superficial scald development may be used as a new model to evaluate the ROP-GAP rheostat function in cold stress. By understanding the molecular aspects underling scald development, it will be possible to predict or identify at harvest, before CA storage, which apple batches may probably develop the disorder allowing rational storage strategies with significant economic gains.

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Chapter II

Cold stress responses (scald) in apple (*Malus domestica*) fruits are associated with ethylene-dependent negative regulation of the ROP-GAP rheostat and disrupted apoplastic ROS homeostasis

Introduction

Cold stress represents one of the major environmental abiotic challenges for plants and results in severe crop losses both in the field and after harvest (Bray *et al*., 2000; Mahajan & Tuteja, 2005). Fruits are artificially subjected to prolonged cold stresses after harvest to extend their storage life and marketing period. Even though fruits tolerate exposures to low-nearly freezing temperatures for relatively long periods of time, they nevertheless undergo a number of cold-induced "physiological disorders", after a certain threshold of cold stress exposure is reached, thus becoming unmarketable (Faust *et al*.,1969; Lyons, 1973; Little *et al*., 1985; DeEll & Prange, 1993). Among cold-induced physiological disorders, apple scald, a chilling stress which is evoked in susceptible cultivars (e.g. Granny Smith, Red Delicious) after a minimum (one to three months) period of cold exposure (1-5°C) is reached (Watkins *et al*., 1995), can cause important losses and has gained significant research attention during the past years (reviewed by Lurie & Watkins, 2012). Manifest apple scald symptoms are represented by irregularly shaped necrotic areas on the fruit's surface, involving epicarp and hypodermal tissues immediately underneath (Bain, 1956; Bain & Mercer, 1963), and they take place when fruits are brought to ambient temperature for some days after having been exposed to cold. The development of these symptoms is thought to be brought about by oxidative phenomena involving the production of conjugated trienols (oxidative products of the sesquiterpene α-farnesene which accumulate during storage in response to cold) together with a burst of H_2O_2 production, finally leading to lipid peroxidation, cell membrane damage and cell death (Lurie & Watkins, 2012). The occurrence of apple scald can be prevented completely by treatments with the inhibitor of ethylene perception 1-methylcyclopropene (1-MCP), providing compelling evidence that scald is a truly ethylene dependent cold stress response (Fan *et al*., 1999; Rupasinghe *et al*., 2000; Watkins *et al*., 2000), while it can be partially controlled by the use of the antioxidant diphenylamine (DPA)(Smock 1955, 1957 and 1961; Lau 1990), reinforcing the hypothesis that oxidative processes are involved in scald development. Besides its economical importance, scald represents an excellent experimental system to model cold stress responses in fruits, since its inductive phase (exposure to prolonged cold stress) can be clearly distinguished and temporarily separated from the phase during which symptoms develop (permanence at ambient temperature)(Lurie & Watkins, 2012). Even though

several studies have focused on development of scald symptoms unraveling some important aspects of this process, the molecular factors responsible for the inductive events of scald are still poorly understood. Many studies have attempted to link the oxidative burst taking place during scald development with the de-regulation of enzymes involved in scavenging of reactive oxygen species (ROS)(Zubini *et al.*, 2007; Du & Bramlage, 1995). However, such studies did not point to a clear relationship between regulation of antioxidant enzyme activities and scald development. Furthermore, no reports have studied in depth the regulation of ROS homeostasis during the inductive phase of scald and, in general, of cold stress responses in plants, despite the fundamental role played by ROS as signaling molecules in several abiotic stress responses adaptation as recently reviewed by Suzuki *et al*., 2012, Shapiguzov *et al*., 2012, Baxter *et al.*, 2013, and Sierla *et al*., 2013. The fine tuning and control of different ROS levels at different subcellular locations can evoke and control local and/or systemic adaptation responses (Baxter *et al.*, 2013; Suzuki *et al*., 2013), thus the balance between ROS production and scavenging, through metabolizing enzymes, plays a major role in this scenario. Recent studies have shown that the superoxide ($O_2^{\cdot -}$) producing enzyme NADPH oxidase (RBOH, Respiratory Burst Plant Homologue) has been identified as a key pivotal element in regulating ROS production and homeostasis during adaptation to several environmental stresses including drought, heat and high light (Suzuki *et al.*, 2013; Mittler *et al.*, 2011; Suzuki *et al.*, 2011). Besides, the fine regulation of the NADPH oxidase activity and, as a consequence, of ROS homeostasis has been shown to be subjected to a tightly regulated feedback control, through the so called ROP-GAP rheostat, and to be essential for defining the capacity of Arabidopsis plants to adapt to low oxygen availability (Baxter-Burrell *et al*., 2002). Based on the latter data, it was hypothesized that this may be a general conserved regulatory hub for plants' adaptation to different types of abiotic stresses but no studies have further pursued this direction and additional evidence in support of hypothesis is lacking. More specifically, whether this regulatory strategy is conserved in the adaptation of plants to cold stress is currently unknown. No data are at present available on a putative involvement of ROPs and RBOHs regulation in response to cold stress nor on the existence of a presumptive action of ethylene on the regulation of the ROP-GAP rheostat in abiotic stresses different from low oxygen. Ethylene is a major regulator of cold stress adaptation, with opposing effects depending on the plant species

being considered (Zhang & Huang 2010; Shi *et al.*, 2012). In Arabidopsis ethylene plays a negative role on cold stress adaptation and recent data have shown that it does so by direct binding of the transcription factor Ein3 to the promoter of several CBF cold stress genes (Shi *et al*., 2012).

In this work we aimed at answering these questions by identifying the essential elements of the *Malus domestica* (apple) ROP-GAP rheostat, and by studying their mode of expression during prolonged exposure to cold stress during storage and in relation to the development of a cold stress related physiological disorder, apple superficial scald. Genes encoding the apple RBOH family, responsible for generation of $O_2^{\cdot -}$ in the apoplast, and the ROP machinery, including ROPs (Vernoud *et al*., 2003; Molendijk *et al*., 2004; Nagawa *et al*., 2010), ROP-GEFs (Berken *et al*., 2005; Gu *et al*., 2006), ROP-GAPs, ROP-GDIs (Berken & Wittinghofer, 2008), essential for the function of the ROP-GAP rheostat, have been identified and their expression has been studied in response to cold stress. We show for the first time that ethylene negatively regulates the ROP-GAP rheostat in response to cold exposure and that this negative regulation is associated with the disruption of apoplastic ROS homeostasis. Our data suggest that the ethylene-dependent control of the ROP-GAP rheostat may be a central pivotal element in cold stress resistance in plants and, possibly, of scald development in apple fruits.

Materials and methods

Sequence identification and analysis

A. Thaliana ROPs (Vernoud *et al*., 2003), ROPGEFs (Berken *et al*., 2005; Shin *et al.*, 2009; Riely *et al.*, 2011), ROPGAPs (Wu *et al*., 2000), ROPGDIs (Bischoff *et al*., 2000), RBOHs (Torres *et al*., 1998) and PLDα (Elias *et al*., 2002; Qin & Wang, 2002) where used as BLASTP queries against *Vitis vinifera*, *Oryza Sativa* and *Populus trichocarpa* sequences present in the Ensembl Plants database (http://plants.ensembl.org/Multi/blastview; http://dx.doi.org/10.1093/nar/gkr895) to retrieve putative orthologous genes. Apple orthologs were identified by means of the BLAST tool provided in the apple genome database (http://www.rosaceae.org; Velasco *et al.*, 2010) using Arabidopsis, grape, rice and poplar genes as queries. Sequences were aligned using CLUSTALX (Jeanmougin *et al*., 1998) and the presence for the typical conserved domains for each gene family was checked, with bootstrapping analysis on the basis of a CLUSTALX alignment (Jeanmougin *et al*., 1998). Rooted phenetic trees for each family were generated by the neighbor-joining method and displayed by MEGA version 5 (Kumar *et al*., 2008).

Plant material and treatments

Apple fruits (*Malus domestica*) cv. Granny Smith, were harvested in Trentino Alto-Adige region (Italy) during the 2009-2010 and 2010-2011 seasons. Batches of apples were treated or not with 625 ppm/m^3 1-methylcyclopropene (1-MCP; Rohm and Haas, Mozzate, Italy) or diphenylamine 2000 ppm (w/v)(DPA, Sigma-Aldrich, Milan, Italy) and stored in controlled atmosphere (CA - 0.8% O_2, 0.8% CO_2, and at 1°C). Samples were taken at four different time points: at harvest (T0) and after one (T1), three (T2) and six months (T3) of storage. After storage the development of scald symptoms was scored, and determination of internal ethylene production was evaluated (not shown). Apple peels were excised with rasor blades, immediately frozen in liquid nitrogen and stored at -80°C for total RNA extraction and ascorbate, glutathione, malonyldialdehyde and H_2O_2 content analyses. Different apple tissues (petals, leaves, anthers, seeds, whole flowers, little fruits) of cv. Gala were collected during April 2011 at the experimental farm of the Univerisity of Padova (Legnaro, Agripolis) and stored at -80°C. For ethylene treatments, apples of cv.

Granny Smith were collected in september 2012 and treated with 100 ppm of ethylene or kept on air (as a control) for 4h and 24h at 20°C in sealed glas jars under continuous flushing. At each time point peels were excised, immediately frozen with liquid nitrogen and stored at -80°C. For ILOS experiments apple of cv. Granny Smith were harvested in Trentino Alto-Adige region (Italy) during the 2010-2011 season. Batches of apples were stored at 1°C and at three different oxygen concentrations: 20% (normoxic), 0.8% (ultra low oxygen ULO) or 0.4% (initial low oxygen stress ILOS). Samples were taken at different time points for each thesis: after one, two, three, four, five weeks of storage and, finally, after eight weeks of storage. One day before the fifth week apples stored at 0.4% oxygen (ILOS) were moved to 0.8% oxygen (ULO). Apple peels and flesh tissues were sampled (three replicates for each thesis with three different apples for each replicate) and stored at -80°C.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from apple peel and different tissues and reverse transcribed as described by Nonis *et al*. (2012). qPCR analysis were conducted as reported by Nonis *et al*. (2012). In order to discriminate between genes with high sequence homology, primers were constructed on the most divergent regions considering also putatives 3' untranslated region (UTR), on the basis of multiple sequence alignments performed with CLUSTALX (http://www.ebi.ac.uk/Tools/clustalw/index.html). PolyA-tail was predicted using HCpolyA (http://zeus2.itb.cnr.it/~webgene/wwwHC_polya.html; Milanesi *et al*., 1996). Primers (Table S1) were designed with Primer3 web-tool (http://frodo.wi.mit.edu/primer3/; Rozen & Skaletsky 2000) and for sequences having high degrees of identity, the PRaTo tool was used to pick the best selective pairs (http://prato.daapv.unipd.it; Nonis *et al*., 2011). All primers were tested with Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/OligoCalc.html; Kibbe 2007) and specificity was confirmed by melting curve analysis. Five ng of reverse transcribed total RNA was used as template for each reaction. Experiments were performed in duplicate. Data were elaborated with DataAssist Software version 2.0 (Applied Biosystems, Monza, Italy) and normalized to Md_8283:1:a (Botton *et al*. 2011) using the Livak & Schmittghen (2001) method.

Chemicals and reagents

Thiobarbituric acid (TBA), malondialdehyde tetrabutylammonium salt (MDA, purity > 98%), diethylenetriaminepentaacetic acid (DTPA), butylated hydroxytoluene (BHT), potassium phosphate and sodium acetate were purchased from Sigma-Aldrich Italy (Milan, Italy). Sodium phosphate and HLPC grade acetonitrile, methanol and ethanol were purchased from Carlo Erba Reagenti (Milan, Italy). Ultrapure water was obtained by means of a Pure-Lab Option Q (Elga Lab-Water, High Wycombe, UK) apparatus. TBA reagent was prepared as a 0.2% (w/v) TBA solution in 0.1 M sodium acetate buffer containing 1 mM DTPA, pH 3.5 (Fukunaga *et al.*, 1995 and 1998), stored up to 2 months (4°C) with light shielding. BHT was dissolved in methanol at a final concentration of 2 mM. The stock solution of MDA (5 mM, stable up to 1 month at 4°C) was prepared by dissolving 40 mg of MDA tetrabutylammonium salt in 25 ml of water:ethanol (60:40, v/v; Lärstad *et al.*, 2002). The MDA working standards were freshly prepared daily by diluting stock solution with water to of 0.1, 0.5, 1.0, 5.0 and 10.0 nmol/ml final concentrations.

For determination of H_2O_2, the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit from Invitrogen™ (Molecular Probes, Eugene, USA) was used. Amplex Red reagent was dissolved in DMSO for a final concentration of 10 mM as described by the manufacter (stable up to six month at -20°C with light and air shielding, Zhou *et al.*, 1997). HLPC grade acetonitrile and perchloric acid were purchased from Carlo Erba Reagenti.

The working standards were freshly prepared daily by diluting 3% H_2O_2 with 50 mM sodium phosphate pH 7.4 to reach H_2O_2 final concentrations of 0.1, 0.5, 1.0, 2.5 and 5.0 nmol/ml.

Determination of Malonyldialdehyde, H_2O_2, Ascorbic acid and Glutathione content

For the determination of *MDA*, a TBA-MDA adduct was measured with high-performance liquid chromatography (HPLC) technique. Frozen apple peels (0.2 g) were ground to a powder under liquid nitrogen and were homogenized in 1:25 (g/ml) 80:20 (v/v) ethanol:water, followed by centrifugation at 3000 g for 10 min at 4°C (Hodges *et al*. 1999). The supernatant was centrifuged again at 13000 rpm for 10 min at 4°C and then filtered through a 0.45 µm micro spin filter tube at 3000 g for 5 min at 4°C.

Samples were prepared from the method described by Lärstad *et al.* (2002): 50 µl aliquots of filtered supernatant were added to 445 µl TBA and 5 µl of 2 mM BHT. The tubes were capped, mixed for 1 s and derivatisation was performed in a water bath at 95°C for 60 min. After cooling in an ice bath for 5 min, the samples were allowed to adapt to room temperature, vortexed centrifuged at 13000 rpm for 10 min at 4°C. The supernatant was transferred into vials and HPLC analysis was performed at ambient temperature.

For the determination of H_2O_2, Resorufin, a product deriving from the oxidation of the Amplex Red reagent (10-acethyl-3,7-dihydroxyphenoxazine) in presence of H_2O_2 by horseradish peroxidase (HRP), was measured by HPLC. Frozen apple fruit peel (0.2 g) were ground to a powder in liquid nitrogen and homogenized in 1 ml of 50 mM sodium phosphate buffer pH 7.4, and after 5 min on ice were centrifuged at 10000 g for 10 min at 4°C (Pilati *et al.*, 2007; Rao *et al.*, 2000). The supernatant was filtered through a 0.45 µm micro spin filer tube at 3000 g for 3 min at 4°C. 50 µl of working reaction (100 µM Amplex Red reagent and 0.2 U/ml HRP in 50 mM sodium phosphate buffer pH 7.4) were added to 50 µl of filtered supernatant. The samples were mixed and the reaction was led in a water bath at 30°C for 30 min under dark conditions (Shin *et al.*, 2005), then blocked with 100 µl of a stop reaction (10 mM HCl and 4 mM BHT dissolved in ethanol). After vortexing, samples were centrifuged at 13000 rpm for 10 min at 4°C before HPLC analysis.

HPLC analysis was performed using a Hewlett-Packard series 1100 HPLC system equipped with degasser, quaternary pump, autosampler and multiple wavelength detector (Agilent, formerly Hewlett-Packard GMBH, Germany). Chromatographic data were collected and integrated by means of Hewlett-Packard ChemStation software (version A.06.03). For determination of MDA, chromatographic conditions were: column: Simmetry

Shield RP8 (4.6 x 250 mm, 5 µm, Waters); mobile phase: 10 mmol/L sodium acetate (pH 4.5)/acetonitrile (80:20, v/v, solvent A) and acetonitrile (solvent B); elution program: isocratic elution with 100% solvent A from start to 15 min, followed by 5 min of linear gradient from 100% to 50% solvent A and from 0% to 50% solvent B, and additional 5 min of isocratic elution with 50% solvent A and 50% solvent B; stop time of 5 min; flow rate 1 ml/min; injection volume 80 µl; column temperature 30°C; pressure 134 bar. TBA-MDA adduct was monitored by fluorescence detection, with excitation at 532 nm and emission at 553 nm. Under the above described conditions the retention time of the adduct was 8.7 min. For determination of H_2O_2 chromatographic conditions were: column: Simmetry C-8 (4.6 x 250 mm, 5 µm, Waters); mobile phase: 10 mmol/L potassium phosphate (pH 7.0)(solvent A) and methanol/acetonitrile (75:25, v/v, solvent B); isocratic elution with 55% solvent A and 45% of solvent B for 11 min; flow rate 0.8 ml/min; injection volume 30 µl; column temperature 40°C; pressure 135 bar. Resorufin was monitored by fluorescence detection, with excitation at 560 nm and emission at 585 nm. Under above conditions retention time of the adduct was 4.5 min.

For ascorbate and glutathione contents frozen peel samples (0.25 g) were ground with a mortar and pestle to extract soluble antioxidants with 0.1 NHCl and 1 mM EDTA. Following centrifugation at 10000 g for 10 min the ascorbate content was rapidly determined spectrophotometrically by measuring the absorbance at 265 nm, according to the Hewitt and Dickes method (1961). Glutathione was measured through HPLC according to Masi *et al*. (2002) method. 50 µL of supernatant were added to a mixture composed of 117 µL of potassium borate buffer (1 mol/L pH 10.5), 33 µL of a freshly-prepared tri-n-butyl phosphine solution (1% in water) and 33 µL 7-fluoro 2,1,3-benzooxadiazole-4-sulfonate (SBD-F) fluorophore (Sigma-Aldrich, St. Louis, USA)(0.3% in water). The mixture was then in C). The test tubes were transferred to an ice bath and derivatisation was terminated by adding 17 µL of 12% HCl. HPLC analysis was performed using a Perkin Elmer Series 400 pump, equipped with a Gilson auto-injector (Mod 234 Gilson, France) and a Jasco 821 FP spectrofluorometer (Jasco, Japan). Data were acquired and processed with the ChromCard board and software (Carlo Erba, Italy). Chromatographic conditions were: column RP C18 column (250 mm×4.6 mm I.D., 5 µm particle size; Luna, Phenomenex, USA); mobile phase: 75 mM $NH4^+$-formiate

(pH 2.9) containing 3% of methanol; flow rate: 1 mL/min; injection volume: 20 μL; column temperature: room temperature. Glutathione was monitored by fluorescence detection, with excitation at 386 nm and emission at 516 nm and identified by comparison with the retention times of standard compound. A calibration curve with concentrations in the range of 0.5 - 25 μmol/L was used to its quantification.

Localization of H_2O_2

Hydrogen peroxide was localized cytochemically on apples peels via determination of cerium perhydroxide formation after a reaction between $CeCl_3$ and endogenous H_2O_2 and visualization of electron-dense precipitates by transmission electron microscopy as described by Bestwick *et al*. (1997).

RNA-seq analysis and data processing

RNA of samples taken at harvest and after 1 or 6 months of storage, treated or not with 1-MCP (as described in plant material) was used for RNA-seq analysis. RNA samples were processed using TruSeq RNA-seq sample prep kit from Illumina (Illumina, Inc., CA, USA). Briefly, the poly-A containing mRNA molecules were purified using poly-T oligo-attached magnetic beads, fragmented into small pieces using divalent cations under elevated temperature, cDNA was synthesized by reverse transcription and standard blunt-ending plus add 'A' was performed. Then Illumina TruSeq adapters with indexes were ligated to the ends of the cDNA fragments. After ligation reaction and separation of not ligated adapters, samples were amplified by PCR to selectively enrich those cDNA fragments in the library having adapter molecules at both ends. Pools of 4 samples were loaded on Illumina flowcell and clusters created by Illumina cBot. Clusters were sequenced at ultra-high throughput on Illumina HiSeq2000 (Illumina Inc.) obtaining 35-40 millions of single-reads per sample, 50 bp long.

Raw data was processed using CLC-Bio Genomics Workbench software (CLC Bio, Denmark) to calculate gene expression levels based on Mortazavi *et al.*(2008) approach. As reference the CDS derived from Malus x Domestica genome sequencing (Velasco *et al*., 2010) and available at Phytozome depot (Phytozome internal release identifier: 196) were

used. All RNA-seq experiments were conducted by a third party external service (IGA Technologies Services, Udine, Italy).

Data Clustering

Hierarchical clusters were generated using the software R (R Core Team, 2013). Heatmaps were obtained with the package gplots (Warnes, 2012). Clustering was done in both linear and log-transformed data. RNA-seq data relative to control and 1-MCP treated samples stored for 1 or 6 months in CA were normalized on T0 data before to transform in logarithmic values.

Results

Identification and sequence characterization of gene families involved in the control of ROS homeostasis in apple: MdROPs, MdROP-GEFs, MdROP-GAPs and MdROP-GDIs, MdRBOHs and MdPLsDα

In order to identify the apple MdROPs (Vernoud *et al*., 2003), their accessory proteins ROP-GEFs (Berken *et al*., 2005), -GAPs (Wu *et al*., 2000) and -GDIs (Bischoff *et al*., 2000), and effectors MdRBOHs (Torres *et al*., 1998) and their regulators MdPLDα (Elias *et al*., 2002; Qin & Wang, 2002), respectively, sequences from *Arabidopsis thaliana* and those retrieved from poplar (Liu *et al*., 2010), grape (Abbal *et al*., 2007; Abbal *et al*., 2010; Liu *et al*., 2010) and rice (Li *et al*., 2007) were used as queries with the BLAST tool provided in the *rosaceae* database (http://www.rosaceae.org). Conserved domains typical of each family were identified on the predicted apple protein sequences. To select *bona fide* protein sequences, only those displaying the typical domains were kept for subsequent studies. The functional domains typical of the ROP family (GTPase (G1 and G3), GDP/GTP-binding (G4 and G5) and effector domains (ED), Rho insert region (RIR), putative serine/threonine-dependent phosphrylation motifs (SYR and SKK) and hypervariable region (HVR)(Zheng & Yang 2000; Jiang & Ramachandran 2006)) could be found in the apple MdROP sequences (Figure 1).

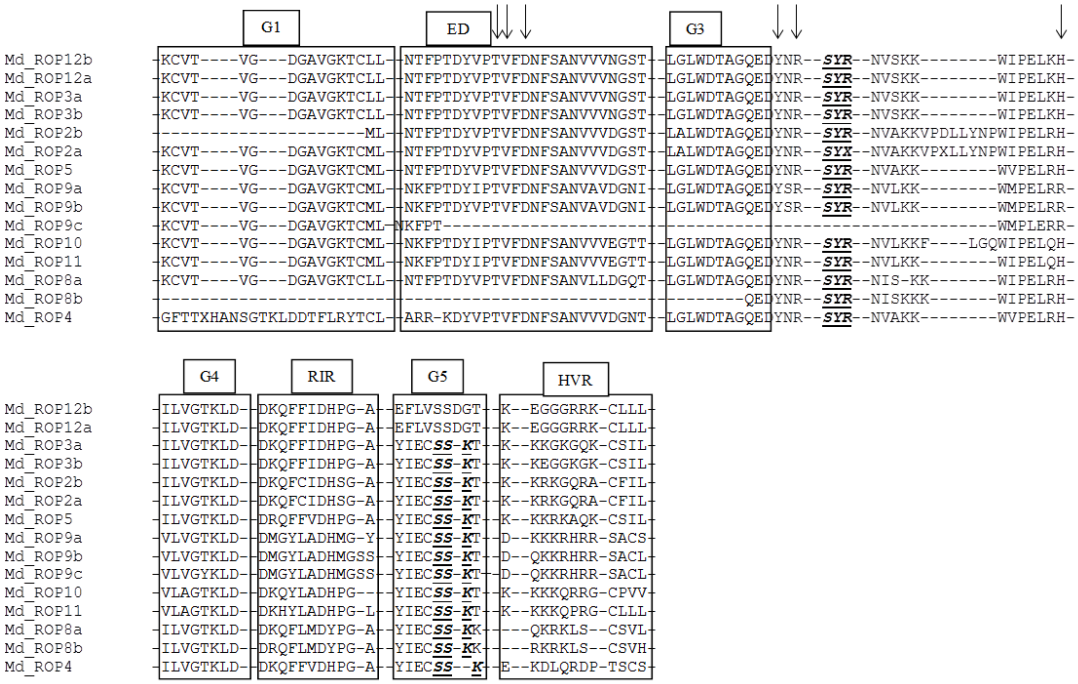

Figure 1 – Alignment of conserved domains of the apple ROP deduced protein sequences: GTPase domains (G1 and G3 boxes), GDP/GTP-binding domains (G4 and G5 boxes), effector domain (ED), Rho insert region (RIR), putative serine/threonine-dependent phosphorylation sites (motifs SYR and SSK, evidenced with bold underlined character) and the hypervariable region (HVR). Arrows show residues putatively involved in ROP/ROP-GDI interaction (Zheng and Yang, 2000).

MdROP-GEFs displayed the three PRONE (Plant-specific ROP Nucleotide Exchanger)(Berken *et al.*, 2005; Shin *et al.*, 2009; Riely *et al.*, 2011) domains (Figure S1), MdROP-GAPs had the Cdc42/Rac-interacting binding (CRIB) motif, the consensus sequence for the SCR homology domain 3-binding motif PXXXXPXXP and the GAP-like domain (Wu *et al.*, 2000)(Figure S2). MdROP-GDIs showed the GDI-like domain (Berken & Wittinghofer, 2007)(Figure S3). MdRBOHs had the typical trans-membrane domains (data not shown) in addition to the two N-terminal EF-hand and the nucleotide binding motifs (FAD-isoalloxazine binding site, motif 2), the NADPH-ribose and NADPH-binding sites (Keller *et al.*, 1998; Torres *et al.*, 1998; Amicucci *et al.*, 1999)(Figure S4). Finally, MdPLDα sequences showed the C2 domains, two HKD motifs and the putative PIP2-binding site (Qin & Wang 2002; Du *et al.*, 2013)(Figure S5). A bootstrap analysis for each family of interest was performed and a distance tree based on the neighbor-joining method for ROPs (shown in Figure 2), ROP-GEFs (Figure S6), ROP-GAPs (Figure S7), ROP-GDIs (Figure S8), RBOHs (Figure S9) and PLsDα (Figure S10) was produced to visualize

relationships between the apple deduced protein sequences, the known sequences from *Arabidopsis thaliana* and the sequences of poplar, grape and rice found in the Ensmbl Plants database (http://plants.ensembl.org/index.html). Examination of the resulting ROP tree showed the distribution of the fourteen MdROPs in the four groups defined by Zheng & Yang (2000)(Figure 2) and of ROP-GEFs in the five groups described previously by Riely *et al*. (2011)(Figure S6). On the base of these results, apple sequences were renamed according to the most similar orthologues in Arabidopsis.

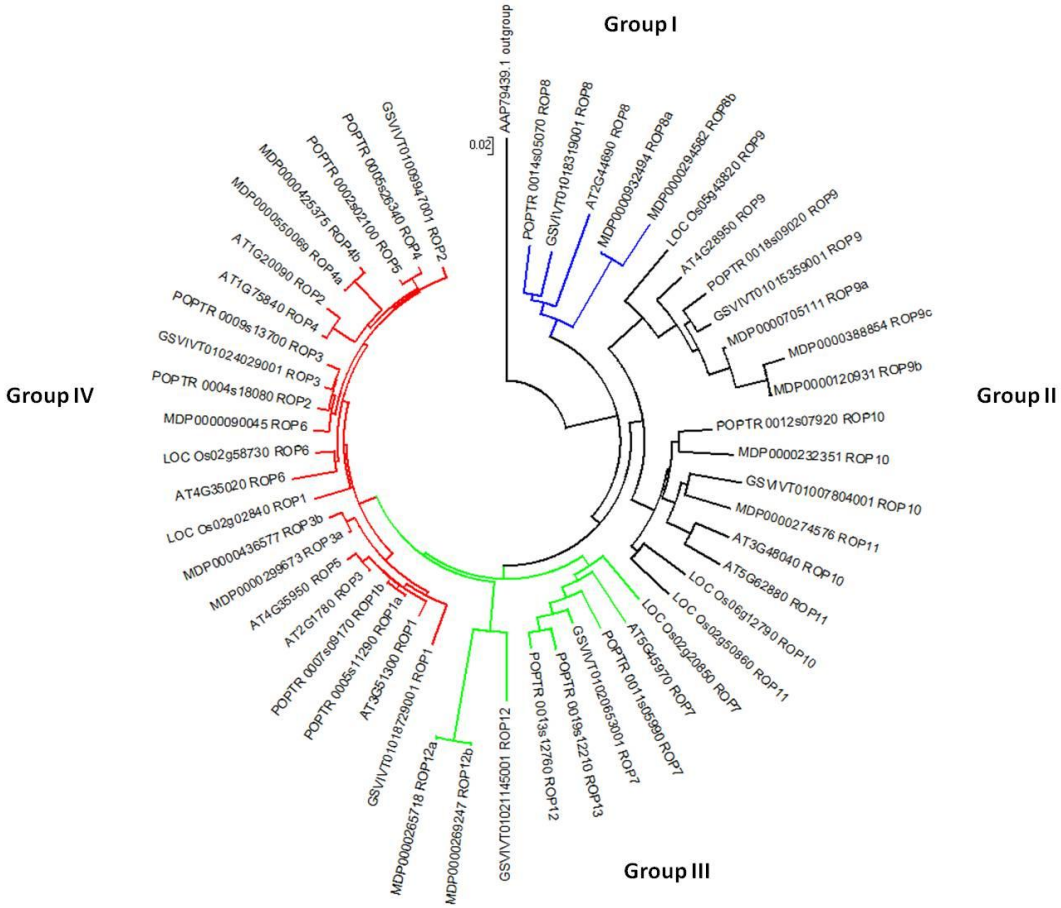

Figure 2 – Phenetic tree showing the relationships among the fourteen identified *Malus domestica* ROP sequences and those belonging to *A. thaliana*, *O. sativa*, *P. thricocarpa* and *V. vinifera* retrieved from the Ensmbl Plants database (http://plants.ensembl.org/index.html). Very short apple ROP sequences were excluded from the analysis. The phenetic tree was constructed by the neighbor-joining method with bootstrapping analysis on the basis of a CLUSTALX alignment (Jeanmougin *et al*., 1998) and was rooted on the *Trichomonas vaginalis* Rac1-putative protein as outgroup (AAP79439.1). The four groups of ROP sequences identified by Zheng & Yang (2000) are highlighted with different colors.

Analyses by real-time PCR on different tissues of cv Gala (leaves at three growth stages, whole flowers, petals, anthers, developing fruitlets and seeds) confirmed the expression of these genes and enabled the obtainment of a tissue-dependent expression clustering, pointing that MdROP3a, MdROP3b, MdROP-GEF11, MdROP-GEF13a, MdROP-GDI2, MdROP-GDI7, MdRBOHH, MdRBOHJ and MdPLDα2 are highly expressed in anthers compared to other considered tissues (Figure S11). On the whole, ten ROPs, fourteen ROP-GEFs, ten ROP-GAPs, seven ROP-GDIs, seven RBOHs and four PLsDα were identified in the *Malus domestica* genome as expressed genes encoding proteins with the typical conserved domains of each respective family (Table S2). These numbers, are consistent with the number of genes found in other species (shown in Table 1)(details for all genes and proteins identified in apple are reported in supplementary Tables S3-S8).

Table 1 – overview of expressed genes encoding ROPs, ROP-GEFs, ROP-GAPs, ROP-GDIs, RBOHs and PLsDα from different species including those identified in apple in this work**.**

	ROP	**ROP-GEF**	**ROP-GAP**	**ROP-GDI**	**RBOH**	**PLDα**
A. thaliana	11	14	6	3	10	3
M. domestica	10	14	10	7	7	4
V. vinifera	8	7	4	4	7	4
P. thricocarpa	12	16	8	6	10	4
O. sativa	6	11	7	3	9	8

Expression analyses of the ROP gene machinery on apple epidermal and hypodermal tissues in relation to prolonged cold stress and to ethylene action

To test whether the ROP machinery and the ROP-GAP rheostat may be involved in the regulation of cold stress responses in apples (and, by extension, in general in cold stress in plants), the expression of the identified genes was studied in the epicarp and hypodermal tissues (peels) of apples of the scald susceptible cv Granny Smith, subjected to one, three or six months of cold stress at 1°C in controlled atmosphere (CA), as described in materials and methods. Granny Smith apples are known to become susceptible to develop cold stress responses (apple scald) after a lag period of 2-3 months of cold stress exposure (Lurie & Watkins, 2012, and references therein). This stress, and the occurrence of symptoms (scald), can be prevented completely by blocking ethylene perception by 1-MCP treatment or, partially, by using the antioxidant molecule diphenylamine (DPA)(Lurie & Watkins, 2012). Thus apples that had been treated with 1-MCP or DPA before cold exposure were considered for this analysis to pinpoint the relationships existing between ethylene action, ROP gene regulation and development of apple scald. After six months of storage, 97% of untreated apples underwent scald development, while this percentage was reduced to 7.4% in apples treated with DPA and almost completely prevented (0.3%) in 1-MCP treated apples (Table 2).

Table 2 – percentage of fruits cv Granny Smith healthy, rotten, pitted, burnt, whitered and scalded after 6 months of storage during 2009/2010 season in controlled atmosphere (0.8% O_2, 0.8% CO_2) at 1°C.

	Healthy fruit	**Rotten fruit**	**Bitter pit**	**Burns**	**Whitering**	**Superficial scald**
Control	2.40%	0.30%	0.00%	0.30%	0.00%	97.00%
1-MCP	94.20%	0.00%	0.00%	5.50%	0.00%	0.30%
DPA	90.00%	0.40%	0.00%	2.20%	0.00%	7.40%

Apple peels were first characterized through the analysis of expression of the ethylene biosynthetic genes, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (MdACS) (Sabban-Amin *et al.*, 2011) and oxidase (MdACO)(Dal Cin *et al.*, 2005), and of the markers of scald development α-farnesene synthase (MdAFS)(Lurie *et al.*, 2005; Sabban-

Amin *et al.*, 2011) and polyphenol oxidase (MdPPO)(Boss *et al.*, 1995 and Sabban-Amin *et al.*, 2011). In peels of untreated control apples the expression of MdACS, MdACO and MdAFS remained steadily high up to three months of cold storage and thereafter started to decline (Figure 3A-C), while transcripts of MdPPO underwent a steady dramatic (up to ca 1000 fold) increase throughout cold stress exposure (Figure 3D). The inhibition of ethylene perception (and of cold stress responses, superficial scald) by 1-MCP treatment significantly down-regulated the transcription of MdACO, MdACS and MdAFS genes to basal levels (Figure 3A-C) and suppressed that of the MdPPO gene (Figure 3D). DPA treatment resulted in an almost complete inhibition of the MdPPO transcript accumulation up to three months, which after six months was partially overcome, while it exerted a stimulatory effect on the transcription of MdACS, MdACO and MdAFS genes from three months of storage onwards (Figure 3A-C). These data are consistent with the magnitude of manifestation of cold stress (scald), showing a complete suppressive effect exerted by the ethylene action inhibitor 1-MCP and a partially suppressive effect by DPA, with a complex action of the latter one on ethylene biosynthetic genes and on the scald markers MdAFS and MdPPO.

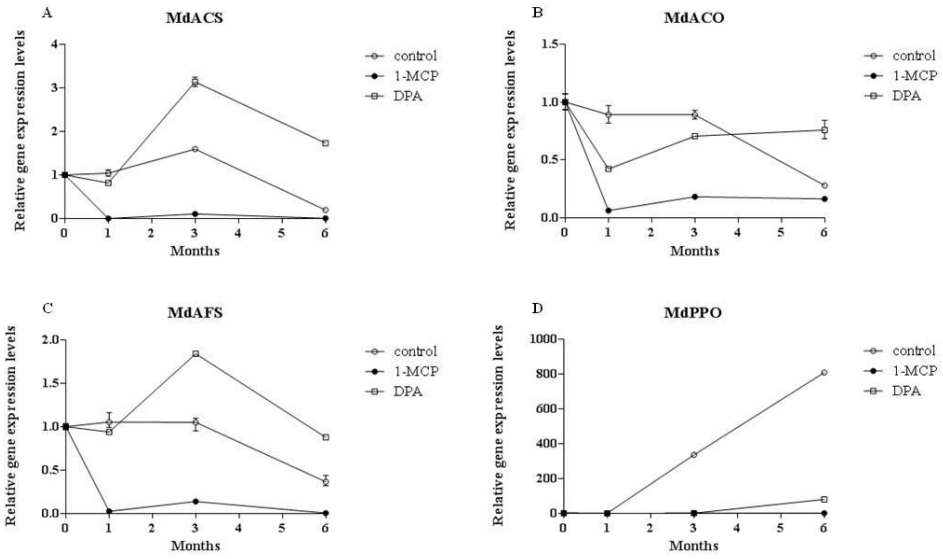

Figure 3 – Relative gene expression levels of different markers genes in apple peels subjected to cold stress evaluated by real-time PCR: **(A-B)** MdACS and MdACO transcripts levels, markers of the main ethylene biosynthetic steps; **(C)** MdAFS and **(D)** MdPPO transcript levels used as markers for oxidative stress induction and superficial scald development. Each value represents two independent biological replicates ± SD.

To gain information about the possible relationships between the regulation of the ROP-GAP rheostat/machinery, of ethylene action and the development of scald in response to cold stress, the transcriptional expression pattern of the identified *Malus domestica* genes has been evaluated by real-time PCR, in apples treated or not with 1-MCP or DPA or subjected to short ethylene treatments. On the whole expression analysis, twenty genes did not show major expression differences between treatments, with the exception of a transient up regulation of MdRBOHD and MdRBOHG in control apples at three months of storage (Figure S12). On the contrary and remarkably, twenty one genes appeared to be de-repressed by inhibition of ethylene perception by 1-MCP treatment, or, in most cases and to a minor extent, by DPA treatment (Figure 4). 1-MCP effect was evident for some of these genes already after one month of cold exposure (i.e. evident for MdROP4a, MdROP-GEF1, MdROP-GEF5b, MdROP-GEF11/13a, MdROP-GAP3, MdROP-GAP5, MdROP-GAP7, MdROP-GAP8a, MdROP-GAP9 and MdRBOHC in Figure 4). Overall, several genes displayed a down-regulation trend of expression in control untreated apples, starting after one month of cold storage, which was partially preventedby DPA or even fully reversed by 1-MCP treatments (particularly evident, for example, for MdROP4a, MdROP6, MdROP-GEF3, MdROP-GEF5b, MdROP-GAP2a, MdROP-GAP5, MdROP-GAP9, MdRBOHC, MdRBOHF, and MdPLDα1 in Figure 4).

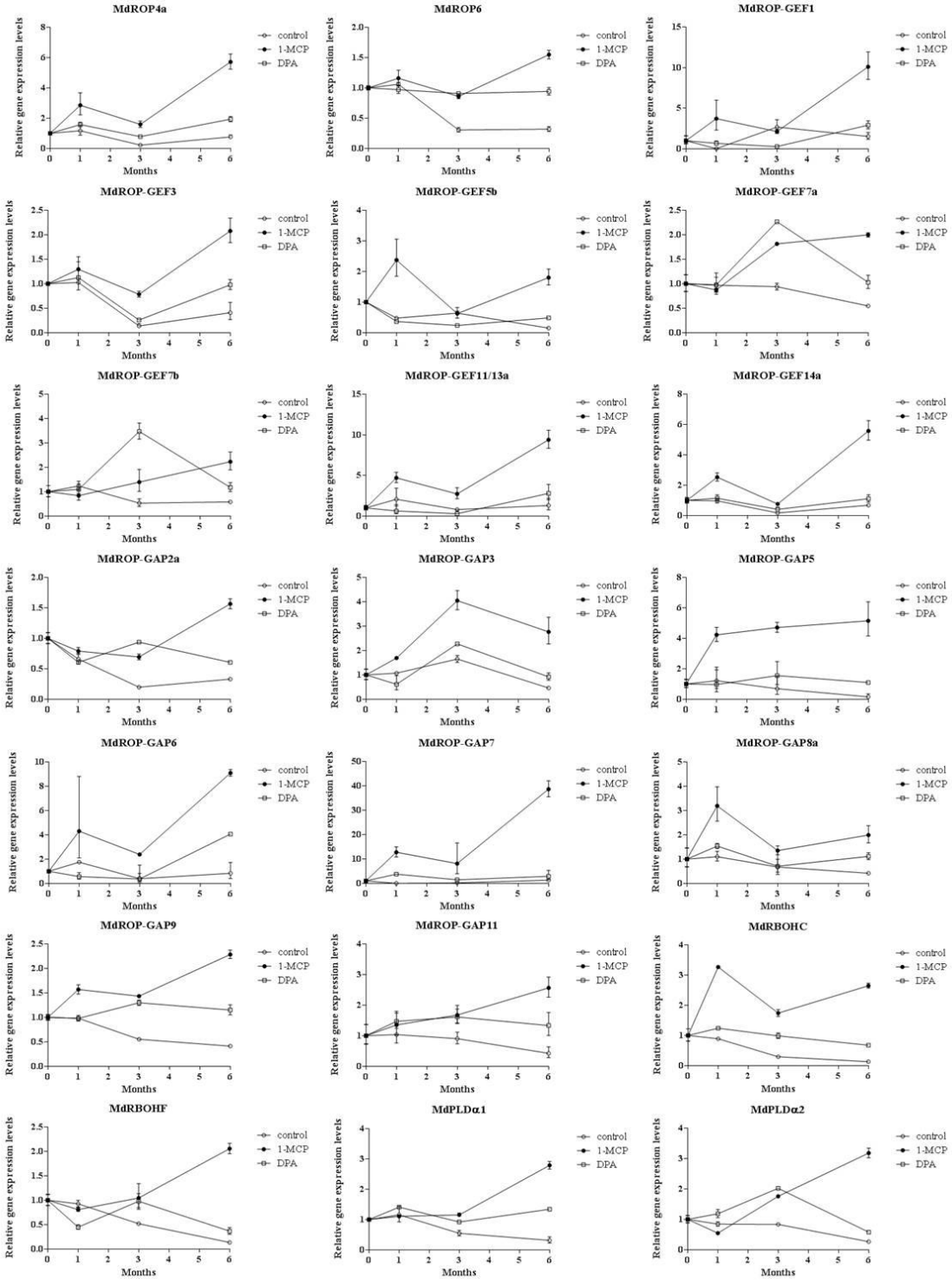

Figure 4 – Relative gene expression levels of MdROPs, MdROP-GEFs, MdROP-GAPs, MdRBOHs and MdPLsDα genes evaluated by real-time PCR on peels collected from control untreated, 1-MCP or DPA treated Granny smith apples at harvest and after 1, 3 and 6 months of cold storage in controlled atmosphere (0.8% O_2, 0.8% CO_2, 1°C). Expression levels were measured relative to Md_8283:1:a (Botton *et al.*, 2011). Each value represents two independent replicates ± SD.

The overall de-repression of the apple ROP-GAP rheostat by 1-MCP was further confirmed on samples collected from an additional year, as described in materials and methods (Figure S13). The de-repression exerted by 1-MCP would suggest a negative effect played by ethylene on the expression of the genes encoding the apple ROP-GAP rheostat. To determine whether this may be a direct effect of the hormone, the expression of the same genes was evaluated by qPCR on peels of apples subjected to short-time treatments with a saturating concentration (100 ppm) of ethylene for 4 and 24 hours. The data obtained showed that ethylene negatively regulates many of the apple ROP-GAP rheostat genes separating one cluster of early ethylene responsive genes (cluster 1a in the heatmap of Figure 5, including MdROP6, MdROP-GEF5b, MdROP-GEF14a, MdROP-GAP5, MdROP-GAP9, MdRBOHC, readily down-regulated after 4 and 24 hours) from a cluster of slower response genes down-regulated after 24 hours of treatment (cluster 2a, Figure 5, including MdROP3b, MdROP4a, MdROP-GEF3, MdROP-GEF11/13, MdROP-GAP8a, MdRBOHF, MdPLDα1). MdROP-GAP6 displayed a strong downregulation after 4 hours of treatment while MdROP-GAP7 showed a complex behaviour, being induced transcriptionally after 4 hours and downregulated after 24 hours, therefore clustering separately from all other genes. Overall, this ethylene-dependent two-step down-regulation of several genes of the apple ROP-GAP rheostat is amply in agreement with the time-course of derepression found in response to inhibition of ethylene perception by 1-MCP treatment during cold storage. Relative gene expression levels of MdACO, MdAFS and MdPPO were evaluated as control of the efficacy of treatment (Figure S14).

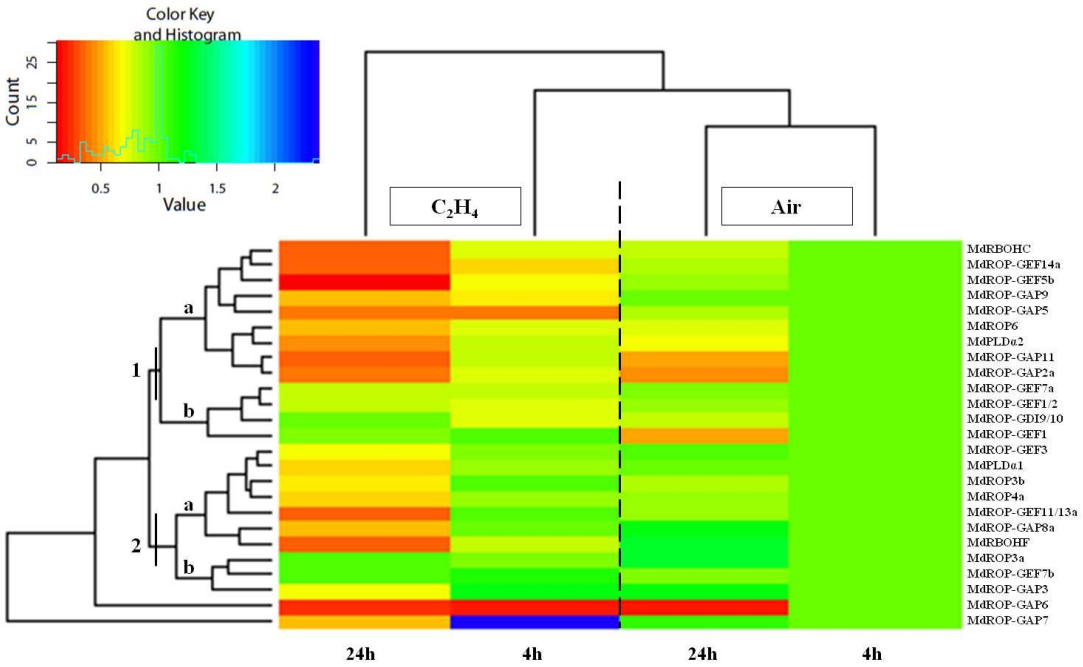

Figure 5 – Heatmap of linear expression data obtained by qRT-PCR for genes of the apple ROP-GAP rheostat/machinery on peels of apples treated for 4h and 24h with 100ppm of ethylene (C_2H_4) or maintained in air (AIR) for the same period of time are represented by a colored scale ranging from red (dow-regulated) to blue (up-regulated) where green identified no expression variation compared to control sample (apples maintained in air for 4h).

Malonyldialdehyde, H_2O_2, Ascorbic acid and Glutathione content and subcellular localization of H_2O_2

Analysis of the marker of lipid peroxidation malonyldialdehyde (MDA)(Frenkel & Neff, 1983; Blokhina *et al*., 2002) showed in peels of untreated (control) apples a transient increase of MDA levels at one and three months of cold storage, thus indicating the occurrence of an oxidative stress (Figure 6A). When ethylene perception was blocked (1-MCP treated samples) MDA levels remained steadily at significantly lower (with respect to control) basal levels up to three months of cold storage, showing an inhibition of the oxidative stress. The same behavior was observed in response to DPA treatment, with the exception of a transient non significant (with respect to control) increase at one month of storage (Figure 6A and Table S9). After six months of storage MDA content appeared equal between treatments. H_2O_2 levels showed an opposite trend, with a tendency to remain

higher in 1-MCP treated samples (significantly higher at one month of cold stress, with respect to control) and, to a lesser extent, in DPA treated samples, while undergoing a slow progressive decay in peels of control untreated apples (Figure 6B and Table S10). Acorbic acid and glutathione levels (the two main antioxidants employed by cells for maintenance of H_2O_2 homeostatic control through the Halliwell-Asada cycle)(Foyer & Halliwell 1976; Noctor and Foyer, 1998; Asada, 1999) displayed a trend to maintain lower levels in 1-MCP and DPA treated samples throughout the experiment, consistent with a higher usage of these components possibly to prevent excessive accumulation of H_2O_2 (Figure 6C-D). The transcriptional expression of the ADH gene, a marker for H_2O_2 responses (Baxter-Burrell, 2002), increased in samples treated with 1-MCP suggested to be induced by the higher levels of H_2O_2 measured in the same samples, while it remained at basal levels in control and DPA treated samples according to the lower content in H_2O_2.

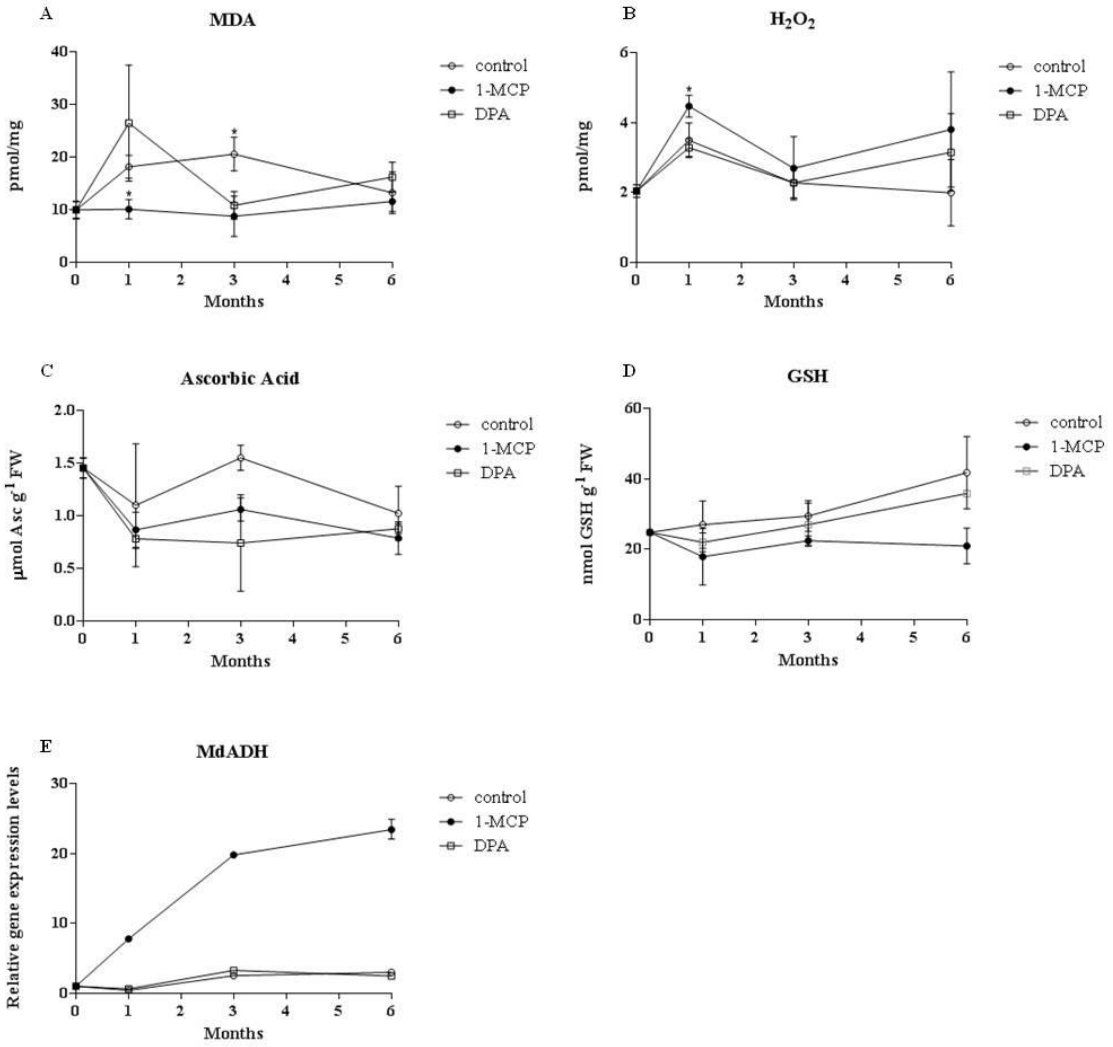

Figure 6 – Levels of the peroxidative marker malonildialdehyde **(A)**(Frenkel & Neff, 1983; Blokhina *et al.*, 2002) and of H_2O_2 **(B)**, ascorbic acid **(C)** and glutathione **(D)** in peels of apples treated or not with 1-MCP or DPA after 1, 3 and 6 months of cold storage. Relative gene expression levels of alchool dehydrogenase (ADH)(E) gene are shown as a marker for H_2O_2 responses (Baxter-Burrell, 2002). Each value represents four **(A-B)** or two **(C-D)** independent replicates ± SD. Asterisks indicate significantly different values obtained by a t-test analysis (for $p < 0.05$, n = 4, **A-B**).

The subcellular localization of H_2O_2, by means of cerium chloride reaction in transmission electron microscopy, revealed remarkably higher levels of apoplastic H_2O_2 in peels of 1-MCP treated fruits in comparison with control fruits, in which H_2O_2 levels resulted to be below detection (Figure 7A-B). No differences could be detected in terms of H_2O_2 for cytoplasmic and organelle localization of $CeCl_3$ deposits between treated an untreated fruits (Figure 7C-D).

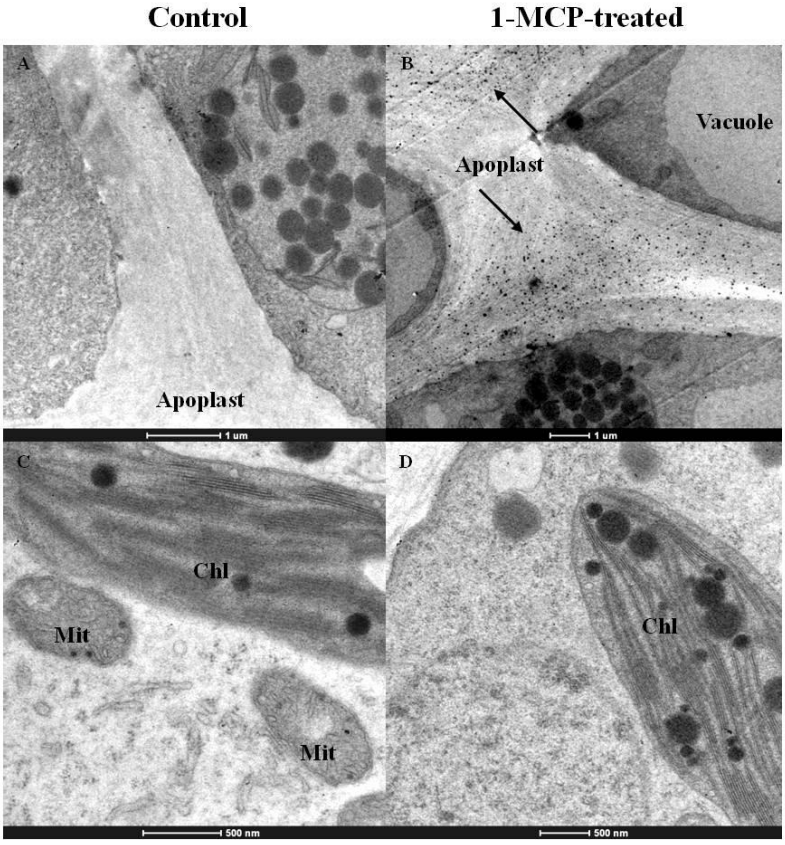

Figure 7 – Representative pictures showing cytochemical localization of H_2O_2 by cerium perhydroxide precipitation (Bestwick *et al*., 1997) in peels of apples treated or not with 1-MCP. Upper panels show localization of apoplastic H_2O_2 in control **(A)** and 1-MCP **(B)** treated apple peels, respectively. Abundant precipitates of cerium perhydroxide in the apoplast of 1-MCP-treated apples are indicated by arrows. Lower panels show representative pictures of intracellular portions of control and 1-MCP treated apple peels **(C-D)**.

Transcriptional rewiring of the apple “ROS gene network”: perception of changes and feedback control of apoplastic ROS homeostasis

To understand the consequences in terms of cellular homeostasis due to the significant changes in apoplastic ROS levels induced by the block of ethylene perception by 1-MCP treatment, we have investigated the regulation of the apple “ROS gene networks”. Evidence obtained from Arabidopsis has pointed that different ROS at different subcellular locations induce diverse and specific transcriptional signatures (Mittler *et al*., 2004). Thus, by using the identified sequences from *A. thaliana* as queries in the BLAST tool of the rosaceae database, we have characterized the apple “ROS gene network” and we have compared it with that described by Mittler *et al*. (2011) (Table S11). On the whole 316 genes were identified including 30 superoxide dismutase (SOD), 26 ascorbate peroxidases (APXs), 8 monodehydroascorbate reductases (MDHARs), 10 dehydroascorbate reductases (DHARs), 3 glutathione reductases (GRs), 5 catalases (CATs), 19 glutathione peroxidases (GPXs), 14 ferritins, 41 hypothetical blue copper proteins, 20 theoretical NADPH oxidase-like, 8 alternative oxidases (AOXs), 18 peroxiredoxins (PrxRs), 66 hypothetical thioredoxins (TRXs) and 48 putative glutaredoxins (GLRs). By RNA-seq analyses on samples taken at harvest and after 1 or 6 months of storage, treated or not with 1-MCP, the expression values (RPKM) of the genes composing the apple “ROS gene network” and the “ROP-machinery” were obtained and used to construct an heatmap, to identify co-regulated transcriptional signatures which may reflect sensing of changes in apoplastic H_2O_2 homeostasis (Figure 8). Remarkably, the specific co-regulation of well-defined clusters of genes could be identified, pointing to differentially regulated ROS network transcriptional signatures in 1-MCP treated versus control samples. In particular, an opposite regulation, was evident for several genes involved in the ascorbate-glutathione cycle, in parallel with that of the genes encoding the ROP-GAP rheostat, following the block of ethylene perception by 1-MCP (Figure 8).

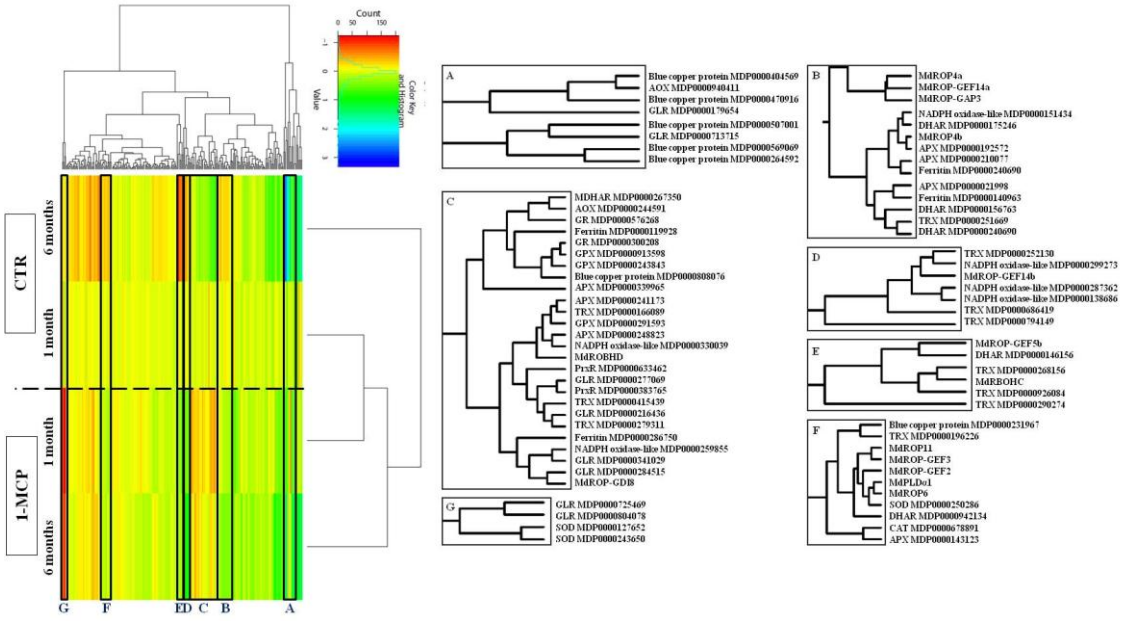

Figure 8 – Heatmap showing expression clustering of genes composing the apple "ROS gene network" and the "ROP-GAP rheostat/machinery", obtained on the basis of RNA-seq analyses on Granny Smith apple peels samples taken after 1 or 6 months of storage, treated with 1-MCP (1-MCP) or not (CTR). Colors (from red down-regulated to blue up-regulated, where yellow identified no expression variation) were assigned on the normalized log-scaled RPKM (reads per kilobase of exon model per million mapped reads) values. In clusters A, C and G (left) are shown genes down-regulated in samples treated with 1-MCP compared to control. In clusters B, D, E and F (on the right) are shown genes up-regulated in 1-MCP treated samples compared to control.

Specifically, three clusters (A, C and G) included genes showing a repressive effect of 1-MCP on transcript abundance, for instance more evident already after one month in cluster A and, to a lesser extent, in cluster C, which did not include the genes composing the apple ROP-GAP rheostat, excepted MdRBOHD (Figure 8). On the contrary, four clusters (B, D, E and F) included the apple ROP-GAP rheostat and their co-regulated genes, whose expression resulted to be down regulated in peels of control untreated apples and de-repressed by inhibition of ethylene action by 1-MCP (Figure 8). These data, besides confirming independently those obtained by qPCR, showed the co-regulation in cluster B with MdROP4a, MdROP4b, MdROP-GEF14a, MdROP-GAP3 of 3 DHAR genes and 3 APXs, representing a transcriptional signature consistent with apoplastic H_2O_2 being sensed and enhancing its own control through a feedback activation of enzymes of the ascorbate-glutathione cycle, and of 3 genes encoding TRXs, involved in the protection of thiol groups

from H_2O_2 action (Buchanan & Balmer, 2005). In cluster F a co-regulation between MdROP6, MdROP11, MdROP-GEF2, MdROP-GEF3 and MdPLDα1 was found together with genes involved in the detoxification of H_2O_2: a Cu/Zn SOD, a CAT, a TRX, an APX and a DHAR. Interestingly no glutathione reductase (GR) encoding genes, involved in the reduction of oxidized glutathione (GSSG), were found up-regulated. Finally 3 genes similar to Arabidopsis Ferric-chelate reductases, a NADPH oxidase-like (Mittler *et al*., 2011), were found co-regulated with 3 TRXs and MdROP-GEF14b in cluster D, while 3 TRX and one DHAR were co-regulated with MdRBOHC and MdROP-GEF5b in cluster E (Figure 8). By contrast in peels of untreated apples, a specific up-regulation was found in cluster C for genes encoding two PrxRs, which metabolize H_2O_2 using TRXs as cofactors (Dietz *et al*., 2006), two GRs and four GLRs, the latter protecting the cysteines from being overoxidized (Lemaire, 2004; Xing *et al*., 2006) and three GPXs (Dixon *et al*., 1998), which seem to act as thioredoxin-dependent peroxidases (Herbette *et al*., 2002). Finally 2 GLRs were found co-regulated with 5 blue-copper proteins in cluster A and with 2 SODs in cluster G.

Regulation of the apple ROP-GAP rheostat in response to low oxygen and cold storage in fruit peel and cortex

Superficial scald development can be prevented by the application of an initial low oxygen stress (ILOS)(Zanella, 2003; Sabban-Amin *et al*., 2011). ROP-GAP rheostat genes were reported to be regulated in response to hypoxia in Arabidopsis (Baxter-Burrell *et al*., 2002). Thus, in order to evaluate the role of the apple ROP machinery and of the ROP-GAP rheostat in response to hypoxia and, more precisely, to the ILOS technique currently used by the apple storage industry to prevent superficial scald, the expression of the identified genes was studied in peel and flesh tissues of apples cv Granny Smith subjected to three different oxygen conditions: 20% (atmospheric - normoxic), 0.8% (ultra low oxygen, ULO) or 0.4% (ILOS) oxygen concentration at 1°C (as described in material and methods and shown in Figure 9).

Figure 9 – Schematic visualization of the experimental plan. Apples of cv Granny Smith were stored at 1°C at three different oxygen concentrations: normoxic (20% oxygen), low oxygen (0.8% oxygen) or subjected to an initial low oxygen stress (ILOS)(0.4% oxygen). Samples were taken weekly at different time points for each experimental thesis up to five weeks of storage and, finally, after eight weeks of storage. One day before the fifth week (indicated by arrow) apples stored at 0.4% oxygen (ILOS) were brought to 0.8% oxygen, for recovery as applied in the currently used ILOS technique.

Apple peel and flesh tissues were characterized through the analyses of expression of the ethylene biosynthetic genes, MdACS and MdACO, and of the marker of oxygen deprivation alcohol dehydrogenase (MdADH)(Baxter-Burrell *et al*., 2002). In both tissues the expression of MdACS and MdACO was higher in apples kept at 20% oxygen and increased already after 2 weeks of storage with a "climacteric-like" rise after 5 weeks. A similar trend was observed also in apples stored at 0.8% oxygen but with a delay of a week and with lower expression levels (Figure 2A-B, top and middle panels). The expression of these genes was almost completely suppressed by the ILOS treatment and started to increase only after four weeks (MdACS) or only when apples were moved from 0.4% to 0.8% oxygen after five weeks (MdACO). Transcript levels of the hypoxic marker MdADH resulted to be higher in peel than in flesh tissues and showed an induction proportional to the degree of the hypoxic stress applied (0.4%>0.8%>20%) with a maximum already after one week and with a decreasing trend that reached a steady basal level after five weeks in all three O_2 conditions (Figure 10A-B, bottom panel).

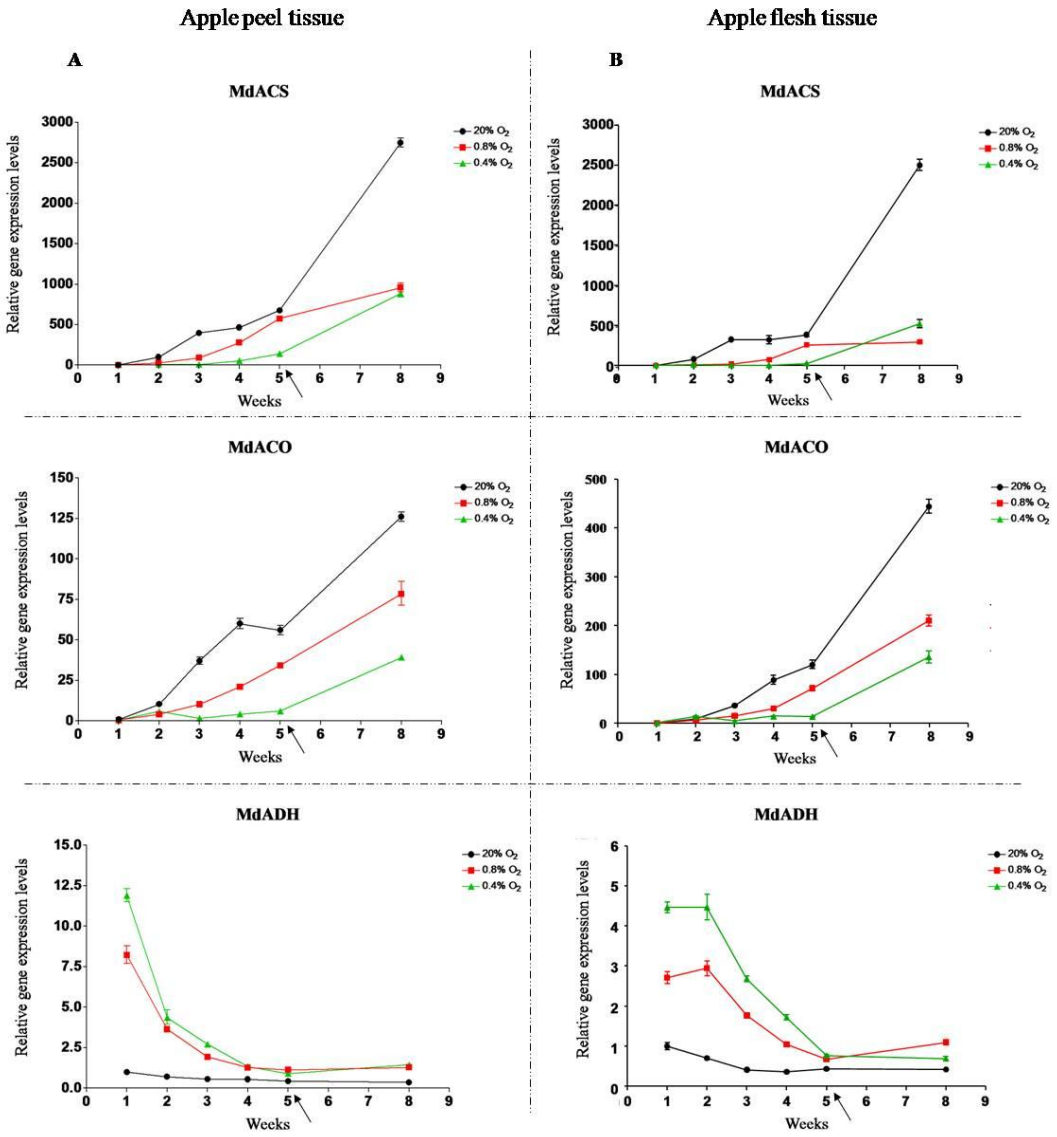

Figure 10 – Top and middle panel, respectively, show relative gene expression levels of MdACS and MdACO genes, markers of the main ethylene biosynthetic pathway; bottom panel shows relative gene expression levels of the hypoxia marker MdADH. Gene expression levels were evaluated by real time PCR on cDNA obtained from peels (**A**) and flesh tissues (**B**) of apples cv Granny Smith stored in 20% oxygen (black line), 0.8% oxygen (red line) or subjected to ILOS (0.4% oxygen)(green line). Expression levels were measured relative to Md_8283:1:a (Botton *et al.*, 2011). A different scale in ordinate between tissues was intentionally maintained to evidence differences between the three oxygen conditions. Arrows indicated the day before the fifth week in which apples that had been subjected to ILOS (0.4% oxygen) were moved to 0.8% oxygen (recovery).

To characterize the mechanism that prevents development of scald symptoms in apples subjected to ILOS, genes involved in the apple ROP-GAP rheostat have been evaluated by real-time PCR, both in peel, the tissue subjected to manifest scald, and flesh of apples stored in 20%, 0.8% or 0.4% oxygen at 1°C. On the whole, the expression profiles of fifteen genes did not show a particularly evident regulation by ILOS in peel nor in flesh tissues (Figure S15-S16, respectively) except for MdROP-GAP3 whose transcript levels increased transiently and specifically only in flesh tissues in samples subjected to ILOS, starting from the second week and peaking at the third week of stress (Figure S16). At this oxygen concentration also MdRBOHF transcripts in flesh tissues resulted higher during the first two weeks of storage and then decreased (Figure 11B). Interestingly an opposite situation was evidenced in peels: MdRBOHF expression was higher in samples stored at ultra low low oxygen (0.8%) compared to those subjected to ILOS while it increased during storage in samples subjected to 20% oxygen (Figure 11A). This suggests that MdRBOHF is under the control of significantly different, or even opposite, oxygen-dependent regulatory pathways in the two tissues (Figure 11A-B). A similar, nearly overlapping behavior, appeared evident for MdRBOHG in both ULO and ILOS but not in normoxic conditions (Figure 12A-B).

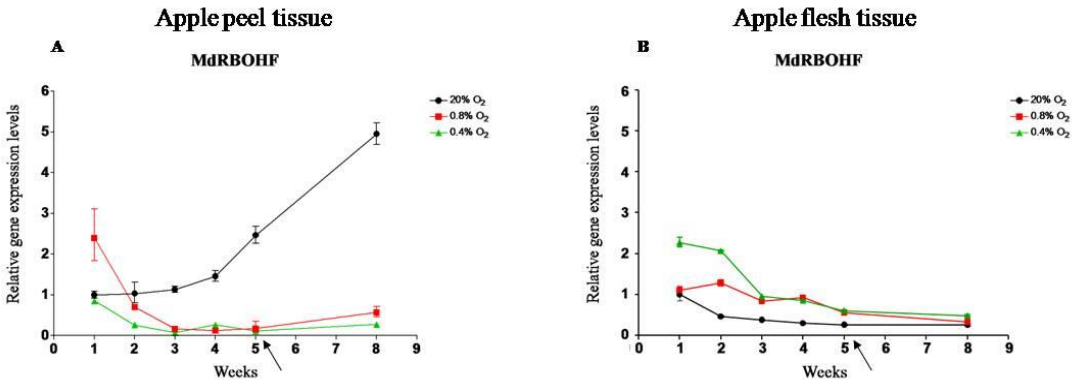

Figure 11 – Relative gene expression levels of MdRBOHF evaluated by real time PCR on RNA obtained from peels (**A**) and flesh tissues (**B**) of apples stored at 20% oxygen (black line), 0.8% oxygen (red line) or subjected to ILOS (0.4% oxygen)(green line) at 1°C. Expression levels were measured relative to Md_8283:1:a (Botton *et al*., 2011).Arrows indicated the day before the fifth week in which apples that had been subjected to ILOS (0.4% oxygen) were moved to 0.8% oxygen (recovery).

MdROP4a, MdROP-GEF1, MdRBOHG and PLDα1 appeared to be up-regulated in peels of apples subjected to ILOS immediately after the first week of storage at 1°C, while this regulation was less evident in flesh tissues, except for MdRBOHG (Figure 12A-B). The up-regulation was evident at the beginning of storage, then transcription followed a down-regulation trend in both tissues and in the three oxygen conditions, as seen for MdADH (Figure 10A-B).

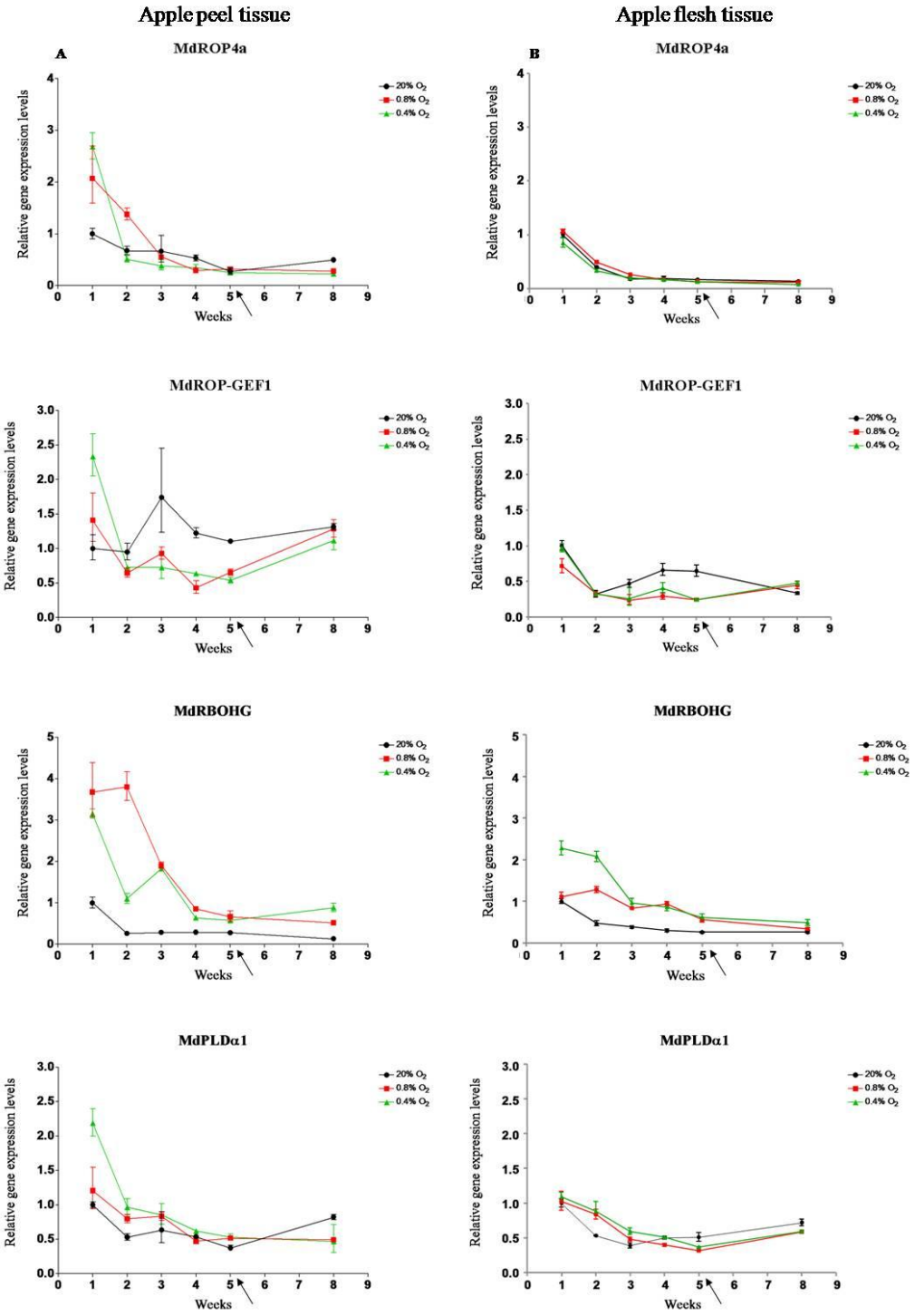

Figure 12 – Relative gene expression levels of MdROP4a, MdROP-GEF1, MdRBOHG and MdPLDα1 evaluated by real time PCR on RNA obtained from peels (**A**) and flesh tissues (**B**) of apples stored at 20% oxygen (black line), 0.8% oxygen (red line) or subjected to ILOS (0.4% oxygen)(green line) at 1°C. Expression levels were measured relative to Md_8283:1:a (Botton *et al.*, 2011). Arrows indicated the day before the fifth week in which apples that had been subjected to ILOS (0.4% oxygen) were moved to 0.8% oxygen (recovery).

All together these results indicate that ILOS, which prevents superficial scald development, is associated with a down-regulation of genes involved in ethylene biosynthesis and upregulates four of the apple ROP-GAP rheostat genes, namely MdROP4a, MdROP-GEF1, MdRBOHG and MdPLDα1. This response resulted to be more evident in peels, was maximal immediately after the first week of storage and overall in concert with MdADH expression, as suggested by Baxter-Burrell *et al.*, 2002.

Discussion

Cold stress is one of the major abiotic challenges in agriculture (Bray *et al*., 2000; Mahajan & Tuteja, 2005). Fruits are usually subjected to prolonged periods of cold stress to extend their storage life. However, prolonged cold stress exposure of fruits to low (near 0°C) temperatures may finally lead to the manifestation of physiological disorders which have large economical impacts. Among cold stress-induced physiological disorders apple scald, besides having a wide economic impact, represents an interesting case study to understand plants responses to prolonged cold stress. Scald occurs only in susceptible apple cultivars after a minimum lag period of 2-3 months of cold exposure, its induction taking place gradually (Watkins *et al*., 1995), and its occurrence can be completely prevented by the ethylene inhibitor 1-MCP, suggesting that it is an eminently ethylene-dependent process. Apple scald is an oxidative process, for which reason research interests have focused mostly on the etiology of symptoms and on the role of the oxidative burst taking place during the development of symptoms (reviewed by Lurie & Watkins, 2012) while little or no information is available on the inductive factors and on the potential role played by ROS during early inductive phases. In addition to 1-MCP diphenylamine (DPA) can be exploited as an additional chemical tool that was shown to prevent the induction of scald by blocking oxidative stress (Whitaker, 2004) and used for several years as scald controller. Besides being a scald prevention tool, DPA provides a means to further characterize scald inductive processes since it partially prevents scald (Smock 1955, 1957 and 1961; Lau, 1990), suggesting that its action impinges on a narrower signaling cascade only partially overlapping with that of ethylene. Thus apple scald represents an interesting model system to study the relationships existing between ethylene and ROS signaling in cold stress

responses. The function of ROS as signaling molecules has been studied only recently while most research on biotic and abiotic (including scald) stress has focused on their role as toxic molecules and, thus, mostly on the function of ROS detoxifying enzymes (Baxter *et al*., 2013). For ROS to act as signaling molecules their levels must be finely and timely tuned through a highly coordinated and precise balancing between ROS production and scavenging systems within various tissue and subcellular contexts (Mittler *et al*., 2011; Suzuki *et al*., 2012; Baxter *et al*., 2013). This implies that ROS perception and signaling differs significantly between scenarios in which homeostatic levels of ROS are produced from those where ROS burst takes place. Consequently, it is implicit that besides scavenging pathways also those responsible for control of ROS production are essential for this fine homeostatic control. Recent works have brought to attention the central role of NADPH oxidases (named in plants as RBOHs, for $gp91^{Phox}$ respiratory burst oxidase homologs) in the generation of apoplastic ROS signals which are in turn perceived and translated by cells into both local or systemic acclimation processes to abiotic stresses such as drought, salt and high light (Suzuki *et al*., 2013, Suzuki *et al*., 2012, Suzuki *et al*., 2011; Mittler at al., 2011). The regulation of RBOH proteins, and of apoplastic ROS, has been shown to be on its own under the control of a finely tuned module including the RHO-like proteins of plants ROPs (Baxter-Burrell *et al*., 2002;), as positive regulators, which are in turn under the feedback control of their negative regulators ROP-GAPs thus defining a complex regulatory module, the ROP-GAP rheostat (Baxter-Burrell *et al*., 2002). A loss of the fine tuning of the ROP-GAP rheostat, due to loss of function of a ROP-GAP and of the negative feedback regulation of NADPH oxidase dependent H_2O_2 production, results in a lack of acclimation of plants to low oxygen (Baxter-Burrell *et al*., 2002). The authors have hypothesized that the ROP-GAP rheostat could be a central module required for plants' adaptation to several abiotic stresses, besides low oxygen. However no works have pursued this hypothesis further so that the role of the ROP-GAP rheostat in abiotic stress in general remains uncharacterized. By adopting apple scald development as a model system, we have studied the regulation of the ROP-GAP rheostat in relation to cold stress and to ethylene action. By using known components identified in other species in similarity BLAST searches we have identified the components of the apple ROP machinery and ROP-GAP rheostat encoded in the apple genome: fifteen *Malus domestica* MdROPs, sixteen MdROP-

GEFs, ten MdROP-GDIs, eleven MdROP-GAPs, ten MdRBOHs and four MdPLsDα, the latter ones responsible for the generation of phosphatidic acids regulating RBOH activity (Zhang *et al*., 2009). Overall, the apple ROP-GAP machinery resembles that found in other species, even though showing interestingly an expansion of ROPs and ROP-GAPs encoding genes. By studying their transcript abundance (both by qPCR and RNA-seq analyses) during cold stress and in response to ethylene inhibition, we could show for the first time that several components of the apple ROP-GAP rheostat are repressed by ethylene. Remarkably, ethylene down-regulated the expression of genes encoding proteins required for both the activation and deactivation of ROPs (ROP-GEFs and ROP-GAPs, respectively), besides down regulating two ROPs (MdROP4a and MdROP6) and two RBOHs (MdRBOHC and MdRBOHF) suggesting that ethylene action in the presence of cold may indeed result in a disruption of maintenance of homeostatic apoplastic H_2O_2. This hypothesis was supported by the fact that not only blocking ethylene perception by 1-MCP treatment resulted in de-repression of these genes but also by the fact that it resulted in the maintenance of higher steady state total content of H_2O_2 which otherwise displayed a progressive decline along with cold storage. This difference appeared more evident at the subcellular level by cerium chloride staining of H_2O_2 in transmission microscopy showing marked signals in 1-MCP treated apples, while no differences could be detected in other subcellular compartments. Consistent with higher apoplastic H_2O_2 levels, lower total contents of ascorbic acid and glutathione were found in the presence of 1-MCP, in agreement with the Halliwell-Asada cycle for the H_2O_2 metabolism (Foyer & Halliwell 1976; Noctor & Foyer, 1998; Asada, 1999) suggesting a faster turn over of these antioxidant molecules required to keep H_2O_2 within homeostatic levels. The fact that this apoplastic H_2O_2 is strictly maintained under control is also further evidenced by the simultaneous de-repression of several ROP-GAPs together with ROPs, RBOHs in response to inhibition of ethylene perception. This is in close agreement with the hypothesis put forward by Baxter-Burrell *et al*. (2002) that for apoplastic H_2O_2 to act as a signaling molecule its levels must be under a fine control through the negative feedback regulation of ROP-GAPs. When this feedback regulation is disrupted, as in the ROP-GAP4 mutant of Arabidopsis (Baxter-Burrell *et al*., 2002), plants fail to acclimate to low oxygen. Moreover the preliminary study on the apple ROP-GAP rheostat in response to ILOS, which prevents

scald development, shows a down-regulation of genes involved in the ethylene biosynthetic pathway and an upregulation of four of the apple ROP-GAP rheostat genes (MdROP4a, MdROPGEF1, MdRBOHG and MdPLDα1) in concert with MdADH expression. Therefore, taken together our data would support the hypothesis that the ROP-GAP rheostat may be a conserved signaling hub required for plants' adaptation to different abiotic stresses and that ethylene may be a previously unseen important hormonal regulator of the rheostat, playing a negative role on the maintenance of apoplastic ROS homeostasis, at least during cold stress in apples. As a consequence, considering the increasing amount of evidence on the central role played by RBOH-dependent apoplastic ROS signaling in regulating plants' acclimation to several abiotic stresses (reviewed recently by Suzuki *et al*., 2013), it is tempting to speculate whether this may also the case for apple scald or for plants resistance to cold stress in general. If such a possibility exists then one would expect that with significantly different apoplastic ROS (H_2O_2), acting as signaling molecules during apple cold stress, in the presence of ethylene or of its inhibitor 1-MCP different ROS transcriptional signatures would be present. Several recent studies in Arabidopsis have shown that divergent "ROS transcriptional networks" exist revealing signatures which represent symptomatic responses to different ROS molecules being produced/metabolized in various subcellular compartments or in the presence of a range of stimuli (Mittler *et al*., 2004; Mittler *et al*., 2011;). We have thus tested this hypothesis by identifying the apple "ROS transcriptional network" according to Mittler *et al*. (2004; 2011), which resulted to be composed of 316 genes. By mining RNA-seq analyses data it was possible to show that blocking of ethylene perception resulted in a significant rewiring of the apple ROS transcriptional network with a specific co-regulation of the ROP-GAP rheostat genes with genes involved in the detoxification of H_2O_2 such as APXs and DHARs and in the protection of thiol groups by H_2O_2 action as TRXs (Holmgren, 1989). Interestingly no glutathione reductase (GR) encoding genes, involved in the reduction of oxidized glutathione (GSSG)(Alscher, 1989; Foyer *et al*., 1991), were found up-regulated thus suggesting a likely accumulation of GSSG in 1-MCP treated apples. The balance between reduced GSH and its oxidized form (GSSG) is a signal of the redox state of the cell and can transmit information to target molecules which may include transcription factors or metabolic enzyme (May *et al*., 1998). Thus it is likely that indeed the ethylene-regulated

ROP-GAP dependent maintenance or loss of apoplastic H_2O_2 homeostasis is perceived by cells and translated in signaling cascades which may lead either to cold stress sensitivity or adaptation, depending on ethylene action or inhibition, respectively. Overall, a model can be drawn, shown in Figure 13, by implementing this evidence on the ROP-GAP rheostat model described by Baxter-Burrell *et al*. (2002).

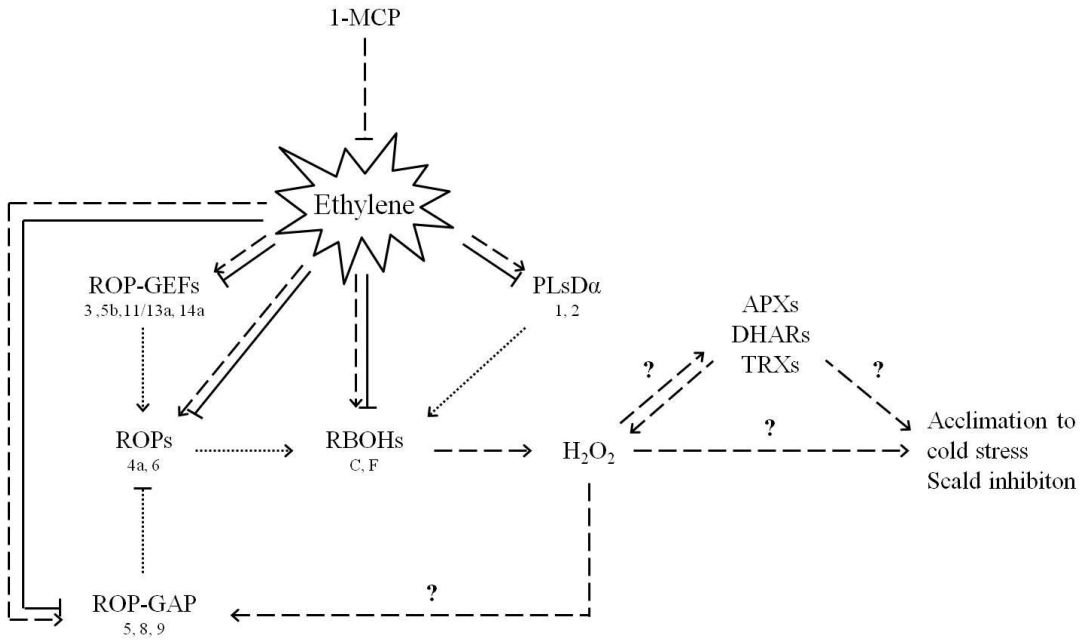

Figure 13 – Schematic model of ethylene action and ROP-GAP rheostat control during apple scald development. Solid and dashed lines indicate, respectively, ethylene or 1-MCP induced regulatory effects on transcriptional gene expressions, and H_2O_2 content. Dotted lines indicate known regulative systems shown in Arabidopsis. Question marks indicate hypothetic mechanisms that may take place as a consequence of H_2O_2-dependent signal transduction.

Conclusions

In this work we provide evidence for the first time showing that the ROP-GAP rheostat and the whole ROP machinery, including RBOHs, may represent an important signaling module in cold stress responses and, by extension, in abiotic stresses in general. In addition, we show that, at least in apple fruits, the rheostat is under hormonal control, since ethylene plays a negative role by down-regulating several components of the rheostat, finally resulting in disruption of homeostatic apoplastic H_2O_2 levels. This ethylene-mediated changes seem to be perceived by cells, since significant rearrangements of the expression of "ROS network genes" take place. Overall, these findings point to a previously overlooked function of ethylene in abiotic stress adaptation, that is to a negative role played by the hormone on RBOH regulation and on ROS signaling completely different from the positive feedback loop in which ethylene and RBOH work in conjunction to promote an oxidative burst and programmed cell death. Besides, it is conceivable that this signaling module may be in important regulatory hub in determining the inductive phase of apple scald development, through the finely-tuned regulation of apoplastic H_2O_2 signals. Future works will be needed to provide further support to this hypothesis and to determine the precise role of RBOH and of ROS signaling in regulation of scald development.

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Supplementary Tables

Table S1 – Primer pairs (forward fw and reverse, rv) used in qRT-PCR experiments are given in 5'-3' orientation. Each primer pair identified a specific apple sequence (ID), re-named in this work (name) as described, with the exception of MdROP-GEF1/2, MdROP-GDI8/10 and MdROP-GDI9/10 for which high sequence identity did not permit design of selective pairs.

ID	*name*	*primer fw*	*primer rv*	*references*
MDP0000299673	MdROP3a	AAAGAAAGGCAAAGGGCAGA	TGTGGGGGAATACATGCAGA	this work
MDP0000436577	MdROP3b	AAGGAAGGCGGCAAGGG	TGTGAGGGAGTACACGCAGG	this work
MDP0000550069	MdROP4a	AGAAGAGAAAGGGGCAAAGG	CAGAGGAGGTAGCGGTGAAG	this work
MDP0000090045	MdROP6	CAGTTCAAAAACGCAGCAGA	CGCACTTTCTTCCCAACACT	this work
MDP0000932494	MdROP8a	CTCTCATTTATACACTTTGTC	TTGCACACTTTCTCTCTGGAA	this work
MDP0000294582	MdROP8b	CATTCTTTCCATCCCTCTTTT	AGTACCCAAAACCAATAACCTTA	this work
MDP0000705111	MdROP9a	TGCTTTCGTTATGTGGTGTTTT	GCAAACTGCTAAACCTCATCAA	this work
MDP0000232351	MdROP10	TCCTTTCGAGTTCCGAGTGT	CAACAGCCTGACGGTGAAG	this work
MDP0000274576	MdROP11	CTGGAGTAACCGCGAACATC	TGACCGCTCAAAACAGAAGC	this work
MDP0000265718	MdROP12a	TTCCTTGCCAACAATCTTCA	AACTCCCAAAGTCGCATTTTA	this work
MDP0000306885	MdROP-GEF1	CCTCTGTGTGTAAAGCGCAGT	CAAAATCAGCCGCGAGTAGTT	this work
MDP0000306885 MDP0000628931	MdROP-GEF1/2	AATCTCTCTGCGACGGTGTT	GCCACATTGCCTTTCTCTG	this work
MDP0000459293	MdROP-GEF3	ATCACCCAGTCCCTTCTCAA	CGGGGCTTCTACCTATCTGA	this work
MDP0000119721	MdROP-GEF4a	GTAAGTGTGCGGGAGAAGGA	GGATGTCTGGGACCATGTCT	this work
MDP0000134252	MdROP-GEF4b	ATAAGGATGTGGGGCAATCA	GAATGGTTGAACTCGGTTGC	this work
MDP0000155158	MdROP-GEF5a	TTGAGGCAAGAAGTGGAAAG	AAACTCCCCCAACTCTCATAA	this work
MDP0000822948	MdROP-GEF5b	TTGAGGCAAAATGAGCTTCT	ACTCCCCTAGCTCTCCTGAA	this work
MDP0000922741	MdROP-GEF7a	GAAACGCTAGGGTCTCACA	GCTGAACACTTCTGCATGGT	this work
MDP0000153185	MdROP-GEF7b	CGAACACAATGCAAGAAACGA	GCGTGAATGAGTCCTGTGATT	this work
MDP0000176388	MdROP-GEF11	GAAATGGTGGAAACCCAAT	GTTCACAGCGTCCTTCTGAG	this work
MDP0000238381	MdROP-GEF13a	CGAAGATGTGCTTTACGCTGATT	CATTGTCTATGTCATTCTCTTCCA	this work
MDP0000176388 MDP0000238381	MdROP-GEF11/13a	AGAGAGAGGGGCAGATAGACA	ACTTCCCAATCCTTCAATAGG	this work
MDP0000233239	MdROP-GEF12	CTGTTGGATTTCATGGGTTG	GTGCCGTTGGACTTCTCAAT	this work
MDP0000186495	MdROP-GEF14a	GACGGTGAACAAGGATGAAC	AACATAGAAATGCTGGGGTCT	this work
MDP0000169427	MdROP-GEF14b	ATTACATTGCTGCCTTGCTG	CCCTACTTGTTCCGCTTTCA	this work
MDP0000684434	MdROP-GAP2a	CTTTCTCTGTTTGGCGTTGCT	GCTCCCTCCTGTCACTCTTG	this work
MDP0000155059	MdROP-GAP2b	GGAGGAAGGAAGGTTGGAGA	GGCGGTCAGTGAAAACAAAG	this work
MDP0000463624	MdROP-GAP3	ACACGGATGAAATGGAGGAG	ACGCTGTAAGCACGAAACCT	this work
MDP0000212513	MdROP-GAP5	GCTGAGGAATCTGTTTTTGTTCT	GTCACTACCCATTTCACTAGACCT	this work
MDP0000674618	MdROP-GAP6	GGGAAGCTGAGGAATCTGTT	ACTTAGGCCCACCCATTTTT	this work
MDP0000237668	MdROP-GAP7	GGACTCTGGCACATCGTTTT	ATGAGGCTTCCCCATCACTC	this work
MDP0000190245	MdROP-GAP8a	GCTGATGTTGCACAAATGGA	TGAAATCGAGGTGCTTTTTAAC	this work
MDP0000279052	MdROP-GAP9	ATGTATGCCGTCCAAGTGAT	CATCCTCGTCAGAAGGCTCT	this work

MDP0000139755	MdROP-GAP10	AACAGAAGCTGCGTTATTGGA	AAATCGGGGTACTTTTCAGTTT	this work
MDP0000163748	MdROP-GAP11	TGAAGAAAAACTCGACCCAAC	ACCACCCAGCAAGACAGAAA	this work
MDP0000934542	MdROP-GDI1	TGGGGAAACTCTTGAACCAG	GCCGGACACAATGTTATTCT	this work
MDP0000257331	MdROP-GDI2	GTGGATTTTGAGAATGTTGGA	ACCGGATACGATGTTATTGC	this work
MDP0000329986	MdROP-GDI3	CATTGTTTTCCCATGCTGAG	AGAGTTGTGCCAAAGCAAGC	this work
MDP0000265024	MdROP-GDI5	AGGGGCTGGTATTGTGTGAG	TTGCTGAAAAACAGATGGAAA	this work
MDP0000661029	MdROP-GDI6	GGCTGTCAAAAACTGCTGGA	GCACCCTCACTTCTGGTTCT	this work
MDP0000320859 **MDP0000860613**	MdROP-GDI8/10	TCGTCAGGTGGGTTTTCATC	TCAGCTTTTCAGCCATTTCC	this work
MDP0000265699 **MDP0000860613**	MdROP-GDI9/10	GGGCATTTATTCAGCAAAGC	TCACCCCTACAAGGAAAAGC	this work
MDP0000703059	MdRBOHC	CGATGCTAGAGTTGGGGTGT	GGGTGGAGGTTTTGTGAGAG	this work
MDP0000262620	MdRBOHD	GTGGGGGTGTTTTACTGTGG	CTTCGTGGTGGTCTTGTGTG	this work
MDP0000273819	MdRBOHE1	AAGAGATACCTTTCCGACTTGA	CTTGTCCACCATCAGCAGTG	this work
MDP0000920069	MdRBOHF	AAGAACTCAGCCAGCTCTGC	GGAAGGGATATGGATTTGAATG	this work
MDP0000421679	MdRBOHG	AGAAACGTGCTCACCAACCT	CGCCGACATACGACTGACT	this work
MDP0000195681	MdRBOHH	TTTCGGGTCTCTTGTTTGTTG	GCTCCCTGAGTGTTTTGGTAAG	this work
MDP0000160005	MdRBOHJ	CAACTTGGCTACCGCACAT	GGCAGAGCTTCCTGAGTGTT	this work
MDP0000300217	MdPLDα1	TGCCAAATCCGACTACCTTC	CCAACCTGCTATGAACATCC	this work
MDP0000233645	MdPLDα2	GTCGCTTGGGTTGTCAAAGT	GCAGGTTGTGAAGCAGATAAG	this work
MDP0000280145	MdPLDα3	TGCGGTAAGCAATAATGGAG	AAACACGAGCCTTGGTATCG	this work
MDP0000274834	MdPLDα4	GTGGCGAAACCAGTGCTC	CGTCCAACTCTTACATTTTCCA	this work
MDP0000375455	Md_8283:1:a	CTCGTCGTCTTGTTCCCTGA	GCCTAAGGACAGGTGGTCTATG	Botton *et al*., 2011

GenBank accession number	*name*	*primer fw*	*primer rv*	*references*
AB030859	MdACO	CAGTCGGATGGGACCAGAA	GCTTGGAATTTCAGGCCAGA	Dal Cin *et al*., 2005
L31347.1	MdACS	AAGTGGCGAACTGGAGTCGA	GGTTTGATGGGTTCGTGACC	Sabban-Amin *et al*., 2011
AY182241.2	MdAFS	AAGATCCTCAGGCAGCATGG	CTTCACCTTCGAAACCCAGG	Sabban-Amin *et al*., 2011
L29450	MdPPO	CTGACTCGGACTGGTTGGAC	CTTCGCTACTTTGCTCAATGC	this work
Z48234.1	MdADH	GGAAGCACTGAAGCCATGAT	CTCCACGACAGAGGGAATGT	this work

Table S2 – MdROPs, MdROP-GEFs, MdROP-GAPs, MdROP-GDIs, MdRBOHs and MdPLsDα genes (ID) (left panel) expressed in different tissues (Petals, Anthers, Flowers, Fruitlets, Seeds, Leaves at three different stage of growth from youngest to oldest names as 1, 2 and 3 respectively)(central panel) and in apple peel of fruits cv Granny Smith (Peel)(right panel) on the whole hypothetical identified genes. Petals are considered as control tissue. Legend: (+) up-regulation compared to control; (=) unchanged expression; (-) down-regulation compared to control; (×) genes expressed in apple peel. Where this symbols are not shown the gene is not expressed.

ID	Petals	Anthers	Flowers	Fruitlets	Seed	Leaves1	Leaves2	Leaves3	Peel
MdROP3a	Control	+	=	=	=	=	=	=	×
MdROP3b	Control	+	+	+	+	+	+	+	×
MdROP4a	Control	-	+	+	+	+	+	+	×
MdROP6	Control	-	-	-	-	-	-	-	×
MdROP8a	Control	+	+	+	+	+	+	+	
MdROP8b	Control	+	+	+	+	+	+	+	
MdROP9a	Control	+	+	+	+	+	+	+	×
MdROP10	Control	=	+	+	+	+	+	+	×
MdROP11	Control	+	+	+	+	+	+	+	×
MdROP12a	Control	-	+	-	+	+	-	-	
MdROP-GEF1	Control	=	+	+	+	+	+	+	×
MdROP-GEF1/2	Control	+	+	+	+	+	+	+	×
MdROP-GEF3	Control	+	+	+	+	+	+	+	×
MdROP-GEF4a	Control	=	+	+	+	+	+	+	×
MdROP-GEF4b	Control	+	+	+	+	+	+	+	
MdROP-GEF5a	Control	+	+	+	+	+	+	+	
MdROP-GEF5b	Control	+	+	+	+	+	+	+	×
MdROP-GEF7a	Control	+	+	+	+	+	+	+	×
MdROP-GEF7b	Control	+	+	+	+	+	+	+	×
MdROP-GEF11	Control	+	+	+	+	+	+	+	
MdROP-GEF13a	Control	+	+	+	+	+	-	-	×
MdROP-GEF11/13a									×
MdROP-GEF12	Control	+	+	-	-	-	-	=	×
MdROP-GEF14a	Control	+	+	+	+	+	+	+	×
MdROP-GEF14b	Control	=	+	+	+	+	+	+	×
MdROP-GAP2a									×
MdROP-GAP2b	Control	+	+	+		+	-	+	
MdROP-GAP3	Control	-	+	+	+	+	+	+	×
MdROP-GAP5	Control	+	+	+	+	+	+	+	×
MdROP-GAP6									×
MdROP-GAP7	Control	-	+	+	+	+	+	+	×
MdROP-GAP8a	Control	+	+	=	+	+	+	+	×
MdROP-GAP9	Control								×
MdROP-GAP10	Control	+	+	-	+	+	+	+	×
MdROP-GAP11	Control	+	+	-	+	+	+	+	×
MdROP-GDI1	Control	+	+	-	-	+	+	+	×
MdROP-GDI2	Control	+	+	+	+	+	+	+	×
MdROP-GDI3	Control	+	+	-	+	+	+	+	×
MdROP-GDI5	Control	-	+	+	+	+	+	+	
MdROP-GDI6	Control	-	+	+	-	+	-	+	

MdROP-GDI8/10									×
MdROP-GDI9/10	Control	+	+	+	+	+	+	+	×
MdRBOHC	Control	+	+	+	+	+	+	+	×
MdRBOHD	Control	+	+	+	+	+	+	+	×
MdRBOHE1	Control	+	+	=	-	+	+	+	×
MdRBOHF	Control	-	+	+	-	+	+	+	×
MdRBOHG	Control	+	+	+	+	+	+	+	×
MdRBOHH	Control	+	+	+	+	+	+	+	
MdRBOHJ	Control	+	+	+	+	+	+	+	
MdPLD*α*1	Control	+	+	+	+	+	+	+	×
MdPLD*α*2	Control	+	+	+	+	=	+	+	×
MdPLD*α*3	Control	+	+	+	+	+	+	+	×
MdPLD*α*4	Control	+	+	+	+	+	+	+	

Table S3 – Putative apple ROP encoding sequences indentified from the apple genome (www.rosaceae.org). The table shows for each hypothetical ROP sequence: gene ID (gene), number of exons, gene and coding sequence (Cds) length (length), chromosome region, strand, corresponding EST(s) found and the proposed name.

GENE	*NUMBER OF EXONS*	*LENGTH*	*CHROMOSOME REGION*	*STRAND*	*EST*	*PROPOSED NAME*
MDP0000388854	5	Gene: 1446bp Cds: 408bp	chr2:4564224..4565669	+		MdROP9c
MDP0000120931	7	Gene: 1443bp Cds: 606bp	chr2:4554919..4556361	-		MdROP9b
MDP0000705111	7	Gene: 1431bp Cds: 603bp	chr15:13122114..13123544	+		MdROP9a
MDP0000436577	7	Gene: 2328bp Cds: 594bp	chr2:10853127..10855454	-	GO569695	MdROP3b
MDP0000299673	7	Gene: 1833bp Cds: 594bp	chr15:17890018..17891850	-		MdROP3a
MDP0000932494	7	Gene: 2552bp Cds: 648bp	chr2:23489373..23491924	-		MdROP8a
MDP0000294582	5 (truncated)	Gene: 1750bp Cds: 407bp	chr2:23785581..23787330	-		MdROP8b
MDP0000090045	7	Gene: 2790bp Cds: 594bp	chr10:7769451..7772240	+	OT041669	MdROP6
MDP0000425375	7	Gene:2338 bp Cds: 552 bp	chr8:758290..760627	-		MdROP4b
MDP0000550069	7	Gene: 2273bp Cds: 615bp	chr8:747195..749467	-	EB142420	MdROP4a
MDP0000232351	8	Gene: 2534bp Cds: 849bp	chr6:20067071..20069604	+	CN857866	MdROP10
MDP0000274576	8	Gene: 3064bp Cds: 783bp	chr14:24843517..24846580	-		MdROP11
MDP0000265718	4	Gene: 1942bp Cds:1356bp	chr12:519514..521455	+		MdROP12a
MDP0000269247	4	Gene: 1942bp Cds:1356bp	chr12:17008838..17010779	-		MdROP12b
MDP0000853669	3	Gene:489bp Cds:249bp	chr2:23792960..23793448	-		Absent in the phenetic tree (Short region)

Table S4 – Putative apple ROP-GEF encoding sequences indentified from the apple genome (www.rosaceae.org). The table shows for each hypothetical ROP-GEF sequence: gene ID (gene), number of exons, gene and coding sequence (Cds) length (length), chromosome region, strand, corresponding EST(s) found and the proposed name.

GENE	*NUMBER OF EXONS*	*LENGTH*	*CHROMOSOME REGION*	*STRAND*	*EST*	*PROPOSED NAME*
MDP0000191912	6	Gene:2390bp Cds: 1593bp	chr6:1566357..1568746	-		MdROP-GEF14c
MDP0000186495	6	Gene:2420bp Cds: 1623bp	chr6:1570160..1572579	+		MdROP-GEF14a
MDP0000922741	7	Gene:3142bp Cds: 2118bp	chr4:14091884..14095025	-		MdROP-GEF7a
MDP0000153185	8	Gene:3160bp Cds: 1989bp	chr12:22516573..22519732	-		MdROP-GEF7b
MDP0000155158	7	Gene:4004bp Cds: 1878bp	chr1:18562289..18566292	-		MdROP-GEF5a
MDP0000822948	7	Gene:3019bp Cds: 1827bp	chr13:21358198..21361216	+	DR996848	MdROP-GEF5b
MDP0000119721	9	Gene:3507bp Cds: 1773bp	unanchored:8472595..8476101	-		MdROP-GEF4a
MDP0000134252	7	Gene:2689bp Cds: 1422bp	chr15:37106075..37108763	+	CN888174	MdROP-GEF4b
MDP0000306885	8	Gene:4790bp Cds: 2187bp	chr10:13104946..13109735	-	CN884517 CN883286 CN882640 CN855821 CN856396 CN856512 CN857418	MdROP-GEF1
MDP0000628931	6	Gene:7759bp Cds: 2055bp	chr9:21337867..21345625	+	CN919478 CN919307	MdROP-GEF2
MDP0000459293	5	Gene:2822bp Cds: 1655bp	chr8:429923..432744	-		MdROP-GEF3
MDP0000176388	7	Gene:2212bp Cds: 1602bp	chr15:11648113..11650324	+	CO052434	MdROP-GEF11
MDP0000238381	7	Gene:2250bp Cds: 1605bp	chr2:4047110..4049359	+		MdROP-GEF13a
MDP0000135163	7	Gene:2254bp Cds: 1605bp	chr2:4047897..4050150	+		MdROP-GEF13b
MDP0000233239	7	Gene:2241bp Cds: 1614bp	chr9:19998242..20000482	-	GO547899 GO512660	MdROP-GEF12
MDP0000169427	6	Gene:3097bp Cds: 1353bp	chr6:1564135..1567231	+		MdROP-GEF14b
MDP0000822990	2	Gene: 778bp Cds: 384bp	chr8:434961..435738	-		Absent in the phenetic tree (Short region)
MDP0000490594	/	Gene: 360bp Cds: 360bp	chr12:20836218..20836577	-		Absent in the phenetic tree (Short region)
MDP0000200158	/	Gene: 360bp Cds: 360bp	chr12:20808431..20808790	-		Absent in the phenetic tree (Short region)

Table S5 – Putative apple ROP-GAP encoding sequences indentified from the apple genome (www.rosaceae.org). The table shows for each hypothetical ROP-GAP sequence gene ID (gene), number of exons, gene and coding sequence (Cds) length (length), chromosome region, strand, corresponding EST(s) found and the proposed name.

GENE	*NUMBER OF EXONS*	*LENGTH*	*CHROMOSOME REGION*	*STRAND*	*EST*	*PROPOSED NAME*
MDP0000190245	5	Gene: 1899bp Cds: 960bp	unanchored:75523280..75525178	-		MdROP-GAP8a
MDP0000434220	4	Gene: 1901bp Cds: 951bp	chr12:2575713..2577613	-		MdROP-GAP8b
MDP0000684434	4	Gene: 2491bp Cds: 750bp	chr3:14057031..14059521	-		MdROP-GAP2a
MDP0000139755	12	Gene: 5823bp Cds: 2331bp	chr14:2029780..2035602	+		MdROP-GAP10
MDP0000463624	4	Gene: 2142bp Cds: 1452bp	chr3:31897140..31899281	-		MdROP-GAP3
MDP0000237668	4	Gene: 2260bp Cds: 1428bp	chr11:33844483..33846742	+	GO593936 GO563182	MdROP-GAP7
MDP0000155059	10	Gene:7462 bp Cds: 2088 bp	chr13:24357067..24364528	+		MdROP-GAP2b
MDP0000163748	3	Gene:1990 bp Cds: 849 bp	chr3:14059777..14061766	+		MdROP-GAP11
MDP0000674618	5	Gene: 3177bp Cds: 1089bp	chr1:11936431..11939607	+		MdROP-GAP6
MDP0000212513	5	Gene: 3292bp Cds: 1494bp	chr4:18589894..18593185	-	GO569418 CN936392 GO568119 CN997758	MdROP-GAP5
MDP0000279052	29	Gene:12220bp Cds: 4650bp	chr12:2564006..2576229	-	CN911016 GO565287	MdROP-GAP9

Table S6 – Putative apple ROP-GDI encoding sequences indentified from the apple genome (www.rosaceae.org) . The table shows for each hypothetical ROP-GDI sequence gene ID (gene), number of exons, gene and coding sequence (Cds) length (length), chromosome region, strand, corresponding EST(s) found and the proposed name

GENE	*NUMBER OF EXONS*	*LENGTH*	*CHROMOSOME REGION*	*STRAND*	*EST*	*PROPOSED NAME*
MDP0000329986	5	Gene:1741bp Cds: 736bp	chr5:548903..550643	+		MdROP-GDI3
MDP0000320859	5	Gene: 2101bp Cds: 840bp	chr10:18100115..18102215	+		MdROP-GDI8
MDP0000460066	8	Gene: 4582bp Cds: 1377bp	chr10:32355452..32360033	-		MdROP-GDI4
MDP0000265024	5	Gene: 1253bp Cds: 762bp	chr15:3106373..3107625	-	CN912568	MdROP-GDI5
MDP0000257331	5	Gene: 2915bp Cds: 687bp	chr17:8884051..8886965	-		MdROP-GDI2
MDP0000934542	5	Gene: 2070bp Cds: 687bp	chr9:8258569..8260638	-	GO549025	MdROP-GDI1
MDP0000661029	3	Gene: 763bp Cds: 741bp	chr9:801618..802380	+		MdROP-GDI6
MDP0000265699	5	Gene: 1695bp Cds: 618bp	chr5:15318145..15319839	+	CO754040	MdROP-GDI9
MDP0000860613	5	Gene: 1806bp Cds: 729bp	chr5:15328097..15329902	-	CO754040	MdROP-GDI10
MDP0000497473	3	Gene: 763bp Cds: 705bp	chr9:810841..811603	-	CO754040	MdROP-GDI7

Table S7 – Putative apple RBOH encoding sequences indentified from the apple genome (www.rosaceae.org). The table shows for each hypothetical RBOH sequence gene ID (gene), number of exons, gene and coding sequence (Cds) length (length), chromosome region, strand, corresponding EST(s) found and the proposed name.

GENE	*NUMBER OF EXONS*	*LENGTH*	*CHROMOSOME REGION*	*STRAND*	*EST*	*PROPOSED NAME*
MDP0000273819	17	Gene: 8807 bp Cds: 3195 bp	chr15:15665392..15674198	-	EH009513	MdRBOHE1
MDP0000264232	19	Gene: 10910 bp Cds: 3438 bp	chr15:23187659..23198573	-		MdRBOHE2
MDP0000195681	14	Gene: 3959 bp Cds: 2661 bp	chr11:9923508..9927466	+		MdRBOHH
MDP0000160005	14	Gene: 3975 bp Cds: 2574 bp	chr3:9125577..9129551	-		MdRBOHJ
MDP0000262620	11	Gene: 4258 bp Cds: 2898 bp	chr4:6919306..6923563	-		MdRBOHD
MDP0000703059	11	Gene: 6479 bp Cds: 2781 bp	chr7:4626933..4633411	+	GO532009 CO723039 CO541075 GO528433	MdRBOHC
MDP0000920069	10	Gene: 2760 bp Cds: 1458 bp	chr2:33394241..33397000	+	CN914800 GO563304	MdRBOHF
MDP0000421679	9	Gene: 3249 bp Cds: 2181 bp	chr8:20040773..20044021	-	CN939426 CN948663 CV085112	MdRBOHG
MDP0000280452	14	Gene :5025 bp Cds: 2451 bp	chr14:26,536,700..26,541,724	-		MdRBOHK
MDP0000261507	12	Gene: 5024 bp Cds: 2469 bp	chr14:26,557,660..26,562,683	-		MdRBOHL
MDP0000302913	2	Gene: 1327bp Cds: 524bp	unanchored:110512965..110514291	+		Absent in the phenetic tree (Short region)
MDP0000303494	4	Gene: 2637bp Cds: 847bp	chr12:28297616..28300252	-		Absent in the phenetic tree (Short region)
MDP0000121332	4	Gene: 982bp Cds: 705bp	chr9:9249129..9250110	-		Absent in the phenetic tree (Short region)
MDP0000289326	5	Gene: 2003bp Cds:1092bp	chr6:21771774..21773776	+		Absent in the phenetic tree (catalytic domain missing)
MDP0000290071	6	Gene: 4277bp Cds: 1425bp	chr1:13747012..13751288	+		Absent in the phenetic tree (catalytic domain missing)
MDP0000832599	6	Gene: 4595bp Cds: 1467bp	chr2:33437666..33442260	+		Absent in the phenetic tree (catalytic domain missing)

Table S8 – Putative apple PLDα encoding sequences indentified from the apple genome (www.rosaceae.org). The table shows for each hypothetical PLDα sequence gene ID (gene), number of exons, gene and coding sequence (Cds) length (length), chromosome region, strand, corresponding EST(s) found and the proposed name.

GENE	*NUMBER OF EXONS*	*LENGTH*	*CHROMOSOME REGION*	*STRAND*	*EST*	*PROPOSED NAME*
MDP0000300217	4	Gene: 3828bp Cds: 2493 bp	chr6:12278109..12281936	+		MdPLDα1
MDP0000280145	3	Gene: 3772bp Cds: 2376 bp	chr15:5997866..6001637	-		MdPLDα3
MDP0000233645	6	Gene: 4876bp Cds: 2550bp	chr13:18895431..18900306	+		MdPLDα2
MDP0000274834	3	Gene: 5620bp Cds: 2430 bp	chr2:3094311..3099930	-		MdPLDα4

Table S9 – MDA peel content (pmol/mg) of apples cv Granny Smith stored 1,3 and 6 months in controlled atmosphere (0.8% O_2 and 0.8% CO_2) at 1°C. sd: standard deviation. Different letters indicate significant differences within the same row (t-test, $p < 0.05$).

	Control	**sd**	**1 MCP**	**sd**	**DPA**	**sd**
T0	9.96	1.64	9.96	1.64	9.96	1.64
1 month	18.15 **(a)**	2.16	10.08 **(b)**	1.82	26.44 **(a,b)**	11.01
3 months	20.55 **(a)**	3.20	8.74 **(b)**	3.83	10.82 **(b)**	2.64
6 months	13.20 **(a,b)**	3.96	11.55 **(b)**	1.86	16.17 **(a)**	2.84

Table S10 – H_2O_2 peel content (pmol/mg) of apples cv Granny Smith stored 1,3 and 6 months in controlled atmosphere (0.8% O_2 and 0.8% CO_2) at 1°C. sd: standard deviation. Different letters indicate significant differences within the same row (t-test, $p < 0.05$).

	Control	**sd**	**1 MCP**	**sd**	**DPA**	**sd**
T0	2.04	0.18	2.04	0.18	2.04	0.18
1 month	3.49 **(a)**	0.49	4.46 **(b)**	0.31	3.28 **(a)**	0.25
3 months	2.27**(a)**	0.43	2.69**(a)**	0.90	2.27**(a)**	0.44
6 months	1.99**(a)**	0.95	3.80**(a)**	1.64	3.14**(a)**	1.10

Table S11 – Overview of apple "ROS gene network" identified on the basis of *A. thaliana* sequences which were used as queries in the BLAST tool provided in the *rosaceae* database (www.rosaceae.org).

Gene family	*Arabidopsis thaliana*	*Malus domestica*
Superoxide Dismutase (SOD)	At4g25100.3	/
$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	At5g51100.1	MDP0000294567
		MDP0000181188
		MDP0000243650
		MDP0000127652
		MDP0000123488
	At5g23310.1	MDP0000374181
		MDP0000272757
		MDP0000162292
		MDP0000222804
		MDP0000187560
	At1g08830.1	MDP0000662094
		MDP0000272510
		MDP0000201158
		MDP0000121919
		MDP0000321336
		MDP0000188546
		MDP0000489706
	At2g28190.1	MDP0000250286
		MDP0000318172
		MDP0000258717
	At5g18100.1	MDP0000364366
		MDP0000315650
	At3g10920.1	MDP0000220086
		MDP0000278922
		MDP0000688410
		MDP0000281277
		MDP0000138103
		MDP0000387371
		MDP0000187714
	At3g56350.1	MDP0000173023
Ascorbate Peroxidase (APX)	At1g07890.1	MDP0000241173
2 Asc + $H_2O_2 \rightarrow$ 2 MDA + $2H_2O$		MDP0000254826
		MDP0000261341
		MDP0000199034
	At3g09640.1	MDP0000126107
		MDP0000192572
		MDP0000210077
		MDP0000701945

		MDP0000399965
	At4g35000.1	MDP0000189320
		MDP0000234905
		MDP0000021998
		MDP0000316890
		MDP0000151342
		MDP0000214851
		MDP0000282062
		MDP0000169497
		MDP0000793434
	At4g09010.1	/
	At4g35970.1	/
	At4g32320.1	MDP0000943804
		MDP0000143123
		MDP0000903820
	At1g33660.1	/
	At4g08390.2	/
	At1g77490.1	MDP0000897274
		MDP0000918790
		MDP0000483271
		MDP0000248823
		MDP0000207771
Monodehydroascorbate Reductase (MDHAR)	At1g63940.4	/
MDHA + NAD(P)H + H^+ → Asc + $NAD(P)^-$	At3g09940.1	/
	At3g27820.1	MDP0000320539
		MDP0000164300
		MDP0000152184
	At3g52880.1	MDP0000140206
		MDP0000157871
		MDP0000267350
		MDP0000261821
		MDP0000199989
	At5g03630.1	/
Dehydroascorbate Reductase (DHAR)	At5g16710.1	MDP0000530903
DHA + 2 GSH → Asc + GSSG		MDP0000240690
		MDP0000175246
		MDP0000156763
	At5g36270.1	/
	At1g75270.1	MDP0000127419
		MDP0000311865
		MDP0000316839
		MDP0000146156
		MDP0000942136

	At1g19550.1	/
	At1g19570.1	MDP0000236168
Glutathione Reductase (GR)	At3g24170.1	MDP0000300208
$GSSG + NAD(P)H \rightarrow 2\ GSH + NAD(P)^+$		MDP0000576268
	At3g54660.1	MDP0000202123
Catalase (Cat)	At1g20630.1	/
$2H_2O_2 \rightarrow 2H_2O + O_2$	At4g35090.1	MDP0000132452
		MDP0000147628
		MDP0000699607
		MDP0000678891
		MDP0000309331
	At1g20620.1	/
Glutathione Peroxidase (GPX)	At2g25080.1	/
$H_2O_2 + 2\ GSH \rightarrow 2H_2O + GSSG$	At2g31570.1	MDP0000251176
		MDP0000291593
	At2g43350.1	MDP0000264931
		MDP0000282034
	At2g48150.1	MDP0000647547
		MDP0000243057
	At3g63080.1	MDP0000751256
	At4g31870.1	MDP0000311291
	At1g63460.1	MDP0000212661
		MDP0000338065
		MDP0000203927
		MDP0000191008
		MDP0000258603
		MDP0000207137
		MDP0000302772
	At4g11600.1	MDP0000365920
		MDP0000913598
		MDP0000180721
		MDP0000243843
Ferritin	At5g01600.1	MDP0000189389
$Fe + P \rightarrow P\text{-}Fe$		MDP0000119928
		MDP0000286750
		MDP0000317816
	At3g56090.1	/
	At2g40300.1	MDP0000152866
		MDP0000385350
		MDP0000325832
		MDP0000252706
		MDP0000870126
	At3g11050.1	MDP0000262639

		MDP0000212807
		MDP0000230140
		MDP0000229741
		MDP0000140963
Blue copper protein	At5g20230.1	MDP0000808076
Cu + P → P-Cu		MDP0000470916
		MDP0000507001
		MDP0000375032
		MDP0000866270
		MDP0000118766
		MDP0000619261
		MDP0000286604
		MDP0000479478
		MDP0000744832
		MDP0000264592
		MDP0000164201
		MDP0000933335
		MDP0000209523
		MDP0000299980
		MDP0000213863
		MDP0000610447
		MDP0000269284
		MDP0000204569
		MDP0000129648
		MDP0000569069
		MDP0000206710
		MDP0000248730
	At1g72230.1	MDP0000873376
		MDP0000163314
	At3g27200.1	MDP0000284556
		MDP0000258325
		MDP0000202045
		MDP0000208735
		MDP0000231401
		MDP0000588940
	At3g60280.1	MDP0000162466
	At4g12880.1	/
	At5g26330.1	MDP0000181736
		MDP0000669625
		MDP0000830099
		MDP0000286477
		MDP0000142111
		MDP0000589057

	At2g33740.1	MDP0000231967
		MDP0000186074
	At4g28365.1	MDP0000161510
	At2g31050.1	/
NADPH oxidase-like	At5g23980.1	MDP0000742438
$NADPH + e^- + O_2 \rightarrow NADP^- + O_2^- + H^+$ (?)	At5g49730.1	/
	At5g50160.1	MDP0000303779
		MDP0000272115
	At1g01580.1	MDP0000214984
		MDP0000226559
		MDP0000225549
		MDP0000144724
	At5g49740.1	MDP0000151434
		MDP0000138686
		MDP0000299273
		MDP0000287362
	At5g23990.1	MDP0000613837
	At1g01590.1	/
	At5g67590.1	MDP0000330039
		MDP0000259855
		MDP0000330038
		MDP0000202050
		MDP0000272802
		MDP0000682771
		MDP0000151721
		MDP0000258055
	At1g23020.1	/
Alternative Oxidase (AOX)	At1g32350.1	/
$2e^- + 2H^+ + O_2 \rightarrow H_2O$	At3g22370.1	/
	At3g22360.1	MDP0000940411
	At3g27620.1	/
	At5g64210.1	MDP0000643331
		MDP0000874020
		MDP0000323076
		MDP0000244591
	At4g22260.1	MDP0000200740
		MDP0000195881
		MDP0000131372
Peroxiredoxin (PrxR)	At1g48130.1	MDP0000159365
$2P\text{-}SH + H_2O_2 \rightarrow P\text{-}S\text{-}S\text{-}P + 2H_2O$		MDP0000232332
	At3g11630.1	/
	At5g06290.1	MDP0000200810

		MDP0000633462
		MDP0000320612
	At3g06050.1	MDP0000258515
		MDP0000884441
	At3g26060.1	MDP0000247659
		MDP0000755936
	At1g65990.1	/
	At1g65980.1	MDP0000519575
		MDP0000293090
		MDP0000244884
		MDP0000705110
	At1g65970.1	/
	At1g60740.1	/
	At3g52960.1	MDP0000673491
		MDP0000383765
		MDP0000148952
		MDP0000614959
		MDP0000188780
	At3g03405.1	/
Thioredoxins (TRX)	At2g04700.1	MDP0000203322
P-S-S-P + $2H^+$ → 2P-SH		MDP0000252195
	At1g62180.1	MDP0000279311
		MDP0000167383
	At1g43560.1	/
	At1g31020.1	/
	At1g52990.1	/
	At1g53300.1	MDP0000226590
		MDP0000553412
		MDP0000501387
	At1g76760.1	MDP0000167378
		MDP0000419574
		MDP0000261677
		MDP0000273793
	At2g33270.1	/
	At2g42580.1	MDP0000146444
		MDP0000252130
	At3g06730.1	MDP0000863789
	At3g08710.1	MDP0000235775
		MDP0000597542
		MDP0000316074
		MDP0000390676
		MDP0000445373
		MDP0000249115

	MDP0000613487
At3g20560.1	MDP0000143707
	MDP0000206232
	MDP0000230558
At3g56420.1	/
At4g04950.1	MDP0000302965
	MDP0000898292
	MDP0000397437
At3g56420.1	/
At4g29670.2	MDP0000279178
	MDP0000595987
	MDP0000138960
	MDP0000209701
At4g32580.1	/
At4g37200.1	MDP0000279928
	MDP0000680639
	MDP0000196226
	MDP0000321876
At2g40790.1	/
At3g51030.1	MDP0000415439
	MDP0000622392
	MDP0000166089
At5g39950.1	MDP0000448333
	MDP0000546099
	MDP0000132688
	MDP000069018
	MDP0000391509
	MDP0000752795
At5g42980.1	/
At1g19730.1	MDP0000322266
	MDP0000562983
At1g45145.1	/
At1g03680.1	/
At4g03520.1	MDP0000794149
	MDP0000686419
At2g15570.1	MDP0000251669
At3g15360.1	MDP0000626628
	MDP0000323884
	MDP0000194903
At4g35460.1	/
At2g17420.1	MDP0000251344
	MDP0000235846
	MDP0000823251

		MDP0000233802
	At2g41680.1	MDP0000525742
		MDP0000845788
		MDP0000314335
		MDP0000394567
	At1g50320.1	MDP0000268156
		MDP0000926084
		MDP0000431533
		MDP0000481941
		MDP0000290274
		MDP0000670699
Glutaredoxin (GLR)	At1g03850.2	/
DHA + 2 GSH → Asc + GSSG	At1g06830.1	/
	At1g28480.1	MDP0000713715
		MDP0000135807
		MDP0000179654
	At2g20270.1	/
	At2g30540.1	MDP0000724699
		MDP0000757379
		MDP0000804081
	At2g47870.1	MDP0000870722
		MDP0000768644
	At2g47880.1	/
	At3g02000.1	MDP0000148389
		MDP0000752328
		MDP0000156398
	At3g62930.1	MDP0000295074
		MDP0000155448
		MDP0000262876
	At3g62950.1	MDP0000244038
		MDP0000406592
		MDP0000272528
		MDP0000804078
		MDP0000725469
	At3g62960.1	/
	At4g15660.1	/
	At4g15660.1	/
	At4g15680.1	/
	At4g15690.1	/
	At4g15700.1	/
	At4g28730.1	MDP0000341029
	At4g33040.1	MDP0000341028
		MDP0000934046

	MDP0000906801
	MDP0000579840
	MDP0000330164
At5g11930.1	/
At5g14070.1	MDP0000781442
	MDP0000432422
	MDP0000177956
	MDP0000788727
	MDP0000823096
At5g18600.1	MDP0000892318
	MDP0000376239
	MDP0000472203
At1g77370.1	MDP0000216436
	MDP0000456271
At5g20500.1	MDP0000850178
At5g40370.1	MDP0000642077
	MDP0000284842
	MDP0000243764
At5g63030.1	MDP0000144735
At3g11920.1	MDP0000284515
	MDP0000277069
	MDP0000183490
At4g10630	MDP0000249252
	MDP0000206005

Supplementary Figures

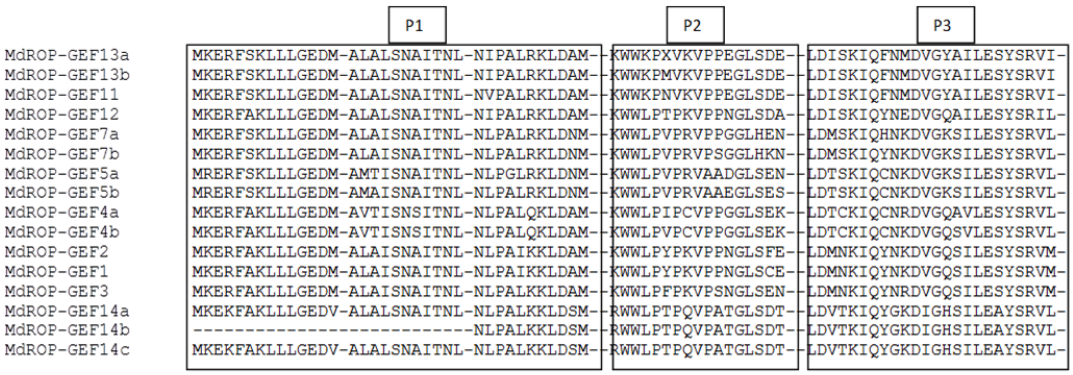

Figure S1 – Alignment of the three PRONE (plant-specific Rop nucleotide exchanger)(P1-P3) conserved domains of the deduced protein sequences of apple ROP-GEFs (Berken *et al*., 2005; Shin *et al.*, 2009; Riely *et al.*, 2011).

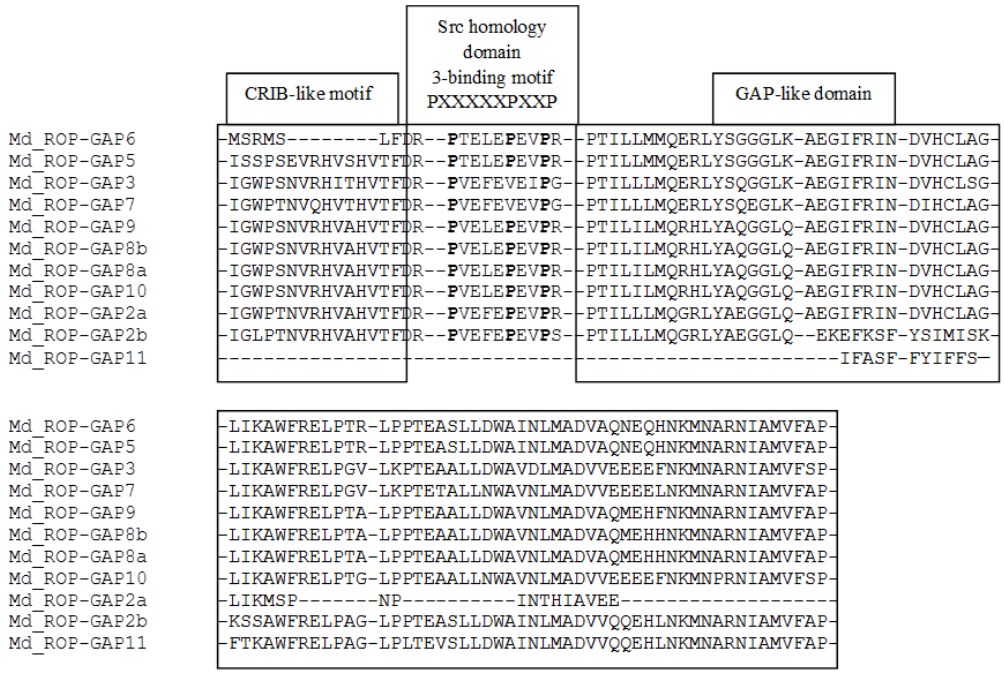

Figure S2 – Alignment of conserved domains of the apple ROP-GAPs deduced protein sequences: Cdc42/Rac-interacting binding (CRIB) motif, consensus sequence for scr homology domain 3-binding motif PXXXXPXXP and GAP-like domain (Wu *et al.*, 2000)

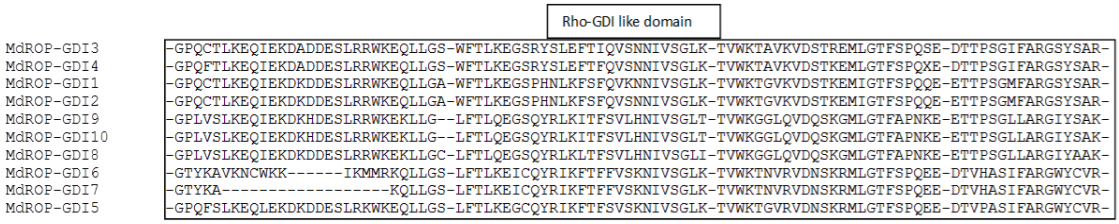

Figure S3 – Alignment of the conserved GDI-like domain of deduced protein sequences from apple ROP-GDIs (Berken & Wittinghofer, 2007).

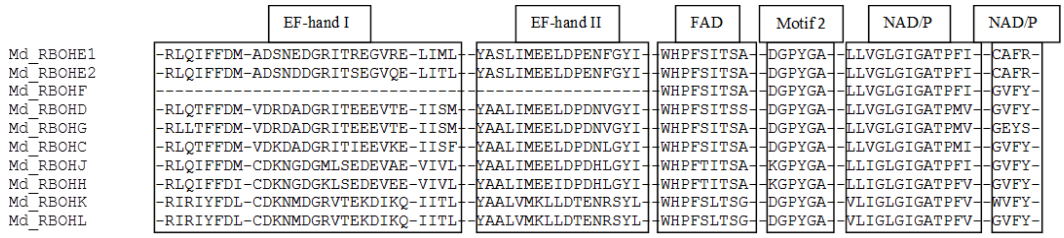

Figure S4 – Alignment of conserved domains of the apple RBOHs deduced protein sequences: EF-hand motifs (EF-hand I and II) and nucleotide binding motifs (FAD-isoalloxazine binding site: FAD; Motif 2; NADPH-ribose and NADPH-binding sites: NAD/P; (Keller *et al.*, 1998; Torres *et al.*, 1998; Amicucci *et al.*, 1999).

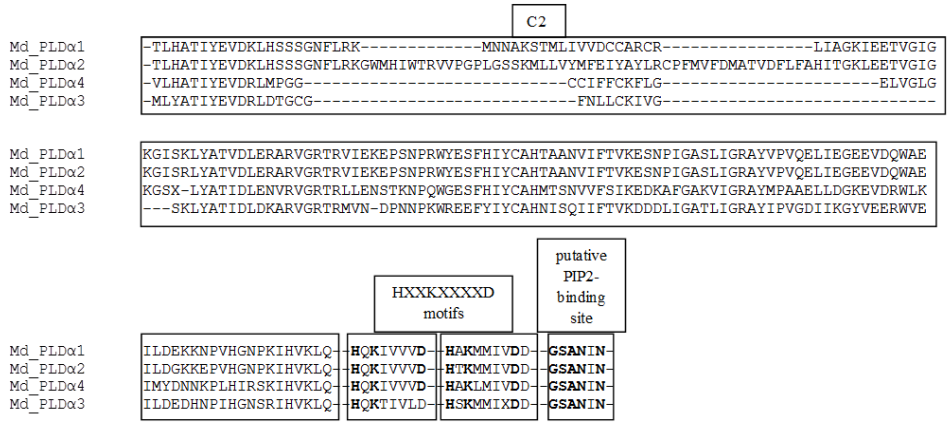

Figure S5 – Alignment of conserved domains of the apple PLsDα deduced protein sequences: C2 domain (C2), two HKD motifs (HXXKXXXXD) and putative PIP2-bnding site (Qin & Wang 2002; Du *et al.*, 2013).

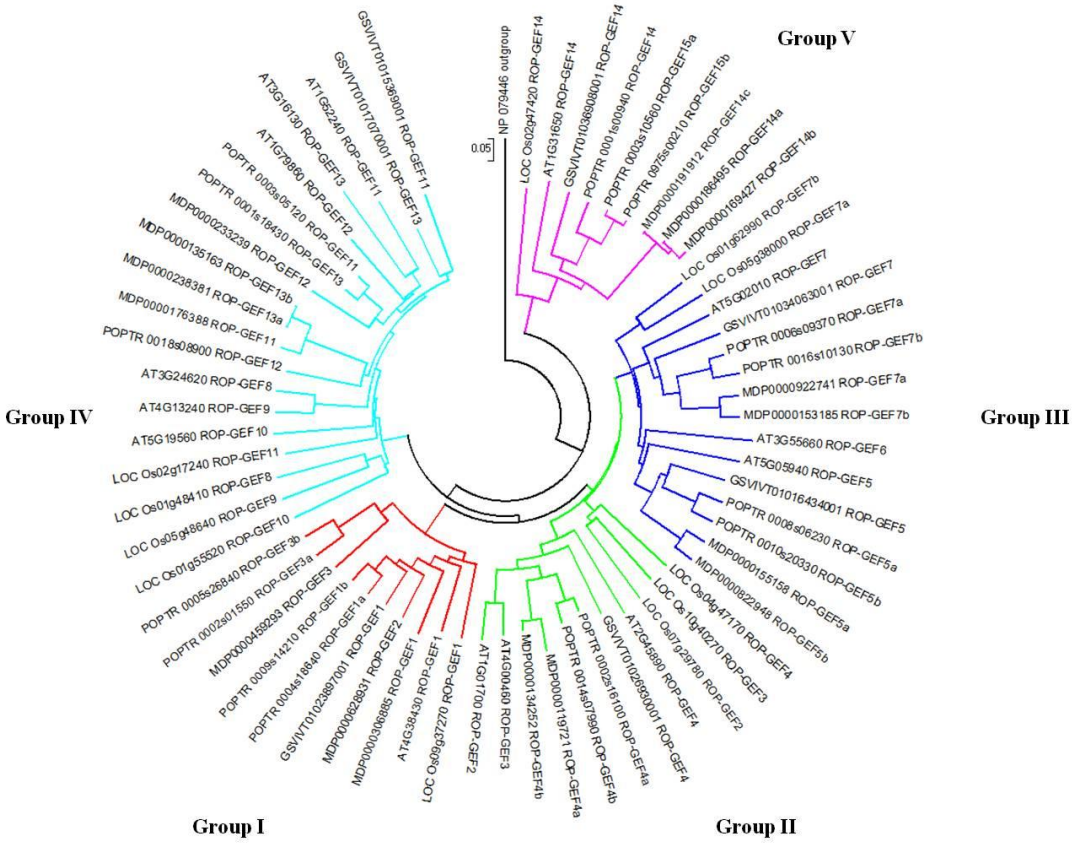

Figure S6 – Phenetic tree showing the relationships among the 16 identified *Malus domestica* ROP-GEF sequences and those belonging to *A. thaliana*, *O. sativa*, *P. thricocarpa* and *V. vinifera* retrieved from the Ensmbl Plants database (http://plants.ensembl.org/index.html). Very short apple ROP-GEF sequences were excluded from the analysis. The phenetic tree was constructed by the neighbor-joining method with bootstrapping analysis on the basis of a CLUSTALX alignment (Jeanmougin *et al*., 1998) and was rooted on the *Homo sapiens* P-REX2 protein as outgroup (NP_079446). The five groups of ROP-GEF sequences identified by Riely *et al*. (2011) are highlighted with different colors.

Figure S7 – Phenetic tree showing the relationships among the 11 identified *Malus domestica* ROP-GAP sequences and those belonging to *A. thaliana*, *O. sativa*, *P. thricocarpa* and *V. vinifera* retrieved from the Ensmbl Plants database (http://plants.ensembl.org/index.html). The phenetic tree was constructed by the neighbor-joining method with bootstrapping analysis on the basis of a CLUSTALX alignment (Jeanmougin *et al*., 1998) and was rooted on the *Xenopus Laevis* Rho-GAP1 protein as outgroup (NP_001080555). Sequence MDP0000163748 (ROP-GAP11) appeared distant from other sequences probably because it didn't present all conserved domains.

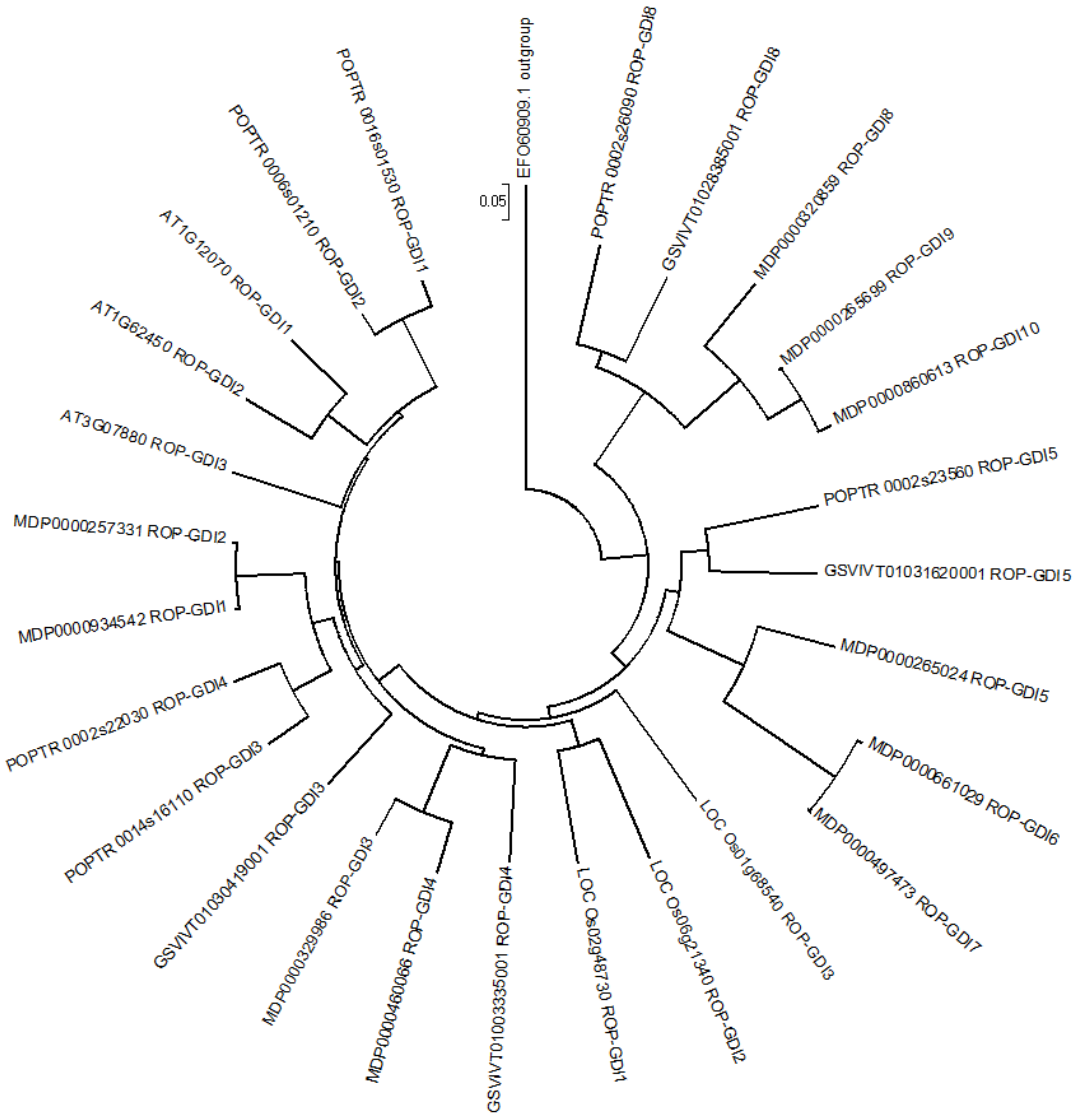

Figure S8 – Phenetic tree showing the relationships among the 10 identified *Malus domestica* ROP-GDI sequences and those belonging to *A. thaliana*, *O. sativa*, *P. thricocarpa* and *V. vinifera* retrieved from the Ensmbl Plants database (http://plants.ensembl.org/index.html). The phenetic tree was constructed by the neighbor-joining method with bootstrapping analysis on the basis of a CLUSTALX alignment (Jeanmougin *et al*., 1998) and was rooted on the *Giardia lamblia* P15 Rho-GDI protein as outgroup (EFO_60909). ROP-GDI tree could be divided into two groups, from one of which the *A. thaliana* sequences were absent, and one that collected apple, grape, poplar and rice genes around Arabidopsis sequences.

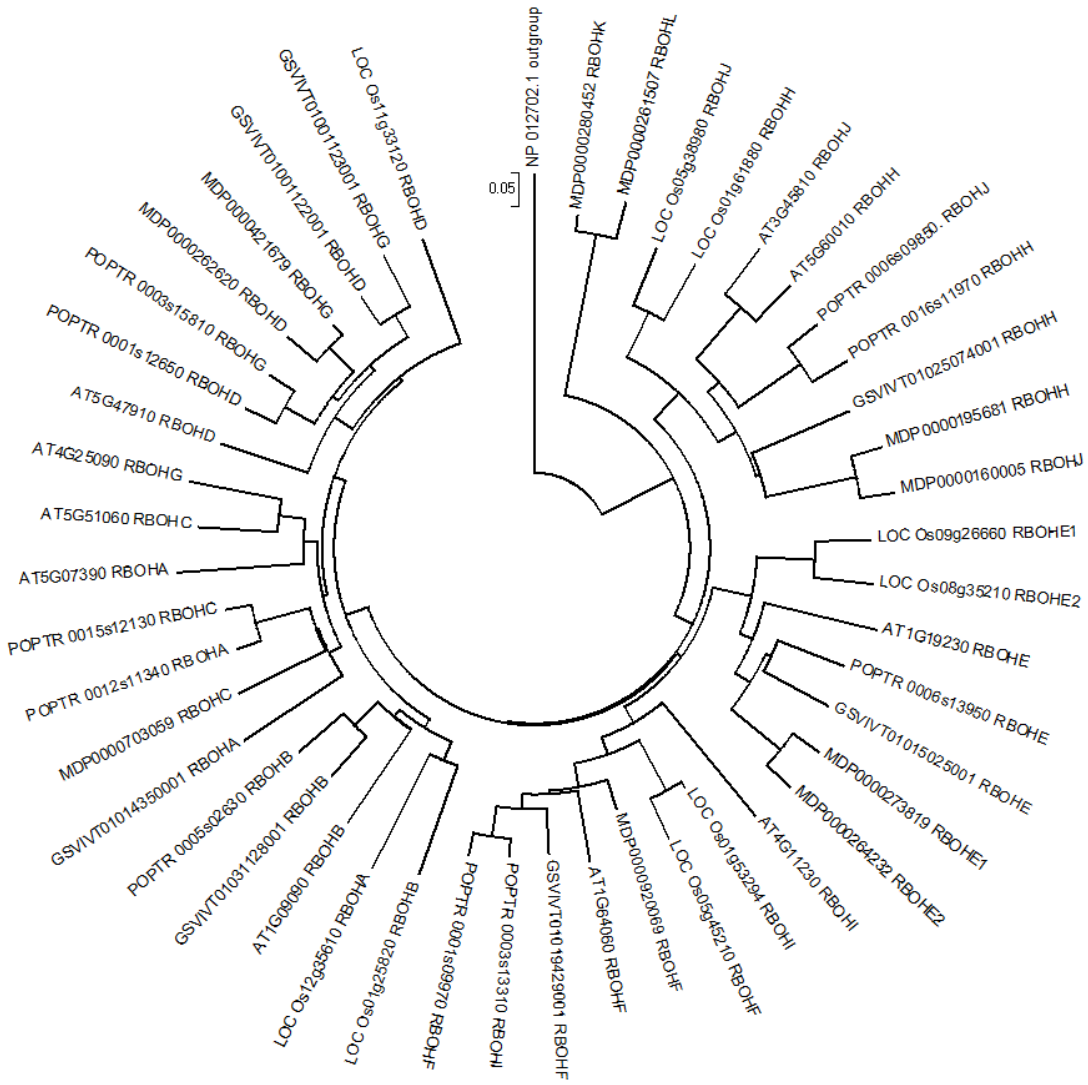

Figure S9 – Phenetic tree showing the relationships among the 10 identified *Malus domestica* RBOH sequences and those belonging to *A. thaliana*, *O. sativa*, *P. thricocarpa* and *V. vinifera* retrieved from the Ensmbl Plants database (http://plants.ensembl.org/index.html). Very short apple sequences were excluded from the analysis The phenetic tree was constructed by the neighbor-joining method with bootstrapping analysis on the basis of a CLUSTALX alignment (Jeanmougin *et al*., 1998) and was rooted on the *Saccharomices cerevisiae* Fre2p protein as outgroup (NP_012702). The RBOH tree could be divided in three groups: the first one contained MdRBOHs similar to AtRBOHH and AtRBOHJ, the second one presented *M. domestica* sequences similar to AtRBOHE, AtRBOHF and AtRBOHI, and in the third one resided those similar to AtRBOHA-D and AtRBOHG. Two MdRBOHs (MdRBOHK and MdRBOHL) remained outside of these groups probably because they presented an extra domain.

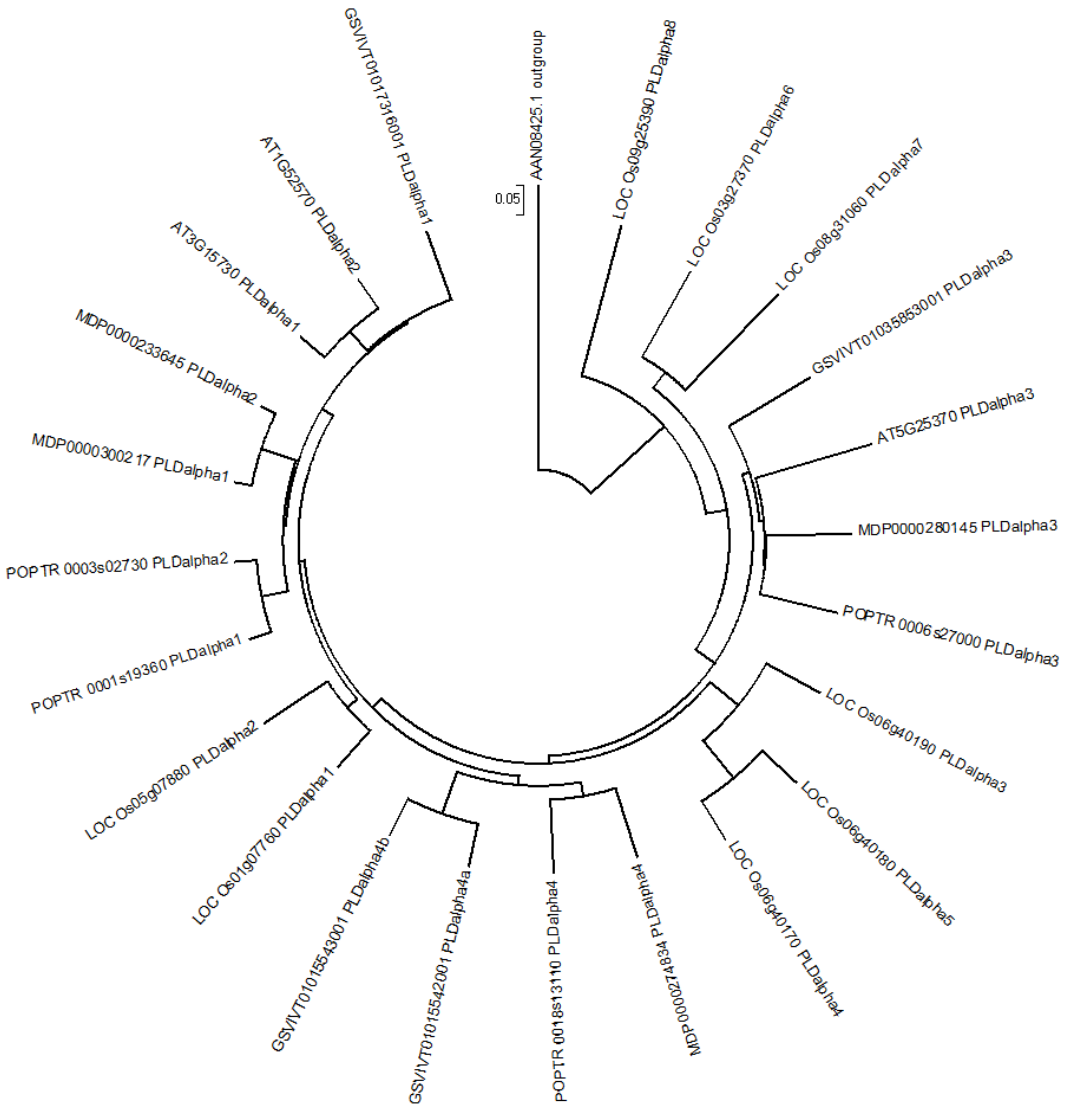

Figure S10 – Phenetic tree showing the relationships among the 4 identified *Malus domestica* PLDα sequences and those belonging to *A. thaliana*, *O. sativa*, *P. thricocarpa* and *V. vinifera* retrieved from the Ensmbl Plants database (http://plants.ensembl.org/index.html). The phenetic tree was constructed by the neighbor-joining method with bootstrapping analysis on the basis of a CLUSTALX alignment (Jeanmougin *et al*., 1998) and was rooted on the *Misgurnus mizolepis* PLDδ1 protein as outgroup (AAN08425).

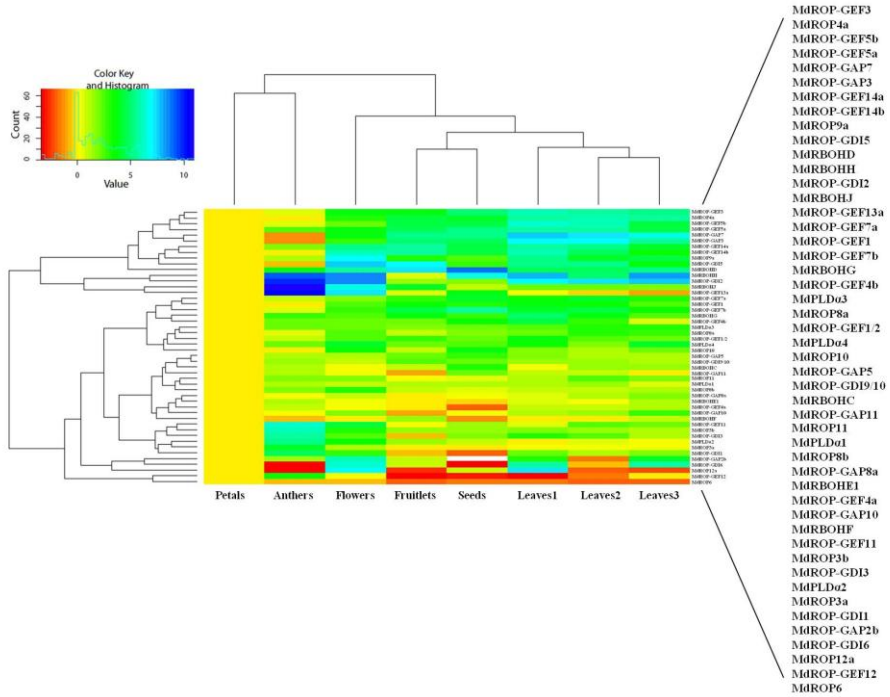

Figure S11 – Heatmap of log-transformed expression data obtained by qRT-PCR for expressed genes MdROPs, MdROP-GEFs, MdROP-GAPs, MdROP-GDIs, MdRBOHs and MdPLsDα in different tissues (Petals, Anthers, Flowers, Fruitlets, Seeds, and Leaves at three different stage of growth, 1, 2 and 3, respectively from youngest to oldest). Colors ranging from red (down-regulated) to blue (up-regulated) where yellow identified no expression variation compared to control sample (Petal tissue).

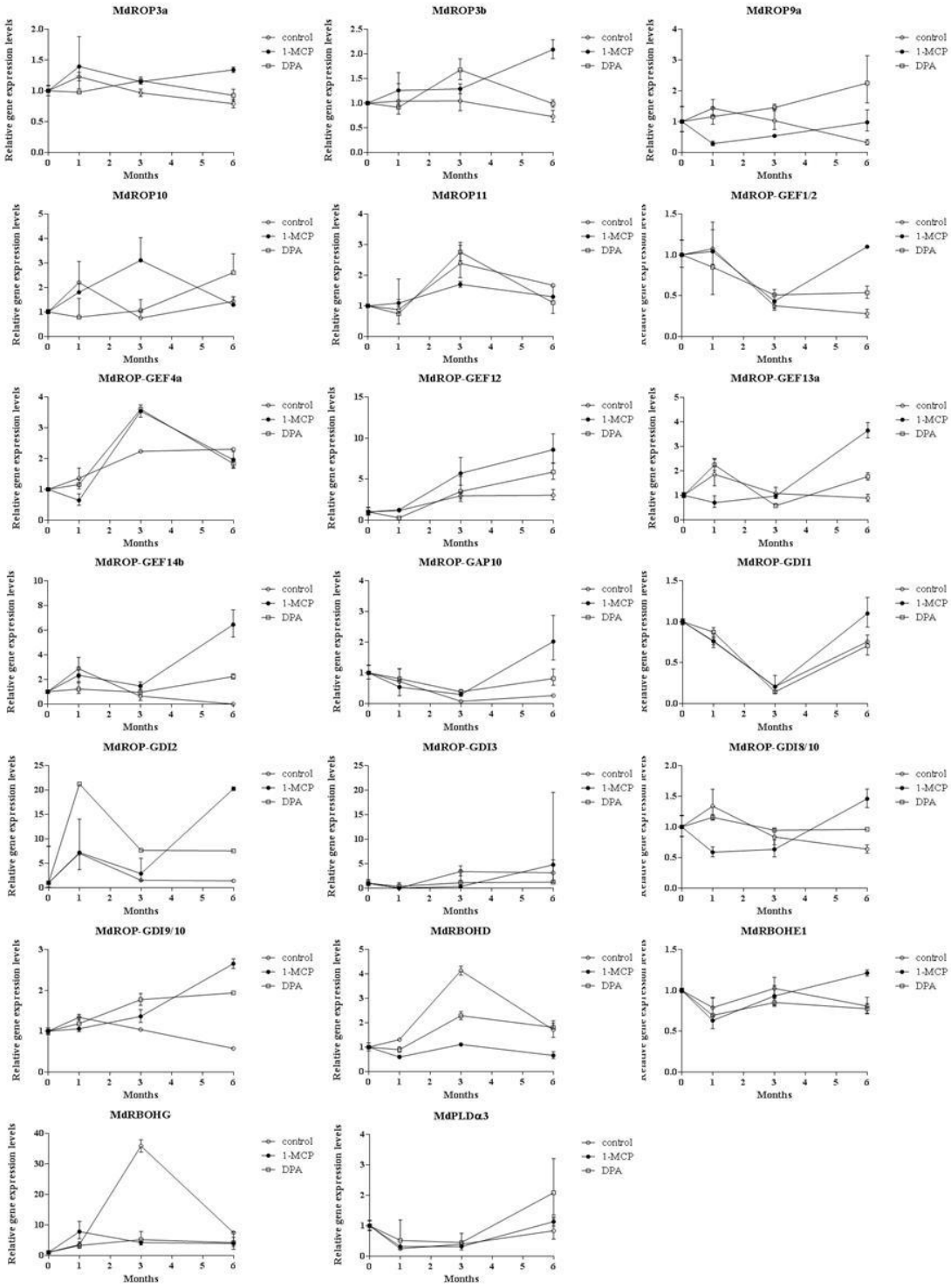

Figure S12 – Relative gene expression levels of MdROPs, MdROP-GEFs, MdROP-GAPs, MdROP-GDIs, MdRBOHs and MdPLsDα genes evaluated by real-time PCR on RNAs obtained from peel tissues from control, 1-MCP or DPA treated Granny Smith apples at harvest and after 1, 3 and 6 months of cold storage (controlled atmosphere: 0.8% O_2, 0.8% CO_2, 1°C). Expression levels were measured relative to Md_8283:1:a (Botton *et al*., 2011). Each value represents two independent replicates ± SD.

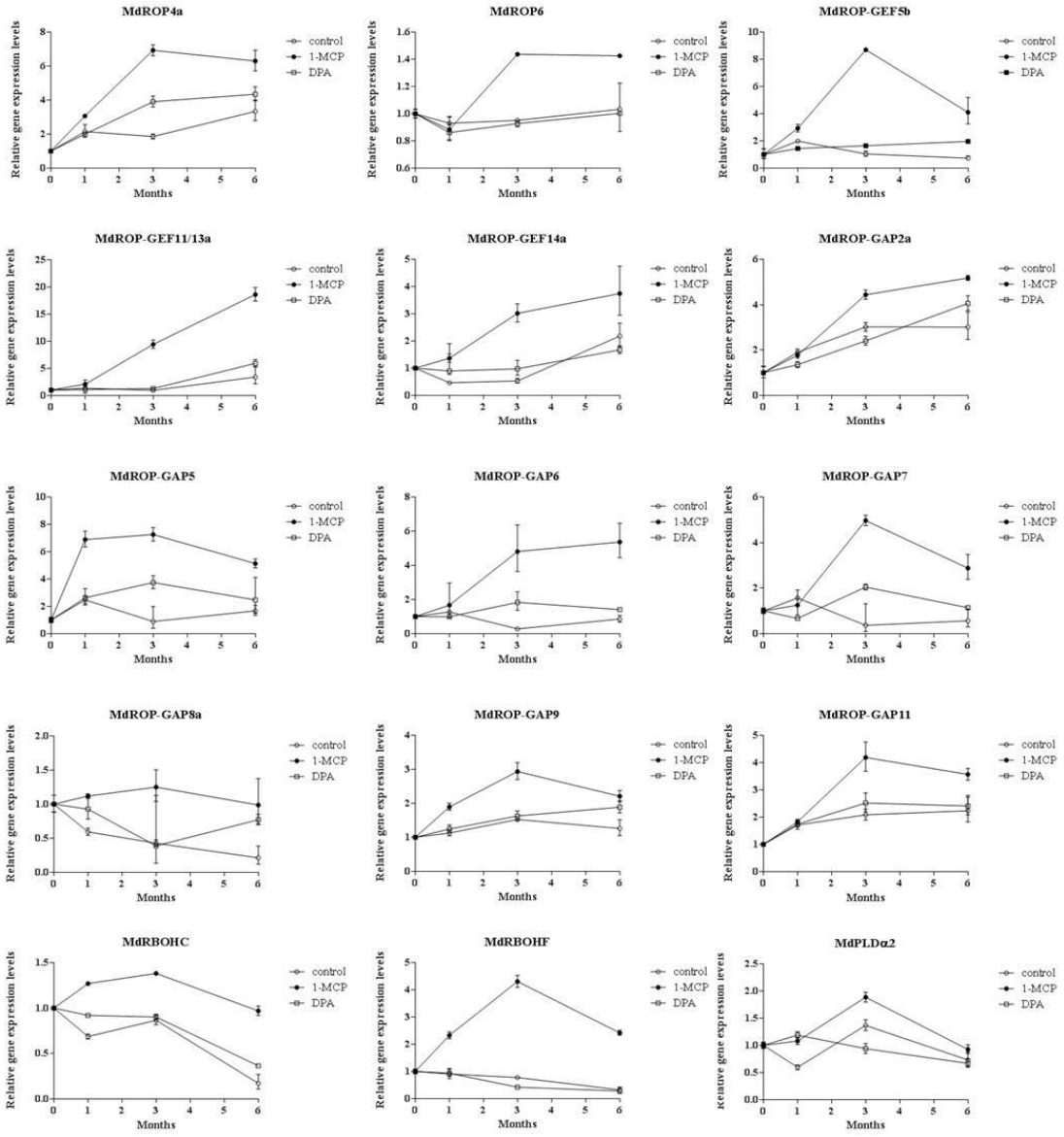

Figure S13 – Relative gene expression levels of MdROPs, MdROP-GEFs, MdROP-GAPs, MdRBOHs and MdPLsDα genes evaluated by real-time PCR on peels collected from control untreated, 1-MCP or DPA treated Granny Smith apples at harvest and after 1, 3 and 6 months of cold storage in controlled atmosphere (0.8% O_2, 0.8% CO_2, 1°C) during season 2010/2011. Expression levels were measured relative to Md_8283:1:a (Botton *et al*., 2011). Each value represents two independent replicates ± SD.

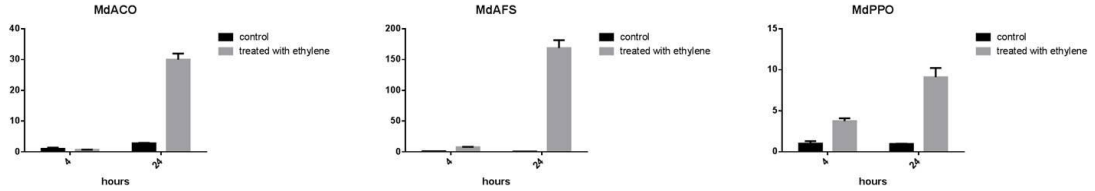

Figure S14 – Relative gene expression levels of MdACO, MdAFS, MdPPO evaluated by real-time PCR on RNAs obtained from peel tissues from Granny Smith treated for 4h and 24h with 100ppm of ethylene or maintained in air (control) for the same period of time. Expression levels were measured relative to Md_8283:1:a (Botton *et al*., 2011). Each value represents two independent replicates ± SD.

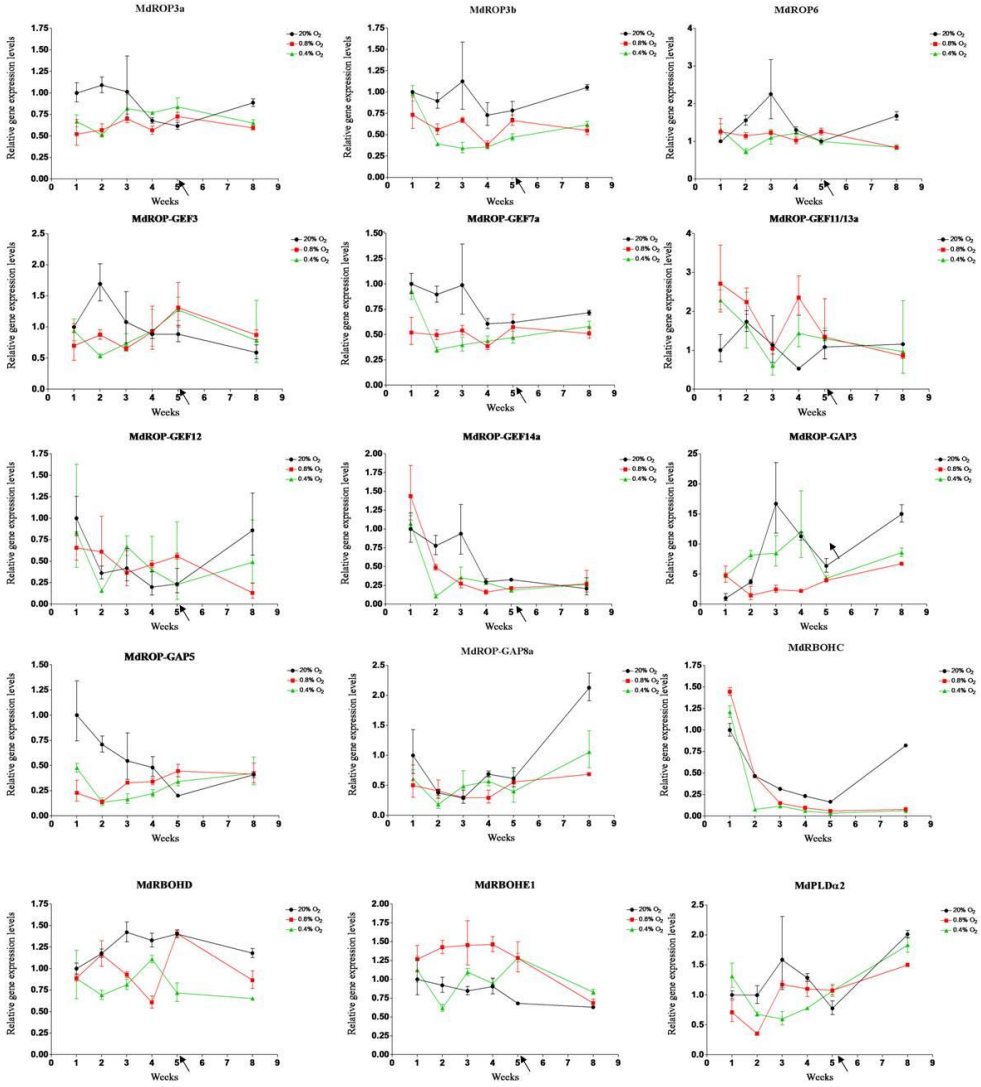

Figure S15 – Relative gene expression levels of MdROPs, MdROP-GEFs, MdROP-GAPs , MdRBOHs and MdPLDα genes evaluated by real time PCR on RNA obtained from peel tissues of apples cv Granny Smith stored at 20% oxygen (black line), 0.8% oxygen (red line) or subjected to ILOS (0.4% oxygen)(green line) at 1°C. Expression levels were measured relative to Md_8283:1:a (Botton *et al*., 2011). Arrows indicated the day before the fifth week in which apples that had been subjected to ILOS (0.4% oxygen) were moved to 0.8% oxygen (recovery).

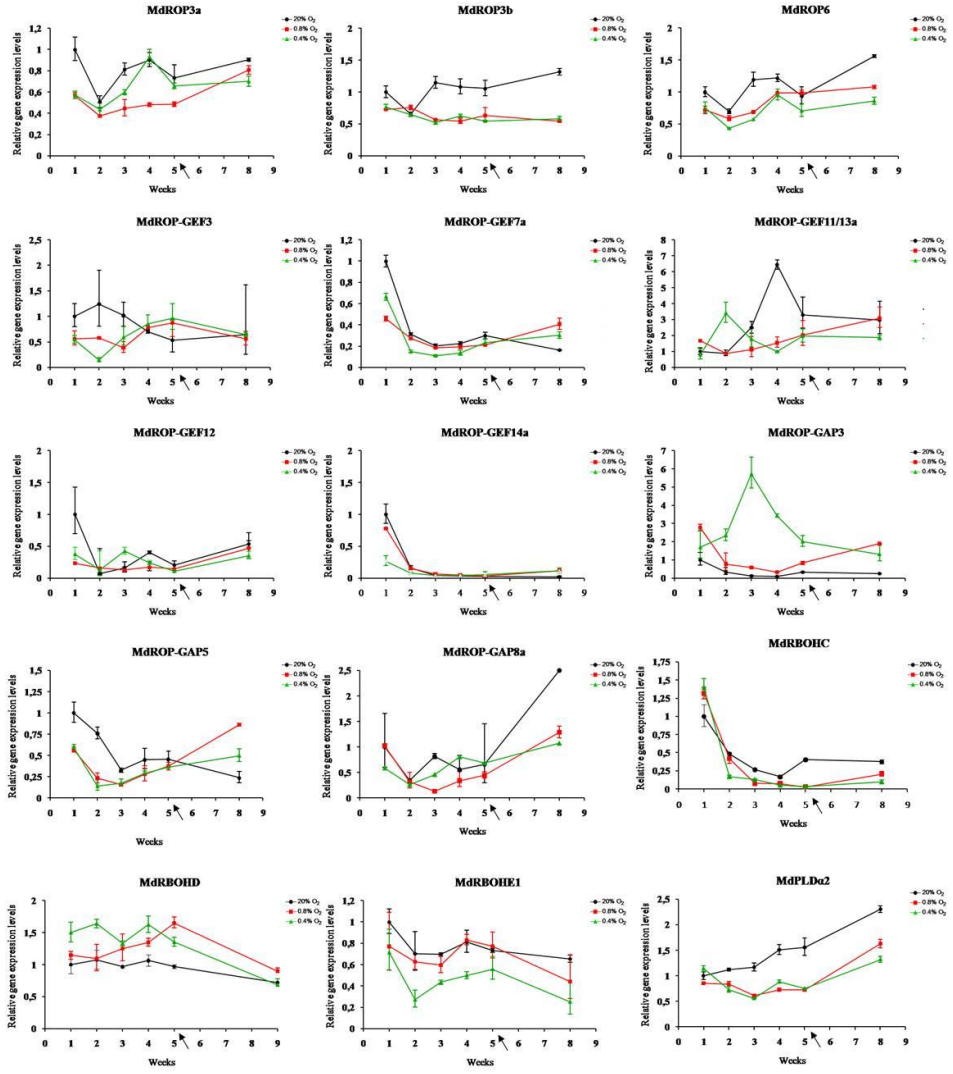

Figure S16 – Relative gene expression levels of MdROPs, MdROP-GEFs, MdROP-GAPs , MdRBOHs and MdPLDα genes evaluated by real time PCR on RNA obtained from flesh tissues of apples cv Granny Smith stored at 20% oxygen (black line), 0.8% oxygen (red line) or subjected to ILOS (0.4% oxygen)(green line) at 1°C. Expression levels were measured relative to Md_8283:1:a (Botton *et al.*, 2011). Arrows indicated the day before the fifth week in which apples that had been subjected to ILOS (0.4% oxygen) were moved to 0.8% oxygen (recovery).