USE OF 1-METHYLCYCLOPROPENE AND HYDROGEN PEROXIDE TO STUDY APPLE RIPENING PHYSIOLOGY

By

PAUL GUY LÉVESQUE

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Department of Horticulture and Landscape Architecture

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To the faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of PAUL GUY LÉVESQUE find it satisfactory and recommend that it be accepted.

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Chair

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USE OF 1-METHYLCYCLOPROPENE AND HYDROGEN PEROXIDE TO STUDY APPLE RIPENING PHYSIOLOGY

Abstract

By Paul Guy Lévesque, Ph. D.
Washington State University
May 2008

Chair: John Keenan Fellman

Unintended consequences of the ethylene action inhibitor 1-methylcyclopropene (1-MCP) are increased susceptibility to rot and decay pathogens, and fruit failure to develop aroma volatiles. In 1-MCP-treated apples, the effects of hydrogen peroxide (H_2O_2) to trigger ethylene biosynthesis and signaling were investigated. Transcription of antioxidative enzyme genes, antioxidant capacity, and catalase activity also were examined. H_2O_2 caused an increase in ethylene emission in 1-MCP-treated 'Golden Delicious' and 'Delicious' apples. In 'Golden Delicious', an increase in transcription of *Md-acs1* in 2005 and *Md-acs3* in 2006 matched the onset of the ethylene burst. In 2005, transcript levels of the putative *ein2*, *Md-acs1*, *Md-etr1*, and *Md-ers1* were lower in fruit treated with 3 $\mu l \cdot l^{-1}$ 1-MCP and dipped for an hour in 30 mM H_2O_2, compared to treated and untreated fruit dipped in water. In 2006, transcription of *Md-ers1* increased in fruit treated with $1 \mu l \cdot l^{-1}$ 1-MCP dipped in 30 mM H_2O_2 compared to treated fruit dipped in water. In 2005, transcript levels of *Md-gpx* and putative *sod* and *cat* increased over time in 1-MCP-treated water-dipped fruit compared to the other treatments. In contrast, transcript levels of putative *apx* varied with treatments and year.

Constantly, 1-MCP-treated fruit always had lower catalase activity compared to untreated ones. In 2006, dipping fruit in H_2O_2 significantly increased catalase activity during the first 12 days overall, however exogenous H_2O_2 did not significantly change catalase activity of 1-MCP-treated fruit. H_2O_2 infiltration in 'Delicious' apples after storage significantly decreased catalase activity. In 2005, dipping 1-MCP- treated fruit in H_2O_2 delayed a decrease in total water-soluble antioxidant capacity by a minimum of nine days. In 2006, 1-MCP significantly prevented a change in total water-soluble antioxidant capacity caused by H_2O_2.

In addition, while 1-MCP-treated apples suffered from higher rot incidence compared to untreated ones, those treated with H_2O_2 had higher induction of PR2 and PR5; two pathogenesis-related proteins linked to heightened disease resistance.

With 1-MCP it is difficult to ascertain treatment status or effectiveness. *Md-acs1* and *Md-pg*, two gene candidates as molecular markers of 1-MCP application were tested using RT-PCR. Both genes are effective molecular markers for 1-MCP application at harvest, after storage, when exposed to exogenous ethylene, and regardless of formulation.

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1. REVIEW OF LITERATURE

ETHYLENE

Ethylene Response and Action

Ethylene is a gaseous plant elicitor that has received great attention in plant biology. It is fundamental to signaling networks associated with numerous responses including seed germination, pollen growth, cell growth regulation, adventitious root development, stress, flower and fruit abscission, climacteric fruit ripening, wounding, programmed cell death, hypersensitive response, and finally senescence (Abeles et al. 1992; Ramina et al. 2007; Yang and Hoffman, 1984; Wang et al. 2002). Elicitors of ethylene include some sugars, auxins, abscisic acid, cytokinins, and ethylene (Stepanova and Alonso, 2005).

Ethylene regulates ripening of fruits known as 'climacteric'. The term climacteric refers to the sigmoidal curve representing the increase in respiration, and often concomitant ethylene burst, of such fruits (Crozier et al. 2000; Kays and Paull, 2004).

Postharvest research has led to the discovery of a plethora of technologies to reduce ethylene biosynthesis and action. Among the safest and most effective inhibitors of ethylene synthesis and action are aminoethoxyvinyl glycine (AVG) and 1-methylcyclopropene (1-MCP), respectively (Kays and Paull, 2004). They are currently employed by the fruit industry to reduce incidence of ethylene-exacerbated disorders and to delay fruit ripening and senescence.

Ethylene Biosynthesis

Ethylene biosynthesis starts by the divergence of *s*-adenosyl methionine (SAM) from a modified methionine cycle by the rate limiting enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS). ACS, catalyzes the conversion of SAM to ACC using pyridoxal-5'-phosphate. Then, ACC is oxidized to ethylene by ACC oxidase (ACO) using O_2, Fe, and ascorbate. By-products of the reaction include CO_2, monodehydroascorbate, and HCN (Yang and Hoffman, 1984; Crozier et al. 2000).

In climacteric fruit, the two committed steps of ethylene biosynthesis, ACS and ACO, are highly regulated by numerous environmental, developmental, and hormonal cues (Oetiker et al. 1997; Sato and Theologis, 1989; Johnson and Ecker, 1998). Both enzymes are parts of multigene families with ethylene inducible with some isoforms part of positive feedback loops (Barry et al. 1996, 2000; Nakatsuka et al. 1998; Hamilton et al. 1990; Oeller et al. 1991; Rottmann et al. 1991).

In apple (*Malus domestica* Borkh. abbreviated Md), ACS homologues *Md-acs1*, *Md-acs2*, *Md-acs3*, and Md-ACO1 are expressed during ripening. *Md-acs1* and Md-ACO1 are responsible for the ethylene burst linked to the climacteric response (Costa et al. 2005; Dong et al. 1991; Harada et al. 1997, 2000; Kim et al. 1992; Lay-Yee and Knighton 1995; Sunako et al. 1999; Wakasa et al. 2006; Wiersma et al. 2007).

In tomato (*Lycopersicon esculentum* Mill. abbreviated Le), Le-ACS2 facilitates autocatalytic ethylene production and continuous ethylene sensitivity is required for its mRNA accumulation. *Le-acs2* transcription was undetectable two days following 1-MCP treatment of tomato at the turning and pink stage; two stages with important *Le-acs2* and *Le-acs4* mRNA accumulation (Nakatsuka et al. 1997, 1998). Both *Le-acs2* and *Le-acs4*

transcription are up-regulated during ripening while *Le-acs6* transcription becomes undetectable (Barry et al. 2000). However, only *Le-acs2* is ethylene inducible (Barry et al. 2000; Nakatsuka et al. 1998). Anti-sense *Le-acs2* tomato reduced ripening-related ethylene levels to 0.5% of wild type plants and did not ripen normally (Oeller et al. 1991). Le-ACS2 may be essential for proper ripening regulation in tomato.

In addition to ripening, biotic and abiotic stress can cause an increase in ACS and ACO expression and activity. For instance, cold or chilling temperatures can increase ethylene production in some crops. In 'Granny Smith', there is an increase in ACO activity during cold storage (Larrigaudière and Vendrell, 1993; Tian et al. 1994). The same can be observed in 'D'Anjou' pear (Blankenship and Richardson, 1985), a cultivar requiring cold storage to trigger ethylene production and proper ripening (Chen and Mellenthin, 1982; Gerasopoulos and Richardson, 1996, 1997). Cooler temperature also increases 'Bartlett' pear ripening (Looney, 1972). In cucumber, ethylene production remains low during chilling, but increases rapidly upon warming (Wang and Adams 1980, 1982). Finally, in citrus, two ACS enzymes are chilling-regulated (Wong et al. 1999).

In most fruit crops, transcription of ripening-related ACO occurs before that of ACS and are known to occur in tomato (Tassoni et al. 2006; Oeller et al. 1991), passion fruit (Mita et al. 1998), winter pear 'Passe-Crassane' (Lelièvre et al. 1997) and apple (Wakasa et al. 2006). In tomato, ACO activity also increased prior to ACS activity (Liu et al. 1985; Nakatsuka et al. 1997). Transcription of ripening-related ACS and ACO in 1-MCP-treated fruit in the absence of ethylene suggests that ethylene evolution is controlled mainly post-translationally and possibly by substrate availability. Post-translational

regulation of some ACS isoforms include phosphorylation, ETO1-mediated stabilization, and degradation by proteases (Matarasso et al. 2005; Yoshida et al. 2005, 2006; Chae et al. 2005; Tatsuki and Mori, 2001; Wang et al. 2004; Spanu et al. 1994).

Ethylene Signal Transduction

Ethylene biosynthesis and signal transduction includes numerous steps as presented in figure 1-1 (abbreviations are explained in table 1-1). The current model regarding ethylene signal transduction (Johnson and Ecker, 1998; Klee, 2004; Alonso and Stepanova, 2004; Stepanova and Alonso, 2005) can be thought to begin with CTR1 on the endoplasmic reticulum (ER). CTR1 shares homology with Raf, a MAPKKK. Because of this, a MAPK cascade is thought to link CTR1 to EIN2. EIN2 is the first positive regulator of ethylene response. CTR1-mediated inhibition of ethylene response is activated by ethylene receptors also on the ER (Chen et al. 2002; Gao et al. 2003) that are always signaling in the absence of ethylene. Upon ethylene interaction with a copper-containing binding site, the receptors are deactivated, leading to ethylene response.

EIN2 relays the signal to the transcription factors EIN3 and EILs, which causes association with the promoter region of the ERF1 gene for expression of ERF1 protein. Subsequently, ERF1 activates secondary ethylene response genes. Ethylene signal transduction is thought to contribute to EIN3 and EILs stability, an event crucial for response. Conversely, EIN3 and EILs ubiquination by EBF1 and 2 would fate them to proteosome degradation (Guo and Ecker, 2003; Potuschak et al. 2003; Gagne et al. 2004). The mechanisms involved in the regulation of EIN3 by EIN2 are obscure at the moment (Stepanova and Alonso, 2005). The wide range of transcription factors and their

regulatory elements then would associate with DNA for transcription of ethylene-induced genes. It has been suggested that EIN-like transcription factors are responsible for the true ethylene response while EIN3 associates with responses related to pathogen attack.

MAPK6 was a candidate protein for relaying CTR1 to EIN2 (Chang, 2003; Ouaked et al. 2003). It was disregarded, as toxic levels of inhibitors were used in the study (Ecker, 2004; Liu and Zhang, 2004) and normal ethylene response occurred in the loss-of-function MAPK6 mutant (Ecker, 2004). It is possible that another MAPK links the two proteins.

Characterization of ethylene receptors and signaling elements in Arabidopsis led to several investigations in fruits. In ripening climacteric fruits, expression of putative ethylene receptors varies greatly and no clear-cut trends regarding induction of ETR and ERS homologs are evident. Expression of certain putative receptors genes follows the climacteric ethylene burst while others seem constitutive. In apples 'Orin' and 'Fuji', both *Md-ers1* and *Md-ers2* transcription was ethylene inducible and followed ethylene evolution post harvest, but not *Md-etr1* transcription (Tatsuki and Endo, 2006). In 'Golden Delicious', *Md-etr1* transcription was lower than *Md-ers1* but continuously increased in parallel with ethylene production, whereas *Md-ers1* transcription had decreased as ethylene levels peaked (Dal Cin et al. 2006).

Table 1-1. List of abbreviations used for naming proteins of the ethylene signal transduction pathway. Most proteins are named after mutants where a mutated form of the protein was found.

Abbr.	Definition
ACS	ACC synthase
ACO	ACC oxidase
CDPK	Ca^{2+}-dependent protein kinase
CTR	Consitutive triple response
EBF	Ethylene binding factor
EIN	Ethylene insensitive
EIL	EIN3-like
ERS	Ethylene response sensor
ETR	Ethylene response
ERF	Ethylene response factor
EBF	Ethylene binding factor
ETO	Ethylene overproducing
MAPK/MPK	Mitogen activated protein kinase
MKK	MPK kinase
MAPKKK	MAPK kinase kinase

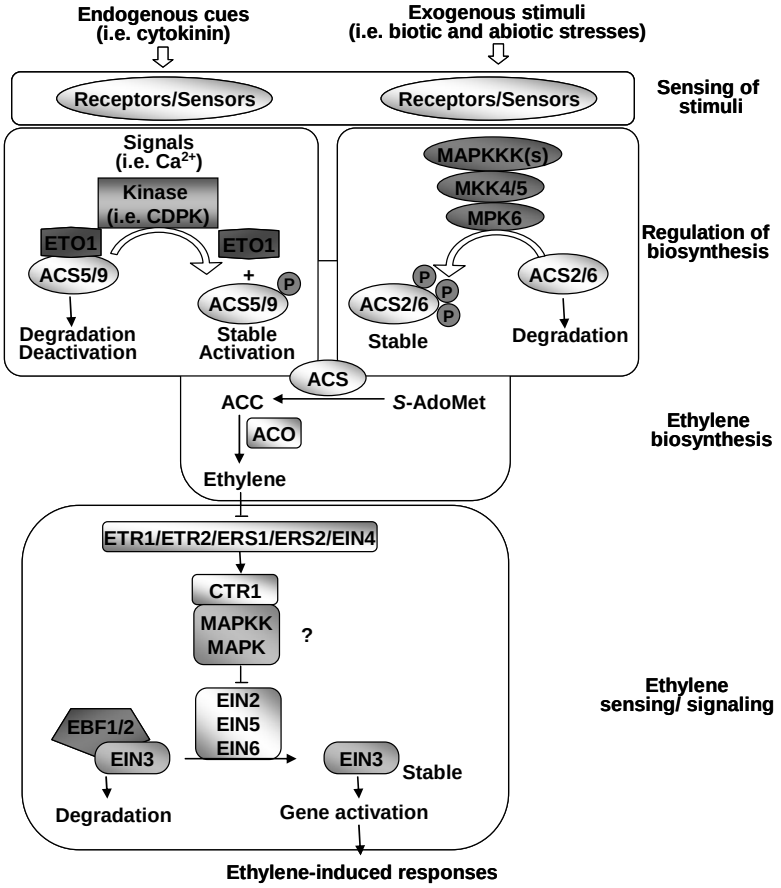

Figure 1-1. A simplified model depicting the induction of ethylene biosynthesis and the downstream ethylene signal transduction pathway. Taken from Ecker, (2004).

In peach (*Prunus persica* (L.) Batsch., abbreviated Pp), though *Pp-ers1* was up-regulated during ripening and by the ethylene homolog propylene, neither changed *Pp-etr1* expression (Rasori et al. 2002). In passion fruit (*Passiflora edulis* (L.) Sims, abbreviated PE), *Pe-ers2* increased during ripening and following ethylene exposure (Mita et al. 2002) and expression of *Pe-etr1* and *Pe-ers1* varied according to fruit tissue and study (Mita et al. 1998, 2002).

In tomato, *Nr* (*Le-etr3*) transcription rose with ripening (Payton et al. 1996; Wilkinson et al. 1995; Tassoni et al. 2006) and was undetectable in the transcription factor and receptor mutants *rin*, and *Nr*, respectively (Lanahan et al. 1994; Payton et al. 1996; Vrebalov et al. 2002; Wilkinson et al. 1995). *Nr* is ethylene-inducible in mature but not immature fruit (Wilkinson et al. 1995). In *Nr* anti-sense plants Le-ETR4 functionally compensated for absence of Nr (Tieman et al. 2000). Transcript levels of *Le-etr4/Le-etr5/Le-etr6* and *Le-etr1/Le-etr2* followed ethylene evolution, though *Le-etr1/Le-etr2* was much lower (Tassoni et al. 2006). Interestingly, Le-ETR1, and Le-ETR2 are thought constitutive and not inducible by stimuli such as ethylene or pathogen attack (Klee, 2002). In muskmelon (*Curcumis melo* (L.), abbreviated Cm), transcription of *Cm-ers1* and *Cm-etr1* increased during ripening. *Cm-etr1* mRNA levels rose markedly compared those of *Cm-ers1*. Moreover, *Cm-etr1* transcription remained high over the course of ripening while that of *Cm-ers1* decreased notably near the end of the study (Sato-Nara et al. 1999). Like for many receptors, transcription of *ctr1* homologues also follows ethylene evolution (Sato-Nara et al. 1999; El-Sharkawy et al. 2003; Zegzouti et al. 1999).

Recently, genetic evidence demonstrated a transitional state for ETR1 in which ethylene is bound to it, yet the receptor remains active prior to conformation change and

deactivation of the receptor (Wang et al. 2006). Also, ligand-mediated receptor degradation is considered partially responsible for increased ethylene sensitivity during ripening onset (Kevany et al. 2007). Because of these discoveries, Kevany et al. (2007) urge caution when interpreting receptor mRNA levels. Undoubtedly, ethylene receptors are under tight regulation including complex synergy with other signals. Thus, explaining receptor expression patterns is difficult and may only provide limited information regarding ethylene signaling.

1-METHYLCYCLOPROPENE (1-MCP)

In the fruit industry, a formulation of 1-methylcyclopropene (1-MCP), SmartFresh®, is used to inhibit the action of ethylene in apple fruit destined for long-term storage. While the use of the ethylene action inhibitor improves firmness retention and reduces ethylene-related disorders, an unintended consequence is the failure of the fruit to ripen normally and attain characteristic fresh quality attributes associated with taste and aroma (Blankenship and Dole, 2003; Watkins, 2006). 1-MCP is effective at the $nl \cdot l^{-1}$ level and considering the greater affinity of 1-MCP for ethylene receptors, a concentration of 100 $\mu l \cdot l^{-1}$ ethylene is required to compete with blocked receptors of apple treated with 1 $\mu l \cdot l^{-1}$ 1-MCP (Blankenship and Dole, 2003).

However, since 1-MCP also has affinity for non-target materials within and surrounding a commodity, reaching an accurate dosage may be difficult in instances when non-saturating responses may be needed (Vallejo et al. 2006; Nanthachai et al. 2007).

Commercial Application and Formulations

Gaseous and liquid formulations of 1-MCP exist. At the moment, only the gaseous formulation is employed in the apple industry. In the US and Canada, 1 $\mu l \cdot l^{-1}$ is the registered dose for use on apple while in some European Union countries such as the U.K. it is registered at 0.626 $\mu l \cdot l^{-1}$ (Watkins, 2006; PMRA, 2004). According to Argenta et al. (2006), a 700-fold higher concentration of 1-MCP is needed for the liquid formulation to be as effective compared to the gaseous.

Effects 1-MCP on Ethylene Biosynthesis

In addition to its effect on ethylene action, 1-MCP also inhibits ethylene synthesis (Blankenship and Dole, 2003; Kays and Paull, 2004; Watkins, 2006; Watkins and Miller, 2007). In tomato, 1-MCP delayed postharvest ACS transcript accumulation (Tassoni et al. 2006). In 1-MCP-treated apple, *Md-acs1* transcription was severely diminished whereas *Md-aco1* transcription was reduced to a lesser degree (Pang et al. 2006; Dal Cin et al. 2006; Tatsuki and Endo, 2006). ACS and ACO activity were diminished by 1-MCP also (Vilaplana et al. 2006a). In contrast, in 1-MCP-treated peach, *Pp-acs1* and *aco* transcription was barely limited (Dal Cin et al. 2006).

Effects of 1-MCP on Ethylene Signal Transduction

Transcription of ethylene receptors associated with signal transduction is also affected by 1-MCP treatment in fruit. In 'Golden Delicious' apple, *Md-ers1* and *Md-etr1* transcript accumulations were suppressed in 1-MCP-treated fruit (Dal Cin et al. 2006). In

'Passe-Crassane' pear (*Pyrus communis* abbreviated Pc), *Pc-etr1a*, *Pc-ers1a*, and *Pc-etr5* were lower in 1-MCP-teated fruit and mRNA levels followed ethylene evolution during ripening (El-Sharkawy et al. 2003). In 'Springcrest' peach, the same was true for *Pp-ers1* but not *Pp-etr1*. The inhibition of *Pp-ers1* transcription, however, lasted only a few hours after treatment (Rasori et al. 2002). In 'Summer Rich' peach, transcript levels of both *Pp-etr1* and *Pp-ers1* were lower in 1-MCP-treated fruit (Dal Cin et al. 2006).

Ctr1 transcription is also influenced by 1-MCP treatment. *Md-ctr1* transcription was greater in 1-MCP-treated 'Golden Delicious' and decreased to minimum levels 12 days after harvest. In untreated fruit a reverse relationship was observed (Dal Cin et al, 2006). In 'Passe Crassane' pear, a rise in *Pc-ctr1* transcription during ripening did not occur in 1-MCP-treated fruit (El-Sharkawy et al. 2003). In peach, a pattern representing *Pp-ctr1* transcript levels over time in 1-MCP-treated fruit was impossible to describe (Dal Cin et al. 2006).

Effects of 1-MCP on ethylene responses and apple quality parameters

As a simplified model, flavor is a combination of both taste and odor. While the four basic sensory elements sweetness, sourness, saltiness and bitterness constitute taste, up to 10,000 different odors/aromas can be perceived by the olfactory epithelium (Kays and Paull, 2004). The involvement of aroma volatiles in flavor is apparent and is important to fruit quality. In climacteric fruit, continuous ethylene exposure is required for aroma volatile development during ripening (Fan et al. 1998). In fruit, alcohol acyl-CoA transferase (AAT), an important, if not rate limiting step for ester production, is believed to be highly regulated by ethylene (Defilippi et al. 2005). Consequently, 1-MCP

treatment severely inhibits aroma volatile production of fruits by its suppression of ethylene action(Argenta et al. 2003; Fan and Mattheis, 1999; Golding et al. 1998, Rizzolo et al. 2005; Li et al. 2006; Ferenczi et al. 2006; Sigal-Escalada and Archbold, 2006).

In 1-MCP-treated 'Jonagold' and 'Delicious' apples, aroma development was inhibited by at least 50% and was partially caused by a decrease in ester precursors and not solely by limitations of AAT (Ferenczi et al. 2006). Inhibiting ethylene action can considerably reduce alcohol and aldehyde development, contributors to fruit aroma (Argenta et al. 2003). The effect of 1-MCP on aroma volatiles can further reduce sensory preference for juice made from treated apples (Drake et al. 2006). Li et al. (2006) claimed that a two-minute 2 mM salicylic acid dip has potential for triggering aroma ester production in 1-MCP-treated apples and demonstrated that this type of elicitor can increase total ester as well as *Md-aat2* transcript, protein, and activity.

1-MCP influences antioxidative mechanisms as well. In 'Delicious' apples, 1-MCP treatment accounted for 6.7 to 14% higher cyanidin 3-galactoside levels, 24% less chlorogenic acid, and 4% more epicatechin than untrated controls (McLean et al. 2006). In 'Golden Smoothee' apples stored in refrigerated air for three months, 1-MCP lowered lipid peroxidation, levels of hydrogen peroxide and ascorbate, and increased peroxidase activity, but had no significant effect on antioxidant capacity (Vilaplana et al. 2006b).

Many physiological disorders can be prevented by blocking ethylene action (Blankenship and Dole, 2003 and Watkins, 2006). However, in 'Empire' and 'Delicious' apples, 1-MCP was not always successful at preventing core and internal browning in (DeEll et al. 2007), two disorders exacerbated by chilling temperatures and high CO_2

concentrations in storage (AAFC, 2003). Also, a grey-brown skin discoloration can develop in 1-MCP-treated 'Golden Delicious' (Argenta et al. 2006).

Although 1-MCP is effective alone, it was more effective when used in synergy with AVG at delaying starch degradation, retarding fruit drop, and hindering ethylene production of untreated and naphthaleneacetic acid-treated 'Golden Delicious' and 'Golden Supreme' apples (Yuan and Carbaugh, 2007). The combination may also increase firmness retention of 'McIntosh' (Moran, 2006b), an apple cultivar prone to softening.

REACTIVE OXYGEN SPECIES (ROS)

ROS are ubiquitous to all living cells. They are high-energy molecules causing DNA, lipid, and protein damage (Foyer et al. 1994; Marnett et al. 2003). ROS are believed the main causative agents responsible for loss of quality in fresh produce and contribute to senescence (Hodges, 2003). Such damage caused to protein, nucleic acids, and lipids can significantly prevent proper metabolism and can cause disorders, diseases, and death (Emongor et al. 1994; Harman, 1956; Hartman, 1981; Ingle and D'Souza, 1989; Whitaker, 2004). The term oxidative stress is used to describe the inability of the innate antioxidant systems to scavenge excessive amounts of ROS. It can occur in response to chilling temperatures, salinity, ultraviolet light, heat, as well as other stress-inducing agents (Bray et al. 2000). Although originally viewed exclusively as detrimental to cell and causes of senescence (Harman, 1956; Hartman, 1981), ROS are now recognized as important signaling molecules regulating numerous responses essential for cell survival (Foyer and Noctor, 2005a,b).

Common ROS are superoxide (O_2-·), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical (·OH). ·OH is particularly damaging since it does not have any enzymatic scavenger (Apel and Hirt, 2004). ROS are short lived, existing only for fractions of seconds (Marnett et al. 2003). In $^1O_2$, the outer shell electron is excited to a higher orbital and its spin reversed. These electronic modifications of O_2 renders it highly reactive, especially with regards to enes and dienes, forming hydroperoxides and endoperoxides resulting in lipid peroxidation and chain reaction free radical propagation (Salin, 1987). In contrast, O_2-· and H_2O_2 are generated by the univalent reduction and oxidation of O_2, respectively (Salin, 1987, Apel and Hirt, 2004). Both are oxidants and mild reductants, with H_2O_2 considered a strong nucleophilic oxidizing agent (Salin, 1987; Elstner, 1982). O_2-· is associated with lipid peroxidation, viral inactivation, membrane damage, and single strand DNA breakage (Salin, 1987). H_2O_2 has lower reactivity and causes damage only at high concentrations. H_2O_2 toxicity is thought to occur principally via oxidation of thiol groups (Salin, 1987). Ironically, this is thought to be a mechanism by which ROS-mediated signaling occurs (Denu and Tanner, 1998; Dröge, 2002, 2006; Paget and Buttner, 2003).

In apples, endogenous H_2O_2 levels have been measured, with various results reported with different cultivars and method used. In 'Golden Delicious' 10 and 15 nmoles·g^{-1} FW was measured one week before and after commercial harvest respectively (Torres et al. 2003). In 'Cortland', ~20 nmoles · g^{-1} FW was measured at harvest, after 2 weeks at 20˚C, and after storage at 0˚C for 24 weeks. However, the harvest level increased to 27 nmoles · g^{-1} FW after one week at 20˚C. The endogenous H_2O_2 levels for 'Delicious' and 'Empire' during storage on average 24 and 31 nmoles · g^{-1} FW, respectively (Du and

Bramlage, 1995). 'Golden Smoothee' also had comparable endogenous H_2O_2 at harvest, ~22 nmoles · g^{-1} FW, however the levels decreased exponentially to ~6 nmoles·g^{-1} FW over a 90-day period at 0˚C. According to this study, 1-MCP-treated fruit had significantly lower H_2O_2 than untreated ones up to ~70 days in storage, when 1-MCP-treated fruit started to have significantly higher H_2O_2 levels (Vilaplana et al. 2006b).

Sources of ROS

In plants ROS have many sources. In the chloroplasts, $^1O_2$ is produced by the transfer of an exciton from the triplet state of chlorophyll. This energy is then transferred to O_2 creating $^1O_2$ (Apel and Hirt, 2004). O_2-· also is generated in the chloroplast. It is produced during the Mehler reaction in photosystem I, as energy escapes the plastoquinone pool, and potentially from an over reduced photosystem II (Apel and Hirt, 2004; Elstner, 1982; Salin, 1987; Suzuki and Mittler, 2006). This occurs more frequently under stress conditions where a loss of membrane integrity can occur (Desikan et al. 2003). Then, O_2-· can react with cellular components or be transformed by superoxide dismutase (SOD) to H_2O_2 (Fridovich, 1974).

Similar to the events occurring in the chloroplast, it is possible for energy to escape an over reduced mitochondrial electron chain reaction to O_2, generating $O_2^{-\cdot}$ (Suzuki and Mittler, 2006). Then, $O_2^{-\cdot}$ is dismutated to H_2O_2 by a mitochondrial SOD (Navrot et al. 2007; Mittler, 2002; Mittler et al. 2004).

Other sources of ROS are peroxisomes and the apoplast. In peroxisomes H_2O_2 is produced by glycolate oxidase, xanthine oxidase, and β-oxidation. However, glycolate oxidase is a major source of H_2O_2 during photosynthesis (Apel and Hirt, 2004). In the

apoplast, H_2O_2 is made from amine oxidase and oxalate oxidase. H_2O_2 generated from cell wall metabolism is also present. Additionally, $O_2^{-\cdot}$ can be generated on the plasma membrane by NADPH oxidase (Mittler, 2002; Torres and Dangl, 2005 ; Sagi and Fluhr, 2001).

Finally, interaction of H_2O_2 with Fe^{2+} creates ·OH, known as the Fenton reaction (Halliwell, 2006). Reaction of ROS with and unsaturated fatty acids causes a chain reaction generating alkyl radicals as well as alkyl H_2O_2. The alkyl radicals then continue the chain reaction and react with other unsaturated fatty acids (Mittler, 2002).

ROS generation increases in response to numerous stresses including cold, heat, freezing, and high light during temperature stress (Suzuki and Mittler, 2006).

Antioxidative mechanisms –vs- ROS

Antioxidants play an important role in protecting fruits from the oxidative damage inflicted by ROS, thus assuring quality and extending produce shelf life (Hodges, 2003). Cellular antioxidant substances consist of ascorbate, α-tocopherol, glutathione, and phenolics (Apel and Hirt, 2004). Crucial to the antioxidant system are a number of enzymes involved in ROS detoxification, and in the regeneration of antioxidant substances in numerous cell compartments. To simplify this review, a comprehensive diagram covering ROS scavenging pathways in plant cells and their localization is presented in figure 1-2. Some of the most studied antioxidant enzymes in fruit are SOD, and the H_2O_2 scavenging enzymes catalase (CAT) and peroxidases (POX) (Hodges, 2003). POX use specific substrates such as ascorbate (abbr. APX for ascorbate POX) and glutathione (abbr. GPX for glutathione POX). CAT, however, simultaneously uses two

H_2O_2 molecules (Noctor and Foyer, 1998). Because of this, CAT is known to have lower higher K_M for H_2O_2 in the mM range compared to a μM range for APX, for example. Thus, APX is thought to fine tune H_2O_2 levels for use in signaling while CAT is a major force in H_2O_2 detoxification during stress (Mittler, 2002) only limited by diffusion of substrate (Voet and Voet, 2003).

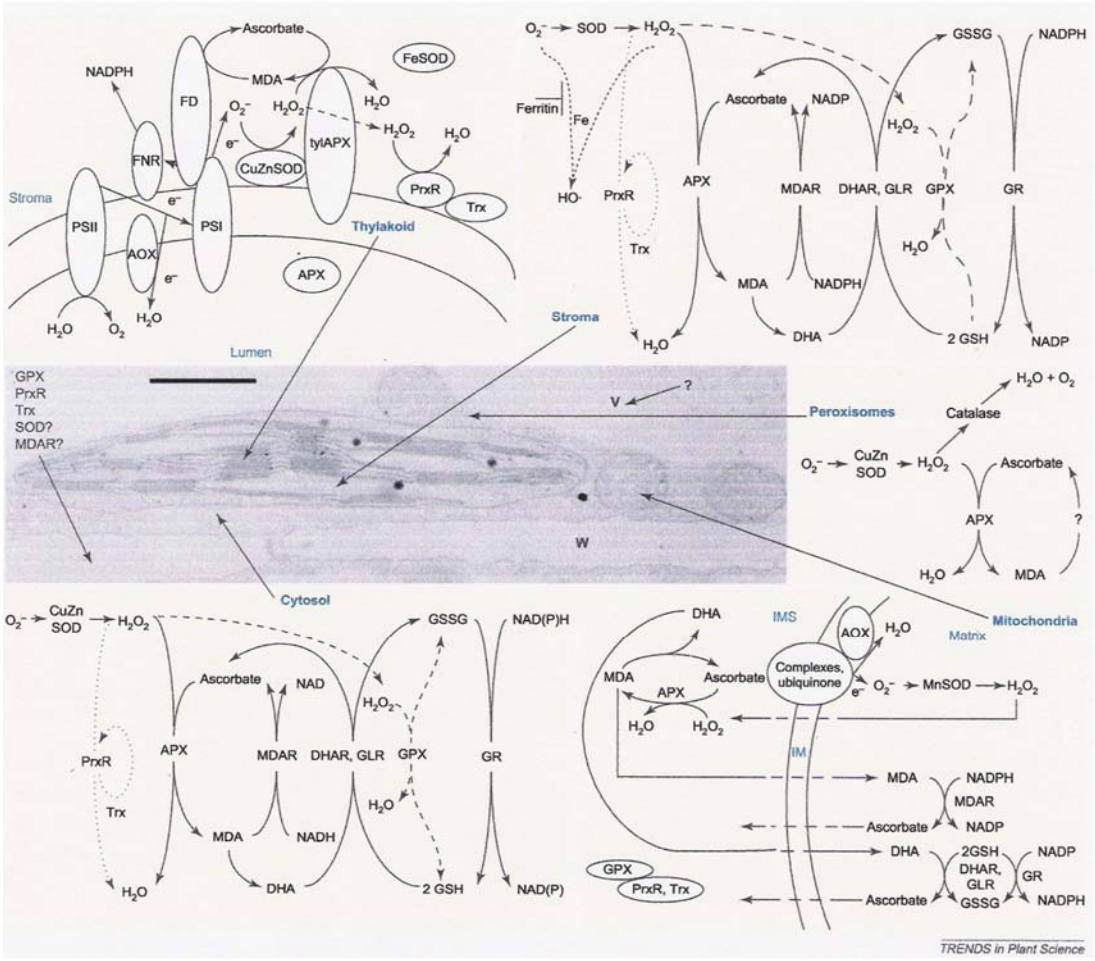

Figure 1-2. Localization of reactive oxygen species (ROS) scavenging pathways in plant cells. A transmission electron micrograph of a portion of a plant cell is used to demonstrate the relative volumes of the different cellular compartments and their physical separation (middle left). The enzymatic pathways responsible for ROS detoxification are shown. The water–water cycle detoxifies O_2·- and H_2O_2, and alternative oxidase (AOX) reduces the production rate of O_2·- in thylakoids [top left; in some plants iron superoxide dismutase (FeSOD) might replace CuZnSOD in the chloroplast]. ROS that escape this cycle and/or are produced in the stroma undergo detoxification by SOD and the stromal ascorbate–glutathione cycle. Peroxiredoxin (PrxR) and GPX are also involved in H_2O_2 removal in the stroma (top right). ROS produced in peroxisomes during photorespiration, fatty acid oxidation or other reactions are decomposed by SOD, CAT, and APX (middle right). SOD and other components of the ascorbate–glutathione cycle are also present in mitochondria. In addition, AOX prevents oxidative damage in mitochondria (bottom right). In principle, the cytosol contains the same set of enzymes found in the stroma (bottom left). However, these are encoded by a different set of genes and the major iron-chelating activity in the cytosol responsible for preventing the formation of HO· radicals is unknown. The enzymatic components responsible for ROS detoxification in the apoplast and cell wall (W) are only partially known, and the ROS-scavenging pathways at the vacuole (V) are unknown. Membrane-bound enzymes are depicted in white, GPX pathways are indicated by dashed lines and PrxR pathways are indicated by dotted lines in the stroma and cytosol. Although the pathways in the different compartments are mostly separated from each other, H_2O_2 can easily diffuse through membranes and antioxidants such as glutathione and ascorbic acid (reduced or oxidized) can be transported between the different compartments. Abbreviations: DHA, dehydroascrobate; DHAR, DHA reductase; FD, ferredoxin; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; IM, inner membrane; IMS, IM space; MDA, monodehydroascorbate; MDAR, MDA reductase; PSI, photosystem I; PSII, photosystem II; Trx, thioredoxin; tyl, thylakoid. Taken from Mittler et al. (2004).

SOD and H_2O_2 scavenging enzymes are crucial in preventing oxidative stress and ·OH formation (Apel and Hirt, 2004). Moreover, SOD, CAT, APX, GPX, and POX are all involved in stress acclimation and tolerance (Anderson et al. 1995; Noctor and Foyer, 1998; Prasad et al. 1994; Yoshimura et al. 2004). In apple callus tissue from 'McIntosh' shoots, activity of CAT and the peroxidases increased during acclimation at 0˚C along with an increase in sugar phosphates (Kuroda et al. 1991).

Water soluble antioxidants such as phenolics and ascorbate play an important role against ROS in apple. Ascorbate is a central antioxidant working at numerous levels. It regenerates α-tocopherol, directly scavenges $^1O_2$, $O_2^{-\cdot}$, and ·OH, and is the most important reducing substrate for APX (Noctor and Foyer, 1998). In addition, the partially oxidized form of ascorbate, monodehydroascorbate (MDHA) can be reduced back to ascorbate by the enzyme MDHA reductase (MDHAR). Two MDHA can also react together yielding ascorbate and dehydroxyascorbate (DHA). DHA self-degenerates, undergoing irreversible hydrolytic ring cleavage in aqueous solution, without damaging the cell, or is reduced back to ascorbate by DHA reductase (DHAR) (Davey et al. 2000; Mittler, 2004). In apple fruits, ascorbate occurs in the range of 20-100 mg · kg^{-1} (Davey et al. 2000) and greatly decreases in cold storage, more particularly in 1-MCP-treated fruit (Vilaplana et al. 2006a). Procyanidins are the major phenolics of most apple cultivars (Tsao et al. 2005).

Like ascorbate, α-tocopherol directly scavenges $^1O_2$, $O_2^{-\cdot}$, and ·OH. Being lipophylic, α-tocopherol scavenges also lipid hydroperoxides (Mittler, 2002) and interacts with carotenoids releasing $^1O_2$ to the triplet state of O_2 (Cunningham and Gantt, 1998).

APX and ascorbate are parts of the ascorbate-glutathione cycle, which consists of series or enzymatic reactions scavenging $O_2^{\cdot-}$ and H_2O_2, and reducing ascorbate and glutathione (Asada, 1992). The enzymes involved in the cycle are SOD, APX, MDHAR, DHAR, and glutathione reductase (GR) and their functions are coordinated as illustrated in figure 1-2. The cycle is ubiquitous throughout cell compartments (Mittler, 2002), and is important to prevent oxidative damage, and delay senescence in the postharvest environment.

ROS Signaling and Response

In some instance, ROS production is desired and required at the cell, organ, or whole plant level. Genetically programmed ROS production occurs via a number of NADPH oxidase isoforms in response to numerous events (Mittler et al. 2004; Torres and Dangl, 2005; Gapper and Dolan, 2006). NADPH oxidase-mediated ROS is involved in auxin-regulated gravitropic responses (Joo et al. 2001), ABA-mediated stomatal closure (Desikan et al. 2005; Kwak et al. 2003), regulation of chlorophyll content, apical dominance, proper plant morphology, flower set and development, fruit and seed development, prevention of blossom end rot of tomato (Sagi et al. 2004), and programmed cell death. Programmed cell death is required for gamete and megaspore formation, embryo development, degeneration of tissue in seed and fruit, tissue and organ development, senescence, and responses to environmental signals and pathogen attack (Dangl et al. 2000). Furthermore, NADPH oxidase and peroxidases are involved in providing ROS which are used as a directed source of energy for cross linking cell wall

polymers and suberin (Croteau et al. 2000; Vance et al. 1980; Bradley et al. 1992; Brisson et al. 1994).

The mode of action for H_2O_2 signaling is via a change in the thiol/disulfide redox state of cysteine residues essential for enzyme activity. Converse to H_2O_2, glutathione reduces the oxidized proteins (Denu and Tanner, 1998; Dröge 2006; Paget and Buttner, 2003). In humans, H_2O_2 is involved in the regulation of the protein tyrosine phosphatases VHR, PTP1, and LAR but not serine/threonine protein phosphatases (Denu and Tanner, 1998). H_2O_2 has a similar signaling power as insulin in regulating PTP1B (Mahadev et al. 2001). Also, direct interaction of H_2O_2 with the insulin receptor kinase increases its activity in the absence of insulin in purified recombinant fragments and intact cells (Schmitt et al. 2005). A positive feedback loop is created by H_2O_2 induction of NADPH oxidase and is involved in signal amplification (Mahadev et al. 2004). The insulin receptor would not only be negatively regulated by protein tyrosine phosphatase, but also directly activated by H_2O_2 (Dröge 2006).

It is possible that H_2O_2 regulates signaling in plants as illustrated in humans. Many signaling and regulatory proteins of plants contain tyrosine kinase and protein tyrosine phosphatase domains (Luan et al. 2000) and oxidative positive feedback loops exist in plants. An example of such a loop in plants is the one formed by H_2O_2 and the NADPH oxidase *AtrbohD* during pathogen attack (Desikan et al. 1998; Torres et al. 2002).

CROSS-TALK BETWEEN ROS AND ETHYLENE SIGNALING DURING RIPENING

Ethylene, although crucial to ripening, is not the only regulatory signal or element to the process. In tomato, the model fruit for climacteric ripening (Alexander and Grierson,

2002), exogenous ethylene triggers autocatalytic ethylene production at the mature but not immature green fruit stage (Lui et al. 1985). In anti-sense ACO tomato and melon lines, ACC accumulates regardless of autocatalytic ethylene inhibition (Picton et al. 1993; Guis et al. 1997), which points to another cue in addition to ethylene for ACC accumulation. Furthermore, although expressed during tomato ripening along with ethylene-induced Le-ACS2, Le-ACS4 is not under ethylene regulation and yet increases in expression during normal ripening (Barry et al. 2000). These observations illustrate that ripening events associated with ethylene are partially regulated by non-ethylene signals.

In plants, ethylene can evolve during stress as a function of ROS (Reddy et al. 1991; Mehlhorn and Wellburn, 1987; Schraudner et al. 1997; Langebartels et al. 1991; Wellburn and Wellburn, 1996). In winter type pears such as 'D'Anjou', a cold stress is required for ethylene-mediated ripening (Blankenship and Richardson, 1985; Chen and Mellenthin, 1982; Chen et al. 1982, 1983). In 'Granny Smith' apple and 'D'Anjou' pear, there is an increase in ACO activity during cold storage (Larrigaudière and Vendrell, 1993a, b; Tian et al. 1994; Blankenship and Richardson, 1985). Cold speeds up ripening of summer type pears such as 'Bartlett' as well (Looney, 1972), and ripening is accelerated or delayed with higher or lower endogenous H_2O_2 levels, respectively (Brennan and Frenkel, 1977). In some climacteric crops, an oxidative burst precedes or follows the rise in respiration and ethylene production (Meir et al. 1991; Masia, 1998).

ROS and ethylene signaling cross-talk with each other to facilitate programmed cell death and in reaction to chilling, drought, UV, and heat stress (Bray et al. 2000). The ethylene signal transduction components EIN2 is a protein linking stress and ethylene

signaling (Alonso et al. 1999). In addition, H_2O_2 regulates protein tyrosine phosphatases (Denu and Tanner, 1998). Protein tyrosine phosphatases oppose protein tyrosine kinases in the regulation of many eukaryotic responses (Voet and Voet, 2003), and are present in plants (Luan et al. 2001; Xu et al. 1998; Huang et al. 2000). Many plant proteins contain tyrosine kinase phosphorylation sites (accession # PS00007). Substrates of protein tyrosine kinases generally have a lysine or arginine seven amino acids to the N-terminal side of the phosphorylated tyrosine. In addition, on the same side, tyrosine phosphorylation sites hold an acidic residue (aspartate or glutamate) at either three or four amino acids away from the tyrosine (Patschinsky et al. 1982; Hunter, 1982; Cooper et al. 1984). Searching the *Malus* taxonomic lineage using ScanProcite (Gattiker et al. 2002) and the protein databases Swiss-Prot, TrEMBL, and PDB (NCBI; NPSA, Combet et al. 2000), 234 tyrosine kinase phosphorylation sites were found in 187 sequences. Among those sequences, both fruit specific Md-ACS3 and Md-ACO1 contain the motif. Other known apple specific elements of the ethylene signal transduction pathway such as Md-ETR1, Md-ERS1, and Md-CTR1, did not have a tyrosine kinase phosphorylation site sequence. In the absence of an Md-EIN2 sequence, the tomato Le-EIN2 was analyzed and showed also a tyrosine kinase phosphorylation site.

Though Md-ETR1 does not have a tyrosine kinase phosphorylation site sequence, a cysteine residue of ETR1 is required to mediate H_2O_2 response in guard cells of *Arabidopsis* (Desikan et al. 2005). In addition, ETR1 has a histidine kinase (HK) domain (Chan et al. 1993; Hua et al. 1995) and HKs may function as H_2O_2 sensors (Desikan et al. 2001). In yeast, some HKs linked to MAPK cascades alternatively function as H_2O_2 sensors (Singh, 2000). In *Arabidopsis,* H_2O_2 activates AtMPK6 (Kovtun et al. 2000), a

MAPK highly homologous to HOG1 (Desikan et al. 2001), a yeast MAPK connected to a HK signaling module (Singh, 2000). H_2O_2 may signal through the HK domain of ethylene receptors.

Exploitation of signaling could be a management tool for improving fruit quality during ripening, either by inducing defense mechanisms against ROS, thus preventing oxidative stress-mediated physiological disorder development, or pathogen attack.

GENE EXPRESSION, REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION

The combined use of reverse transcription (RT) with polymerase chain reaction (PCR) is effective at examining gene expression. RT-PCR readily provides information regarding ethylene metabolism of fruits. In a first step, mRNA coding for up regulated proteins is used as a template to create complementary DNA (cDNA) using reverse transcriptase. In any sample, the cDNA pool accurately represents the transcriptome the moment of sampling and provides a snapshot of gene regulation. In a second step, *Taq* polymerase, a heat resistant enzyme, amplifies the cDNA of interest primed with specific forward and reverse DNA sequences, known as primers. Following 30 amplification cycles, the products of the PCR are loaded on an agarose gel and via electrophoresis migrate according to size; the smaller the product, the greater the distance travelled. Finally, the products within the gel are stained with ethidium bromide and are viewed using UV-induced fluorescence emission. The level of fluorescence of the PCR product is proportional to the degree of transcription of the gene of interest (Ausubel et al. 2002; Sambrook and Russell, 2001).

HYPOTHESES AND OBJECTIVES

The following hypotheses were investigated in this study:

1. H_2O_2 reverses ethylene action inhibition caused by 1-MCP in apples by initiating ethylene signaling;
2. H_2O_2 is an important ripening signal functioning through induction of ethylene biosynthesis and signal transduction genes;
3. H_2O_2 induces stress tolerance mechanisms;
4. *pg* and *acs1* transcripts are good molecular markers for detection of 1-MCP application, regardless of formulation, storage, and exposure to exogenous ethylene.

The objective for the first study was to examine H_2O_2 as a signal of ripening-related ethylene production and restoration of signaling in 1-MCP-treated apple by studying: a) ethylene evolution using gas chromatography, and b) ethylene related gene transcription using RT-PCR. The objective for the second study was to improve understanding of ROS metabolism with regards to ethylene and antioxidative mechanisms in fruit after harvest. The objective for the third study was to test *pg* and *acs1* transcripts as molecular markers for detection of 1-MCP application with regards to formulation, storage, cultivars, and exogenous ethylene exposure.

Finally a *post hoc* hypothesis was tested. The hypothesis was that instigation of systemic resistance as illustrated by induction of PR proteins by H_2O_2 is responsible for

preventing disease incidence in 1-MCP-treated H_2O_2-dipped fruit as compared to those dipped in water.

2. HYDROGEN PEROXIDE INDUCES ETHYLENE BIOSYNTHESIS AND SIGNALING IN 1-MCP-TREATED 'GOLDEN DELICIOUS' AND 'DELICIOUS' APPLES

Ethylene plays a crucial role of climacteric fruits by orchestrating ripening-associated events such as color changes, flavor development, and softening (Kays and Paull, 2004). In the commercial fruit industry, formulations of 1-methylcyclopropene (1-MCP) are used to inhibit ethylene action in apples destined for long-term storage. While the use of 1-MCP improves firmness retention and reduces ethylene-related disorders, an unintended consequence is failure of fruit to ripen normally and attain characteristic flavor (Blankenship and Dole, 2003).

As a simplified model, flavor is a combination of both taste and odor. While taste is constituted of the four basic sensory elements sweetness, sourness, saltiness, and bitterness, up to 10,000 different odors/aromas can be perceived by the olfactory epithelium (Kays and Paull, 2004). The involvement of aroma volatiles in flavor is apparent and is important to fruit quality. In climacteric fruit, continuous ethylene exposure is required for ripening-related volatile development (Fan et al. 1998, Argenta et al. 2003). Defilippi et al. (2005) believe that alcohol acyl-CoA transferase (AAT) is a rate limiting step for ester production and is highly regulated by ethylene. Inhibiting ethylene action in 'D'Anjou' pears considerably reduced ester, alcohol, and aldehyde development (Argenta et al. 2003) which are major contributors to fruit aroma. Volatile esters produced during ripening provide some of the signature aromas of apples (Flath et

al. 1967, Fellman et al. 2000, Mattheis et al. 1991). Consequently, 1-MCP can severely inhibit volatile production of fruits (Argenta et al. 2003; Fan and Mattheis, 1999; Golding et al. 1998, Rizzolo et al. 2005).

In plants, ethylene is synthesized by conversion of *S*-adenosylmethionine by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) to generate ACC. Then, ACC is oxidized by ACC oxidase (ACO) generating ethylene. In climacteric fruit ripening, ethylene is a key factor regulating its own synthesis as well as other ripening-related processes, and contributes to a rise in ethylene exhibiting a climacteric response (Kays and Paull, 2004).

Ethylene, although crucial to ripening, may not be the only regulatory signal or element to the process. In tomato, the model fruit for climacteric ripening, ethylene exposure triggers autocatalytic ethylene production at the mature but not immature green fruit stage (Lui et al. 1985). In anti-sense ACO tomato and melon lines, ACC accumulates regardless of autocatalytic ethylene inhibition (Picton et al. 1993; Guis et al. 1997), which points to another cue in addition to ethylene for ACC accumulation. Furthermore, although expressed during tomato ripening along with ethylene-induced Le-ACS2, Le-ACS4 is not under ethylene regulation and yet increases in expression during normal ripening (Barry et al. 2000). These observations illustrate that ethylene physiology during ripening is partially regulated by other signals than ethylene.

In pome fruits, cold-induced ethylene production is well documented (Larrigaudière and Vendrell, 1993a, b; Tian et al. 1994; Looney, 1972; Blankenship and Richardson, 1985; Chen and Mellenthin, 1982; Chen et al. 1982, 1983; El-Sharkawy et al. 2003; Gerasopoulos and Richardson, 1997; Knee et al. 1983; Knee, 1987; Lelièvre et al. 1995;

Sfakiotakis and Dilley, 1974). Plants exposed to cold stress accumulate hydrogen peroxide (H_2O_2) (Fadzillah et al. 1996; Prasad et al. 1994; Okuda et al. 1991; Omran, 1980; Wise and Naylor, 1987a,b), an important plant signal (Desikan et al. 2001; Laloi et al. 2004; Neill et al. 2002; Uhrig and Hülskamp, 2006; Van Breusegem et al, 2006; Vranová et al. 2002). In addition, in some climacteric fruits, an oxidative burst precedes or follows the rise in respiration and ethylene production (Meir et al. 1991; Masia, 1998). Also, a study by Brennan and Frenkel (1977) showed higher endogenous H_2O_2 levels associated with advancing of ripening in pear while inhibition of H_2O_2 accumulation delayed ripening events. Under different ripening scenarios, endogenous H_2O_2 levels displayed an ethylene-like response curve over the course of ripening. Moreover H_2O_2 enhanced ethylene production while ethylene increased endogenous H_2O_2. This study implied H_2O_2 as a regulating agent of ethylene response as well as part of cross-talks between ethylene signaling and oxidative metabolism. It is conceivable that ethylene signal transduction leading to autocatalytic ethylene production is partially regulated by H_2O_2.

Exploitation of cross-talk signaling between the ethylene signal transduction and oxidative metabolism could be a management tool for proper aroma development in 1-MCP-treated apples by restoring ripening processes related to ethylene. This study examined the potential role of H_2O_2 as a signaling molecule of ripening-related ethylene production and its possible involvement in restoring ethylene sensitivity of 1-MCP-treated ‘Golden Delicious’, by examining ethylene-related gene expression using reverse transcription and polymerase chain reaction.

MATERIALS AND METHODS

Plant material and treatments

In 2005, 'Golden Delicious' apples (*Malus domestica* Borkh.) harvested at optimum maturity (0.02 ppm internal ethylene; n=10) were treated with 1-MCP (0 or 3 ppm) overnight at 1°C. Subsequently at room temperature, 1-MCP-treated fruits were dipped for an hour in either 0, or 30 mM H_2O_2, while the control (no 1-MCP, no H_2O_2) was dipped in water only. Each treatment had three replicates. Five fruit per replicate per treatment were sealed Plexiglas chambers and ethylene production was measured over a 28-day period at room temperature to monitor the ripening response. A gas sample from each chamber was automatically analyzed via gas chromatography every 8 hours. In parallel, the skin of three fruit per replicate was sampled on day 1, 10, and 28, immediately frozen and ground in liquid nitrogen to create a homogenous composite sample representative of the physiological state of the treated fruit. The sample was stored at -80°C for further analyses.

Some experimental changes were made in 2006 for 'Golden Delicious'; the 1-MCP concentration was adjusted to 1 $\mu L \cdot L^{-1}$, which is the standard concentration employed by the industry in the US (Blankenship and Dole, 2003), and fruit were dipped in 0 or 30 mM H_2O_2. In addition, peel tissue was sampled on day 1, and 12.

Agrofresh® and Stemilt Growers Inc. generously provided SmartFresh-treated 'Delicious' apples and their equivalent control after roughly four months of storage in refrigerated air at 1°C. The fruit were treated with 1$\mu l \cdot l^{-1}$ 1-MCP by SmartFresh® representatives within a week from harvest. The fruit were transported by Agrofresh from Wenatchee, WA, to the Postharvest Laboratories in Pullman, WA. On arrival, the cold

fruit was placed in storage at 1˚C overnight. On the next day the fruit was warmed to room temperature. Treated and untreated fruit from three replicate/boxes were infiltrated with 0, 1, or 10 mM H_2O_2 for 12 minutes with >60 kPa of vacuum. Subsequently, five fruit from each replicate/box were placed in Plexiglas chambers for ethylene analysis as previously described. Skin tissue was sampled and frozen in liquid nitrogen on day 1, 7, and 14 as indicated earlier.

Measurement of Internal Ethylene Concentration

Internal ethylene concentration was used as a harvest index for optimum maturity of apples for long-term storage. A 0.5 cm^3 gas sample was withdrawn with a 50-gauge syringe using a hypodermic needle from the core of 10 fruit per assessment day. The gas sample was injected into a gas chromatograph (HP 5830A, Hewlett Packard, Palo Alto, CA) equipped with a PLOT-Q column (Agilent, Avondale, PA.) (15 m x 0.53 mm I.D.), . The injector was a packed type with a flame ionization detector (FID). The pre-purified nitrogen carrier gas flow rate was to 8 ml· min^{-1}. Injector and detector temperatures were 200 °C and the oven temperature 100 °C.

Measurement of Ethylene Evolution

Ethylene evolution from fruit was measured by placing the fruit into airtight flow chambers and the headspace was sampled using an automated system (Patterson and Apel, 1984). The Plexiglas chambers (18 L) were supplied with ethylene-free air at approximately 100 ml·min^{-1}. Ethylene concentration from each chamber was measured

automatically every 8 h using a HP 5890A gas chromatograph (Hewlett-Packard Co., Palo Alto, CA, USA). The system was equipped with a FID connected to a 0.53 mm x 15 m GS-Q-PLOT column (Agilent Technologies, Inc., Santa Clara, CA) with a flow rate of 10 $ml \cdot min^{-1}$. The oven, injector, and FID temperatures were 30, 200, and 200˚C, respectively.

RNA extraction

RNA was extracted according to Manning (1991) with minor modifications. RNA was extracted by homogenizing 1.2 g of frozen skin tissue in 6 ml of extraction buffer containing 0.2 M boric acid, 10 mM Na_2EDTA, and adjusted to pH 7.6 with Tris base. Both β-mercaptoethanol and SDS were added to the extraction buffer in 1/50 (v:v) of final volume. The extract was thawed and centrifuged at 20,000 x g for five minutes at room temperature. After decanting the supernatant, two phenol: chloroform: isoamyl alcohol (25:24:1) extractions were done. Following centrifugation at 3,000 g for 10 minutes at room temperature, the upper phase containing total nucleic acids was diluted 2.5 times using diethyl pyrocarbonate (DEPC) -treated water and adjusted to a final sodium concentration of 80 mM using 1 M sodium acetate. To this, 0.4 volume of 2-butoxyethanol (2-BE) was added and the solution was placed on ice for 45 minutes prior to centrifugation at 20,000 g for 10 minutes at 0°C. The supernatant was collected without touching the gel-like pellet containing polysaccharides and other carbohydrates. The total nucleic acids were precipitated by adding 1 volume of 2-BE and by placing the solution on ice for 45 minutes prior to centrifugation at 20,000 g for 15 minutes at 0°C. The pellet was washed three times with 70 % (v/v) ethanol. Residual ethanol was

evaporated at room temperature in a fume hood for five minutes and the pellet solublized in DEPC-treated water. The RNA was precipitated in 3M LiCl overnight at 0˚C. Then, the solution was centrifuged at 11,600 g for 20 minutes. The pellet was rinsed three times with 70 % (v/v) ethanol followed by a wash with 100% ethanol and the residual ethanol was evaporated for five minutes in a fume hood at room temperature. Finally, the pellet was dissolved in 30 µl of DEPC-treated water. RNA was quantified at 260 nm using a Cary 100 Bio UV-visible spectrophotometer (Varian, Walnut Creek, CA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Following DNase treatment (DNA-free kit, Ambion, Inc., Austin, TX, USA). First-strand cDNA synthesis was done using 0.75 µg of total RNA in 2005 and 1 µg in 2006, and oligo$(dT)_{20}$ primer using the ThermosScript™ RT-PCR system (Invitrogen, Carlsbad, CA, USA). Thirty PCR cycles were done using GoTaq® DNA Polymerase following manufacturer's instruction (Promega Corporation, Madison, WI, USA) using the gene-specific primers listed in Table 2-1. Conditions for the PCR reactions are noted in the caption of the appropriate figure. Gene sequences and expressed sequence tags (EST) were obtained from the National Center for Biotechnology Information (NCBI, 2007) and primers were designed using the software Vector NTI Advance™ 10.3 (Invitrogen Carlsbad, CA, USA).

Experimental design and statistical analysis

The experiments were split-plot designs with repeated measures. In 2006, 1-MCP was the whole plot treatment and Box(1-MCP) was the whole plot error, and H_2O_2 as the

subplot treatment and residual error as the subplot error. The data were analyzed using the GLM procedure of SAS version 9.1 for Windows (SAS Institute Inc., Cary, North Carolina). In 2005, statistical analysis only included 1-MCP-treated fruit. In the vacuum infiltration study, 1-MCP-treated and untreated fruit were analyzed separately. In every study, the ANOVA assumptions were met.

Table 2-1. Forward (F) and reverse (R) primer sequences used for PCR amplification of ethylene-related genes in apple. Sequence and identity from NCBI (2007).

Gene name		Sequence (5' → 3')	Size (bp)	Accession No.	Reference
Ethylene biosynthesis					
Md-acs1	F	ATGCGCATGTTATCCAGAAACG	1079	U89156	Harada et al. 1997
	R	AACAAGCCAGCATTGCCGTT			Direct submission
Md-acs2	F	TGAAATCTATGCAGCAACCG	491	U73815	Rosenfield et al. 1996
	R	ATGAACAACCAGGTGAAACG			Direct submission
Md-acs3	F	ACCCACTTGGTGCAACAATC	230	U73816	Rosenfield et al. 1996
	R	AACTCGAAAGCCTGGAAGGC			Direct submission
Md-aco1	F	GACTTTCCCAGTTGTCGACTTGAG	736	AJ001646	Castiglione et al. 1997
	R	TATCGACATTCTGGTCCCATCC			Direct submission
Ethylene Signal Transduction					
Md-etr1	F	TGTTGGCATGCAACTGTATTGAGC	1721	AF032448	Lee et al. 1998
	R	GCCAAGACCTTCACTTTCAATCCA			Direct submission
Md-ers1	F	ATGGAGTCCTGTGATTGCATAGACG	1375	AY083169	Trivedi and Solomos, 2002
	R	GATTCTCATCTCCAACGGCATACA			Direct submission
Md-ctr1	F	GGCATGGATCTGATGTTGCTGT	479	AY670703	Cin et al. 2005
	R	ATTTGATGGCTCATCACGCAGGAC			Direct submission
Putative *EIN2* (Arabidopsis; 63.2% id.)	F	GAGCCGCAGTACCATTCTTCAGAT	482	CV084546	Korban et al. 2004
	R	TTTGGGAAAGCTACATCACCGGCT			Direct submission
Volatile Ester Synthesis					
Md-aat1	F	ATACTATGGCAATGCATTTG	451	AY707098	Souleyre et al. 2005
	R	GCGGCAACTGAAATTTAATTT			
Md-aat2	F	ATACTATGGCAATGCATTTG	455	AY517491	Li et al., 2006
	R	AGGATGGCAAACACATTGGTA			
Other					
Putative *act3* (Arabidopsis; 98.4% id.)	F	GATGAAAATCTTGACTGAGCGTGG	515	CN927806	Beuning et al. 2004;
	R	CAGCTTTGGCAATCCACATCTG			Direct submission

RESULTS

1-MCP is an excellent inhibitor of ethylene action and is effective at stopping autocatalytic ethylene production in climacteric fruits (Blankenship and Dole, 2003; Watkins, 2006). Reversal of 1-MCP-mediated ethylene inhibition in apple was investigated using H_2O_2 as an initiator of ethylene metabolism.

Ethylene production

1-MCP inhibited ethylene production and delayed the rise in ethylene concomitant with the climacteric response (Fig. 2-1, 2-2, and 2-3). Dipping 'Golden Delicious' in H_2O_2 did not significantly increase ethylene production, even though ethylene production appeared greater or advanced when 1-MCP-treated fruit were dipped in H_2O_2 (Fig. 2-1 and 2-2). Vacuum infiltration of H_2O_2 in 'Delicious' apple after commercial storage significantly advanced the ethylene burst of 1-MCP-treated fruit and also decreased the magnitude of the response in untreated fruit (Fig. 2-3). As a main factor, H_2O_2 significantly affected ethylene production in 1-MCP-treated fruit but not in non-treated apple. However, an interaction between 'Time' and 'H_2O_2' was significant in both, illustrating the influence of H_2O_2 over time on ethylene production.

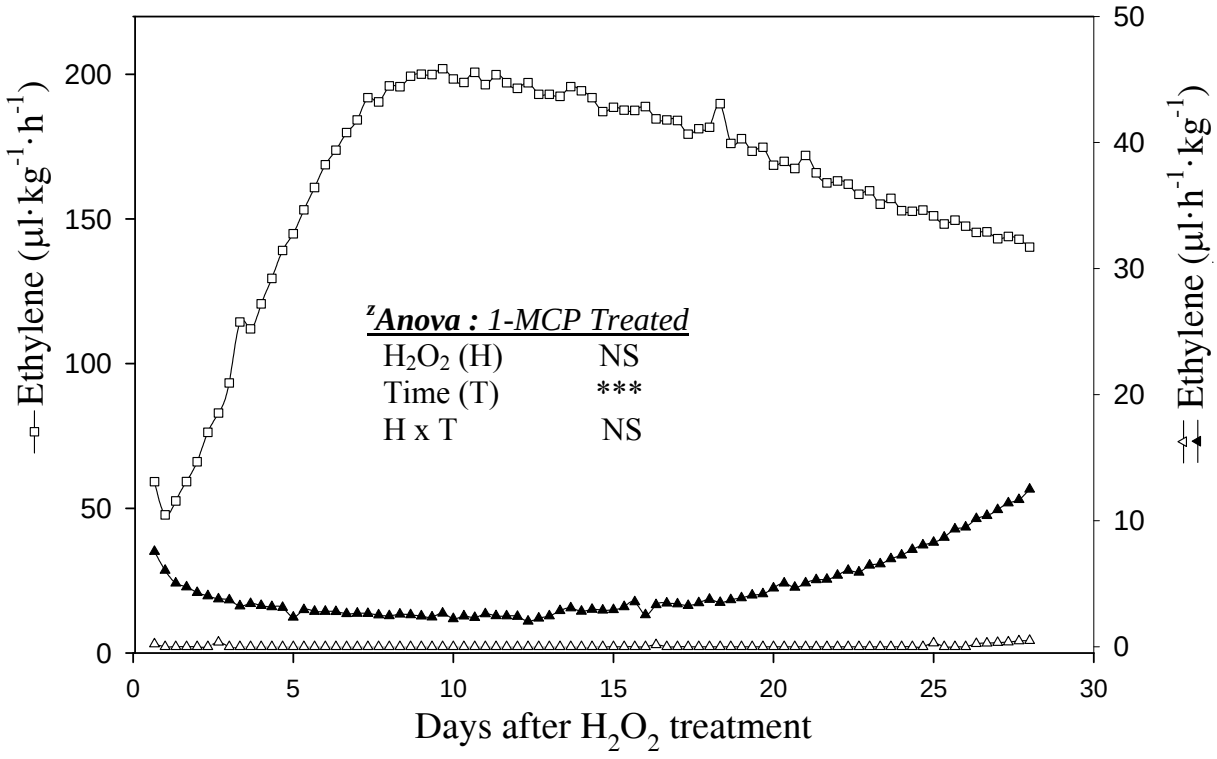

Figure 2-1. Postharvest ethylene evolution of 'Golden Delicious' apple at 23°C after treatments. Fruit were treated with 0 (□) or 3 µl·l^{-1} 1-MCP (△). In addition, 1-MCP-treated fruit were dipped in 0 or 30 mM H_2O_2 (▲). ^{z}Significance: NS and ***; Non significant and significant at $P \leq 0.001$, respectively.

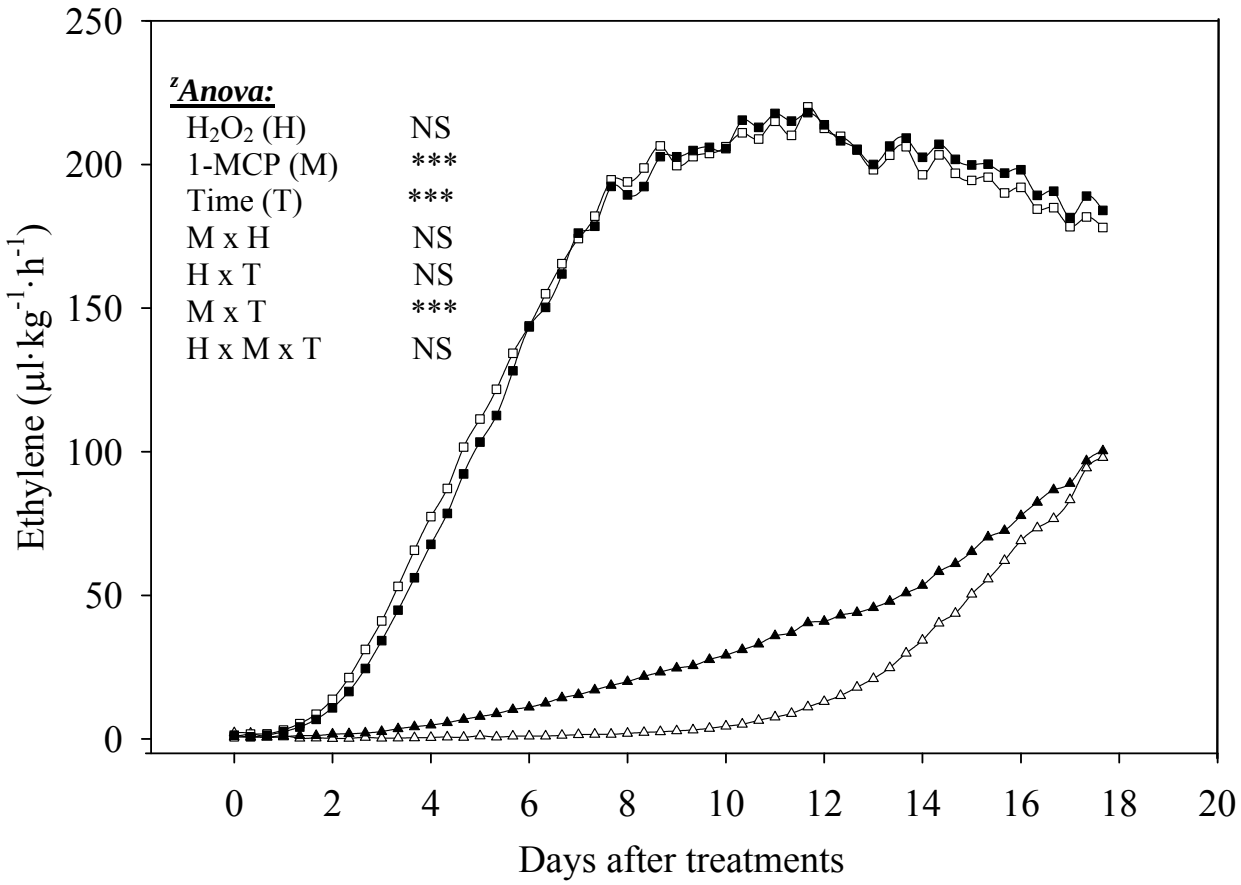

Figure 2-2. Postharvest ethylene evolution of 'Golden Delicious' apple at 23°C after treatments in 2006. Fruit were treated with 0 (□) or 1 µl·l^{-1} 1-MCP (△). White and black symbols represent fruit dipped in 0 and 30 mM H_2O_2, respectively. [z]Significance: NS and ***; Non significant and significant at $P \leq 0.001$, respectively.

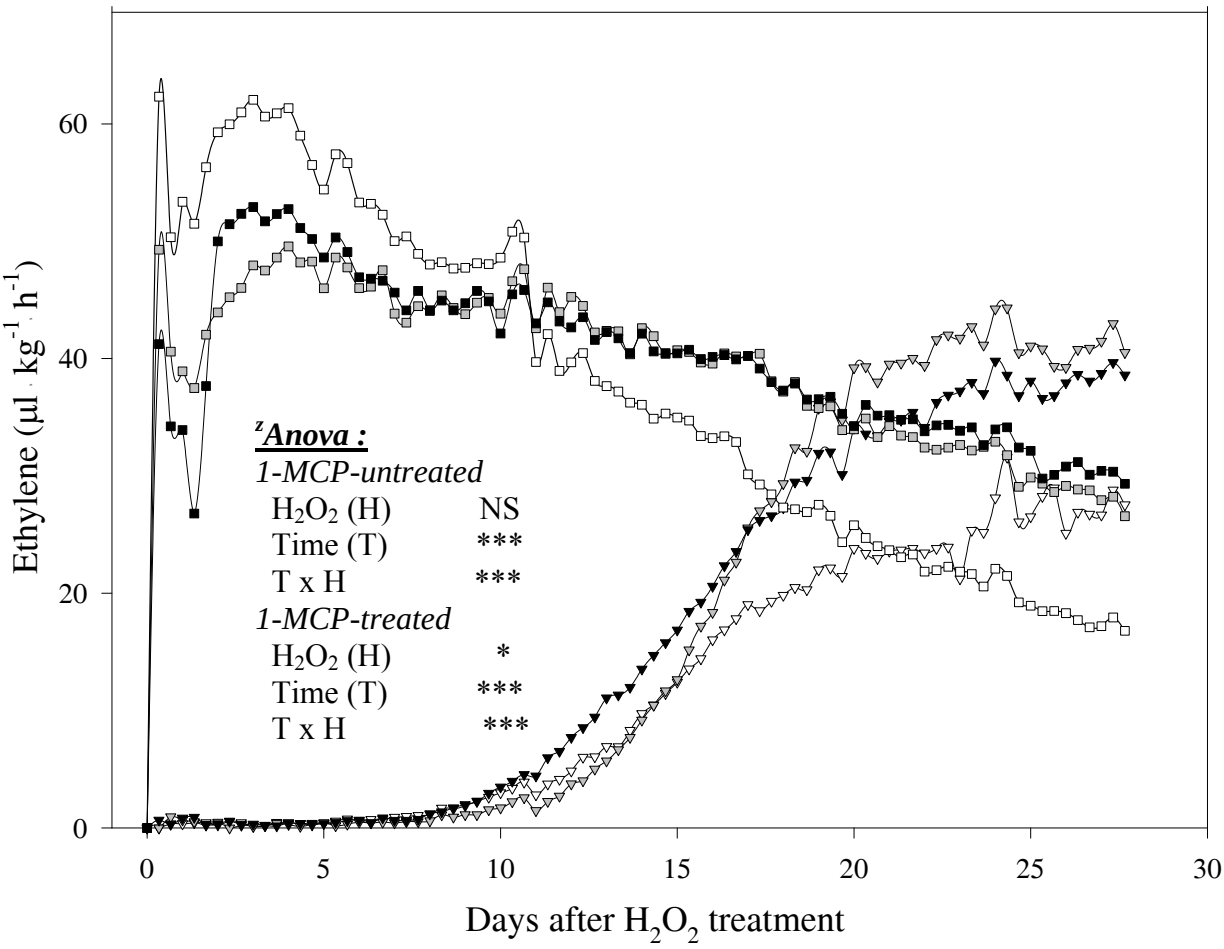

Figure 2-3. Ethylene evolution of 'Delicious' apple at 23°C following vacuum infiltration of H_2O_2 after storage in 2006. Fruit were treated with 0 (□) or 1 $\mu l \cdot l^{-1}$ 1-MCP (△). White, grey, and black symbols represent fruit infiltrated with 0, 1, and 10 mM H_2O_2, respectively. [z] Significance: NS, *, and ***; Non significant and significant at P=0.05 and $P \leq 0.001$, respectively.

Gene transcription

The impact of 1-MCP and H_2O_2 on the transcription of ethylene biosynthetic enzymes ACS and ACO was studied. 1-MCP and H_2O_2 influenced transcription pattern of *Md-acs1*, but had limited influence on *Md-acs3* and *Md-aco1* (Fig.2-4 and 2-5). Transcript levels of *Md-acs1* increased from day1 to 12, and were higher in fruit not treated with 1-MCP. In 1-MCP-treated fruit, *Md-acs1* transcription was not detectable in H_2O_2 -dipped fruit on day 1 and 10 but was higher on day 28 in 2005 (Fig.2-4). The higher *Md-acs1* transcript level in H_2O_2 -dipped fruit coincided with the increase in ethylene production (Fig. 2-1). In 2006 the transcript levels were similar between all 1-MCP-treated fruit. In fruit not treated with 1-MCP, *Md-acs1* was lower in H_2O_2 -dipped ones on day 1and 10. *Md-acs2* was not transcribed in any of the fruit during the studied period. Transcription patterns of *Md-acs3* differed from those of *Md-acs1* and were low in all fruit. In 2005, *Md-acs3* transcription was the greatest in 1-MCP-treated H_2O_2 -dipped fruit one day after treatment. By day 10, *Md-acs3* was only detectable in 1-MCP-treated fruit dipped in water. In 2006, one day after treatment, *Md-acs3* was more prominently induced in 1-MCP-treated fruit compared to untreated ones and was higher in H_2O_2 -dipped fruit. This up-regulation of *Md-acs3* could explain the higher ethylene levels in 1-MCP-treated H_2O_2 -dipped fruit at that point in time (Fig. 2-2). By day 12, *Md-acs3* transcript level in 1-MCP-treated water-dipped fruit was lower than any other treatment. Among all possible treatment combinations, 1-MCP-untreated H_2O_2-dipped fruit had the lowest transcription. Transcription of *Md-aco1* was high and similar in fruit of all treatment combinations in both years, with the exception of 1-MCP-treated H_2O_2 -dipped fruit 10 days after treatments where it was lower.

In addition, the long-term effect of 1-MCP and H_2O_2 on transcription of genes associated with the ethylene signal transduction pathway elements was examined. Drought conditions were present during the 2005 growing season and seem to have influenced expression of ethylene signal transduction components. Therefore results from both years are presented separately to improve text flow.

In 2005 transcription of *Md-etr1* was undetectable in H_2O_2-dipped fruit one day after treatments (Fig 2-4), while transcript levels were similar in fruit dipped in water regardless of 1-MCP. Nine days later, 1-MCP-treated water-dipped fruit had higher transcript levels whereas fruit from the other two treatments had comparable transcription levels. *Md-ers1* transcription was lower in 1-MCP-treated H_2O_2 -dipped fruit. One and 10 days after treatment, fruit not treated with 1-MCP had higher *Md-ers1* transcription than treated ones. By day 10, 1-MCP-treated fruit had initiated *Md-ers1* transcription while those dipped in H_2O_2 still had undetectable levels. *Md-ctr1* transcription was higher in untreated fruit than 1-MCP-treated fruit, where the levels were comparable in both water- and H_2O_2 -dipped fruit. By day 10, only 1-MCP-treated fruit had detectable *Md-ctr1* levels. Transcription of the putative *ein2* was lower in fruit dipped in H_2O_2 both one and ten days after treatment.

In 2006, one day following treatments, *Md-ers1* transcription was lower in 1-MCP-treated fruit (Fig. 2-5). H_2O_2-dipped fruit showed also lower *Md-ers1* transcript level. By day 12, fruit from all treatments had increased *Md-ers1* transcription and fruit not treated with 1-MCP showed higher levels than treated ones. Also on day 12, 1-MCP-treated fruit those dipped in H_2O_2 had greater *Md-ers1* transcript levels in comparison to water-dipped ones. No difference in *Md-ers1* transcript levels were noticed in fruit not treated with 1-

MCP, regardless of the dipping treatment on day 12. On day one after treatment, *Md-etr1* transcript level was higher in 1-MCP-untreated H_2O_2-dipped fruit. Eleven days afterwards, fruit not treated with 1-MCP had higher transcript levels with fruit dipped in H_2O_2 still showing greater induction. *Md-ctr1* transcription seemed unaffected by 1-MCP or H_2O_2 during the first 12 days. Neither 1-MCP nor H_2O_2 seemed to influence expression of the putative *ein2* either one day after treatment while eleven days later, H_2O_2-dipped fruit had lower induction of *ein2*.

Similarly, we investigated the consequence of ethylene inhibition and H_2O_2 on expression of alcohol acyl-CoA transferase (AAT) during ripening, a gene involved in aroma volatile development. 1-MCP and H_2O_2 did not seem to affect transcription of *Md-aat1* and *Md-aat2* in the first 12 days following application. Cues other than ethylene and H_2O_2 seemed to regulate *Md-aat1* transcription in ripening 'Golden Delicious' as it decreased with time. *Md-aat2* levels did not change.

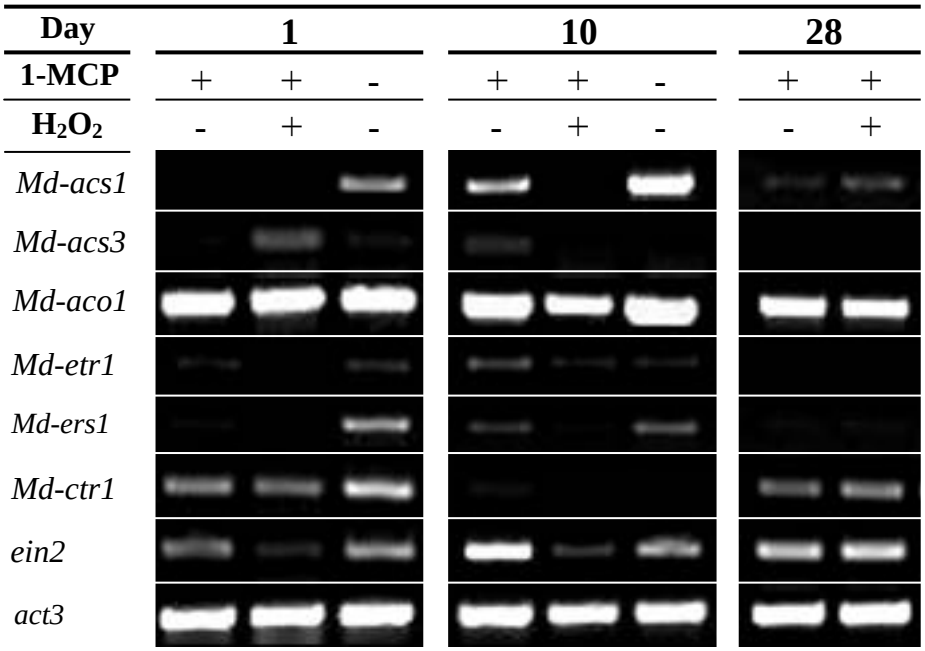

Figure 2-4. RT-PCR analysis of transcription of genes from 'Golden Delicious' in 2005 following post harvest treatment with (+) and without (-) 3 $\mu l \cdot l^{-1}$ 1-MCP and dipped with (+) or without (-) 30 mM H_2O_2.
It was impossible to extract RNA from fruit not treated with 1-MCP at the 28 day stage. Therefore no result could be presented. Putative *act3* was used as a PCR control.

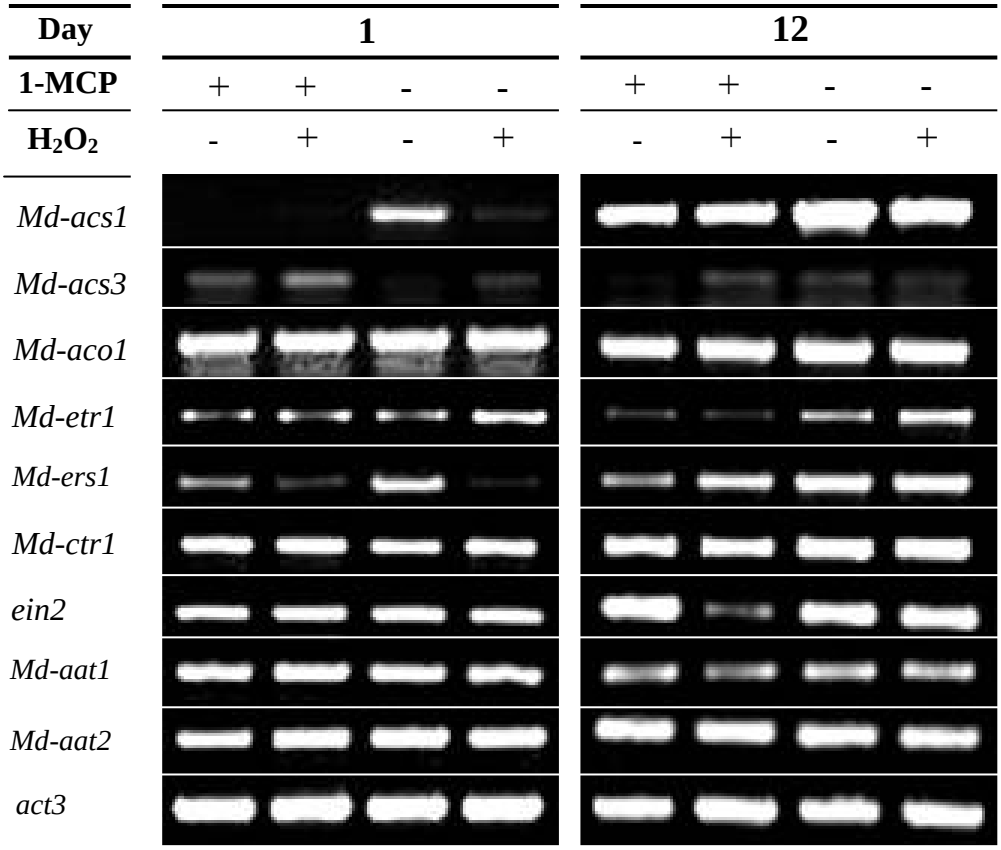

Figure 2-5. RT-PCR analysis of transcription of genes from 'Golden Delicious' in 2006 following post harvest treatment with (+) and without (-) 1 $\mu l \cdot l^{-1}$ 1-MCP and dipped with (+) or without (-) 30 mM H_2O_2. Putative *act3* was used as a PCR control.

DISCUSSION

Ethylene can evolve as a function of ROS in plants in response to stress (Reddy et al. 1991; Mehlhorn and Wellburn, 1987; Schraudner et al. 1997; Langebartels et al. 1991; Wellburn and Wellburn, 1996). In winter type pears such as 'D'Anjou', a cold stress is required for ripening (Blankenship and Richardson, 1985; Chen and Mellenthin, 1982; Chen et al. 1993). In 'Granny Smith' apple and 'D'Anjou' pear, there is an increase in ACO activity during cold storage (Larrigaudière and Vendrell, 1993a, b; Tian et al. 1994; Blankenship and Richardson, 1985). Cold speeds up ripening of summer type pears such as 'Bartlett' as well (Looney, 1972), and ripening is accelerated or delayed with higher or lower endogenous H_2O_2 levels, respectively (Brennan and Frenkel, 1977). In some climacteric crops, an oxidative burst precedes or follows the rise in respiration and ethylene production (Meir et al. 1991; Masia, 1998).

To initiate ripening of 1-MCP-treated fruit, dipping fruit in H_2O_2 to trigger initiation of ethylene metabolism was investigated. In 2005, to assure a complete shutdown of ethylene action and therefore to illustrate the potential of H_2O_2 in reversing 1-MCP-mediated ethylene inhibition, 3 $\mu l \cdot l^{-1}$ was used instead of the U.S. commercial norm of 1$\mu l \cdot l^{-1}$ (Blankenship and Dole, 2003).

1-MCP-treated fruit a H_2O_2 dip always advanced the ethylene burst when compared to a water dip; however the effect was not statistically significant (Fig. 2-1 and 2-2). H_2O_2 -induced ethylene synthesis has been also observed in all preliminary studies using both apple and 'D'Anjou' pears (Appendix Fig. A-1, A-2, and A-3). Considering potential limitations with regards to H_2O_2 administration, vacuum infiltration was used to provide a uniform H_2O_2 administration potentially reducing variation at the delivery

level. To maximize the ability of H_2O_2 in reversing ethylene inhibition by 1-MCP, various H_2O_2 concentrations, close to those reported to inhibit protein tyrosine phosphatase and increase tyrosine kinase signaling (Dröge, 2002), were investigated. Finally, gene expression was examined using RT-PCR to confirm the potential effect of a H_2O_2 dip on ethylene biosynthesis and signaling of 'Golden Delicious'. In 2005, drought conditions during the growing season could be responsible for variations in results between 2005 and 2006.

ACS and ACO transcription and ethylene evolution during fruit ripening

In climacteric fruit, the two committed steps of ethylene biosynthesis, ACS and ACO, are highly regulated by numerous environmental, developmental, and hormonal cues (Oetiker et al. 1997; Sato and Theologis, 1989; Johnson and Ecker, 1998). Both enzymes are part of multigene families (Barry et al. 1996; Rottmann et al. 1991) while some isoforms are ethylene inducible and part of positive feedback loops, others are not (Barry et al. 2000; Nakatsuka et al. 1998; Hamilton et al. 1990; Oeller et al. 1991; Rottmann et al. 1991).

In 2006, transcription of *Md-acs1* (Fig. 2-5) coincided greatly with the climacteric-related ethylene burst and with delayed ethylene production caused by 1-MCP (Fig. 2-2). In 2005, 1-MCP-treated fruit had *Md-acs1* transcript levels were undetectable one day after treatment some level was observable by day 10 (Fig.2-4). However, no ethylene was detectable at that point (Fig. 2-1). This could be caused by a lack of substrate availability. However, in 'Orin' apple, *Md-acs1* transcription paralleled ACC accumulation (Pang et al. 2006).

In other studies (Dal Cin et al. 2006; Pang et al. 2006), a decrease in *Md-acs1* transcript in 1-MCP-treated apple was observed but *Md-acs1* transcription was reinitiated after 12 to 35 days following treatment. In treated fruit, *Md-aco1* was transcribed immediately following treatment but decreased to undetectable levels until a few days before *Md-acs1* transcription was reinitiated (Pang et al. 2006). Both gene transcription for ACS and ACO and activity were lowered in 1-MCP-treated apple (Dal Cin et al. 2006; Vilaplana et al. 2006a). In peach, 1-MCP seems to have limited influence on *Pp-acs* and *aco* transcript accumulation (Dal Cin et al. 2006).

In this study, in general *Md-acs3* transcription did not show a relationship with ethylene evolution in any year and was expressed at much lower levels than *Md-acs1*. Because of this, it seems that Md-ACS1 is the main isoform responsible for the ethylene burst during the climacteric period confirming the results of Wiersma et al. (2007). However, *Md-acs3* transcript levels were higher in 1-MCP-treated H_2O_2 -dipped fruit in both years, with the exception of 1-MCP-treated fruit which had higher transcription 10 days after treatment in 2005 compared to the other treatments. The difference in *Md-acs3* transcription between 1-MCP-treated in H_2O_2 and treated fruit dipped in H_2O_2 corresponds with the difference in ethylene evolution observed on day 12 (Fig. 2-2).

Transcription of *Md-aco1* during ripening occurs pre-climacteric and seems unaffected by ethylene inhibition suggesting induction via a different signal. In 'Golden Delicious', *Md-aco1* transcript accumulated before *Md-acs1* (fig. 2-4, and 2-5). Commonly, in climacteric fruit ripening, ripening-related ACO isoforms show transcription prior to ACS and rise in ethylene. Such transcription patterns are observed in tomato (Tassoni et al. 2006; Oeller et al. 1991), passion fruit (Mita et al. 1998), winter

pear ‘Passe-Crassane’ (Lelièvre et al. 1997) and apple (Pang et al. 2006; Wakasa et al. 2006). In tomato, ACO activity also increased prior to that of ACS (Liu et al. 1985; Nakatsuka et al. 1997).

Transcription of ripening-related ACS and ACO in 1-MCP-treated fruit in the absence of ethylene suggests that ethylene evolution is controlled post-translationally and possibly by substrate availability. Post-translational regulation of some ACS isoforms include phosphorylation, ETO1-mediated stabilization, and proteolysis (Matarasso et al. 2005; Yoshida et al. 2005, 2006; Chae et al. 2005; Tatsuki and Mori, 2001; Wang et al. 2004; Spanu et al. 1994).

Transcription levels of ethylene signal transduction components and ethylene evolution

Characterization of ethylene receptors and signaling elements in Arabidopsis led to several investigations in fruits. In ripening climacteric fruits expression of putative ethylene receptors varies greatly and no clear-cut trends regarding induction of ETR and ERS-type receptors is evident. In this study, in fruit dipped in water, transcription of *Md-ers1* was lower in 1-MCP-treated than untreated fruit (Fig. 2-4 and 2-5). However, factors other than 1-MCP seemed responsible for *Md-etr1* transcription as seen by variations between the two years. In other studies with ‘Orin’ and ‘Fuji’ apples, both *Md-ers1* and MdERS2 transcription was ethylene inducible and followed postharvest ethylene evolution, but not *Md-etr1* transcription (Tasuki and Endo, 2006). In another study with ‘Golden Delicious’ (Dal Cin et al. 2006), *Md-etr1* transcription was lower than *Md-ers1* but continuously increased in parallel with ethylene whereas *Md-ers1* transcription had decreased as ethylene levels peaked.

In ‘Passe-Crassane’ pear (*Pyrus communis* (L.), abbreviated Pc), *Pc-etr1a*, *Pc-ers1a*, and *Pc-etr5* were lower in 1-MCP-teated fruit and mRNA levels followed ethylene evolution during ripening (El-Sharkawy et al. 2003). In ‘Springcrest’ peach (*Prunus persica* (L.) Batsch, abbreviated Pp), the same was true for *Pp-ers1* but not *Pp-etr1*, and accumulation was only delayed by a few hours after treatment (Rasori et al. 2002). In ‘Summer Rich’ peach, both transcript levels were lower in 1-MCP-treated fruit (Dal Cin et al. 2006). In passion fruit (*Passiflora edulis* Sims, abbreviated Pe), *Pe-ers2* increased during ripening and following ethylene exposure (Mita et al. 2002) and expression of *Pe-etr1* and *Pe-ers1*varied according to fruit tissue and study (Mita et al. 1998, 2002).

In tomato, *Nr* (*Le-etr3*) transcription rose with ripening (Payton et al. 1996; Wilkinson et al. 1995; Tassoni et al. 2006) and was undetectable in the transcription factor and receptor mutants *rin*, and *Nr*, respectively (Lanahan et al. 1994; Payton et al. 1996; Vrebalov et al. 2002; Wilkinson et al. 1995). *Nr* is ethylene-inducible in mature but not immature fruit (Wilkinson et al. 1995). In *Nr* anti-sense plants *Le-etr*4 functionally compensated for *Nr* absence (Tieman et al. 2000). Transcript levels of *Le-etr*4/ *Le-etr*5/ *Le-etr*6 and *Le-etr1/ Le-etr2* followed ethylene evolution, though *Le-etr*1/ *Le-etr*2 was much lower (Tassoni et al. 2006). Interestingly, *Le-etr1*, and *Le-etr*2 are thought constitutive and not inducible by stimuli such as ethylene or pathogen attack (Klee 2002). In muskmelon (*Curcumis melo* (L.), abbreviated Cm), transcription of *Cm-ers1*and *Cm-etr1* increased during ripening. *Cm-etr1*mRNA levels rose markedly compared those of *Cm-ers1*. Moreover, *Cm-etr1* transcription remained high over the course of ripening while that of *Cm-ers1* decreased notably near the end of the study (Sato-Nara et al. 1999).

Recently, genetic evidence demonstrated a transitional state for ETR1 in which ethylene is bound to it, yet the receptor remains active prior to conformational change and deactivation of the receptor (Wang et al. 2006). Also, ligand-mediated receptor degradation is considered partially responsible for increased ethylene sensitivity during ripening onset (Kevany et al. 2007). Because of the discovery of the active transitional state of the receptor bound to ethylene and considering receptor degradation as a means to mediate ethylene response, Kevany et al. (2007) urge caution when interpreting mRNA levels of receptors. Undoubtedly, ethylene receptors are under tight regulation including complex synergy with other signals. Thus, explaining receptor expression patterns is difficult and may only provide limited information regarding ethylene signaling.

In most climacteric crops, CTR1 homologs seem to follow ethylene evolution (Sato-Nara et al. 1999; El-Sharkawy et al. 2003; Zegzouti et al. 1999). In the 2005 study, *Md-ctr1* had higher transcription in fruit not treated with 1-MCP one day after treatment however, only 1-MCP-treated fruit had detectable levels on day 10. By 28 days, 1-MCP-treated fruit dipped in H_2O_2 had slightly higher transcription levels. In 2006 *Md-ctr1* transcript levels were similar in fruit from all treatments. Again the complexity of ethylene signaling and network of cross-communication renders data interpretation difficult. According to Dal Cin et al (2006), *Md-ctr1* transcription was greater in 1-MCP-treated 'Golden Delicious' and decreased to an ultimate low 12 days after harvest. In untreated fruit a reverse relationship was observed.

In most instances, mRNA levels for the putative *ein2* were lower in 1-MCP-treated H_2O_2 -dipped fruit. The decrease in its transcription may be responsible for the lower transcription of *Md-ers1* and *Md-ctr*1 observed in 2005 since it contributes positively to

the ethylene response. EIN2 is a bifunctional protein linking ethylene signal transduction and stress responses (Alonso et al. 1999). According to the transcription results (Fig. 2-4 and 2-5), both the presence of ethylene sensitivity and oxidative stress are required for *ein2* expression. Further research is needed to confirm this observation.

Potential elements linking ROS and ethylene metabolism in ripening apple

The mode of action for H_2O_2 signaling is via a change in the thiol/disulfide redox state of cysteine residues essential for enzyme activity (Dröge, 2006). In humans, H_2O_2 is involved in the regulation of the protein tyrosine phosphatases VHR, PTP1, and LAR, but not serine/threonine protein phosphatases (Denu and Tanner, 1998). H_2O_2 has a similar signaling power as insulin in regulating PTP1B (Mahadev et al. 2001). Also, direct interaction of H_2O_2 with the insulin receptor kinase increases its activity in the absence of insulin in purified recombinant fragments and intact cells (Schmitt et al. 2005). A positive feedback loop is created by H_2O_2 induction of NADPH oxidase and is involved in signal amplification (Mahadev et al. 2004). The insulin receptor would not only be negatively regulated by protein tyrosine phosphatase, but also directly activated by H_2O_2 (Dröge, 2006).

It is possible that events similar to H_2O_2 signaling in humans occur in plants. Protein tyrosine phosphatases are present also in plants (Luan et al. 2001; Xu et al. 1998; Huan et al. 2000). Many plant proteins contain tyrosine kinase phosphorylation sites (accession # PS00007). Substrates of protein tyrosine kinases generally have a lysine or arginine seven amino acids to the N-terminal side of the phosphorylated tyrosine. In addition, on the same side, tyrosine phosphorylation sites hold an acidic residue (aspartate or glutamate)

at either three or four amino acids away from the tyrosine (Patschinsky et al. 1982; Hunter, 1982; Cooper et al. 1984). Searching the *Malus* taxonomic lineage using ScanProcite (Gattiker et al. 2002) and the protein databases Swiss-Prot, TrEMBL, and PDB (NCBI; NPSA, Combet et al. 2000), 234 tyrosine kinase phosphorylation sites were found in 187 sequences. Among those sequences, both fruit specific *Md-acs3* and Md-ACO1 contain the motif. Other known apple specific elements of the ethylene signal transduction pathway such as Md-ETR1, Md-ERS1, and Md-CTR1, did not have a tyrosine kinase phosphorylation site sequence. In the absence of an Md-EIN2 sequence, the tomato LeEIN2 was analyzed and showed also a tyrosine kinase phosphorylation site. Finally, H_2O_2 and the NADPH oxidase *AtrbohD* form an example of an oxidative positive feedback loop during pathogen attack (Desikan et al. 1998; Torres et al. 2002).

In this study, *Md-etr1* and *Md-acs3* transcript levels appeared higher in H_2O_2–dipped 1-MCP-treated fruits. Post 1-MCP-induction suggests that these genes are part of a stress response and perhaps an intersection between the ethylene and ROS signaling pathways. Though Md-ETR1 does not have a tyrosine kinase phosphorylation site sequence, a cysteine residue of ETR1 is required to mediate H_2O_2 response in guard cells of *Arabidopsis* (Desikan et al. 2005). In addition, ETR1 has a histidine kinase (HK) domain (Chang et al. 1993; Hua et al. 1995) and HKs may function as H_2O_2 sensors (Desikan et al. 2001). In yeast, some HKs linked to MAPK cascades alternatively function as H_2O_2 sensors (Singh, 2000). In *Arabidopsis,* H_2O_2 activates AtMPK6 (Kovtun et al. 2000), a MAPK highly homologous to HOG1 (Desikan et al. 2001), a yeast MAPK connected to a HK signaling module (Singh, 2000). H_2O_2 may signal though the HK domain of ethylene receptors. In apple, it is plausible that ETR1 integrates response to H_2O_2 in this manner.

In this study, putative *ein2* was down regulated in 1-MCP-treated fruit (Fig. 2-4 and 2-5). *Ein2* offers another integration point for both ethylene and ROS pathways (Alonso et al. 1999). The exact implications of its transcription pattern in this study remain obscure. Further work is needed to examine cross-communication between H_2O_2 and ethylene metabolism in ripening fruit.

CONCLUSION

Ethylene production did not significantly increase in 1-MCP-treated fruit by H_2O_2, even under severe ethylene inhibition conferred by 3 $\mu l \cdot l^{-1}$ 1-MCP treatment. An increase in transcription of *Md-acs1* in 2005 and *Md-acs3* in 2006 match the onset of the increase in ethylene in 1-MCP-treated fruit. Whether the small shift in magnitude and offset of the ethylene response mediated by H_2O_2 importantly impact fruit ripening remains to be demonstrated. The differences in ethylene emission caused by H_2O_2 were so small that it is doubtful that H_2O_2 alone could be exploited to recover 1-MCP-conferred inhibition of aroma development. *Md-acs3*, *Md-etr1*, and the putative *ein2* all show some promise in linking ethylene and H_2O_2 signaling in ripening apple. Peroxide did not cause any visual symptoms of physiological disorders in these studies.

3. HYDROGEN PEROXIDE AFFECTS ANTIOXIDATIVE MECHANISMS AND GENE TRANSCRIPTION OF PR PROTEINS DURING RIPENING OF 1-METHYLCYCLOPROPENE-TREATED APPLES

Reactive oxygen species (ROS) are high-energy molecules existing for fractions of seconds and are linked to DNA, lipid, and protein damage when unregulated (Bray et al. 2000; Mittler, 2002; Apel and Hirt, 2004; Dröge, 2002; Temple et al. 2005). ROS causes postharvest quality loss and senescence of fresh produce (Hodges, 2003). Common ROS in plants include superoxide radical, hydrogen peroxide (H_2O_2), singlet oxygen, and the highly reactive hydroxyl radical (reviewed by: Desikan et al. 2003; Elsner, 1982; Apel and Hirt, 2004; Salin, 1987; Mittler, 2002; Temple et al. 2005). ROS are constantly produced as byproducts of regular cell metabolism, and increase under stress conditions (Neill et al. 2002). It has been reported that a base level of H_2O_2 in the range of 60 μM to 7 mM is always present within living plant cells (Velijovic-Jovanovic et al. 2001; Karpinski et al. 1999; Jian and Zhan, 2001; Lin and Kao, 2001). Neill et al. (2002) explain such a wide range of basal H_2O_2 concentrations to technical difficulties measuring H_2O_2. Nonetheless, some levels of ROS exist in cells under normal metabolism.

Antioxidants protect fruits from ROS-induced oxidative damage, assure quality, and extend produce shelf life. Antioxidants are substances or enzymes, at low levels, competing against oxidizable substrates and thus significantly delaying or inhibiting oxidation of such substrate (Halliwell and Gutteridge, 1989; Dröge, 2002). A number of

enzymes are involved in cell detoxification and in the regeneration of antioxidant substances. Some examples include superoxide dismutase (SOD), catalase (CAT), and ascorbate and glutathione peroxidases (APX and GPX, respectively). SOD reduces superoxide radical to H_2O_2, and CAT, APX, and GPX are involved in H_2O_2 removal (Bray et al. 2000).

The term oxidative stress describes an equilibrium shifting in favor of ROS accumulation caused by an inability of the antioxidant system to scavenge excessive amounts of ROS. Such stress occurs in response to chilling, heat, salinity, ultraviolet light, ozone, and other stressing agents (Bray et al. 2000; Desikan et al. 2001). Oxidative stress significantly affects cell metabolism and possibly leads to disorders and/or death (Hodges, 2003).

Ironically, although originally viewed exclusively as detrimental to cells (Harman, 1956; Harman, 1981), controlled ROS metabolism is now recognized important to cell signaling and regulation of numerous essential responses for an organism's survival and development (Uhrig and Hülskamp, 2006; Denu and Tanner, 1998; Dröge, 2002; Mahadev et al. 2001). Among ROS, H_2O_2 is a prime contender as a signaling molecule since it is relatively long-lived, has high membrane permeability (Allan and Fluhr, 1997; Neil et al. 2002), and overlaps various stresses possibly mediating cross-tolerance (Bowler and Fluhr, 2000). In addition, H_2O_2 is part of other signaling pathways such as programmed cell death, stomatal closure, peroxisome biogenesis, induction of ROS scavenging enzymes (Bray et al. 2000; Foyer and Noctor, 2003; Mittler et al. 2004), and appears involved in ripening (Brennan and Frenkel, 1977; Larrigaudière and Vendrell, 1993a, b; Tian et al. 1994; Blankenship and Richardson, 1985; Chen and Mellenthin,

1982; Looney, 1972). In 'Bartlett' and 'Bosc' pears, Brennan and Frenkel (1977) observed increased and delayed ripening with higher and lower endogenous H_2O_2 levels, respectively.

In some instances, ROS synthesis is genetically programmed (Foyer and Noctor, 2005a,b), with a number of enzymes such as NADPH oxidase, peroxidases, and amine oxidases capable of ROS generation. H_2O_2 seems able to reversibly inactivate signaling proteins such as protein tyrosine phosphatase by changing the thiol/disulfide redox state within their catalytic site (Denu and Tanner, 1998). In Arabidopsis, H_2O_2 induces At-MPK3 and activates At-MPK3 and At-MPK6, mediators of oxidative stress responsive genes (Desikan et al. 1999; Kovtun et al. 2000). Furthermore, microarray analysis revealed that H_2O_2 regulates many genes, including genes related to metabolism, energy, transcription, protein distribution and transport, cell organization and biogenesis, signal transduction, and cell rescue/ defense (Desikan et al. 2001).

In plant-pathogen interactions, ROS are produced at the site of infection (Bowles, 1990; Baker and Orlandi, 1995; Lamb and Dixon, 1997). A short burst occurs immediately after inoculation for all plant-pathogen interactions while in incompatible interactions, those causing diseases, another longer burst occurs 1.5 to 3 hours afterwards (Baker and Orlandi, 1995). These ROS are part of signal transduction events, antimicrobial mechanisms, cell wall modification and membrane lipoxidation, and can lead to induced resistance and eventual hypersensitive death locally (Apostol et al. 1989; Baker and Orlandi, 1995; Bradley et al. 1992; Heil and Bostok, 2002; Kombrink and Schmelzer, 2001). Transgenic potato with constitutive glucose oxidase exhibited higher levels of endogenous H_2O_2 and greater resistance to pathogens compared to the wild type

(Wu et al. 1995). Also, they had higher levels of salicylic acid, and acidic chitinase and anionic peroxidase transcription (Wu et al. 1997). Some suggest that ROS signal directly or indirectly via lipid peroxidation (Bowles, 1990) and/or a change in cellular redox potential (Dron et al. 1988, Wingate et al. 1988).

In addition to ROS, other signal molecules such as salicylic acid (SA), jasmonic acid (JA), and ethylene confer plant protection by invoking defense responses involving systemic resistance and possibly hypersensitive death near the infection site. Two types of systemic resistance have been characterized: systemic acquired resistance (SAR) and induced systemic resistance (ISR). Even though SAR is thought to be an ethylene-independent process (Durrant and Dong, 2004; Lawton et al. 1994) while ISR is JA/ethylene dependent (Dong, 1998; Ecker and Davis, 1987; Penmetsa and Cook, 1997; Penninckx et al. 1996; Thomma et al. 1999, Vallad and Goodman, 2004), some evidence shows cross-talk and cross-resistance between both pathways, downstream of SA, ethylene, and JA (Dong, 2004; Heil and Bostock, 2002; Spoel et al. 2003; van Wees et al. 2000).

Since ethylene is involved in certain defense mechanisms (Toppan and Roby, 1982; Broglie et al. 1986; Ecker and Davis, 1987; Thomma et al. 1999), plant organs insensitive to ethylene show greater susceptibility to some pathogens (Thomma et al. 1999). Accordingly, fruits and vegetables treated with 1-methylcyclopropene (1-MCP) have increased vulnerability to decays and rots (Blankenship and Dole, 2003; Watkins, 2006).

Ethylene is responsible, individually or synergistically, with other signals to trigger defense mechanisms upon pathogen attack. This includes induction and translation of certain pathogenesis-related (PR) proteins (Bowles, 1990), such as β-1,3-glucanase (PR2)

and endochitinase (PR3 and 8), two hydrolases involved in the degradation of cell wall components of some pathogens (Toppan and Roby, 1982; Broglie et al. 1986; Medina-Suarez, 1997; Clendennen and May, 1997).

The protein EIN2 (ethylene insensitive 2) joins ethylene signal transduction and stress responses (Alonso et al. 1999). Both functionality of EIN2 and ethylene sensitivity are required for induction of PR3 and 4 (hevein-like protein) and e*in2* mutants are more susceptible to *Botritys sp.* infection than wild-type plants (Thomma et al. 1999). Other PR proteins, such as PR1, are not regulated by ethylene (Lund et al. 1998; Lawton et al. 1994).

Either as a mechanistic driving force or as signaling molecules, ROS implication in fruit quality retention, pathogen response, and senescence can no longer be ignored. Little is known regarding ROS/ H_2O_2 signaling in ripening and senescing produce. Since exposure to low stress conditions can confer tolerance toward other and greater stress (Prasad et al. 1994, Anderson et al. 1995), H_2O_2 seems a perfect candidate for acclimation and induction of cross-tolerance (Bowler and Fluhr, 2000) in fresh produce. Cell signaling exploitation using H_2O_2 to increase CAT activity, antioxidant capacity, and to induce antioxidant enzyme and PR proteins could prolong shelf-life and improve postharvest quality retention of apples.

To complement the H_2O_2 -induced ethylene study and to improve understanding of ROS metabolism with regards to fruit postharvest life, the effects of a low H_2O_2 dose on transcription of *sod*, *cat*, *apx*, and *gpx*, as well as *pr2*, *pr3*, *pr5*, and *pr8* genes was examined. In addition, the effect of H_2O_2 on CAT activity and total water soluble

antioxidant capacity on 1-MCP-treated and untreated 'Golden Delicious' at harvest and 'Delicious' after storage in air for a few months was studied.

MATERIALS AND METHODS

Plant Material and Treatments, RNA extraction, and Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Fruit was treated, RNA extracted, and RT-PCR performed as described in chapter 2. The primers used in this study are listed in Table 3-1. For genes *Fa-apx* (*Fragaria ananassa*), *Pp-cat* (*Prunus persica*), and *Mx-sod* (*Malus xiaojinensis*), primers were designed on apple expressed sequence tags to nucleic acid sequences corresponding to signature and catalytic domains specific to each of the proteins of interest. The domains were found using ScanProsite (Gattiker et al. 2002). From this point on *Fa-apx*, *Pp-cat*, and *Mx-sod* will be referred to as *apx*, *sod*, and *cat*, respectively.

Total Water Soluble Antioxidant Capacity

Frozen skin tissue was ground with a mortar and pestle using liquid nitrogen. The homogenate was weighted and mixed in five volumes of 0.5 M Tris buffer pH 7, and centrifuged at 20 000 *g* for 30 minutes at 4˚C. The supernatant was centrifuged again at 20 000 g for 1 minute and total water soluble antioxidant capacity was assessed by measuring absorbance at 695 nm using a PowerWave$_x$ microplate scanning spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT, USA) quantifying the formation of phosphomolybdenium complex upon molybdenum reduction by antioxidant

substances (Prieto et al. 1999). Optical density was converted to ascorbate equivalence using a standard curve.

Catalase Activity

Catalase activity was measured according to Aebi (1983) with minor modifications. A crude extract was made by adding 3 ml of 200 mM phosphate buffer pH 7.2 to 1 g of frozen skin tissue ground to a fine powder in liquid nitrogen. To this extract was added 5% PVPP (w: v), 1% triton-x-100 (v: v), and 4 µl of a protease inhibitor cocktail. The cocktail stock consisted of 2.5 mg leupeptin, 2.5 mg chymostatin, 2.5 mg pepstatin, 2.5 antipain, 10 mg pNH2-benzamindine, and 10 mg benzamindine mixed in 1 ml ethanol. The crude extract was centrifuged for 20 minutes at 27,000 x *g* and the supernatant was transferred to new tubes and centrifuged for another 15 minutes. The supernatant was filtered using glass fiber prefilters (Milipore, Bedford, MA, USA) attached to a 3 ml plastic syringe. Catalase activity was measured at 240 nm using a Cary 100 Bio UV-visible spectrophotometer (Varian, Walnut Creek, CA) by adding 1.2 µl of 30% H_2O_2 to a reaction cuvette containing 50 µl of extract and 1 ml of the extraction buffer against a blank cuvette containing the extract in buffer. Catalase activity was expressed as units (U) of catalase per minute per mg protein. Protein concentration was measured using the Bradford method using Bio-Rad protein assay dye (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Standard curves were generated using commercial catalase (Sigma-Aldrich, USA) and bovine serum albumin as protein standards.

Statistical analysis

Data were analyzed as previously described in chapter 2 with the exception of the 2005 data. The 2005 data were analyzed as a complete randomized design using Box(1-MCP) as the error term for “treatment”, residual error for all other analysis. The “lsmeans' statement was added to the GLM procedure in SAS for all analysis to compute least significant differences (α=0.05).

Table 3-1. Forward (F) and reverse (R) primer sequences used for PCR amplification of apple antioxidant enzyme and pathogenesis related genes. Sequence and identity from NCBI (2007).

Gene name		Sequence (5' → 3')	Size (bp)	Accession No.	Reference
Antioxidant Enzymes					
Mx-sod	F	ATGCTTTCGGAGACACAACA	304	DQ431473	Zhang et al. 2006
	R	ATAATACCACAAGCTACCCTGC			Direct submission
Fa-apx	F	GCTCCTCTCATGCTCCGTCT	391	AF159632	Kim et al. 2001
	R	ACCACCAGAGAGAGCAACAAT			Direct submission
Md-gpx	F	TCCGAGAGTAAATCAATCCACG	258	AF403707	Sung and Yu, 2001
	R	GCAGGCAAACTCTACAATCTC			Direct submission
Pp-cat	F	ATCCCAGAGCGTGTTGTCCA	878	AJ496418	Bagnoli et al. 2004
	R	GTATCAGAATAGGCGAAGAGTC			Direct submission
Defense Related Proteins					
pr2	F	ACTCACAGTCACCATCCTCAACA	525	AY548364	Bonasera et al. 2006
	R	GGTGCACCAGCTTTTTCAA			
pr3	F	AGTGATTATCTATTGGGGCC	525	AY548364	Bonasera et al. 2006
	R	GATTGCATGGGGGATTGTTG			
pr5	F	GGCAGGCGCAGTTCCACCAG	299	DQ318213	Bonasera et al. 2006
	R	GACATGTCTCCGGCATATCA			
pr8	F	CCTAGCCCAATGCCAGTATG	376	DQ318214	Bonasera et al. 2006
	R	CGCCATCATCCCTAACACACTT			
Other					
Putative *act3* (*Arabidopsis* ; 98.4% id.)	F	GATGAAAATCTTGACTGAGCGTGG	515	CN927806	Beuning et al. 2004;
	R	CAGCTTTGGCAATCCACATCTG			Direct submission

RESULTS

Ethylene and H_2O_2 are important signal molecules responsible for induction of numerous responses including defense mechanisms against stress and pathogen attack (Yang and Hoffman, 1984; Alonso et al. 1999; Wang et al. 2002; Ludwig et al. 2005). In this study, the influence of 1-MCP and H_2O_2 treatment was examined on total water-soluble antioxidant capacity (antioxidant capacity), catalase activity, and transcription of induction of antioxidant enzymes and pathogenesis related (PR) proteins in apples.

Catalase activity

Catalase activity was measured to evaluate the H_2O_2 removal capability and assess potential acclimation of 1-MCP- and H_2O_2-treated fruit. Only statistically significant effects are presented. 1-MCP decreased catalase activity post harvest and after 4 months of storage (Fig. 3-1, 3-2, and 3-3). Postharvest dipping of 1-MCP-treated fruit in 30 mM H_2O_2 did not significantly affect catalase activity of 'Golden Delicious' in 2005. In 2006, although dipping fruit in H_2O_2 significantly increased catalase activity during the first 12 days overall (Fig. 3-2B), H_2O_2 did not significantly change catalase activity in 1-MCP-treated fruit according to a LSD mean comparison analysis (Appendix Fig. A-4). However, infiltrating 10 mM H_2O_2 under vacuum in 'Delicious' apple after four months in storage significantly decreased catalase activity (Fig. 3-3A). No synergic effect was observed between 1-MCP application and H_2O_2 treatment in either study.

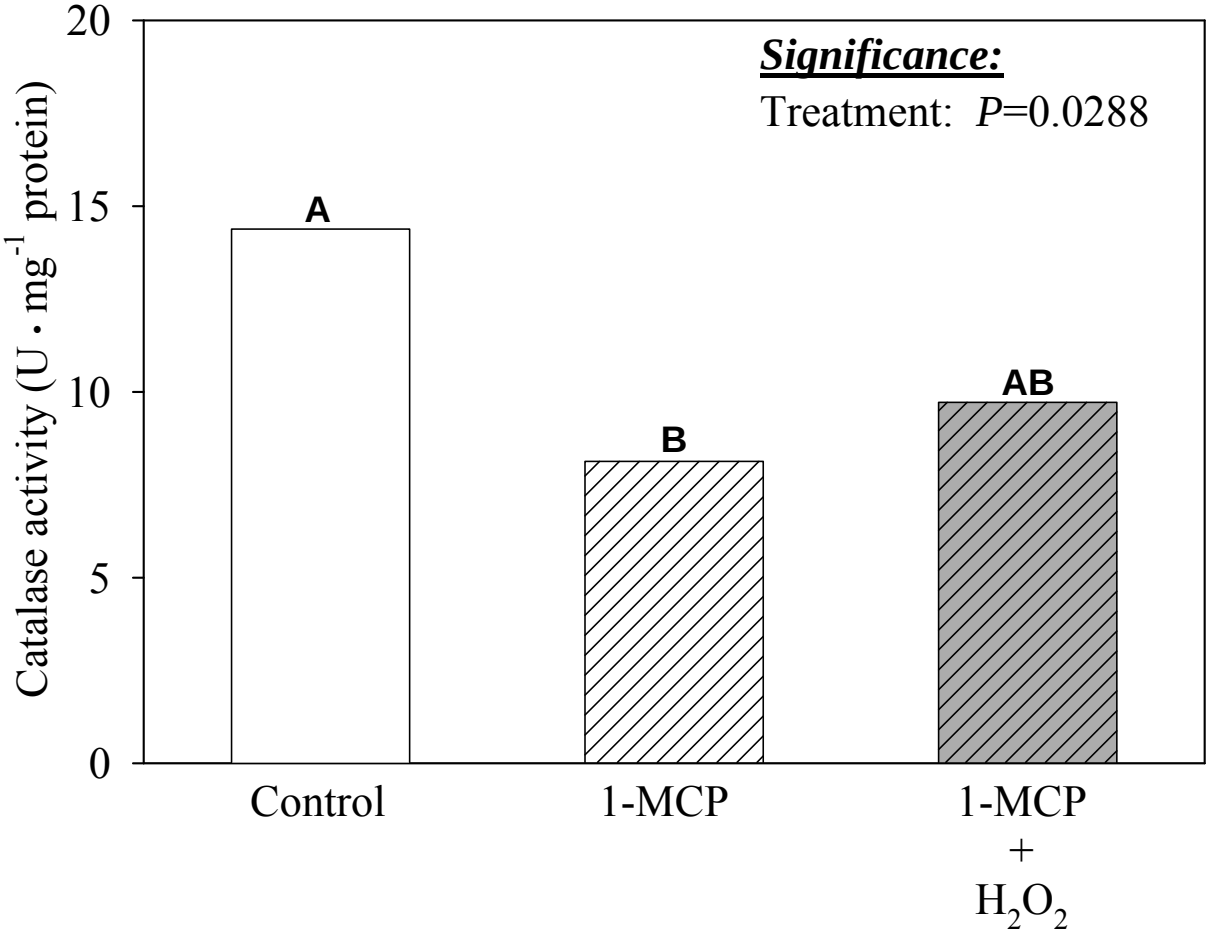

Figure 3-1. Effects of 1-MCP and peroxide on catalase activity of 'Golden Delicious' postharvest in 2005. Cross-hatching represents treatment with 3 $\mu l \cdot l^{-1}$ 1-MCP while grey bars represent an hour dip in 30 mM H_2O_2. *P* values are from an analysis of variance. Means with the same letter are not significantly different based on a least significant difference analysis (α=0.05).

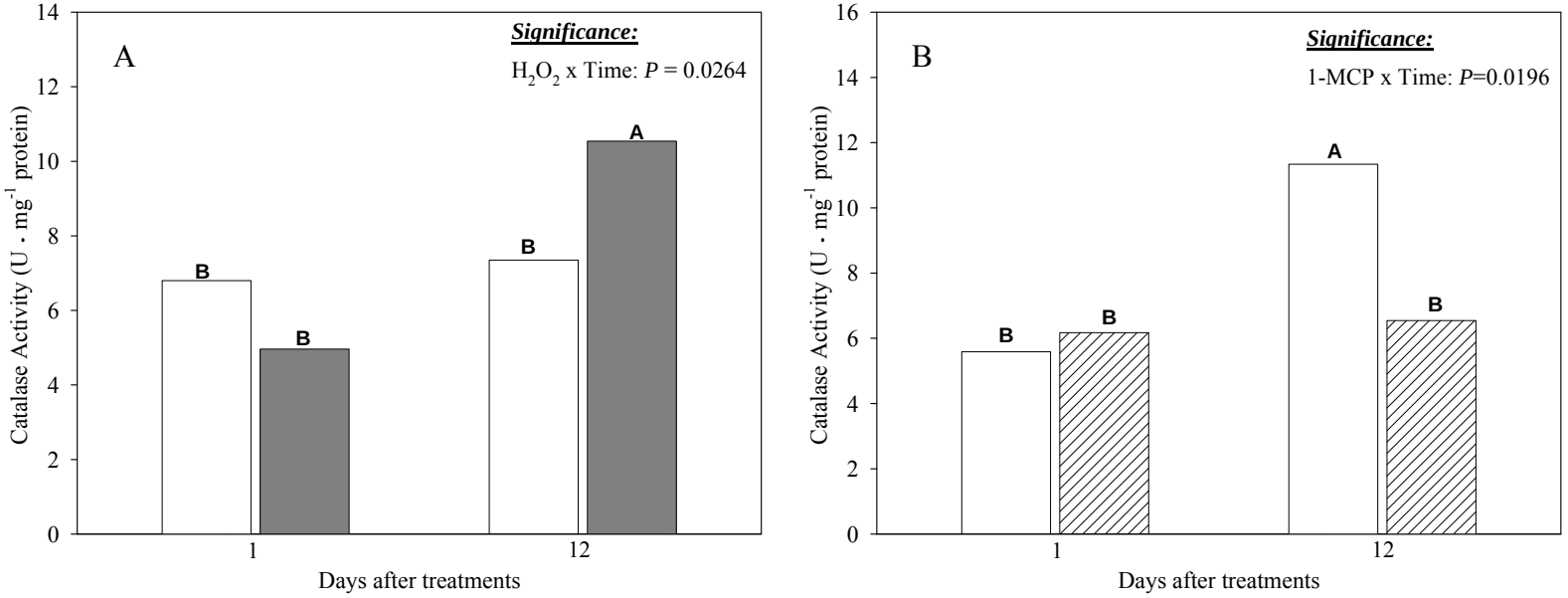

Figure 3-2. Catalase activity of 'Golden Delicious' postharvest in 2006 as affected by peroxide and time (A) and 1-MCP and time (B). Grey bars represent activity of fruit dipped in 30 mM H_2O_2 for an hour while Cross-hatching represent fruit treated with 1µl·l^{-1} 1-MCP. *P* values are from an analysis of variance. Means with the same letter within a graph are not significantly different based on a least significant difference analysis (α=0.05).

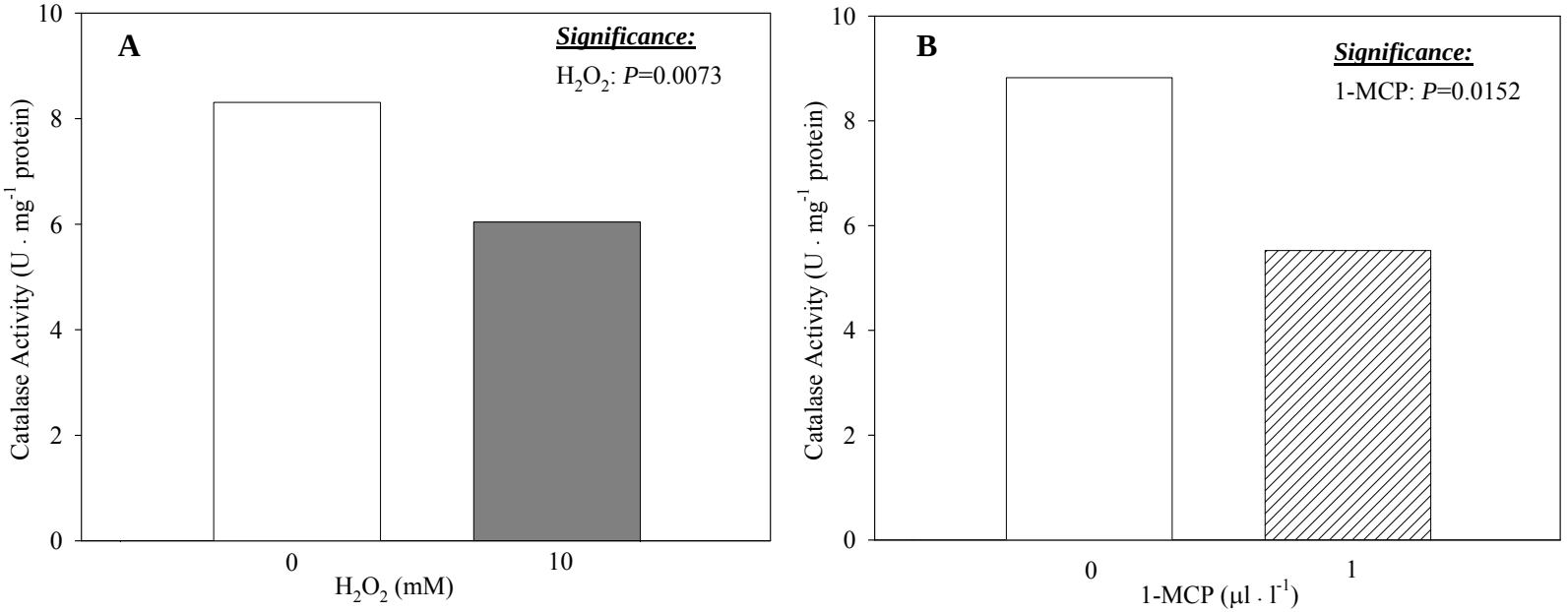

Figure 3-3. Catalase activity of 'Delicious' apple after four months in refrigerated air storage at 1˚C in 2006 as affected by vacuum infiltration of H_2O_2 after storage (A) and 1-MCP treatment at harvest (B). *P* values are from an analysis of variance.

Total Water Soluble Antioxidant Capacity

Antioxidant capacity was used to estimate oxidation of antioxidant substances by H_2O_2 (Fig. 3-4, 3-5, and 3-6). Again, only statistically significant effects are presented. 1-MCP-treated fruit showed a significant decrease in antioxidant capacity in 'Golden Delicious' in 2005 (Fig. 3-4). Dipping 1-MCP- treated fruit in H_2O_2 delayed this decrease by a minimum of nine days. In the same cultivar in 2006, 1-MCP significantly prevented a change in antioxidant capacity caused by H_2O_2 (Fig. 3-5A). In 'Delicious' apple after storage in 2006, 1-MCP blocked an increase in antioxidant capacity induced by vacuum infiltration of 10mM H_2O_2 within the fruit (Fig. 3-6A). In fruit not treated with 1-MCP a 30 mM H_2O_2 dip significantly decreased antioxidant capacity (Fig. 3-5A); however in general, antioxidant capacity of H_2O_2-dipped fruit was significantly higher 23 days after treatment (Fig 3-5B). Vacuum infiltration of 10 mM H_2O_2 after storage significantly increased antioxidant capacity (Fig. 3-6B).

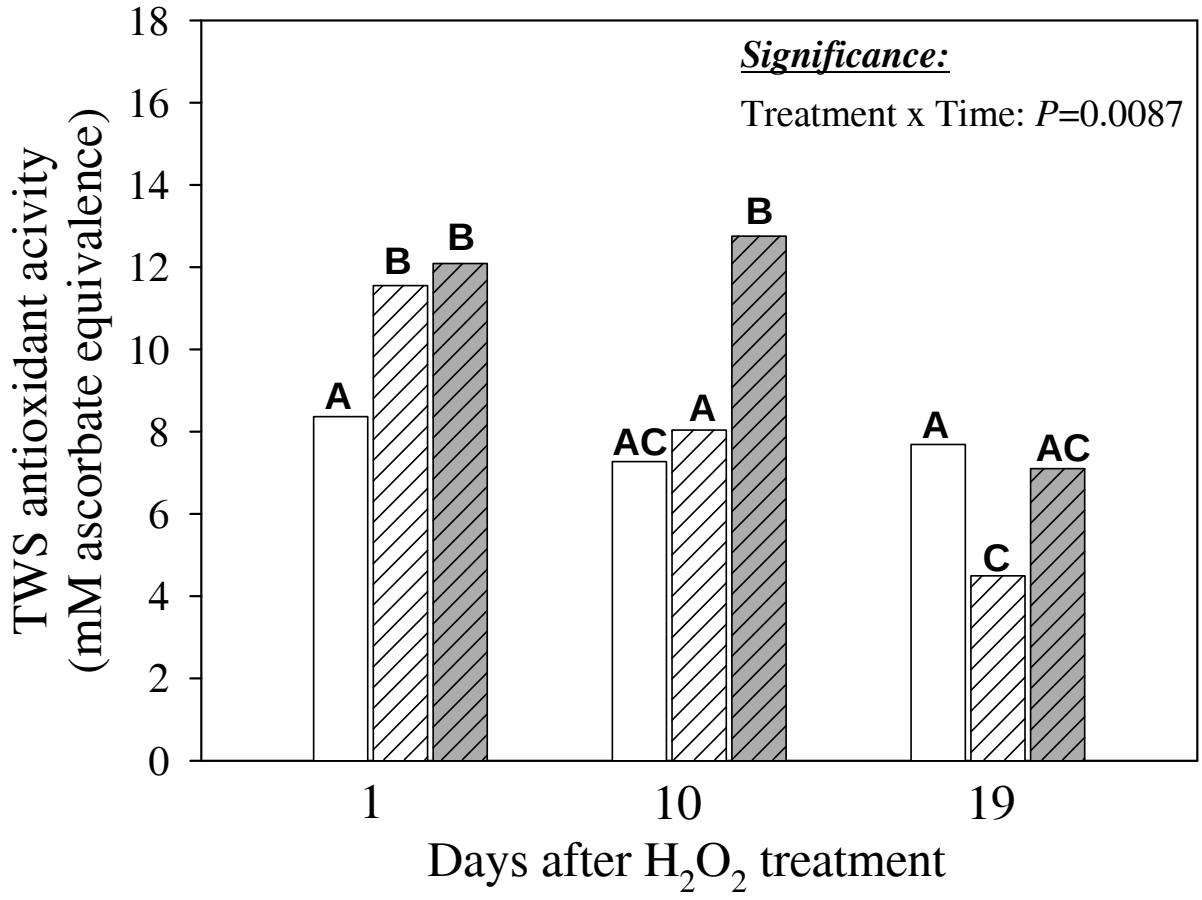

Figure 3-4. Effects of 1-MCP and peroxide on total water soluble antioxidant capacity of 'Golden Delicious' postharvest in 2005. Cross-hatching represents treatment with 3 $\mu l \cdot l^{-1}$ 1-MCP while grey bars represent an hour dip in 30 mM peroxide. *P* values are from an analysis of variance. Means with the same letter within a graph are not significantly different based on a least significant difference analysis (α=0.05).

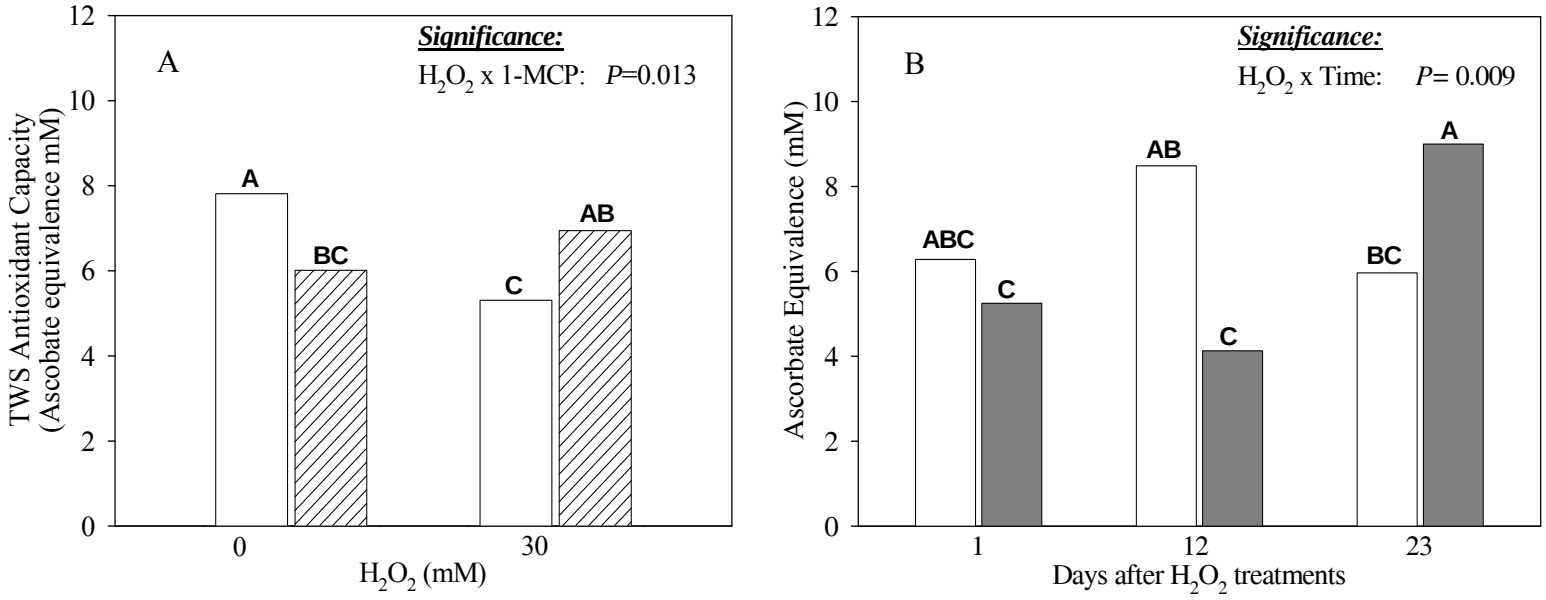

Figure 3-5. Total water soluble antioxidant capacity of 'Golden Delicious' postharvest in 2006 as affected by 1-MCP and peroxide (A), and H_2O_2 and time (B). Cross-hatching represents treatment with 1 $\mu l \cdot l^{-1}$ 1-MCP while grey bars represent an hour dip in 30 mM H_2O_2 following 1-MCP application. *P* values are from an analysis of variance. Means with the same letter within a graph are not significantly different based on a least significant difference analysis (α=0.05).

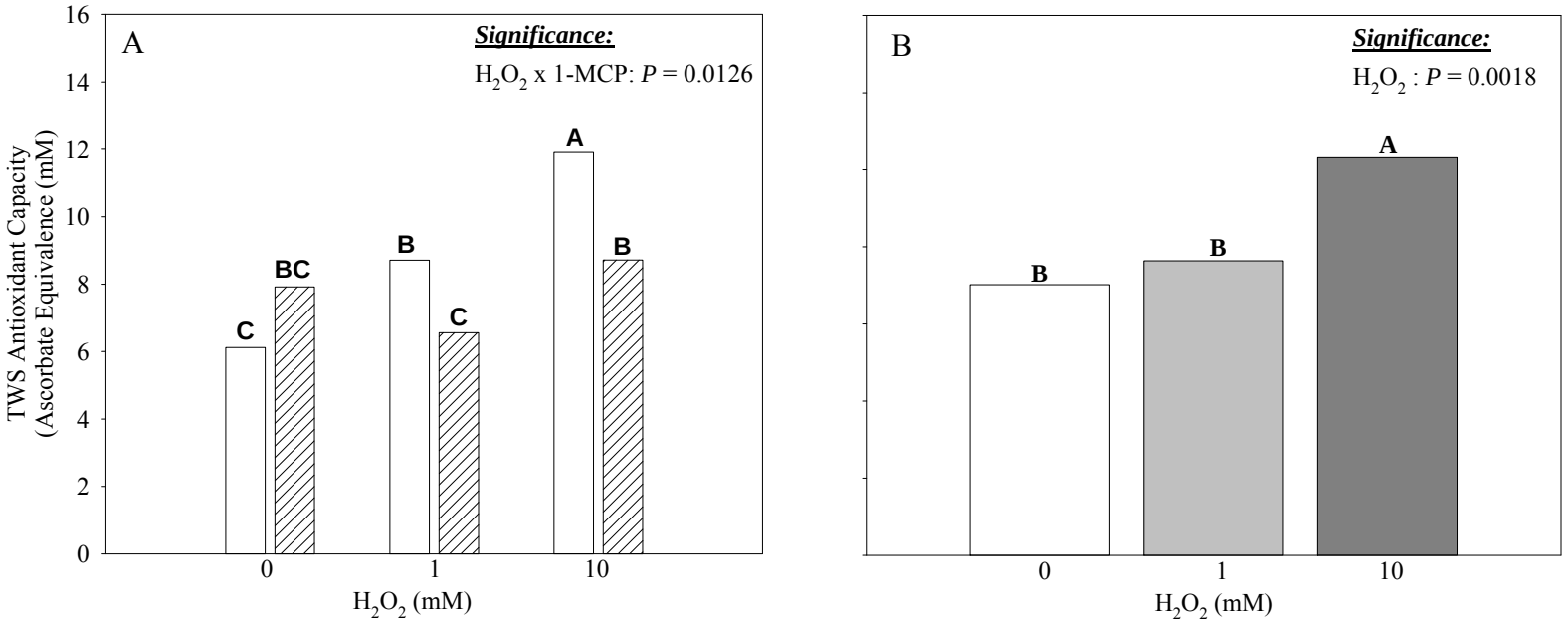

Figure 3-6. Total water soluble antioxidant capacity of 'Delicious' after four months in storage in 2006. Synergic effect of 1-MCP and vacuum infiltration of H_2O_2 (A), and effect of vacuum infiltration (B). Cross-hatching represents fruit treated with 1 $\mu l \cdot l^{-1}$ 1-MCP by AgroFresh prior storage. H_2O_2 was infiltrated after storage. *P* values are from an analysis of variance. Means with the same letter within a graph are not significantly different based on a least significant difference analysis (α=0.05).

Transcript examination

The effects of 1-MCP and H_2O_2 on transcription of the antioxidant enzyme genes *sod*, *cat*, *apx*, and *Md-gpx* in apples were examined. Primers specific to sequence coding for conserved motifs such as catalytic and signature domains were designed using apple expressed sequence tags. In both years, neither 1-MCP treatment nor H_2O_2 dip affected *sod*, *Md-gpx*, and *cat* transcription one day after treatments (Fig. 3-7 and 3-8). In 2006, their transcription was comparable to each other on day 12 also regardless of treatments. In 2005 however, their transcript levels were all higher on day 10 later in 1-MCP-treated water-dipped fruit compared to the other treatments. In 2005, one day after treatment *apx* transcription levels were low but were the highest in fruit not treated with 1-MCP. On day 10 1-MCP-treated fruit had the highest levels. In contrast, in 2006, *apx* transcription was higher in 1-MCP-treated fruit than untreated ones and *apx* transcription was higher in 1-MCP-untreated H_2O_2-dipped fruit one day after treatment when compared to the other treatments. On day 12, 1-MCP-untreated H_2O_2-dipped had the lowest transcription levels.

Pathogenesis-related (PR) proteins are induced in response to pathogen attack and are considered useful molecular markers of systemic resistance against pathogens (Ward et al. 1991; Durrant and Dong, 2004). They are induced also by elicitors including jasmonic acid, salicylic acid, ethylene, and H_2O_2 (Vallad and Goodman, 2004; Ferreira et al. 2007). In this study the effects of 1-MCP and H_2O_2 on transcription of *pr2*, *pr3*, *pr5*, and *pr8* were also investigated (Fig. 3-9).

Transcription levels of *pr3* and *pr5* were the highest in 1-MCP-treated fruit compared to untreated ones one day after treatment (Fig.3-9). In 1-MCP-treated fruit, those dipped in H_2O_2 had higher *pr5* transcription levels, while *pr3* levels were unaffected. In apples

not treated with 1-MCP, *pr3* was higher in H_2O_2-dipped fruit compared to those dipped in water. No difference in levels of *pr5* was observed in fruit not treated with 1-MCP between H_2O_2 or water-dipped fruit after one and 12 days after treatments. The expression patterns of *pr3* as affected by treatment were similar on day 1and 12 and only fruit not treated with 1-MCP dipped in water had lower transcription. Induction levels of *pr2* were lower in fruit not treated with 1-MCP compared to treated ones. Dipping fruit in H_2O_2 did not affect *pr2* induction in 1-MCP-treated fruit, but decreased its level in untreated ones dipped in H_2O_2. On day 12, *pr2* levels were higher in all fruit dipped in H_2O_2 and any 1-MCP influence disappeared. A constant level of *pr8* transcript was found in fruit regardless of treatment and sampling date.

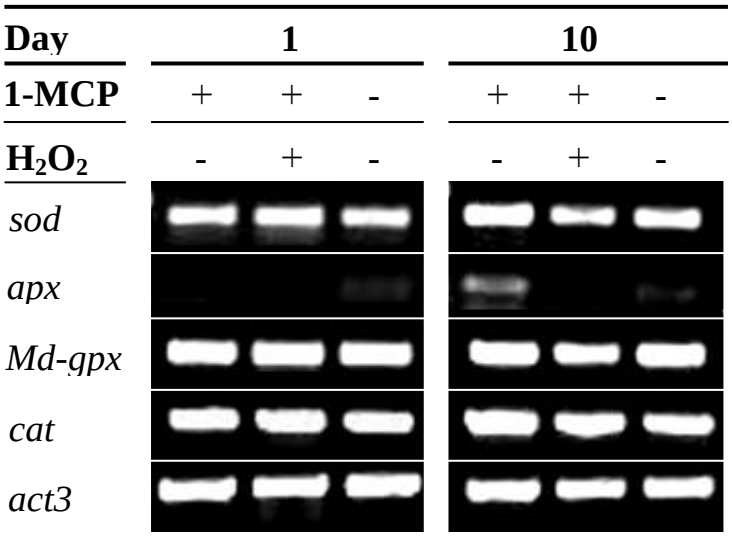

Figure 3-7. RT-PCR analysis of putative antioxidant enzymes genes transcription of 'Golden Delicious' in 2005 following post harvest treatment with (+) and without (-) 3 $\mu l \cdot l^{-1}$ 1-MCP and dipped with (+) or without (-) 30 mM H_2O_2. Putative *act3* was used as a PCR control.

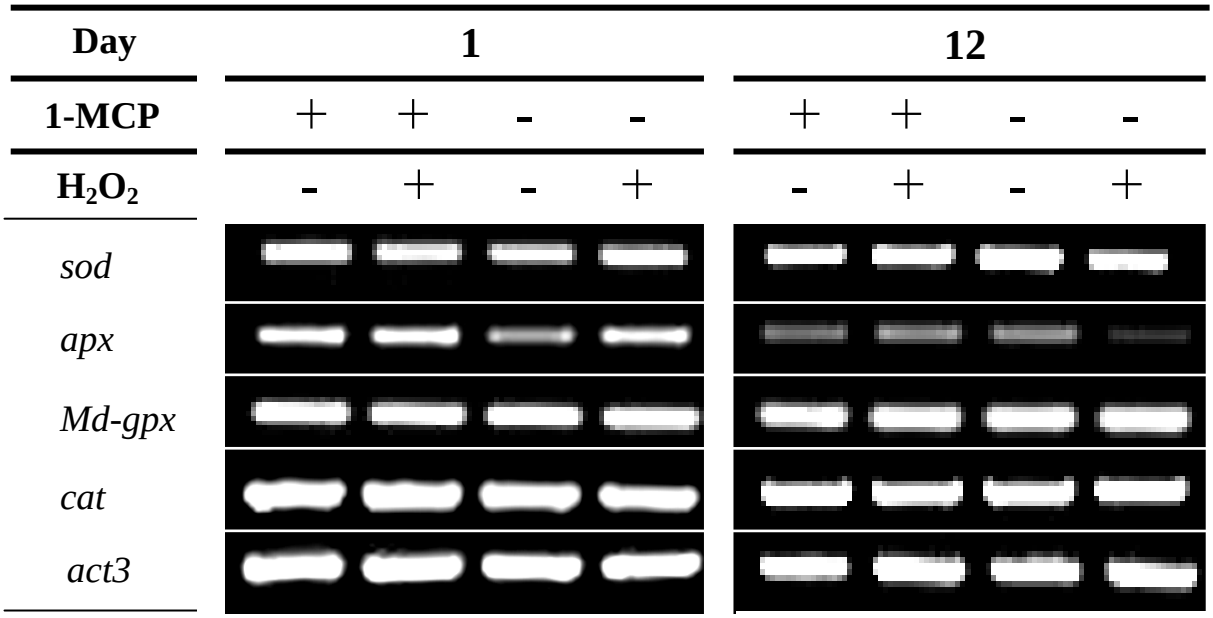

Figure 3-8. RT-PCR analysis of putative antioxidant enzymes genes transcription of 'Golden Delicious' in 2006 following post harvest treatment with (+) and without (-) 1 $\mu l \cdot l^{-1}$ 1-MCP and dipped with (+) or without (-) 30 mM H_2O_2. Putative *act3* was used as a PCR control.

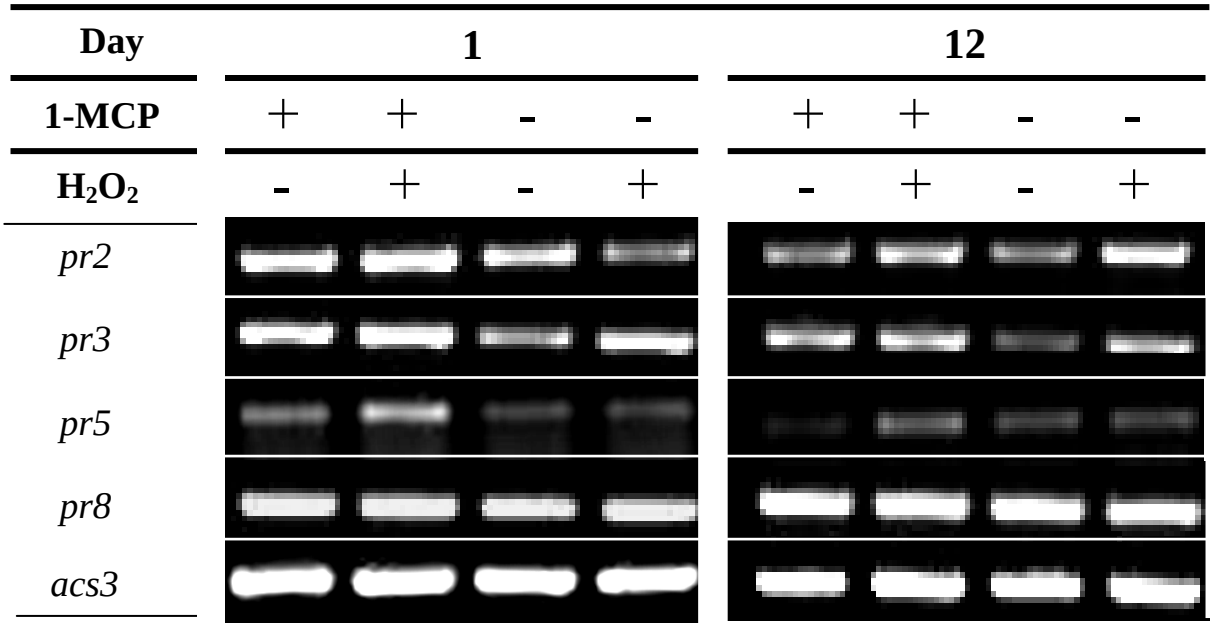

Figure 3-9. RT-PCR analysis of putative pathogenesis related protein gene transcription of 'Golden Delicious' in 2006 following post harvest treatment with (+) and without (-) 1 $\mu l \cdot l^{-1}$ 1-MCP and dipped with (+) or without (-) 30 mM H_2O_2. Putative *act3* was used as a PCR control.

DISCUSSION

1-MCP lowers oxidative stress but may prevent acclimation to stress

Ethylene evolves as a reaction to stress (Yang and Hoffman, 1984) and is involved in mediating the response (Alsonso et al. 1999; Wang et al. 2002; Ludwig et al. 2005). Since 1-MCP inhibits ethylene action, 1-MCP should interfere with the response to stress. In this study, oxidative pressure on fruit was evaluated by measuring antioxidant capacity, catalase activity, and antioxidant enzyme transcription. ROS-mediated oxidation was greater in 2005 than 2006 in 1-MCP-treated fruit as seen by a significant decrease in antioxidant capacity in 2005 while it remained constant over the study period in 2006. Catalase activity was more than twice as high in fruit not treated with 1-MCP in 2005 compared to 2006 (Fig. 3-1 and 3-2). Catalase increases in response to stress (Prasad et al. 1994; Anderson et al. 1995) and is central to acclimation (Apel and Hirt, 2004). In 2005, ROS-mediated oxidation was lower in 1-MCP-treated H_2O_2-dipped fruit compared to water-dipped ones as demonstrated by a delay in the decrease in antioxidant capacity (Fig. 3-4). Not only was antioxidant capacity higher by day 10 in 1-MCP-treated H_2O_2-dipped fruit but they had lower *sod*, *Md-gpx*, and *apx* transcript levels also (Fig. 3-7). This suggests a lower requirement for ROS detoxification. 1-MCP-treated water-dipped fruit appeared to have a higher requirement for ROS removal than fruit from other treatments as *sod*, *apx,* and *cat* transcription was higher by day 10 (Fig. 3-7). This ability of 1-MCP-treated H_2O_2-dipped fruit to cope with stress was not mediated via catalase activity, considering H_2O_2 did not significantly increase catalase activity of 1-MCP-treated fruit (Fig. 3-1). Induction of antioxidant enzymes does not appear to have contributed to the coping mechanisms (Fig. 3-5). It is possible however that H_2O_2

increased SOD and GPX activity augmenting ROS scavenging which resulted in lower antioxidant oxidation. It is also plausible that H_2O_2 simply increased antioxidant synthesis or regeneration. Fruit not treated with 1-MCP had lower antioxidant capacity than 1-MCP-treated ones one day after treatment (Fig. 3-4). Unlike 1-MCP-treated fruit, however, antioxidant capacity remained stable and did not decrease over time. Higher catalase activity (Fig. 3-1) and increased transcription of *apx* genes (Fig. 3-7) seems responsible for preventing further oxidation of antioxidant substances (Fig. 3-4) and acclimating the fruit. Furthermore, phenylalanine ammonia-lyase, a crucial step in the synthesis of flavonoids requires ethylene (Blankenship and Unrath, 1988; Faragher and Chalmers, 1977; Riov et al. 1969; Rhodes and Wooltorton, 1971).

In 2006, fruit were not exposed to drought conditions during the growing season and different observations were witnessed. Antioxidant capacity and catalase activity were significantly greater overall in fruit not treated with 1-MCP than treated ones (Fig. 3-5A and 3-2B, respectively). However, H_2O_2 significantly decreased antioxidant capacity of fruit not treated with 1-MCP compared to treated ones (Fig. 3-5A). In addition, while H_2O_2 generally increased catalase activity over time (Fig. 3-2A and 3-8), dipping 1-MCP-treated fruit in H_2O_2 did not significantly increase activity over time according to LSD analysis (Appendix Fig. A-4). *Apx* was induced by H_2O_2 in fruit not treated with 1-MCP showing potential for stress acclimation. *Apx* transcript levels were higher in 1-MCP-treated than untreated fruit though they were not up regulated over time (Fig. 3-8). Higher *apx* levels in 1-MCP-treated fruit may be responsible for counteracting the H_2O_2 dip (Fig. 3-5) and preventing a superfluous acclimation. Vilaplana et al. (2006b) found higher peroxidase activity and lower ascorbate levels in 1-MCP-treated ‘Golden Smoothee’

apple fruit which suggests that this may be the case. Measurement of APX activity in this study could have supported this claim.

In 'Delicious', infiltration of H_2O_2 after four months of storage in 2006 significantly increased antioxidant capacity of fruit not treated with 1-MCP in a dose response manner, but not in 1-MCP-treated ones (Fig. 3-6). Also, catalase activity was lower in fruit infiltrated with 10 mM H_2O_2 and all of those treated with 1-MCP (Fig. 3-3A and B). It is possible that higher antioxidant capacity is responsible for lower catalase activity or simply that lower overall ROS-mediated oxidation resulted in higher antioxidant capacity and lower catalase activity.

In some instances, 1-MCP-treated fruit failed to respond positively and negatively to H_2O_2 (Fig. 3-1, 3-5A, and 3-6A). Although this illustrates a need for both ethylene and H_2O_2 signaling, this was not true in all situations (Fig. 3-4). The intricacies of ethylene and H_2O_2 signaling during acclimation and stress tolerance are complex. The use of 1-MCP on apple may prevent stress acclimation and tolerance in some situations. The varying results between 2005 and 2006 suggest that once fruit are stressed at the moment of 1-MCP treatment, they may not be able to induce antioxidative mechanisms in response to subsequent stress conditions. Further research is needed to confirm this point.

In all studies, 1-MCP-treated fruit appeared in a lower oxidative state than untreated ones as illustrated by higher catalase activity and lower antioxidant capacity (Fig. 3-1, 3-2, 3-4, and 3-5). Higher catalase activity in H_2O_2-dipped fruit over time also suggests higher oxidative state as well as ability to survive the stress (Fig. 3-2A). Furthermore, it is possible that ethylene sensitivity is required for regulation of catalase activity (Fig. 3-1, 3-2, and 3-3) but not transcription (Fig. 3-4 and 3-5).

1-MCP treatment kept catalase activity low

In this study, treatment with 1-MCP prevented an increase in catalase activity. It was surprising to observe significantly lower catalase activity in 1-MCP-treated compared to untreated fruit since other studies showed no difference or higher activity in 1-MCP-treated fruit (Tsantili et al. 2007; Vilaplana et al. 2006b) using similar assays (Aebi 1983,1984). Using EDTA during extraction and reaction (Tsantili et al. 2007) could have chelated iron out of catalase ferriprotoporphyrin groups since the group can be easily lost (Aebi, 1983) and decreased catalase activity and precision of the assay. Furthermore, according to Aebi (1983), the use of Triton-x-100® during the extraction of catalase is necessary to assure sufficient activity for the assay.

Another variable in extraction and assay protocols is the use of protease inhibitors. In our study we used inhibitors to various proteases. Ethylene is linked to increased proteolytic activity in some reproductive tissues (Jones et al. 2005). Using a protease inhibitor to only one type of protease or not using a protease inhibitor cocktail could have resulted in higher proteolytic degradation of catalase in fruit not treated with 1-MCP compared to treated ones, and caused lower activity. This *post hoc* hypothesis was tested using 1-MCP-treated and untreated fruit which were extracted with and without use of a protease inhibitor cocktail. Under the conditions used for this essay, only 1-MCP significantly affected catalase activity (P=0.0480), while the use of protease inhibitors did not (Appendix Fig. A-5).

It is possible that sampling fruits directly in cold storage without warming them could have prevented an increase in catalase activity of fruit not treated with 1-MCP as seen by

Tsantili et al. (2007). Response to stress can be prevented during chilling (Prasad et al. 1994). Vilaplana et al. (2006b) observed a significant increase in catalase activity in cold storage in 1-MCP-treated fruit compared to untreated ones, but by three months, the relationship was reversed. To confirm that catalase activity is lower in 1-MCP-treated fruit, it was measured on 'Delicious' apples provided by AgroFresh® after four months in commercial refrigerated air storage. Again, catalase activity was significantly lower in 1-MCP-treated fruit. Time at room temperature may be required to witness this difference as the significant difference was observed 12 and seven days after treatments (Fig. 2B and 3B).

While a general approach to the estimation of the scavenging capacity of antioxidant substances provides some information regarding ROS scavenging capabilities, it does not provide information regarding the profile of antioxidant substances. Moreover it is impossible or difficult to discern the influence specific factors have on the regulation of the synthesis or regeneration of antioxidants when antioxidant capacity is examined globally. However, since lipid-soluble antioxidant capacity is linked to water-soluble antioxidant capacity via α-tocopherol and ascorbate (Noctor and Foyer, 1998), total water-soluble antioxidant capacity measurement offers an estimate of the impact of oxidizing agents on cells.

Most of the genes studied were strongly expressed regardless of treatment. It is possible that using Q or TaqMan PCR differences could be observed as they are more precise and sensitive PCR methods. In this study, examination of transcription of all isozymes was performed using primers to sequences representing signature domains of translated proteins, with the exception of GPX. In apple, with the exception of *Md-gpx*,

no nucleic acid sequence of antioxidant enzymes was available at the time of this study. Examination of the expression profile of homologs could have revealed differences in expression patterns of treated fruit. 1-MCP-treated fruit had slightly higher levels of *apx* transcription than untreated ones in 2006 and ten days after treatments in 2005. Lower ascorbate levels were found in 1-MCP-treated than untreated 'Golden Smoothee' during storage (Vilaplana et al. 2006b). This difference could be caused by higher *apx* expression in 1-MCP-treated than untreated fruit as seen in ten days and one day after treatment in 2005 and 2006, respectively (Fig. 3-5, and 3-6). One day after treatment in 2005 however, 1-MCP-treated fruit had undetectable *apx* transcription while untreated fruit had very low transcript levels. During the growing season in 2005, fruit were exposed to drought conditions and fruit appeared acclimated as higher catalase activity was measured in fruit not treated with 1-MCP in 2005 (Fig. 3-1) compared to 2006 (Fig. 3-2). Catalase activity is crucial to acclimation (Apel and Hirt, 2004; Willekens et al. 1997) and is a good marker of acclimation. Higher endogenous H_2O_2 levels in response to stress (Prasad et al. 1994; Anderson et al. 1995; Fadzillah et al. 1996; Okuda et al. 1991; Omran, 1980; Wise and Naylor, 1987a,b) at the moment of harvest could explain why *apx* transcript levels were similar to 1-MCP-treated fruit regardless of the H_2O_2 dip. Both catalase activity and antioxidant capacity data show this relationship and point to higher H_2O_2 levels.

Ethylene and peroxide signaling during fruit ripening and systemic resistance

Ethylene and ROS/ H_2O_2 signaling appear important to fruit ripening. An oxidative burst seems to occur in tandem with onset of ethylene production as witnessed in apples

by a significant increase in SOD and CAT activity in apples (Masia, 1998) and an increase in lipid peroxidation in avocado (Meir et al. 1991). Decreasing and fostering this oxidative burst delayed and advanced ripening processes in pears (Brennan and Frenkel, 1977). Furthermore, like ethylene in climacteric fruits, ROS signaling and synthesis can also create a positive feedback loop (Desikan et al. 1998; Torres et al. 2002). ROS seems to positively affect ethylene synthesis and/or signaling and vice-versa (Brennan and Frenkel, 1977). In the current study, whether or not the ethylene loop is interrupted by 1-MCP, ROS appear to have at best limited influence on ethylene biosynthesis and signaling (Fig. 2-3).

Both ethylene and H_2O_2 are part of signaling pathways leading to systemic resistance (Durrant and Dong, 2004; Spoel et al. 2003; van Wees et al. 2000). Inhibiting ethylene action leads to an increase in the incidence of certain diseases (Blankenship and Dole, 2003; Watkins and Miller, 2007). Accumulation of PR mRNA tightly correlates with the onset of systemic resistance which makes most PR genes, with the exception of *pr1* (Bonasera et al. 2006; Heil and Bostok, 2002; Greenberg et al. 2000), useful molecular markers for systemic resistance (Ward et al. 1991; Durrant and Dong, 2004). In this study, considerable rotting of 1-MCP-treated fruit was observed; however, not in 1-MCP-treated fruit dipped in H_2O_2. To determine whether this difference in rot incidence between 1-MCP-treated fruit dipped in water or H_2O_2 was caused by contaminated deionized water or fruit, and by H_2O_2-mediated asepsis, transcription of PR proteins was examined (Fig. 3-9). H_2O_2 induced PR proteins transcription; *pr2* and *pr5* were higher in 1-MCP-treated H_2O_2-dipped fruit. These results are promising; however a more rigorous

study using fruit inoculation with a rot-causing pathogen after H_2O_2 treatment would greatly support this observation.

Although recognition and perception of a certain pathogen is highly specific, defense mechanisms expressed are general; defenses against one pathogen thus can convey resistance against a wide spectrum of pathogens. Since signaling is a broader process than perception, resistance to a great number of pathogens could be obtained by exploiting host signals either chemically or genetically. Most systemic resistance is long lasting and confers generalized protection to plants (Bowles, 1990; Heil and Bostok, 2002; Vallad and Goodman, 2004), which may last for the plant's lifetime (Durrant and Dong, 2004).

While in general senescing tissue are more susceptible to ethylene and decay (Boller et al. 1991), the effects of systemic resistance are long lasting and could decrease decay and rot in long-term storage as ripening and aging fruits are more susceptible to pathogen invasions (Brown and Burns, 1998).

CONCLUSION

A decrease in antioxidant capacity suggests an increase in ROS-mediated oxidation which could lead to loss of fruit quality and senescence (Hodges, 2003). H_2O_2 has potential as a dip to increase antioxidative mechanisms by increasing catalase activity and antioxidant capacity, or at least delaying a decrease in antioxidant capacity. However, H_2O_2 did not increase transcription of genes of key antioxidant enzymes. The 2006 results indicate that H_2O_2 had a lower oxidative and signaling impact on 1-MCP-treated fruit. Furthermore, although 1-MCP-treated fruit are less prone to stress, they may suffer more from stress by not acclimating to it.

The ethylene-catalase relationship is obscure and whether ethylene directly increases catalase activity or indirectly via increased ROS production requires more research. In any case, at room temperature fruit not treated with 1-MCP repeatedly had higher catalase activity than treated ones. Results regarding the effect of 1-MCP on antioxidant capacity were more variable as a function of the study year and so was the effect of H_2O_2. Changes in antioxidant capacity and catalase activity were prevented in 1-MCP-treated fruit, however, which may prevent acclimation to subsequent stress conditions. This was exemplified in 2005 as a consequence of drought conditions during the growing season where 1-MCP-treated fruit significantly decreased in antioxidant capacity but not in untreated ones. More research is needed regarding acclimation of 1-MCP-treated fruit, especially considering the introduction of new 1-MCP liquid formulations applicable directly to an orchard at the mercy of the stress conditions.

Finally, while 1-MCP-treated had higher rot incidence, those treated with H_2O_2 did not and had higher induction of pathogenesis related proteins PR2 and PR5. Both proteins have been linked to heightened disease resistance (Ferreira et al. 2007). H_2O_2 is an elicitor activating defense genes (Apostol et al. 1989; Bi et al, 1995) and originates in the apoplast (Bowles, 1990). Considering this, apoplastic infiltration of H_2O_2 using a dip or under vacuum could trigger systemic resistance and thus decrease rot and decay incidence in 1-MCP-treated apples. Using H_2O_2 on fruit to prevent disease would greatly benefit an industry relying heavily on pesticides to control disease.

4. MOLECULAR MARKERS FOR 1-METHYLCYCLOPROPENE TREATMENTS OF POME FRUITS

The active ingredient of SmartFresh®, 1-methylcyclopropene (1-MCP), is a safe and effective ethylene action inhibitor working at the receptor level. It is currently used by the fruit industry to maintain postharvest fruit quality by preventing or delaying ripening and reducing incidence of ethylene-related disorder (Blankenship and Dole, 2003; Watkins, 2006). Efficacy of application is a function of dose, storage, cultivars (Watkins et al. 2000), duration, and temperature (DeEll et al. 2002). At the moment the registered doses for use in the apple industry in the US and Canada are 1 $\mu l \cdot l^{-1}$ (Watkins, 2006; PMRA, 2004). Although not yet registered, a liquid formulation exists (Hamrick, 2001) in addition to the standard gas formulation.

Recent studies regarding the impact of 1-MCP on fruit ripening reported changes in hydroxycinnamic acid and flavonoids (McLean et al. 2006), redox metabolism (Vilaplana et al. 2006b), aroma volatiles (Li et al. 2006; Ferenczi et al. 2006; Sigal-Escalada and Archbold, 2006), elements of ethylene biosynthetic and signal transduction pathways (Dal Cin et al. 2006; Pang et al. 2006; Tatsuki and Endo, 2006; Tassoni et al. 2006; Vilaplana et al. 2006a), polyamines metabolism (Pang et al. 2006; Tassoni et al. 2006), rate of absorption by numerous fresh produce (Nanthachai et al. 2006), and synergy with bioregulators such as naphthaleneacetic acid, aminoethoxyvinylglycine, and ethephon (Drake and al. 2006; Yuan and Cabaugh, 2007).

An inconvenience using 1-MCP, however, is the difficulty to ascertain treatment status or effectiveness. Vallejo et al. (2006) reported sorption of 1-MCP by non-target material, reducing the 1-MCP concentration available to commodity and possibly compromising the effectiveness of a treatment. 1-MCP is widely used by the apple industry and scientists, and use of molecular markers to ascertain efficacy of treatment would support scientific and commercial claims.

Envisioning the use of reverse-transcription polymerase chain reaction (RT-PCR), to investigate ethylene-induced genes as potential molecular markers of 1-MCP led to examination the genes of *Malus domestica* Borkh (Abbreviated *Md*), *Md-etr1*, *Md-ers1*, *Md-ctr*1, *Md-aat*1, *Md-aat*2, *Md-pg*, *Md-acs1*, *Md-acs2*, and *Md-acs3* (data not shown). Initial selection criterion for candidates was to demonstrate a difference in expression between treated and non-treated fruit over ten days at room temperature. The genes *Md-pg* and *Md-acs1*, coding for an apple endo-polygalacturonase and 1-aminocyclopropane 1-carboxylic acid synthase, respectively, were selected for further examination.

Md-pg and *Md-acs1* markers were tested on four cultivars at harvest after treatment with either gas or liquid 1-MCP formulation. Also, the markers were tested on organically grown ‘Granny Smith’, treated commercially by SmartFresh®, after four months of commercial storage. The impact of exogenous ethylene after storage on marker expression also was examined.

MATERIALS AND METHODS

Plant Material and Treatments

The cultivars 'Improved Red McIntosh', 'Gala', 'Jonagold', and 'Golden Delicious' were harvested at the WSU Tukey farm at optimum maturity for long-term storage as indicated in each cultivar by a ten-fruit average internal ethylene concentration of 0.1 - 1 $\mu l \cdot l^{-1}$. Internal ethylene concentration was measured as described in chapter 2.

For each cultivar at optimum maturity, a total of 36 fruit were harvested for the study. Then, the fruit were treated overnight at 1°C in sealed 11-l jars with 0 or 1 $\mu l \cdot l^{-1}$ 1-MCP (SmartFresh®, Philadelphia, PA, USA). Following treatment, the fruit were left at room temperature in ambient air. One and ten days after treatment, for each 1-MCP concentration, a composite consisting of the skin of nine fruit was sampled and immediately frozen and homogenized in liquid nitrogen prior to storage at -80°C for RT-PCR analysis.

In October 2006 at the WSU Tukey farm, SmartFresh® representatives sprayed or dipped 'Jonagold' apples on trees with a new liquid 1-MCP formulation. Each apple was sprayed, or dipped for 30 seconds and left to dry in air prior to harvest. A composite skin sample from nine harvested fruit per treatment was sampled after six days at room temperature and stored as previously described for RT-PCR analysis.

At the beginning of January 2007, SmartFresh® and Stemilt Growers Inc. generously provided SmartFresh®-treated 'Granny Smith' apples and their equivalent control which were stored in refrigerated air for four months. The fruit were treated with 1-MCP by SmartFresh® representatives within a week of harvest. After commercial storage, fruit was transported from Wenatchee, WA, USA, to the Postharvest Laboratories in Pullman,

WA, USA. On arrival, the cold fruit was stored at 1˚C overnight prior to warming to room temperature the next day. Subsequently fruit treated with 0 and 1 $\mu l \cdot l^{-1}$ 1-MCP were exposed to 0 or 20 $\mu l \cdot l^{-1}$ ethylene, released from ethephon and fruit for two 2 hours, in a 2 $ml \cdot h^{-1}$ flow through system. The skin of four fruit per treatment was sampled and stored as described above after six days at room temperature following ethylene treatment.

RNA extraction and RT-PCR

RNA extraction, RT-PCR, and primer design were done as mentioned in chapter 2. The primers used for PCR are listed in Table 4-1.

Table 4-2. Forward (F) and reverse (R) primer sequences used for PCR amplification of apple genes used as molecular markers of 1-MCP treatment. Sequence and identity from NCBI (2007).

Gene	Sequence (5' - 3')	Accession No.	Reference
Putative *act3* (Arabidopsis; 98.4% id.)	F GATGAAAATCTTGACTGAGCGTGG R CAGCTTTGGCAATCCACATCTG	CN927806	Beuning et al. 2004;
Md-pg	F ATGCGCATGTTATCCAGAAACG R AACAAGCCAGCATTGCCGTT	U89156	Harada et al. 1997
Md-acs1	F AGGCCGATTGAATTCTCAGG R GGGAAGCTTGAACGTTGATG	AY821543	Asif and Solomos, 2004

RESULTS

The reliability of the *Md-pg* and *Md-acs1* markers was tested in fruit from four apple cultivars treated with 1-MCP after harvest, one and ten days after treatment using RT-PCR. For all four cultivars, expression patterns of the markers showed effective 1-MCP treatments by the tenth day. Markers showed considerably less transcription in 1-MCP-treated (Fig. 4-1). However, this trend was not always observed one day after treatment; pattern of transcripts showed variation among treatments and cultivars. For example while the markers worked well in 'Golden Delicious', however, in 'Gala' and 'Jonagold' had not detectable marker transcription. In 'Improved Red McIntosh', only *Md-acs1* had detectable transcription levels.

In addition, the markers were tested on fruit treated with the liquid 1-MCP formulation. In comparison to untreated fruit, transcription of the markers was considerably less in treated fruit six days after treatment in 'Jonagold' apple (Fig. 4-2).

To ascertain that the markers also worked for commercial certification of 1-MCP treatment, the markers were tested on 1-MCP-treated and untreated 'Granny Smith' apples from commercial storage. The impact of exogenous ethylene on marker expression was examined also. The markers were not transcribed by storage or a high exogenous ethylene concentration (Fig. 4-3). In 1-MCP-treated 'Granny Smith', expression of the markers was insignificant, even six days after exposure to exogenous ethylene. Untreated fruit, however, had extremely high marker expression, regardless of ethylene exposure (Fig. 4-3).

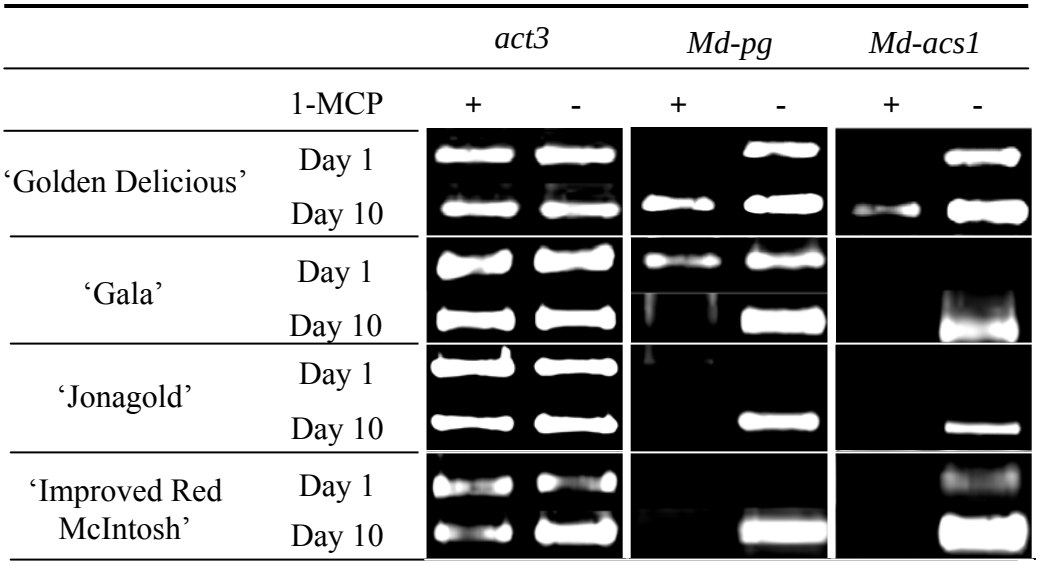

Figure 4-1. RT-PCR analysis of gene transcription used as molecular markers of 1-MCP treatment in various cultivars. Fruit were treated with 1 $\mu l \cdot l^{-1}$ 1-MCP overnight at 0°C on the day of harvest in 2006. Putative *act3* is used as a PCR control.

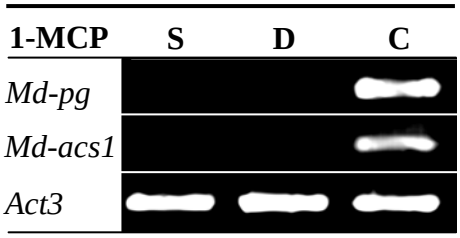

Figure 4-2. Assessment of *Md-pg and Md-acs1* in ascertaining application of liquid 1-MCP on 'Jonagold'using RT-PCR analysis. Fruit peel was sampled six days after 1-MCP spray (S), dip (D), or untreated (C).

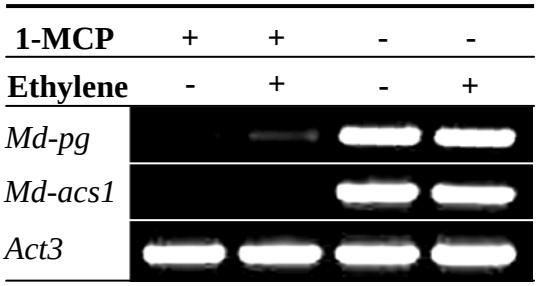

Figure 4-3. RT-PCR analysis of *Md-pg* and *Md-acs1* transcription of 'Granny Smith' apple after four months of storage in refrigerated air, to ascertain pre-storage application of gaseous 1-MCP and to assess the effects of ethylene exposure after storage on *Md-pg* and *Md-acs1* transcription. 1-MCP was applied at 1 $\mu l \cdot l^{-1}$ 1-MCP and ethylene at 20 $\mu l \cdot l^{-1}$. Putative *act3* was used as a PCR control.

DISCUSSION

Two ethylene inducible genes, *Md-pg* and *Md-acs1*, were tested as potential molecular markers of 1-MCP treatment. The markers effectively illustrated 1-MCP treatment in various cultivars regardless of formulation, as long as a delay at room temperature longer than a day occurred between treatment and sampling (Fig 4-1, 4-2, and 4-3). Furthermore, the markers were just as effective in fruit after storage following exposure to high exogenous ethylene (Fig.4-3).

Although the markers are effective, timing of sampling following treatment may be critical, since a carryover effect of ethylene metabolism on gene induction may decrease confidence in interpretation of the results. Some variation in transcription of the markers in fruit of different treatments and cultivars occurred one day after 1-MCP application (Fig. 4-1). This was not surprising, as no marker transcription had yet occurred in control fruit. Fruit maturity and ethylene metabolism at the moment of 1-MCP application are important factors influencing marker transcription. However in every cultivar, a substantial difference in expression of the markers indicated a successful application by the tenth day following application (Fig. 4-1). An increase in *Md-pg* and *Md-acs1* transcription had taken place by the tenth day in 1-MCP-treated 'Golden Delicious'. Return of ethylene sensitivity caused by fruit overcoming ethylene inhibition with addition of functional ethylene receptors could be responsible for the initiation of *Md-pg* and *Md-acs1* transcription in 1-MCP-treated 'Golden Delicious' (Fig. 4-1). Numerous studies in various crops have demonstrated that though one application is effective at delaying ethylene production or its action, normal ethylene metabolism can be eventually reestablished (Feng et al. 2000; Watkins and Miller, 2007 reference therein) and multiple

applications may be more desirable (Able et al. 2002; Pesis et al. 2002; Hoeberichts et al. 2002; Wills and Ku, 2002; Sisler et al. 1996). *Md-pg* and *Md-acs1* as molecular markers would be an excellent tool to study the effect of a single or multiple 1-MCP applications at various stages of climacteric ripening as well as the specificity/reactivity of apple cultivars to 1-MCP.

Considering the absence of marker transcription in fruit not treated with 1-MCP caused by fruit physiology at the moment of treatment, timing of sampling at harvest following treatment needs to be reassessed. Sampling tissue three to seven days following 1-MCP treatment might be ideal to eliminate carryover effects of ethylene on fruit metabolism prior to treatment and to escape potential reestablishment of ethylene metabolism. Using exogenous ethylene to advance maturity of untreated fruit may also be useful in inducing the markers in fruit not treated with 1-MCP.

Md-pg and *Md-acs1* as molecular markers of 1-MCP application were effective in ascertaining application of liquid 1-MCP (Fig. 4-2). The markers could help develop an effective field application methodology including dosage per acre, required coverage, timing of application, and assessment of spray equipment. Additionally, the molecular markers could be used to study the potential translocation ability of liquid 1-MCP formulations from the tree to the attached fruit providing information on the mode of action of 1-MCP.

Difficulties extracting good quality RNA from post-storage ‘Fuji’ apples were experienced. This illustrates that limitation using a molecular marker system using transcription of gene lies with RNA quality. All post storage ‘Fuji’ samples had degraded RNA which prevented further testing using the marker system. It is unclear at the

moment whether fruit maturity at harvest, handling, and storage are responsible for the outcome of this experiment.

Further investigation is needed to determine the 1-MCP dose to marker expression relationship. Furthermore, the determination of an effective time period between application and fruit sampling is crucial to overcome the absence of marker transcription caused by fruit immaturity at the moment sampling. Perhaps an exposure to exogenous ethylene could increase transcription of the markers in fruit not treated with 1-MCP. Also, the markers could be an excellent tool to determine the half-life of 1-MCP effects using a one-time treatment followed by various storage conditions.

CONCLUSION

The use of *Md-pg* and *Md-acs1* as molecular markers of 1-MCP application was examined in various apple cultivars at harvest and after storage. Storage and ethylene did not appreciably induce marker expression once ethylene action was blocked by 1-MCP, which renders the markers ideal to ascertain 1-MCP application. In addition, the markers were effective at confirming 1-MCP application regardless of the current formulations. Finally, the primers used in these studies are viable for all apple cultivars tested thus far.

5. CONCLUSIONS

Ethylene is a signal involved in the ripening of climacteric fruit, abiotic and biotic stress, as well as numerous other responses (Yang and Hoffman, 1984; Crozier et al. 2000). In pome fruit, ethylene action is required for development of aromatic esters and defense against certain rot- and decay-causing pathogens. The use of the ethylene action inhibitor 1-MCP in the apple industry, therefore, affects these responses negatively (Argenta et al. 2003; Blankenship and Dole, 2003; Watkins, 2006). Another issue with 1-MCP is the apparent difficulty to ascertain application or its effectiveness.

The ethylene signal pathway alone is not responsible for the regulation of ethylene responses. It is interlinked with numerous other pathways and signals. Ethylene and ROS signaling are tied in reaction to stress and cell death program such as senescence, which overlaps ripening (Watada et al. 1984). In climacteric fruit, ethylene is autocatalytic. In the absence of ethylene action caused by 1-MCP-mediated receptor blockage (Blankenship and Dole, 2003), to stimulate the ethylene signal transduction pathway downstream of the receptor could reintegrate ethylene action and thus improve aroma development and resistance to rot and decay pathogens. The use H_2O_2 to reinitiate ethylene signaling and its impact on the physiology of ripening apple was investigated.

To ascertain application of 1-MCP in pome fruit, a molecular biology approach was developed comparing transcription of various ethylene-related genes of 1-MCP-treated and untreated apple. The candidate genes resulting from this search were *Md-pg* and *Md-acs1*. The efficacy of both genes as molecular markers of 1-MCP treatment were tested

first in various cultivars, then as a function of formulation, and finally post storage in the presence of exogenous ethylene.

Ethylene metabolism can be induced by H_2O_2 in 1-MCP-treated fruit, even at a 1-MCP concentration three times that registered in U.S. and Canada. RT-PCR analysis demonstrated that transcription of *Md-acs1* and *Md-acs3* in 2006 matched the increase in ethylene production in 2005 and 2006, respectively. *Md-acs3*, *Md-etr1*, and the putative *ein2* all show promise to link ethylene and H_2O_2 signaling in ripening apple. Whether the small shift in magnitude and offset of the ethylene response mediated by H_2O_2 importantly impact fruit physiology remains to be demonstrated. Although no visual symptoms of physiological disorders were observed using H_2O_2, the differences in ethylene production caused by H_2O_2 were small and it is doubtful it could be exploited to recover 1-MCP-conferred inhibition of aroma development.

H_2O_2 has potential as a dip to increase antioxidative mechanisms by increasing catalase activity and antioxidant capacity, or at least delaying a decrease in antioxidant capacity. However, H_2O_2 did not increase gene transcription of key antioxidant enzymes in this study. The 2006 results indicate that H_2O_2 had a lower oxidative and signaling impact on 1-MCP-treated fruit than in 2005, a year when trees were exposed to drought conditions.

The ethylene-catalase relationship is obscure and whether ethylene directly increases catalase activity or indirectly via increased ROS production requires more research. In any case, at room temperature, fruit not treated with 1-MCP repeatedly had higher catalase activity than treated ones. Results regarding the effect of 1-MCP on antioxidant capacity were more variable according to the year and so was the effect of H_2O_2. In some

instance, changes in antioxidant capacity and catalase activity were prevented in 1-MCP-treated fruit. Such a behavior may prevent acclimation to subsequent oxidative stress caused by an increase in ROS production. This was exemplified in 2005 as a consequence of drought conditions during the growing season when 1-MCP-treated fruit had significantly decreased in antioxidant capacity as catalase activity and transcription levels remained unchanged.

It is possible that 1-MCP-treated fruit are less prone to stress when treated in the absence of stress. However by not responding to factors promoting stress, 1-MCP-treated fruit may be more susceptible to stress damage. The state of the fruit at the moment of the treatment with 1-MCP could be a determining factor in the efficacy of H_2O_2 in fruit acclimation and deserves further investigations.

SmartFresh® recently developed a liquid formulation for orchard application. Spraying fruit at the mercy of elements causing stress during the growing season may affect quality retention as 1-MCP-sprayed fruit are subsequently subjected to stress-causing conditions in cold storage.

While 1-MCP-treated apples had high rot incidence, those treated with H_2O_2 did not and had higher gene transcription of pathogenesis related proteins PR2 and PR5. Both proteins have been linked to heighten disease resistance (Ferreira et al. 2007). Considering H_2O_2 is an elicitor activating defense genes (Apostol et al. 1989; Bi et al, 1995) and that the apoplast is where signals originate to elicit defense responses (Bowles, 1990), apoplastic infiltration of H_2O_2 within fruit using a dip or under vacuum could trigger systemic resistance and thus decrease rot and decay incidence in 1-MCP-treated

apples. Using H_2O_2 to reduce disease incidence in fruit could greatly benefit an industry relying heavily on pesticides to control diseases.

Finally, *Md-pg* and *Md-acs1* were effective molecular markers for ascertaining 1-MCP application in various apple cultivars at harvest and in the 'Granny Smith' cultivar after storage. In addition, the markers were successful at establishing 1-MCP application when the inhibitor was applied using the liquid formulation. Storage and exogenous ethylene did not appreciably induce marker expression once ethylene action was blocked by 1-MCP. This renders the markers ideal for commercial treatment certification.

APPENDIX.

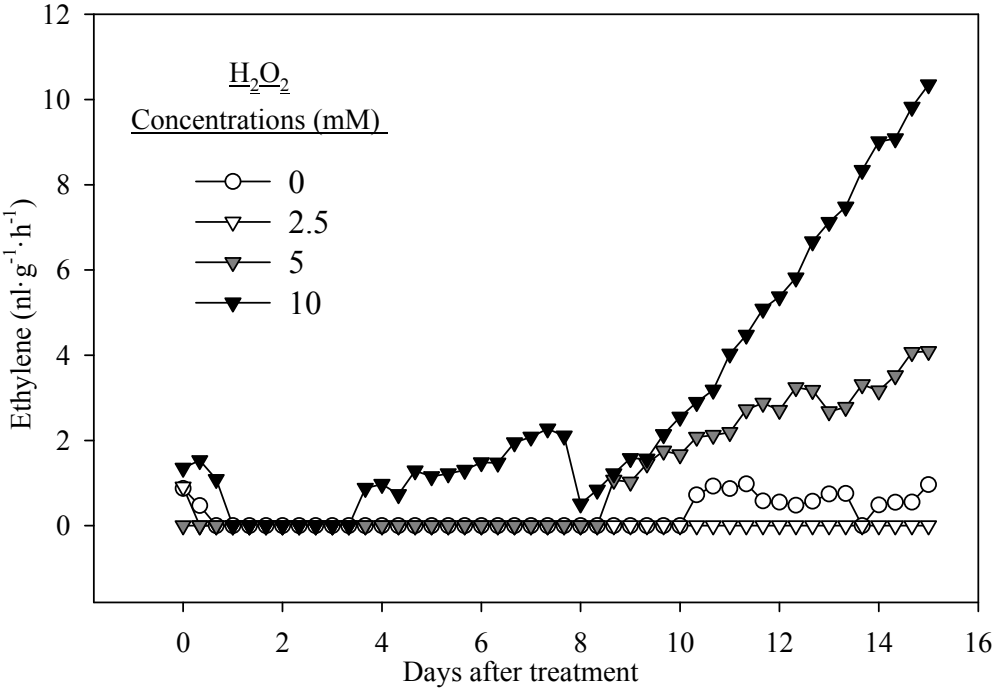

Figure A-1 Ethylene production of D'Anjou pears following 21 days at 1°C after a dip in various H_2O_2 concentrations for an hour. Data represent only one replicate.

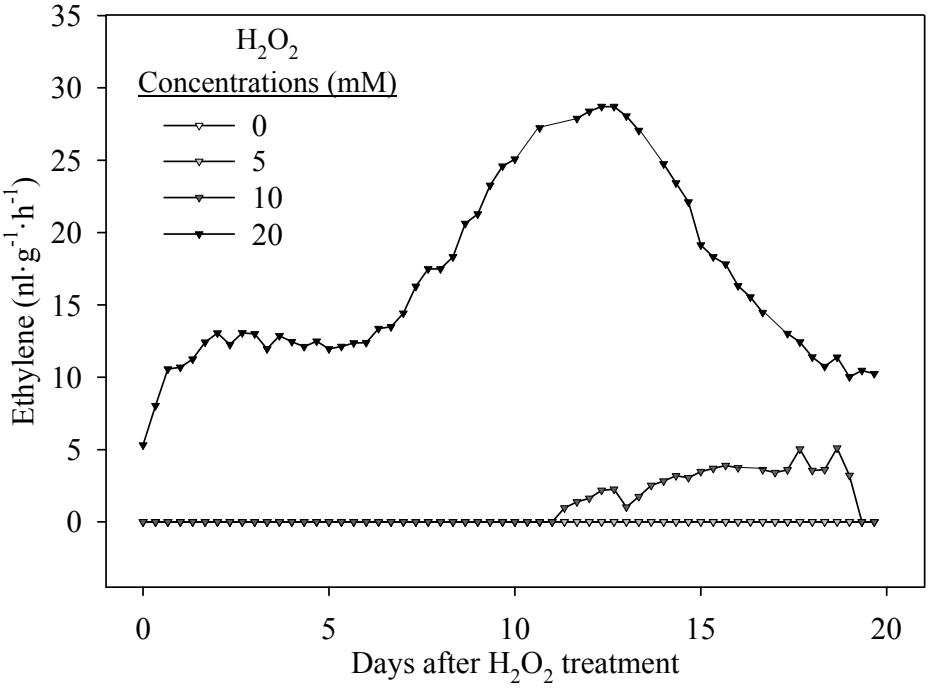

Figure A-2. Ethylene production of D'Anjou pears following 28 days at 1°C after a dip in various H_2O_2 concentrations for an hour. Numbers represent one replicate.

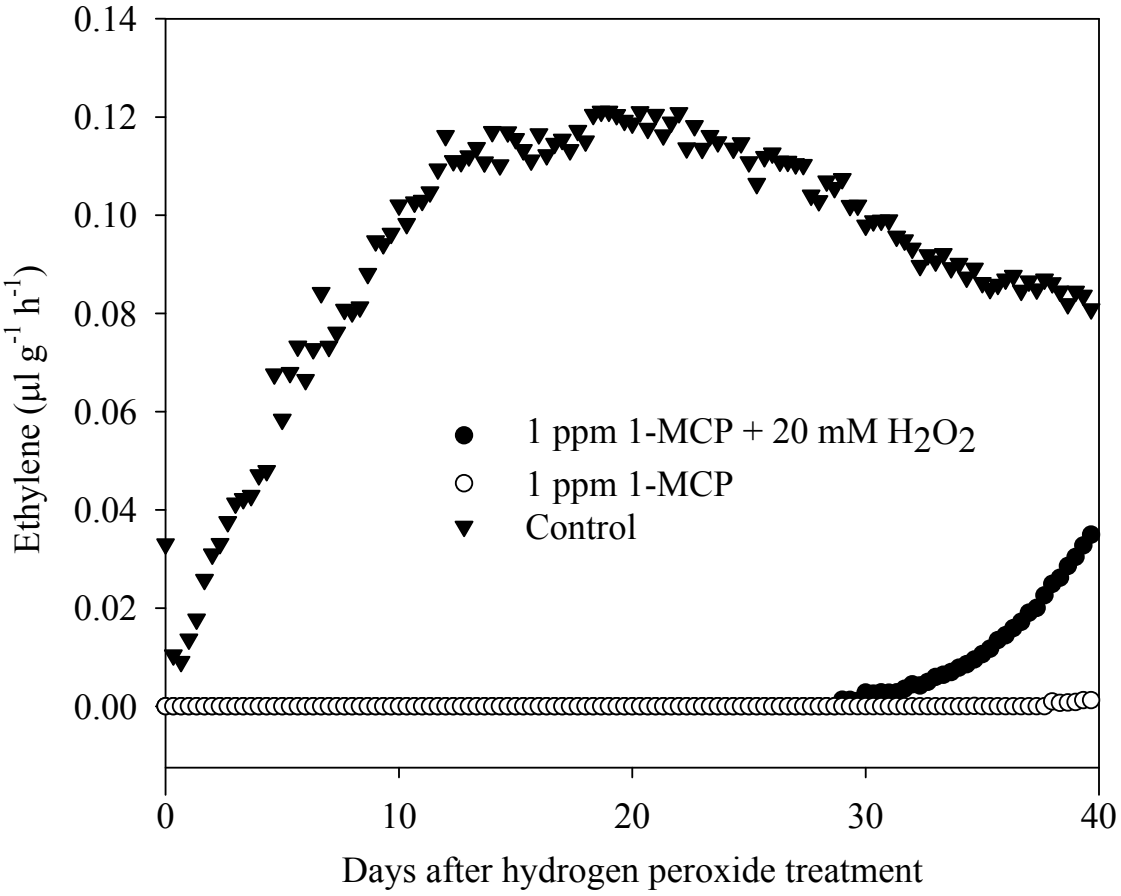

Figure A-3. Ethylene evolution of 'Golden Delicious' five months after standard controlled atmosphere following a dip in H_2O_2 for an hour. Numbers represent one replicate.

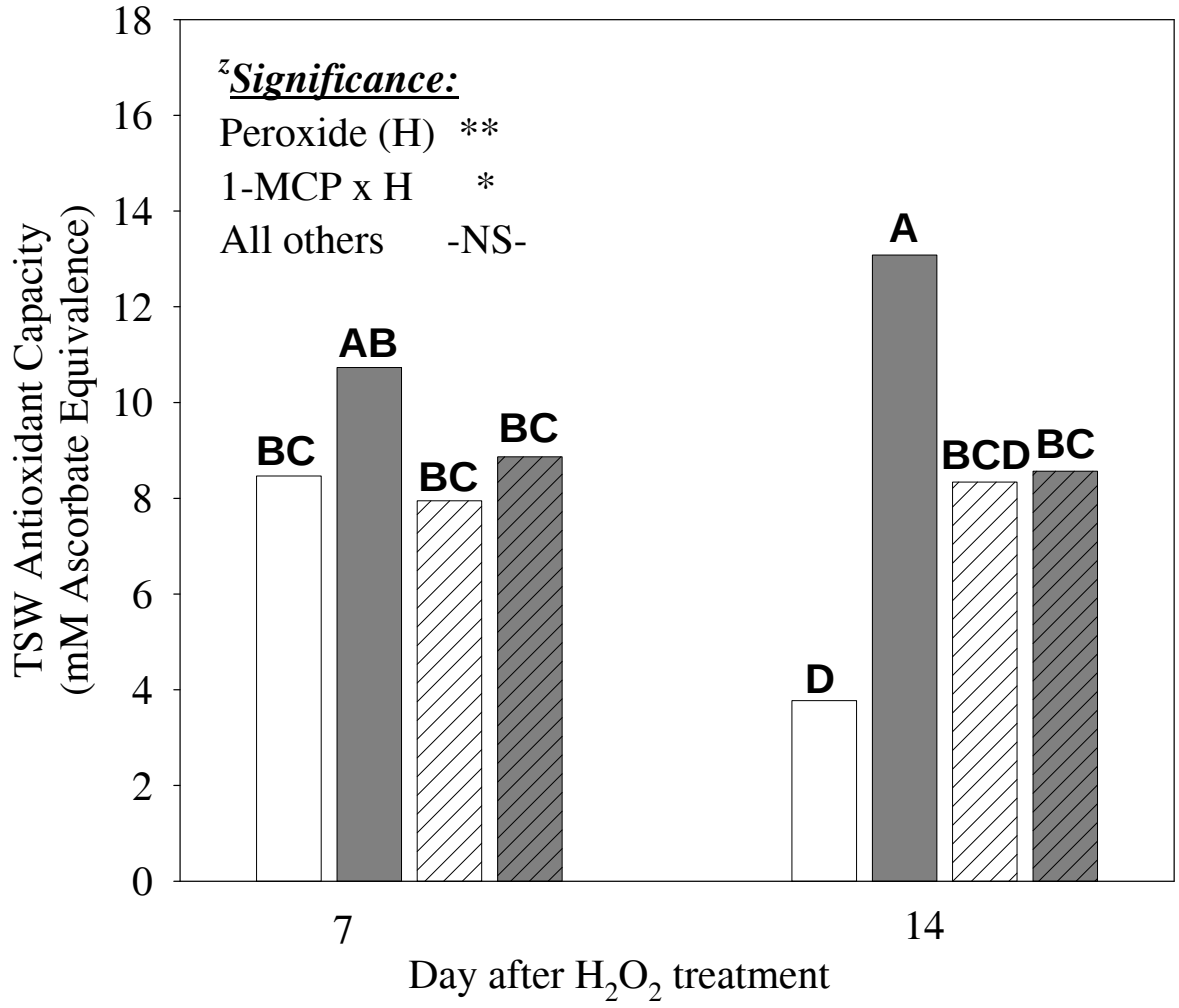

Figure A-4. Antioxidant capacity of 'Delicious' after four months of storage a 1˚C. An LSD analysis of the analysis of variance effect "Peroxide x 1-MCP x Time" was computed. Means with the same letter are not significantly different based on a least significant difference analysis (α=0.05). Cross-hatching represents treatment with 1 $\mu l \cdot l^{-1}$ 1-MCP while grey bars represent an hour dip in 30 mM peroxide following 1-MCP application. NS, *, and ** mean non significant or significant at *P*=0.05 and *P*=0.01, respectively.

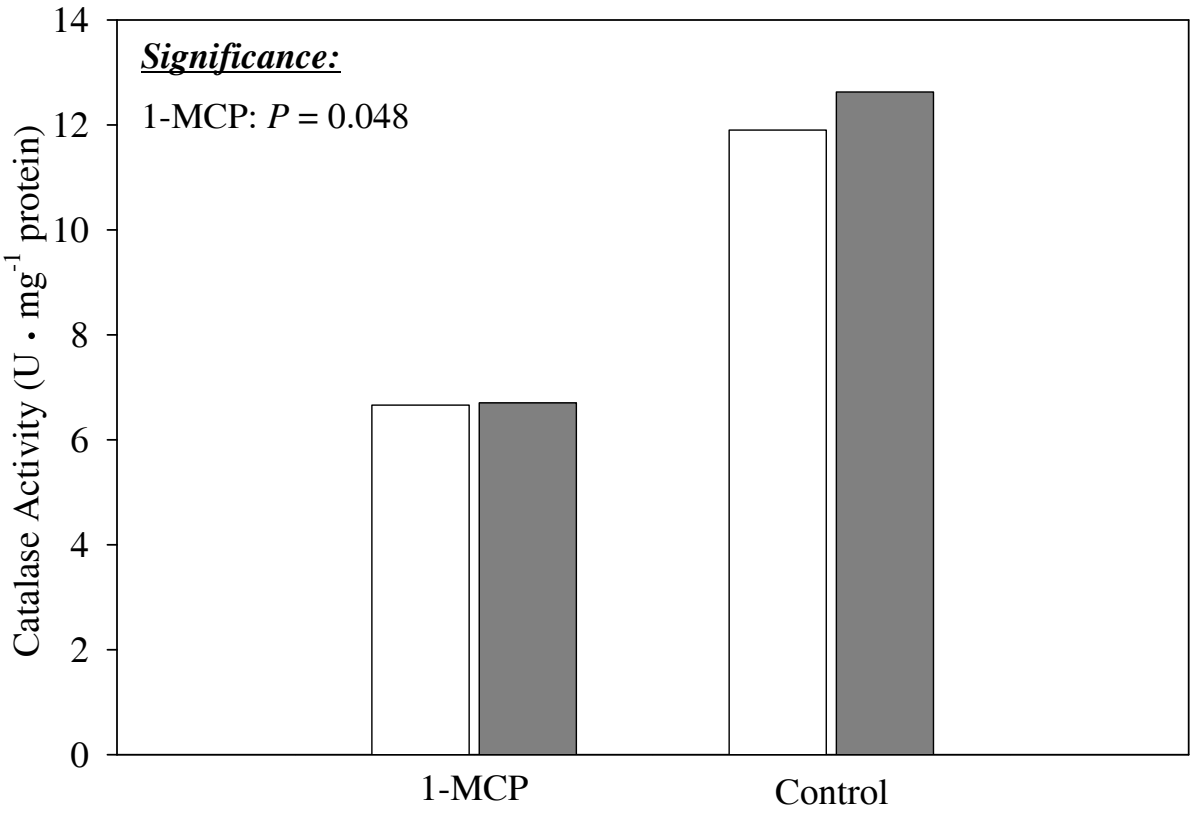

Figure A-5. Effect of protease inhibitor cocktail on catalase activity protocol using 'Golden Delicious' 12 days after treatments in 2006. Grey bars represent catalase activity of samples containing the protease inhibitor cocktail described in chapter 3 during extraction of the enzyme. *P* value is from an analysis of variance.

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